

**Intervention methods to control the transmission
of noroviruses and other enteric and respiratory viruses**

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**Intervention methods to control the transmission
of noroviruses and other enteric and respiratory viruses**

Era Tuladhar

Thesis

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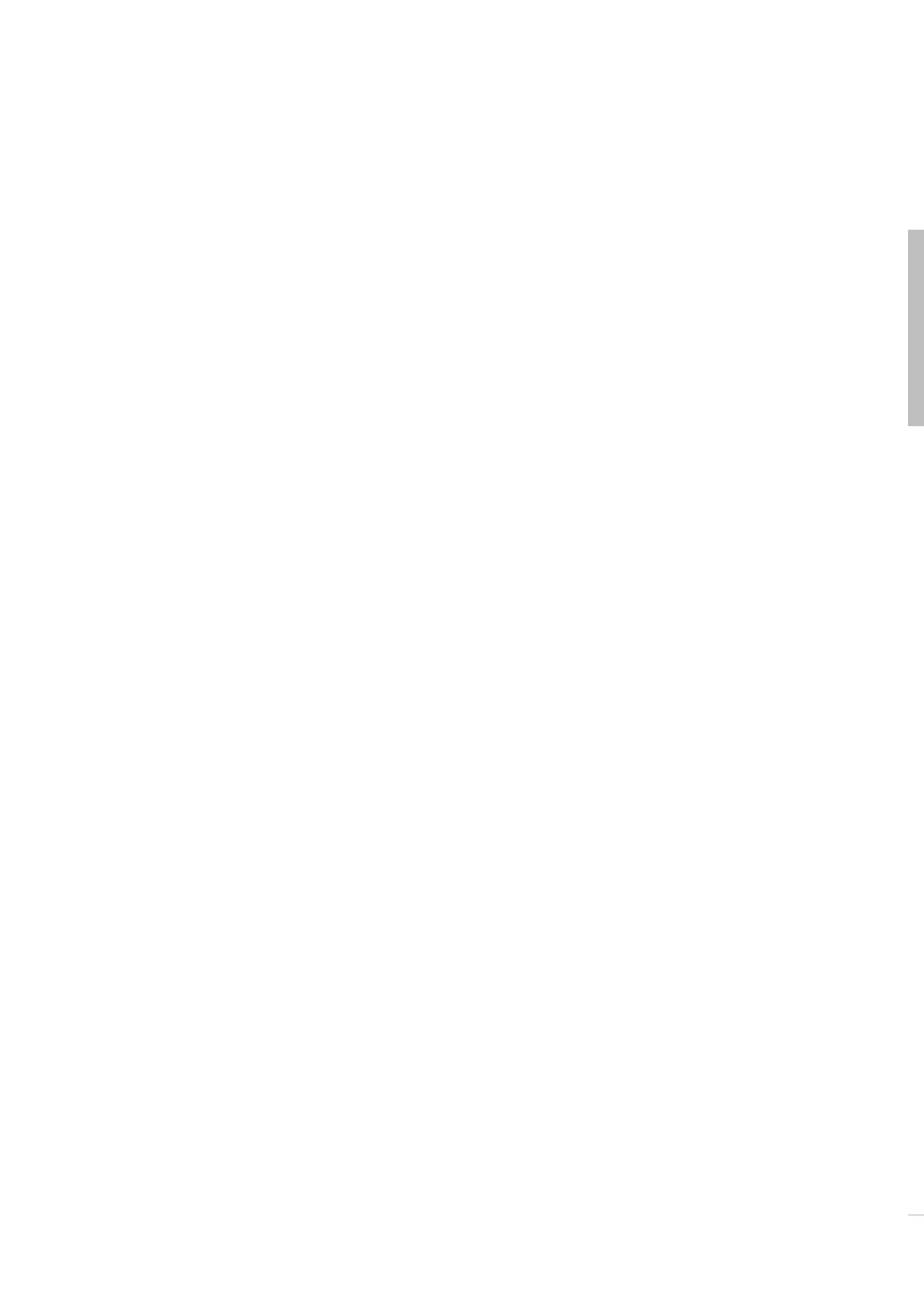
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Abstract

Human noroviruses are the leading cause of acute and outbreak associated gastroenteritis worldwide. The outbreaks occur often in hospitals, nursing homes, health care centers as well as in individual homes. Due to the high number of outbreaks and frequency of infection, the burden of disease is high. The virus transmission takes place from person to person directly through the fecal oral route or indirectly through contaminated surfaces or consumption of contaminated food. This study aimed to investigate methods to reduce the burden of disease caused by norovirus and focuses on reducing the transmission through hands and contaminated surfaces. Besides human norovirus, other enteric and respiratory viruses are also transmitted through these routes and were included in the study. Enteric viruses included in the study were human norovirus GI.4 and GII.4, poliovirus Sabin 1, rotavirus SA11, parechovirus 1 and murine norovirus 1 (MNV1). The respiratory viruses were adenovirus type 5 and influenza A (H1N1) virus. These viruses can contaminate food through food handler-related contamination. Heating and pasteurizing is a commonly used method for inactivation of microorganisms in food. Heating at commonly used temperatures of 56°C and 73°C showed that thermo-stability of parechovirus and influenza virus was found to be similar to that of proven foodborne viruses and heating at 73°C for 3 min is sufficient to reduce the infectivity of the tested viruses $> 4 \log_{10}$. Not only food, contaminated hands can also transfer viruses to different surfaces, which are then sources of further transmission of the viruses. Cleaning and disinfection of contaminated surfaces are common intervention methods used in health care and kitchen facilities. The enteric and respiratory viruses showed varying susceptibility to cleaning and disinfection procedures applied. Data on infectious doses and transfer efficiencies was used to estimate a target level to which the residual contamination should be reduced and it was found that a single wipe with liquid soap followed by a wipe with 250 ppm free chlorine solution was sufficient to reduce the residual contamination to below the target level for most of the pathogens tested. Spot disinfection by 1000 ppm chlorine solution after wiping with liquid soap was sufficient to reduce the genomic copies of human norovirus GI.4 and GII.4 ($> 5 \log_{10}$ PCRU) completely within 10 min. In addition to manual cleaning and disinfection, non-touch disinfection of contaminated surfaces by vaporized hydrogen peroxide disinfection at 127 ppm for 1 h, as approved disinfection in the Netherlands for hospitals and health care centers, is effective against poliovirus Sabin 1, rotavirus SA11, adenovirus type 5 and MNV1 on stainless steel, framing panel ($> 4 \log_{10}$ infectivity reduction) and gauze ($> 2 \log_{10}$ infectivity reduction). Beside non-touch disinfection, immobilized biocidals have been tested for disinfection of contaminated surfaces. Immobilized quaternary ammonium compound coatings were virucidal against the influenza virus within 2 min but no virucidal effect against poliovirus was found in 6 h. Thus the coating can be used against the influenza A virus to prevent the transmission through frequently touched sites but not for non-enveloped viruses. Our study demonstrated that a norovirus contaminated hand can transfer the viruses to different surfaces, including food, even after the virus is dried. As an intervention method to prevent the transmission of the virus, washing hands with soap and water ($> 5 \log_{10}$ PCRU reduction) is better than using alcohol based hand disinfectants in removing noroviruses from hands.

This research has delivered new insights in methods to reduce transmission of human norovirus and produced comparative quantitative data on intervention methods to control transmission of other enteric and respiratory viruses. The study has additionally contributed to a better understanding of human norovirus transmission intervention efficiency. The new insights allow for the development of science based guidelines to control norovirus and other enteric and respiratory viruses in outbreak settings and thus help to reduce the burden of illness caused by these viruses.



Chapter 1

Introduction and thesis outline

Approaches for prevention and control of enteric viruses like norovirus, rotavirus, enterovirus, and respiratory viruses like adenovirus and influenza A virus have been described in a number of guidelines (4, 30-32, 119-121). Most guidelines provide similar sets of control measures; however, these are often based on “common sense”, educated guesses, or protocols for bacteria. They are rarely science based methods and their effectiveness to limit virus transmission is often not proven and sometimes even disputed. Thus it is important to validate the effectiveness and applicability of transmission intervention methods for enteric and respiratory viruses. This thesis focuses on effective methods to prevent transmission via environmental surfaces and hands of noroviruses, other enteric viruses, and some respiratory viruses.

Human norovirus outbreaks in hospitals and healthcare facilities

Norovirus has a high infectivity (i.e. low infectious dose), high level of shedding, asymptomatic shedding, and continued shedding after clinical recovery. Furthermore, norovirus has high genetic and antigenic variability and the immunity against the virus is short lived. In addition, the infective virus persists on inanimate substances. These features make it possible to cause

frequent outbreaks, sometimes even recurring at the same place/facility. Hospitals and health care facilities are the most commonly reported settings of norovirus outbreaks (89, 114, 191, 192). Such reported outbreaks in health care settings are frequent from developed countries. Besides the frequently reported outbreaks in developed countries, there are even more unreported outbreaks and sporadic cases. But in developing countries, the reported outbreaks are less: often not because norovirus is not a problem but because there are far less health care settings in developing countries (52, 164) and diagnostic capabilities are limited. Therefore, higher underreporting than in developed countries is likely. Outbreaks reported in long term care facilities in different developed countries show that approximately 60% of the outbreaks of gastroenteritis are caused by norovirus (81). In the Netherlands, 75% of norovirus outbreaks reported in 2002 were from nursing homes and 9% from hospitals (205). We now know this was hugely biased due to underreporting by hospitals (14, 15). In the US 43% of norovirus outbreaks were reported from nursing homes and hospitals (63). The viruses can be introduced into health care facilities by contaminated food, infected staff, visitors, or patients who are infected with the virus on admission. The outbreaks in these closed or semi closed settings often last for months (35). Outbreaks affecting multiple institutions have also been reported (193, 226) where the infection has reported to be spread from infected staff to patients (226).

Mode of transmission

Transmission of the virus takes place through different routes such as hands, aerosols, food and water and environmental surfaces as is shown in figure 1.

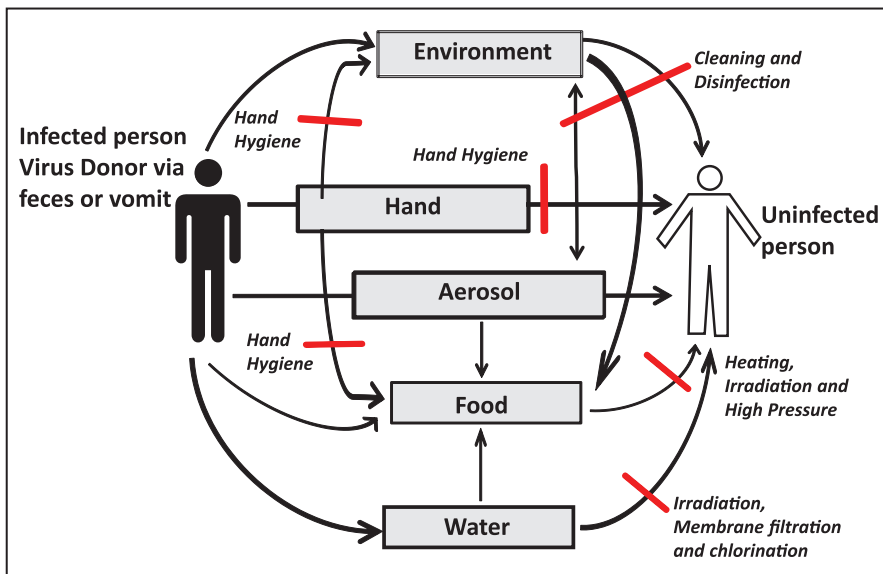


Figure 1: Transmission routes of human norovirus and the possible intervention points. Adapted from Lopman *et al.*(4, 132).

Person to person

Human noroviruses have a great potential for transmission through direct person to person contact through the fecal oral route. Direct person to person transmission is believed to be the primary mode of spread of human norovirus (132, 227). However, since norovirus is a human pathogen and all transmission is per definition from person to person, sometimes transmission is indirect when foods or surfaces are first contaminated. We define direct person to person transmission as virus transfer that takes place from feces to donor-hand to recipient-hand to mouth/nose or via ingestion of aerosols or droplets produced by vomiting, speaking and coughing. Direct person to person transmission is shown in figure 2 and the uninfected person is the virus recipient.

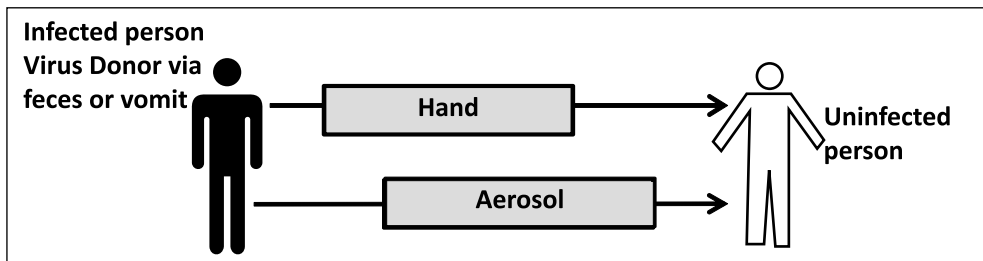


Figure 2: Transmission route of human norovirus by person to person contact.

Due to its high infectivity and high level of shedding in stool and vomit, the majority of norovirus outbreaks reported are associated with person to person transmission, especially in closed settings like nursing homes, hospitals, day care centers, long term care facilities and in large gatherings (39, 81, 141). A Foodborne Viruses in Europe network (FBVE) study also reported this route of transmission (116) as one of the main causes of transmission (88%) in norovirus outbreaks. Evidence of person to person transmission was shown in epidemics where infection began with infected staff in hospitals and health care facilities which was then spreading to patients (74, 126, 175, 224, 226) and vice versa (181, 206, 233). Some of these studies have shown clear association with number of staff in contact with patients and the risk of illness (176, 207). The risk of spreading disease among patients has also been demonstrated (145). The transmission of norovirus within two health care facilities has also been shown and symptomatic patients and health care workers have been more often involved in transmission events than asymptomatic shedders. It has been shown that asymptomatic health care workers rarely contribute to the transmission in hospitals (with highly trained personnel) (193).

An outbreak in a concert hall has also been linked to transmission through vomit (61), where the viral gastroenteritis outbreak affected more than 300 people who attended a metropolitan concert hall over a 5-day period suggesting also environmental transmission: aerosols basically set in an hour resulting in environmental contamination. The people with highest risk were among those seated close to the place where the vomiting incident occurred. A similar situation has been described on a cruise ship where 6 consecutive cruises were affected (100) that may also indicate environmental contamination and inadequate cleaning. However in this incident, there was person to person transmission and most likely the crew members were carriers of the virus.

In person to person transmission, contaminated hands play a major role as virus donor and receiver. Even though hands are the main vehicle for transmission, there is still limited data on persistence of norovirus on hands and transfer from hands to environmental surfaces and vice versa. Due to this lack of data, risk assessment models for human norovirus are often non-quantitative or based on estimated data, or based on personal observations and assumptions (151). Data on norovirus transfer from hand to surfaces and vice versa are needed for better estimation of transmission during handling and food preparation or in health care facilities during care taking and care giving. The data on the transfer is also needed to set minimal requirements for intervention methods to be considered effective.

Transmission via droplets and aerosols

Transmission of human norovirus through droplets and aerosols is also suggested. Production of viral aerosols and droplets has been suspected following vomiting (29, 95). A number of studies have reported transmission of the virus occurring during vomiting (37, 141, 142, 218). In such cases, transmission might occur due to direct aerosol contact from person to person. In addition to that, widespread contamination of surrounding environmental surfaces may also occur during the vomiting due to settlement of the aerosols (40). Transmission of the virus through contaminated aerosols was demonstrated in outbreaks in restaurants where the attack rate had an inverse correlation with the distance from the infected persons to the person who started vomiting. In both cases (141, 142) no contaminated food was involved or present. Besides vomiting, flushing of toilets also generates droplets that can contaminate surrounding toilet surfaces. In such cases, further transmission may take place through droplet contaminated surfaces (75).

Environmental surfaces

Surfaces contaminated with viruses serve as a vehicle for transmission to inanimate objects or vice versa (20). The surfaces may become contaminated with viruses by contact with body secretions through contaminated hands or via aerosols through talking and vomiting or via

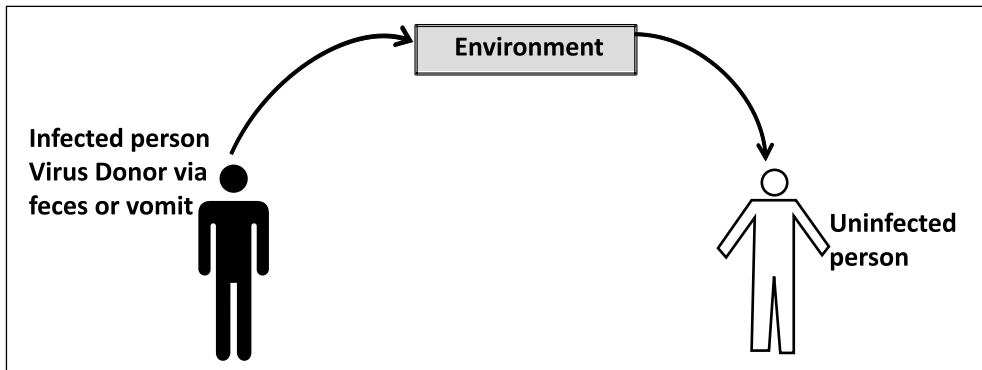


Figure 3: Transmission route of human norovirus through contamination on environmental surfaces.

diapers etc. (Figure 3). When hands touch contaminated material/fomites or fecal material when handling patients, the virus is transferred to the hand touch sites, and thus transmission through contaminated environmental surfaces is an indirect method. The viruses may be easily transferred between hands and surfaces (13) and this facilitates in completing a transfer cycle. Norovirus is shed in feces and vomitus in high numbers and after contamination the virus is persistent on surfaces (54). Moreover, a low number of the virus is sufficient for infection (196) and norovirus has a short incubation period and potentially long infectious period (91, 159). Therefore, the surfaces contaminated with feces, vomits and droplets containing norovirus may remain as a potential source for transmission for a considerably longer time than in case of direct transmission through aerosols during a vomiting incidence.

Noroviruses have been found on different types of surfaces (porous and non-porous) both in hospitals and in the community (1, 20, 21, 61, 72, 224) and have been linked with different environmental surfaces touched by hands: toilet taps, door knobs, hospital switches, and telephone receivers (13, 61, 72, 224). Besides that, hospital floors and bed sides of patients are important surfaces for transmission of the virus and many norovirus outbreaks in hospitals and health care facilities have been implicated due to transmission through environmental surfaces (1, 11, 48, 153, 215). Furthermore, health care workers not providing direct care to infected patients have been reported to contract the infection, probably due to contaminated surfaces (215).

Projectile vomiting has been shown to contaminate large areas due to the settlement of aerosolized viruses on different surfaces (28). Environmentally mediated transmission of norovirus after a vomiting incident has also been demonstrated in a number of cases (61, 130, 131, 134, 197) and norovirus outbreaks have also been frequently reported in aircrafts (124, 190).

Food and waterborne transmission

Food and water borne transmission of the virus is shown in figure 4. Food borne transmission is defined as transmission of pathogens by consumption of a contaminated food item. The transmission of human norovirus via food is common and causes the majority of diagnosed food borne disease outbreaks (30%) in the US (136). Foods are most commonly contaminated by the pathogens either by contamination from environmental sources or through infected food handlers. Zoonotic foodborne transmission is not important for human norovirus, though it is one of the important routes for some foodborne bacteria.

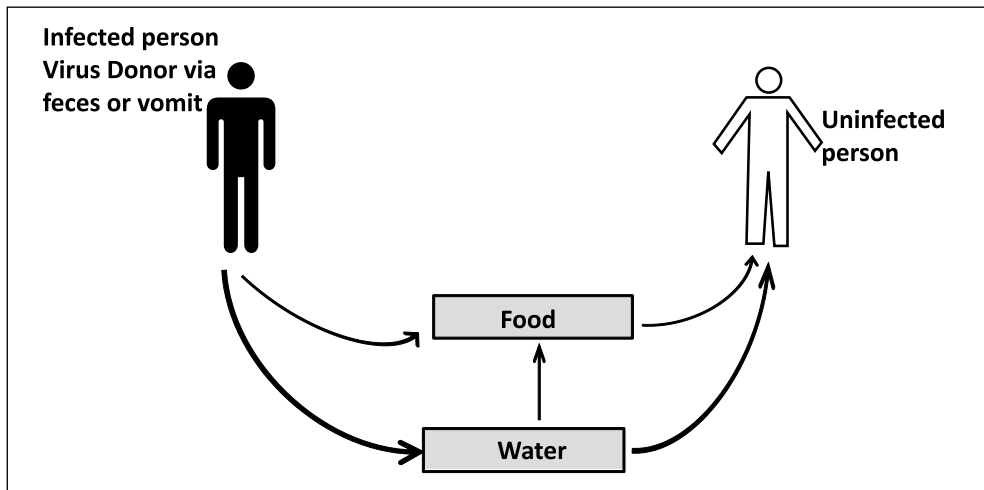


Figure 4: Transmission route of human norovirus through food and water.

Fresh produce are considered to be a main vehicle for norovirus contamination. Such products normally undergo little to no processing before consumption. Fresh produce, such as lettuce, tomatoes, melons, green onions, strawberries, raspberries, blueberries, peppers, fresh-cut fruits (5, 47, 62, 90, 94), ready to eat food like sandwiches (9, 79) and salads (8) are implicated in norovirus outbreaks. Sandwiches are often contaminated by food handlers; salads and soft fruits and other fresh products are commonly contaminated by food handlers or irrigation or wash waters or both (139, 146).

Apart from these sources of contamination, uptake (internalization) of virus by fruits and vegetables in agricultural fields has been described as another possible source of contamination and dissemination of norovirus (93, 200, 216). However, the detailed mechanism of internalization and the relevance are still unclear.

Seafood, particularly bivalves such as mussels and oysters, is another group of high risk foods for norovirus contamination (9, 23, 49, 111, 211, 213). The bivalves are filter feeding and filter

water with suspended matter and food particles over a specialized internal filtering structure. If pathogens like noroviruses, salmonellae and vibrios are present in water contaminated by sewage or waste water effluents (19, 71), the pathogens are retained in the shellfish, and then the seafood remains contaminated. High numbers of norovirus particles have been detected in shellfish and oyster tissues (6, 43, 122). Specific binding of norovirus genogroups to the gastrointestinal tract of oysters have also been described (123, 137). Depuration is a commercial process where live shell fish stocks are kept in a clean sea water tank for several days to get rid of bacteria and viruses. This process is only partially successful for reducing the virus numbers and appears to be inadequate in itself (58). Depuration may result in decrease of bacterial contamination in shellfish and it results in shellfish approved for consumption but still contains loads of viruses. Specification for shellfish is still based on bacteria like coliforms and *Salmonella* species (67, 217). But the WHO, FAO and codex alimentarius commission have recently (217) suggested sewage treatment to ensure adequate reduction in viral loads and to achieve significant reduction of noroviruses and hepatitis A virus. In addition, during heavy rainfall and in case of possible contamination with human sewage, testing of water or bivalve molluscs for presence of indicators of fecal contamination and / or norovirus or hepatitis A virus has been advised to ensure safety prior to reopening of growing and harvesting bivalve molluscs for human consumption.

Human noroviruses can also be transmitted directly via water (172, 182). In general contamination of water occurs via sewage overflows, discharge of sewage treatment plants, or any other way where human norovirus contaminated feces or vomit comes into contact with water. This can result in transmission via drinking water (92, 96, 146, 158), recreational water (96, 148), ground water (198) or wash water used for vegetables (8, 10).

Prevention and control

Virus transmission takes place through different means as described above. This project focuses on studying methods to prevent the transmission through environmental surfaces and hands.

Environmental disinfection

Cleaning and disinfection are widely used to interrupt the transmission chain via contaminated environmental surfaces. After cleaning, sodium hypochlorite solution with chlorine concentration of 200 to 250 ppm (mg/L) is recommended for routine disinfection in hospitals and health care facilities (32). For stringent disinfection chlorine concentrations of more than 1000 ppm are often recommended to disinfect contaminated environmental surfaces (54, 77, 162). This solution should be in contact with the contaminated surfaces for at least 5 min to inactivate hepatitis A virus and presumably norovirus (45). Manual cleaning in hospitals and health care facilities is common and routinely used. The manual cleaning is generally

performed with liquid soap and water and occasionally with chlorine solutions. In case of norovirus, largely due to the uncertainty from *in vitro* studies, CDC recommends chlorine bleach solution at a concentration of 1000–5000 ppm for disinfection of hard, nonporous, environmental surfaces whenever feasible (36, 163). However disinfection with higher concentrations than 200 ppm of chlorine solution is generally not preferred by the working personnel, resulting in low compliance to the guideline. Thus studies on new approaches to achieve reduction of contamination from surfaces are still necessary.

Electrochemically oxidized water (EOW) is an electro-chemically activated water solution produced by the electrolysis of ordinary tap water containing dissolved sodium chloride. The electrolyzed oxidized water has been recommended for surface disinfection in food industries and also for fresh fruits and vegetables (97, 171, 229). Compounds containing phenolic (e.g. Lysol) and or quaternary ammonium compounds (alkyl dimethyl benzyl ammonium, dodecyl dimethyl ammonium) are less effective against non-enveloped viruses such as Feline Calicivirus (FeCV) (57, 82) and parvovirus (30). Acids like lemon juice and vinegar have been described to be less effective against the non-enveloped enteric viruses than against bacteria, however the acids have been shown to give enhanced physical removal of viruses from surfaces (51).

The problem with manual cleaning and disinfection is that it is difficult to reach all surfaces with an appropriate concentration of the disinfectant. Room disinfection using gaseous chemicals is another method used for surface disinfection which is less labour intensive than manual cleaning. Room disinfection by ozone gas, chlorine fog, and hydrogen peroxide has been described. Room disinfection, using ozone gas with a concentration of 25 ppm for one hour has been shown to be effective against FeCV and reduced the infectivity with a \log_{10} factor > 3 (99). Vaporized hydrogen peroxide (30% for 90 min) reduced bacteriophage MS2 with a \log_{10} factor 6 in presence of blood on stainless steel carriers (167). Similarly, fog based hypochlorous acid (180 to 200 ppm free chlorine) has been shown to reduce the infectivity of Murine norovirus 1 (MNV1) and MS2 and RNA titers of norovirus on ceramic tile and stainless steel with $3\log_{10}$ (150). Among those, vaporized hydrogen peroxide is described to be the most promising due to its nontoxic end products; however, more research is needed to decide about the applicability of hydrogen peroxide vaporization against norovirus, and other enteric and respiratory viruses to control spread via contaminated surfaces. Other disinfection approaches, such as coating surfaces with antimicrobial materials have been proposed for environmental transmission control. Photo-catalytic antimicrobial activity of thin surface films of TiO_2 , CuO and TiO_2/CuO dual layers have been shown to be effective against bacteriophage T4 (53). However, more studies on coated antimicrobial materials for environmental surfaces are still needed and a proper choice, based on scientific evidence, for cleaning and disinfection should be made to reduce the indirect transmission through contaminated surfaces as indicated in figure 1.

Hand hygiene

The main purpose of hand hygiene should be removal and / or inactivation of the contaminating microorganism from hands and to control transmission through hands. Hand hygiene is relevant to prevent person to person, food handler and environmental transmission. It is better that hand hygiene reduces at least $4 \log_{10}$ infective microorganisms for effectiveness; however the required level of reduction may differ. Hand hygiene is regarded as one of the most important measures in prevention of health care associated infections (103). Different types of hand hygiene products and procedures are applied in various health care facilities. Hand hygiene procedures such as washing with soap and rubbing hands with alcohol based hand disinfectants are used. Hand hygiene applied should be effective against norovirus and other viruses. Use of proper hand hygiene can reduce the transmission of viruses through several routes as shown in figure 1. For personnel, who are likely to spread viruses, suitable hand hygiene procedures may reduce the number, size and perhaps duration of outbreaks by minimizing transmission.

Antimicrobial soaps have been shown to be effective against microorganisms (156). Beside the soaps, alcohol based hand disinfectants are widely used in hospital settings because of their easy application and acceptability (105, 195). Alcohol based hand disinfectants have been shown to have a varying degree of efficacy towards norovirus and other enteric viruses (73, 129, 140). Some studies have shown inadequate effectiveness against non-enveloped viruses including norovirus (117, 138). In addition to that efficacy of alcohol based hand disinfectants against norovirus and other enteric viruses remains controversial due to mixed evidence of effectiveness obtained with suspension tests, carrier tests and finger pad tests using different viruses (140, 179, 180, 183, 188). Moreover, health care personnel in hospitals and health care facilities using alcohol based hand disinfectant, have been suspected to spread the virus through their contaminated hands in several outbreaks (18). A study reported that among 45 long term care facilities in the United States using alcohol-based hand sanitizers, 53% experienced a confirmed outbreak of norovirus, compared with 18% of 17 facilities that used hand sanitizers less often than soap and water. Three facilities with multiple norovirus outbreaks reported that staff were more likely to use hand sanitizers than soap and water, both routinely and during an outbreak (18). These findings indicate that alcohol-based hand sanitizers might be suboptimal in controlling the spread of noroviruses as shown by lab based studies (86, 96). However, the direct link to alcohol hand sanitizer and outbreaks has not been confirmed yet. This shows the need for a virus specific outbreak study focusing on hands and hand sanitizers. In another study, it has been shown that washing hands with soap and water removes viruses and bacteria by mechanical action (183). With this evidence, it might be important to test the effectiveness of washing hands with soap and water and rubbing hand with alcohol in removing or reducing enteric viruses in one setting, to have a close comparison.

Human norovirus

Viruses are probably the main causal agent of infectious disease acquired in indoor environments, causing considerable impact on health (12). Among several groups of viruses, human noroviruses are recognized as the most common cause of epidemic gastroenteritis affecting people of all age groups, and norovirus outbreaks occur frequently in health care facilities in industrialized countries. Norovirus infection was initially described as a cause of illness in 1929 (230) and the virus was first found in stool samples from a gastroenteritis outbreak in an elementary school in Norwalk, Ohio in 1968 where 50% of students and teachers developed gastroenteritis (107). The virus was identified as a small round structured viral particle under the electron microscope (Figure 5). The virus received its name as Norwalk like virus or small round structured virus and was later renamed as norovirus. The disease caused by human norovirus is popularly known as winter vomiting disease because of norovirus outbreaks following the winter seasonality (2), however epidemics during summer and spring have also been reported in the Northern Hemisphere (185). Illnesses caused by norovirus are also known as stomach flu or gastric flu. A high number of outbreaks among travellers in cruise ships also resulted in the name cruise ship virus. The unavailability of a cell culture system for norovirus has hindered further advance in knowledge. However, cloning and sequencing of the norovirus in the 1990s has led to the development of molecular tools to study the virus (118, 225).

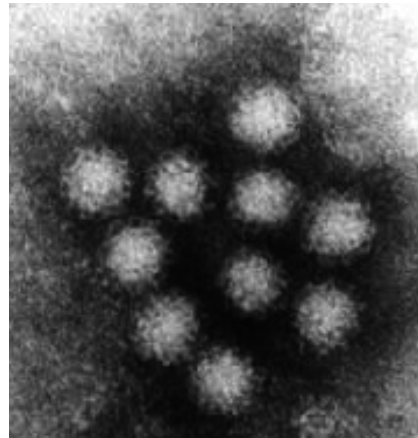


Figure 5: Electron microscopic picture of human norovirus. Picture from Wikipedia (234).

Characteristics of noroviruses

Noroviruses are classified within the genus norovirus in the family *Caliciviridae*, they are nonenveloped viruses of about 27-32 nm in size with an icosahedral capsid (Figure 5). They are RNA viruses with a single-stranded positive-sense RNA genome. The genome is 7.5 to 7.7 kb in length and contains three open reading frames (ORFs; Figure 6). ORF1 encodes a polyprotein of six nonstructural proteins: p48, NTPase, p22, VPg, protease and RNA dependent RNA polymerase. ORF2 encodes for a major structural protein (VP1) of approximately 60 kDa comprising a major hyper variable region of the genome and is also responsible for receptor binding (17, 42). Virions contain 180 copies of the ORF2 encoded protein assembled into icosahedral particles (168, 169). The protein can be divided into two domains, the shell (S) and the protruding (P) domains. The S domain forms a scaffold

surrounding the RNA, whereas the P domain forms the arch-like protrusions, is further divided into P1 and P2 subdomains and is thought to contain the determinants of cell attachment and strain diversity (24, 101, 168, 194). The P2 subdomain is located at the outmost surface of the viral capsid and comprises a hyper-variable region. ORF3 encodes a minor structural protein (VP2). VP2 protein is a basic protein and it is suggested that VP2 may function in RNA genome packaging (78). In-vitro studies showed that it up-regulates VP1 expression and it is also involved in stability of VP1 (17). VP2 has been shown to be essential for production of infectious particles when evaluated in FeCV (186).

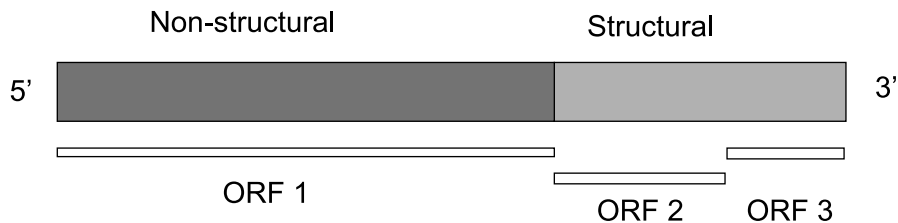


Figure 6: Schematic representation of norovirus genome.

The norovirus genome is replicated by the virus encoded RNA-dependent RNA polymerase, which lacks proof reading activity. This leads to production of many progeny viruses with mutations, making noroviruses flexible and diverse as other RNA viruses. Noroviruses can be classified into five genogroups, GI to GV based on amino acid identity at the VP1 region (232). The viruses of GI, GII and GIV are known to infect humans (232), whereas GIII viruses infect cattle and sheep (221), GIV viruses have been detected in lion cubs and dogs (143, 144) and GV viruses infect mice (98, 109). Each genogroups can be further divided into genotypes based on > 80% identity of amino acids on VP1 (212). GI contains 8 genotypes, GII contains 19 genotypes and GIII contains 3 genotypes (80). Noroviruses of genetic cluster GII.4 are predominant in human outbreaks worldwide (44, 86, 116, 128, 185, 207).

The disease

Common symptoms associated with norovirus infection are diarrhoea, (projectile) vomiting, abdominal pain, cramps and nausea and sometimes fever (170, 173). Clinical symptoms usually start 12-72 hours after exposure and last for 1-3 days in adults and up to 5 days in children and the elderly (133, 173). The gastroenteritis caused by norovirus is usually mild and self-limiting in healthy individuals. However, in risk groups such as young children, elderly people, immunocompromised patients and patients with renal disorder the symptoms may become more severe and infection and shedding can be prolonged (66). In these groups more severe gastrointestinal symptoms and even extra intestinal manifestations have been reported (84). Human norovirus is now recognized as one of the major pathogens causing

hospitalization because of gastroenteritis (68, 70). Additionally, fatal cases and mortality due to norovirus are reported in countries like England, Canada, and The Netherlands (88, 178, 201). In the United States of America many cases of gastroenteritis associated death per year have been reported (85) and an overall mortality of 6.8% among elderly people (85+ years) due to the seasonal viruses including norovirus has been reported in the Netherlands (202).

Shedding of viruses

Norovirus is shed in high quantities in stool of infected persons with on average $> 10^7$ PCR units (PCRU) per g (160) and up to 10^{11} PCRU per g (76). The shedding of the virus by clinically recovered persons may last for three to four weeks and can be even longer in young children (7, 173). High numbers of virus shedding are often associated with old aged hospitalized patients (124) and long-time shedding is associated with immuno-compromised patients (27, 113, 135, 155, 184). Up to 1 year of shedding has been reported from a patient with chronic disease and impaired immunity (192). A study has shown that for norovirus GI.1 (norovirus 8Fla inoculum) peak shedding from infected volunteers may occur (in some it was concomitant with symptoms, in one it was later) after the symptoms had disappeared and the level of shedding was high in symptomatically and asymptotically infected persons (7). The median peak amount of virus shedding was approximately 1.0×10^{10} genomic copies per g feces, and remained above 1.0×10^7 genomic copies per g feces for up to 2 weeks. In another study (228), median loads of norovirus GII.4 were over 3.0×10^8 genomic copies per g, while in that same study norovirus GI was excreted at 8.4×10^5 genomic copies per g. The high loads of shedding of the GII.4 strain might be a cause of the high transmission and prevalence of this strain. However, further confirmation is required as detection and quantitation of genomic copies do not necessarily indicate the presence of infectious viruses. Furthermore, quantitation of PCRU must be confirmed as it might vary according to different PCRs performed by different individuals and might be defined differently according to different fragment sizes of DNA amplified.

Projectile vomiting is a typical symptom of norovirus infection and shedding of the virus in vomit has been reported to contain $> 10^{10}$ particles per g (153). Due to the vomiting, the oral cavity is likely to become contaminated with norovirus during episodes of gastroenteritis. Recently in a hospital study, norovirus was detected in mouth wash samples from approximately 25% of the hospitalized patients a week after symptoms had disappeared (112). This suggests that coughing and speaking may contribute to virus spreading.

Infectivity

To estimate the infectivity, Teunis et al. (196) used volunteer studies with human norovirus GI.1 and showed norovirus to be more infectious than any other known human virus. The

probability of infection by a single norovirus particle was estimated to be 0.5 and the median infective dose (ID₅₀) was at 18 virus particles (196). The reproduction number (RO) has been described for norovirus outbreak situation in absence of hygiene measures as 14 (91), which means that on average every primary case infects 14 secondary cases. The RO of norovirus is thus higher than the RO of 2-3 estimated for pandemic influenza virus (149).

Burden of disease estimates

Human norovirus infections and outbreaks cause considerable economic burden to society, due to workday loss, health care costs and through costs to ensure food safety. The high costs are not due to the severity of the disease or death but due to the high frequency of these infections. Data on the economic impact of norovirus disease is limited. However, in The Netherlands more than 80% of the costs associated with gastroenteritis is the high nonmedical costs of food borne illness (203). Data on cost estimates based on severe cases of gastroenteritis in The Netherlands published in 2012 varied from €611-695 million including both direct medical costs and non-medical costs (69). The cost of a large outbreak in a 946 bed tertiary care hospital in the United States was estimated over 650 000 US dollars (102). Norovirus was reported to cause the highest closing rate of wards in health care facilities among many nosocomial outbreaks (33). In 44.1% of the norovirus outbreaks, the ward had to be closed whereas this was 38.5% for influenza, 25.9 % for rotavirus, and 11.8% for *Clostridium* spp. (87). Therefore, the number of norovirus outbreaks in hospitals and health care centres causes a great burden for patients and staff as well as great financial costs.

In addition to norovirus reported outbreaks, the number of non-reported family outbreaks and sporadic cases is even higher. To estimate the incidence of infection on a population level, population based studies are required. Such population based studies have been done only in the US, Australia, the UK and the Netherlands, resulting in burden estimates for viral gastroenteritis varying between 1.3 to 31 infections per 1000 inhabitants per year (210). The community based cohort studies in England and The Netherlands have estimated incidence in general population between 4.1 and 4.6 cases per 100 person-years (50, 165). A study in the Netherlands in 2006 estimated the burden of norovirus in 2004 to be 450 DALYs (the number of Disability Adjusted Life Years) (110), whereas the burden in 2009 was estimated to be 1622 DALYs (209). In another study it was calculated that norovirus infections cost the Dutch society 25 million euros in 2004 and in the same population, the burden of disease for *Salmonella* and *Campylobacter* were estimated to be 8.8 million euros and 19.6 million euros respectively (210). A study (209) showed that the burden of norovirus institutional outbreaks is relatively small (< 5% of norovirus cases occurs in reported outbreaks) compared to the burden of community acquired infections.

Cell culture model

Despite numerous efforts to cultivate human norovirus, no cell line is available yet (56). Straub et al. (189, 190) reported the use of a three dimensional small intestinal epithelium cell line (INT-407) for infectivity assay of human norovirus GI and GII. This cell culture method described could not be reproduced in other laboratories. The method described is also time consuming, and costly and it is laborious to maintain the cells on collagen-I-coated porous micro-carrier beads in rotating-wall vessel (RWV) bioreactors for three weeks to generate three dimensional differentiated cells for further infection. The authors showed cytopathic effect (CPE) and RNA of the human norovirus virus at each five passages after infection on the three dimensional cells. However, the level of replication and amount of new synthesized viruses are questionable. Duizer et al. (56), also showed PCR positivity after 5 passages of cells lines, but no reproducible norovirus-induced CPE was observed. This shows that RNA may remain in the cell and is apparently not degraded. So far, neither a cell culture method nor a small animal model for human norovirus infectivity determination is available. However, norovirus GII.4 infection in pigs and non-human primates is reported (41, 104, 174).

Murine norovirus 1 (MNV1) is widely used as cultivable surrogate of human norovirus. MNV1 was first isolated from severely immunocompromised mice (93). The virus causes systemic infection and lethal disease in mice (93). However, the MNV1 does not cause the typical symptoms of norovirus infection, such as vomiting and diarrhoea but the virus can be found in feces of infected mice. It shares some biochemical features with human norovirus. MNV1 and human norovirus are similar in size (28-35 nm), shape (icosahedral), and also in buoyant density (109, 220). Both MNV1 and human norovirus belong to the genus *Norovirus* and have a similar genome size and gene organization. In addition to that MNV1 is resistant to acid and heat, and highly stable and persistent in the environment (26, 109, 220). However, it differs in many aspects from human norovirus such as viral receptor binding, pathogenesis, and immunity (109, 220). It does not cause clinical manifestation of gastroenteritis as human norovirus does. In addition, comparative murine norovirus studies showed a lack of correlation between intestinal virus titer and enteric pathology. MNV1 showed significantly lower titer in the intestine than MNV3 (another strain of MNV) but the MNV1 titer has been shown to be higher than MNV3 in the stomach (106). The virus has tissue tropism in macrophage and dendritic cells. It was found to be stable across the pH range of 2–10, whereas FeCV, another cultivable surrogate of human norovirus, was rapidly inactivated at a pH less than 3 and greater than 9. FeCV was more stable than MNV1 at 56°C, but they both exhibited similar stability at both 63°C and 72°C. Long-term persistence studies found that MNV1 was more stable than FeCV at room temperature when both viruses were suspended in a fecal matrix and inoculated onto stainless steel coupons (26).

Porcine sapovirus has been recently described as a surrogate (214), and the virus has genetic relatedness with human norovirus being in the same family and the virus is causing enteric gastroenteritis in pigs (83). The cell culture adapted Tulane virus has also been suggested as an improved surrogate for human norovirus, as the virus recognizes the type A and B histo-blood group antigens (HBGAs) similar to human norovirus (65). The virus is a member of the family *Caliciviridae*, however the stability of Tulane virus to pH and chemicals has not been reported yet.

Other enteric and respiratory viruses

In addition to human norovirus, other enteric viruses such as poliovirus, parechovirus, rotavirus and respiratory viruses such as adenovirus type 5 and influenza A (H1N1) virus were included to study methods of reduction of transmission of these viruses. Besides norovirus, these enteric and respiratory viruses are also transmitted through contaminated hand and surfaces, thus they are included this study.

Enteric viruses

Poliovirus belongs to the family *Picornaviridae*, genus enterovirus. Poliovirus is small in size, about 27-30 nm, non-enveloped with a single stranded positive-sense RNA genome. The virus causes acute infection involving the oropharynx, gastrointestinal tract and occasionally the central nervous system. Polioviruses are shed in stool and they spread through fecal-oral transmission. Poliovirus may be a foodborne virus (45) and is also reported to be persistent on environmental surfaces (1, 157) and used as a model for enteroviruses.

Parechovirus is grouped under the family *Picornaviridae* and the genus Parechovirus. Parechovirus is a small, non-enveloped virus with a single stranded positive sense RNA genome of approximately 7.3 kb. Parechovirus causes gastroenteric and respiratory infection (16, 204, 208). It can also cause serious infections like necrotizing enterocolitis, encephalitis, myocarditis acute flaccid paralysis, and aseptic meningitis (127, 176, 187). As the virus is shed in feces, direct person to person transmission through the fecal oral route and indirectly through environmental spread is also possible (64). Evidence for food and water borne transmission of parechovirus is not available but as the virus is shed in stool, fecal oral transmission is likely.

Rotaviruses belong to the family *Reoviridae* and the genus Rotavirus. The virus has a non-enveloped icosahedral structure with wheel like appearance, a diameter about 60 to 80 nm and a segmented RNA genome with a total size of about 16 to 27 kb (219). Rotaviruses are the leading cause of gastroenteritis among infants and young children worldwide (161). Rotavirus infection causes symptoms of watery diarrhoea, severe dehydration, fever, and vomiting. The disease is more severe among children under five years of age in developing

countries and is causing approximately 111 million episodes of gastroenteritis requiring home care, 25 million clinic visits and 2 million hospitalizations each year (161). The virus is shed in feces for 5 to 7 days in high numbers above $> 10^7$ genomic copies per g of stool (46). The virus is transmitted person to person mainly by the fecal oral route and also through contaminated fomites, water, and food (25, 64). The cell culture adapted simian rotavirus SA 11 is the recommended model virus to test antivirals (60).

Respiratory viruses

Adenovirus belongs to the family *Adenoviridae* and measures 80 to 100 nm in size. The virus is non-enveloped with a double stranded DNA genome. The genome has a size of about 28-45 kb (22). Adenoviruses are associated with gastroenteritis and respiratory disease like acute respiratory disease, pharyngitis, pneumonia in humans (223) and also conjunctivitis (3, 59, 150). The virus transmission takes place person to person, through the respiratory route, via contaminated environmental surfaces (1, 219) and food (108, 166). Human adenovirus type 5 was included in the virucidal activity testing since it is a non-enveloped DNA virus that can be detected in respiratory secretions and in feces (177). The European Committee for Standardisation suggests testing the adenovirus type 5 to assess the efficacy of disinfectants (60).

Influenza virus is a genus of the *Orthomyxoviridae* family of viruses. Influenza A viruses are large (~ 300 nm), enveloped with negative sense, single-stranded, segmented-RNA genomes. The influenza virus is associated with acute respiratory diseases. The virus is shed in respiratory secretions in humans; however the avian influenza viruses are shed in high numbers in feces (38). Influenza viruses are transmitted mostly via person to person contact, aerosols and via contaminated environmental surfaces, however zoonotic transmission does occur since influenza A is able to cross the host barrier from animal reservoir species to humans (34). Consequently, zoonotic foodborne transmission, e.g. via eggs (125, 199, 222) or edible parts of poultry and ducks (115, 147, 154) cannot be excluded (55, 152, 231).

Outline of this thesis

The aim of this thesis is to test methods for transmission intervention of norovirus, and other enteric and respiratory viruses, to support the development of science based intervention guidelines to be able to reduce the health burden caused by these viruses. Specific objectives of the study are to develop and validate tests to study intervention methods, to test intervention methods in a laboratory setting and to produce data that can assist in risk assessment of the transmission of these viruses in health care or food production settings.

The study focuses on methods to prevent transmission of these viruses through hands and environmental surfaces. The enteric viruses included in the study were poliovirus Sabin 1, rotavirus SA 11, parechovirus 1, MNV1, human norovirus GI.4 and GII.4 and respiratory viruses were adenovirus type 5 and influenza A (H1N1) virus. The enteric and respiratory viruses may contaminate food through food handler-related contamination. Heating and pasteurizing are common methods used in the household and industries for inactivation of microorganisms in food. Therefore in **chapter 2** thermal stability of enteric and respiratory viruses were tested at different temperatures. Contaminated hands can directly or indirectly contaminate environmental surfaces which can then be a source of transmission of the viruses and chemical disinfection is a commonly used prevention method. Cleaning and disinfection of contaminated surfaces daily and during outbreaks are common in health care facilities. Efficacy of the cleaning and disinfection procedures needs to be tested, therefore in **chapter 3** effectiveness of cleaning and commonly used disinfection methods were assessed by quantitative carrier tests against human noroviruses, different enteric and respiratory viruses. In addition to manual cleaning and disinfection, non-touch disinfection of contaminated surfaces in critical areas of health care facilities by vaporized hydrogen peroxide (VHP) has been approved for decontamination of hard surfaces in rooms for humans in the Netherlands. Virucidal efficacy of VHP was tested in a real life situation against different enteric and model viruses, which is described in **chapter 4**. In addition to VHP, self-decontaminating surfaces can be helpful in preventing transmission in health care settings, food production areas and in general from frequently touched and contaminated surfaces. Therefore, efficacies of immobilized quaternary ammonium compounds were tested against poliovirus Sabin 1 and influenza A (H1N1) virus and these results are described in **chapter 5**. Contaminated hands are thought to be a principle vehicle for transmission of enteric viruses. However, limited data is available on transfer of noroviruses, thus to fill those gaps and for better understanding of transmission of the virus through hands, transfer of MNV1, human noroviruses GI.4, and GII.4 from hands to different surfaces and vice versa, and also to food products were quantified and the results are presented in **chapter 6**. Hand hygiene is one of the most important measures to prevent transmission of noroviruses through contaminated hands. In **chapter 7**, use of alcohol based hand disinfectant is compared to washing hands with soap and water for removing the noroviruses from human hands. Finally, **chapter 8** includes a summarizing discussion and concluding remarks.

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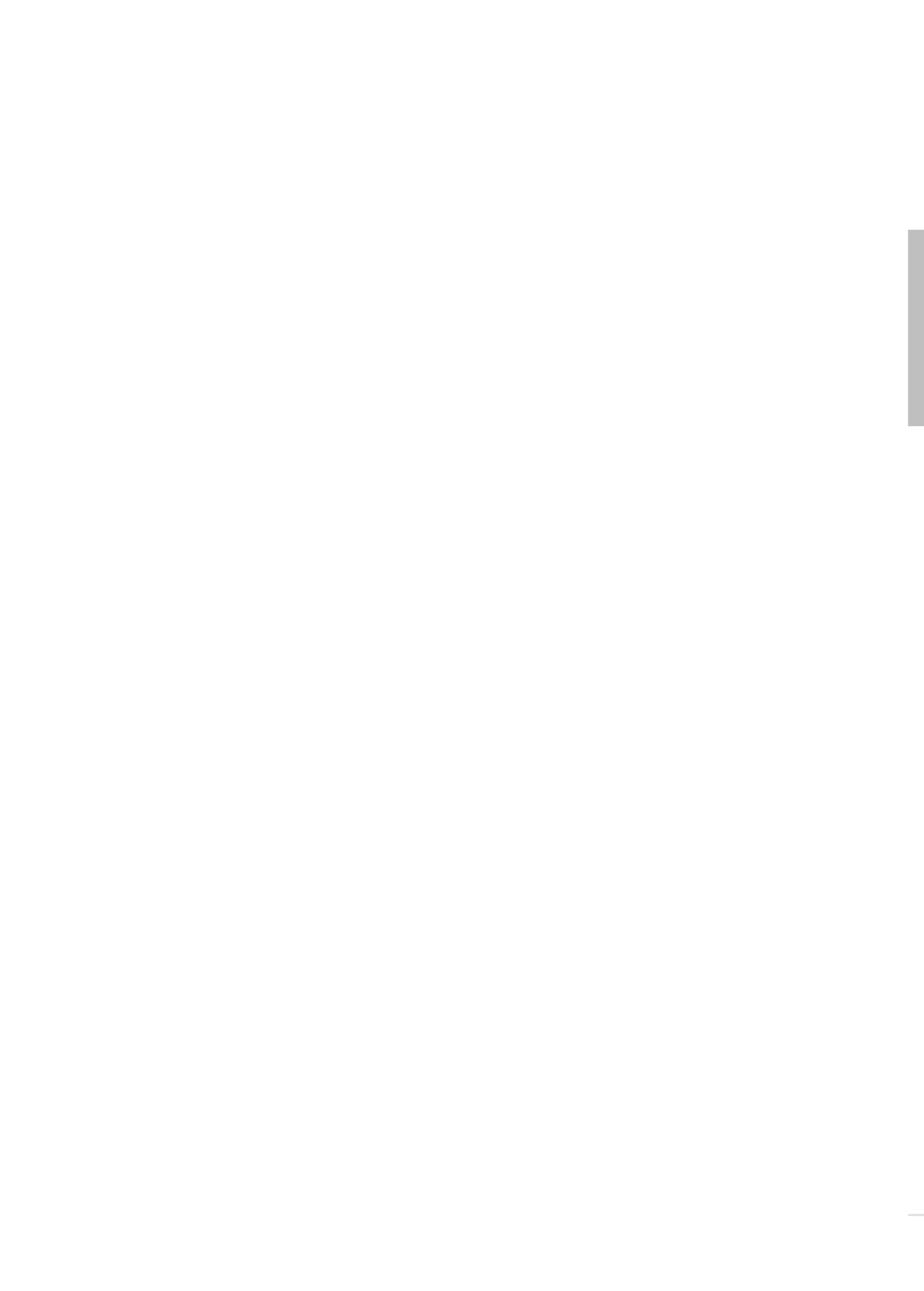
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Chapter 2

Thermal stability of structurally different viruses with proven or potential relevance to food safety

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Abstract

The aim of the study was to collect comparative data on thermal stability of structurally different viruses with proven or potential relevance to food safety. Suspensions with poliovirus Sabin 1, adenovirus type 5, parechovirus 1, human norovirus (NoV) GII.4, murine NoV (MNV1) and human influenza A (H1N1) viruses were heated at 56°C and 73°C. Infectivity was tested by culture assay for all but human NoV GII.4 which cannot be cultivated in vitro. Time to first \log_{10} reduction (*TFL*-value) was calculated based on best fit using the monophasic, biphasic, or Weibull models. The Weibull model provided the best fit at 56°C for all viruses except influenza virus. The *TFL* at 56°C varied between a high value of 27 min (parechovirus) to a low value of 10 s (adenovirus) and ranked parechovirus > influenza > MNV1 > poliovirus > adenovirus. The monophasic model best described the behaviour of the viruses at 73°C, in which case the *TFL* was in order of value MNV1 (62 s) > influenza > adenovirus > parechovirus > poliovirus (14 s). Viruses do not follow log-linear thermal inactivation kinetics and the thermo-stability of parechovirus and influenza virus is similar to that of proven foodborne viruses. Thus resistant fractions of viruses may remain infectious in thermal inactivation processes and inactivation of newly discovered or enveloped viruses in thermal food preparation processes should not be assumed without further testing.

Introduction

In recent years, viruses have been increasingly recognized as important cause of foodborne diseases. The viruses most frequently reported as involved in foodborne outbreaks are the human noroviruses (NoVs) and hepatitis A virus (HAV) (14, 21, 34). Other viruses such as rotavirus, hepatitis E virus (HEV), astrovirus, Aichi virus, sapovirus, enterovirus, coronavirus, parvovirus, and adenovirus can also be transmitted by food, and anecdotal evidence suggests that the list of foodborne viruses may be even longer (11, 13, 16). Most recognized viruses transmitted by foods are non-enveloped small spheres (around 30 nm in diameter) with single stranded positive sense RNA genomes. The primary mode of transmission is person-to-person spread, but indirect transmission via fecally contaminated food is common.

The purpose of this study was to obtain data on the thermal stability of a variety of viruses, specifically human NoV (and the cultivable surrogate, murine NoV (MNV1) (6, 35, 36)) poliovirus, parechovirus, adenovirus and influenza A virus. These viruses represent different families that are shed via feces or respiratory secretions and because of this, might ultimately contaminate foods via food handler related contamination. Only human NoV and poliovirus have been confirmed to be transmitted by foods and/or water. Foodborne transmission of adenoviruses is considered likely (11) and the serotype 5 strain is interesting because it can be detected in respiratory excretions as well as feces, and has a different genome structure than the small RNA viruses (26). Adenovirus is a non-enveloped virus, approximately 80 nm in diameter with a double stranded DNA genome. We also evaluated the thermal stability of parechovirus, which causes a mild gastrointestinal illness in young children (4).

The respiratory influenza A (H1N1) virus was included, since these viruses are shed in respiratory secretions and may thus contaminate foods. Since the oropharynx is a common replication site for many respiratory viruses including influenza virus (23) infection resulting from ingestion, while not considered likely, cannot be excluded. Furthermore, a relatively high thermo stability for highly pathogenic avian influenza viruses has been shown (30) and foodborne transmission has been suggested (11). However, influenza A viruses are much larger (around 300 nm), do have an envelope and a different genome (negative strands RNA) than the common foodborne viruses. Temperatures of 56°C and 73°C for different time intervals were evaluated because these are commonly used in cooking and pasteurization processes (6, 18).

Since the observed reduction in virus number under progressing treatment (e.g., time-temperature) can follow different patterns depending on the different inactivation mechanisms or changing experimental circumstances (7), we analysed our data using different models. The simplest reduction pattern involves a constant reduction rate, with a log-linear decrease in virus numbers (i.e., monophasic reduction) (8). Alternatively, the initial population of viruses

in the sample can consist of several virus fractions and/or several different experimental circumstances, with each fraction displaying a particular reduction rate. For example, when two such rates are observed, then the reduction is biphasic (9). Alternatively, the reduction rate(s) need not be constant over time, but may continuously change under progressing treatment. This situation is modelled with the Weibull model (32). By applying statistical modelling, hypothesis can be tested regarding constant or variable inactivation rates or presence of mixture virus populations to support data interpretation in terms of intervention measures.

Comparable data on thermal stability from different viruses and characterization of the kinetics of inactivation will help assessing the likelihood of virus survival through food production processes and it points to the need for considering foodborne transmission of viruses for which this route is unexpected, such as the enveloped influenza viruses.

Materials and methods

Viruses and cells

Viruses used for the study were poliovirus Sabin 1 (vaccine strain), adenovirus type 5 (Hu/adenovirus/type 5/6270/1988/Ethiopia), influenza A (H1N1) virus (Hu/influenza A/266/2008/Netherlands (H1N1) virus), parechovirus 1 (Hu/parechovirus/type 1/147/2008/Netherlands), MNV1 (Mu/NoV/GV/MNV1/2002/USA), and human NoV GII.4 (stool sample, Hu/NoV/GII.4/1803/2008/Netherlands).

For all but human NoV, virus stocks were prepared by infecting monolayers of respective host cells (31). Poliovirus and adenovirus were cultivated on human epidermoid cancer (Hep-2) cells, MNV1 on raw mouse macrophage (Raw-264.7) cells, parechovirus 1 on human colon adenocarcinoma (HT-29) cells (1) and influenza A virus was cultivated on Madin-Darby canine kidney (MDCK-1) cells. Human NoV suspension was prepared as 10% w/v stool homogenates in Dulbecco's Modified Eagle Medium (DMEM) as described before (29) that was filtered through a 0.2 µm pore size filter. The suspension was free of all other enteric viruses tested (rotaviruses, enteric adenoviruses, astroviruses and sapoviruses) as determined before by PCR assays (29). All the virus stocks were stored at -70°C until used in experiments.

Preparation of sterile stool suspension

A 20% (w/v, wet weight) stool suspension from a healthy volunteer was prepared in phosphate buffer (0.01M, pH 7.2) and sterilized by autoclaving at 121°C for 15 min. The suspension was vortexed, centrifuged at 1500 x g for 20 min, and the supernatant recovered, aliquoted, and stored at -20°C. As described above (29), the stool suspension was tested and found to be free of evidence of viral RNA or DNA corresponding to human NoVs GI and GII,

rotaviruses, enteric adenoviruses, astroviruses and sapoviruses. The stool suspension was further diluted to 6% in sterile phosphate buffer to perform the heat inactivation experiments at the final concentration of 1% stool in the sample.

Thermal inactivation of viruses

Thermal inactivation experiments were done using a suspension assay design in which viruses were suspended with and without stool. The virus stocks titers used were as follows: poliovirus Sabin 1: 6.3×10^8 50% Tissue Culture Infective Dose (TCID₅₀) per ml (1.6×10^{10} PCR unit (PCRU) per ml), adenovirus type 5: 6.3×10^7 TCID₅₀ per ml (3.2×10^9 PCRU per ml), parechovirus 1: 1.3×10^8 TCID₅₀ per ml (2.0×10^9 PCRU per ml), influenza A (H1N1) virus: 1.3×10^6 TCID₅₀ per ml (1.5×10^8 PCRU per ml), MNV1: 1.7×10^7 PFU per ml (5.0×10^8 PCRU per ml) and human NoV GII.4 was at a concentration of 1×10^8 PCRU per ml. The virus stocks were dispensed in 100 μ l fractions in reaction tubes and 100 μ l stool suspension (6% w/v) or DMEM (control) was inoculated separately. Since human stool is not the natural matrix for influenza A virus, this virus was suspended in DMEM only. The virus suspensions were preheated to 30°C, followed by the addition of 400 μ l of DMEM preheated to 69°C or 94.5°C to instantaneously achieve temperatures of 56°C or 73°C, respectively. The final temperatures obtained were recorded using a digital thermometer (Sling, China). The suspensions were maintained at the desired temperatures in a digitally controlled water bath at 56°C for 0, 5, 10, and 30 min and at 73°C for 0, 30 s, 1, and 3 min. At each time point, a suspension was removed and cooled immediately by ice immersion. The samples were stored at -70°C prior to analysis by infectivity assay or quantitative PCR, as appropriate. The infectivity reduction was determined by cell culture assays. As human NoV cannot be cultured (12), only the viral nucleic acid reduction was determined. Each experiment was performed in triplicate.

Enumeration of viruses

Infectivity (Plaque) assay

Plaque assays for MNV1 enumeration were performed in 6-well culture plates as described by Wobus *et al.* (2004) except for the addition of 2 ml of warm DMEM complete medium on top of the agarose after solidification. After removing the medium, plaques were visualized by adding 2 ml of freshly prepared 0.015% w/v neutral red solution in DMEM at room temperature. After 2 h, plates with 5 to 50 plaques were counted. The results were expressed as plaque forming unit (PFU) per ml of sample.

Infectivity (TCID₅₀) determination

The other viruses were enumerated by titration in 96 well plates using the TCID₅₀ approach. For poliovirus Sabin 1, parechovirus 1, adenovirus type 5 and influenza A (H1N1) virus, 10 fold serial dilutions were prepared followed by inoculation in 96 well plate using cell monolayers as previously described (31). For Hep-2, MDCK-1 and HT-29 cells, seeding density was 2×10^5 cell per ml. Poliovirus Sabin 1 and adenovirus type 5 virus suspensions were added on freshly trypsinized Hep-2 cells. Parechovirus 1 was titrated on one-day old HT-29 cells. Influenza A (H1N1) virus was titrated on three 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 DMEM with 2.5 g per ml TPCK (L-(tosylamido-2-phenyl) ethyl chloromethyl ketone) treated trypsin (Sigma porcine pancreatic type IX). Cytopathic effect was observed after 5 or 6 days of incubation at 37°C in 5% CO₂. Virus titers were calculated by the Spearman-Kärber method (20).

Viral Nucleic acid extraction and Real time (RT) PCR

In addition to infectivity assay, semi quantitative PCR or RT-PCR was performed on all virus suspensions before and after heat treatment. Viral nucleic acid extraction was performed using the MagNA Pure Light Cyclor total nucleic acid isolation kit as described previously (29). Amplifications for MNV1 (2), poliovirus Sabin 1 (10), adenovirus type 5 (29), human NoV GII.4 and influenza A (H1N1) virus have been previously described (31). For parechovirus 1, forward and reverse primers were designed by our laboratory and are as follows: 5'GCCATCCTCTAGTAAGTTTG3' and 5'TACCTTCTGGGCATCCTTC3' (location 326-582) respectively. The probe sequence, which was labelled with 6-carboxyfluorescein (FAM) at the 5' end, and conjugated with black hole quencher (BHQ) at the 3' ends, was TAACAGGTGCCTCTGGGGCCAA. The amplifications consisted of 95°C for 10 s for denaturation and annealing temperature of 50°C for 20 s. PCR or RT-PCR amplifiable units (PCRU or RT-PCRU) were determined by slopes of standard curves made for each virus. The standard curve was made by plotting cyclic threshold (Ct) value versus log PCRU or RT-PCRU of 10 fold dilutions of the virus stock. The highest dilution giving a positive result was assigned a value of 1 amplifiable unit. The log₁₀ reduction in PCRU or RT-PCRU was calculated by subtracting the 30 min time point value from 0 min value for 56°C treatment, and subtracting the 3 min value from the 0 min value for the 73°C treatment.

Data analyses

For the infectivity data, virus inactivation was expressed as reduction of the infectious units (TCID₅₀ or PFU). The infectious units were log₁₀-transformed and assumed to be distributed normally after transformation. The data were fitted to three potential mathematical models: a monophasic using model (1) as described by Chick (1908):

$$\log_{10}(C(t)) = \log_{10}(C_0) - \lambda t \quad (1)$$

a biphasic model (2) as described by De Roda Husman *et al.* (2009):

$$\log_{10}(C(t)) = \log_{10}\left[C_0\left(w e^{-\lambda_1 t} + (1-w)e^{-\lambda_2 t}\right)\right] \quad (2)$$

and a Weibull model (3) as described by Van Boekel (2002):

$$\log_{10}(C(t)) = \log_{10}(C_0) - (\lambda t)^p \quad (3)$$

where C_0 is the \log_{10} number of infectious viruses at time zero, the λ 's and p are inactivation parameters (with λ in model (3) being $1/\delta$ as described by Van Boekel (2002), and w is a parameter that directs the biphasic shape in equation 2. Models (1) & (2) and models (1) & (3) are nested, justifying the likelihood ratio test for assessing statistically superior fits (α set at 0.05). Models (2) and (3) are non-nested and therefore model selection was based on the corrected Akaike Information Criterion (5). Parameter values were estimated by maximizing the likelihood of the respective models. Zero-counts were included as censored observations, with contributions to the total likelihood based on the cumulative density function using a detection limit of a single infectious virus. Parameter uncertainty was assessed by Markov Chain Monte Carlo sampling using the Metropolis and Hastings algorithm (15). The time to the first \log_{10} reduction (*TFL*-value) was estimated by solving t from eqn. (1-3) using the maximum likelihood estimates for the parameters. The 95% confidence intervals were generated likewise using parameter values from the Markov Chain Monte Carlo posterior and taking the 2.5 and 97.5 percentiles. The best fitting model was chosen to describe the inactivation for each virus-temperature-matrix combination. Mathematica software (version 8, Wolfram Research, Champaign, IL, USA) was used for the analysis.

Results

Infectivity reduction

No decrease in virus titer was detected after heating to 30°C for 20 min in a heat block (data not shown). The time-dependent infectivity reductions observed at 56°C and 73°C are presented in figure 1. The inactivation data were fitted to the monophasic, biphasic and Weibull models and the model providing the best fit was chosen to calculate *TFL*-values. *TFL*-values for the viruses both in DMEM and 1% stool at 56°C are presented in Table 1. In most cases, the Weibull model provided the best fit for the 56°C treatment, except for influenza A (H1N1) virus (monophasic reduction). When the viruses were suspended in DMEM, the *TFL*-values at 56°C ranked (from highest or most resistant, to lowest or least resistant) as follows: parechovirus 1 > influenza A (H1N1) > MNV1 > poliovirus Sabin 1 > adenovirus type 5 (Table 1).

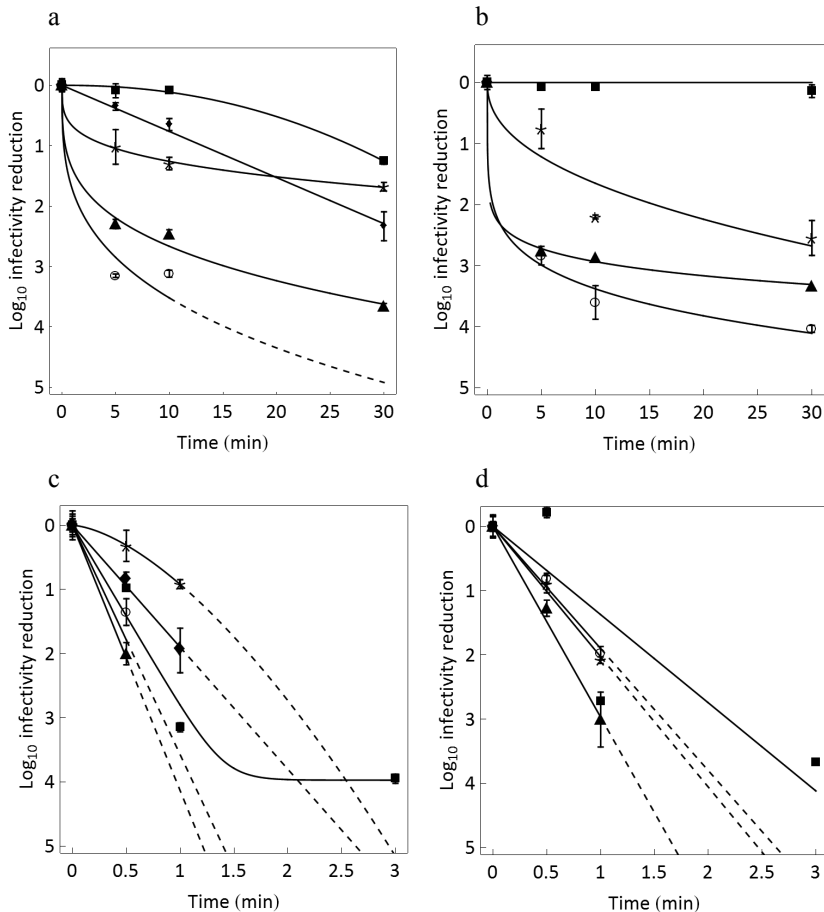


Figure 1: Infectivity reduction of viruses suspended in DMEM and treated at 56°C (a) or 73°C (c), or suspended in 1% stool and treated at 56°C (b) or 73°C (d). Markers indicate the mean ($n=3$) (○) adenovirus type 5, (▲) poliovirus Sabin 1, (*) MNV1, (◆) influenza A (H1N1) virus, (■) parechovirus 1. Dotted lines indicate complete inactivation and error bars show standard deviation.

After the maximum treatment time of 30 min at 56°C, log_{10} reductions of viruses suspended in DMEM ranked as follows: parechovirus 1 > MNV1 > influenza A (H1N1) virus > poliovirus Sabin 1 > adenovirus type 5 with less than 1 log_{10} reduction for parechovirus 1 to over 4 log_{10} reduction for adenovirus type 5 (Figure 1a). After 30 min at 56°C there is a moderate stabilizing effect of stool on the thermal stability of parechovirus 1 and adenovirus type 5, but no clear effect on thermal stability of MNV1 and poliovirus Sabin 1 (Figure 1 a and b). Parechovirus 1 showed no significant reduction in infectivity at 56°C in stool.

The *TFL*-values at 73°C calculated from the best fitting model are presented in Table 2. The monophasic model provided the best fit for poliovirus Sabin 1, adenovirus type 5 and

Table 1: Best fitting inactivation model per virus-matrix combination at 56°C, and estimated required time (min) and 95% confidence interval for the first \log_{10} reduction (*TFL*) and the estimated values for the parameter *p*

Virus	Matrix	Best Fitting model	<i>TFL</i>		<i>p</i>	
			Mean	95% interval	Mean	95% interval
Adenovirus type 5	DMEM	Weibull	0.16	0.05 – 1.61	0.30	0.21 – 0.47
Poliovirus Sabin 1		Weibull	0.30	0.16 – 0.5	0.28	0.27 – 0.37
MNV1		Weibull	4.21	2.7 – 13.4	0.27	0.06 – 0.36
Influenza A virus		Monophasic	13.1	11.6 – 15.4	1	–
Parechovirus 1		Weibull	27.0	23.0 – 29.9	2.18	1.24 – 3.06
Poliovirus Sabin 1	1% stool	Weibull	< 0.01	10 ⁻⁸ – <0.01	0.11	0.05 – 0.12
Adenovirus type 5		Weibull	0.01	0.006 – 0.17	0.18	0.16 – 0.33
MNV1		Weibull	3.20	2.03 – 11.4	0.44	0.23 – 0.86
Parechovirus 1		No decay	–	–	–	–

Table 2: Best fitting inactivation model per virus, matrix, estimated time (min) to first \log_{10} reduction (*TFL*) at 73°C, 95% confidence interval (a value of 0.5 means 30 s), and the estimated values for the parameter *p*

Virus	Matrix	Best Fitting model	<i>TFL</i>		<i>p</i>	
			Mean	95% interval	Mean	95% interval
Poliovirus Sabin 1	DMEM	Monophasic	0.24	0.17 – 0.36	1	-
Parechovirus 1		Biphasic	0.35	0.27 – 0.61	–*	-
Adenovirus type 5		Monophasic	0.40	0.25 – 0.57	1	-
Influenza A virus		Monophasic	0.53	0.40 – 0.77	1	-
MNV1		Weibull	1.06	0.78 – 1.77	1.57	1.32 – 12.6
Poliovirus Sabin 1	1% stool	Monophasic	0.34	0.29 – 0.53	1	-
MNV1		Monophasic	0.49	0.41 – 0.62	1	-
Adenovirus type 5		Monophasic	0.53	0.43 – 0.74	1	-
Parechovirus 1		Monophasic	0.73	0.51 – 6.26	1	-

* parameter values were λ_1 : 6.5 (4.3 – 8.7), λ_2 : ~0 (97.5% upper limit: 10⁻¹⁴), w: 0.9999 (0.99 – 0.9999)

influenza A (H1N1) virus at 73°C in DMEM. The biphasic model was most appropriate for parechovirus 1, while the Weibull model was best for MNV1 (Figure 1c). At 73°C all the viruses tested, except parechovirus 1, were inactivated completely (defined as $> 4 \log_{10}$ infectivity reduction) within 3 min. *TFL*-values at 73°C ranked (from highest to lowest) as MNV1 $>$ influenza A (H1N1) virus $>$ adenovirus type 5 $>$ parechovirus 1 $>$ poliovirus Sabin 1 and varied between 14 s for poliovirus Sabin 1 to 64 s for MNV1. Based on infectivity reduction after 3 min at 73°C in DMEM, thermal stability of the tested viruses rank as parechovirus 1 $>$ MNV1 $>$ influenza A (H1N1) virus $>$ adenovirus type 5 $>$ poliovirus Sabin 1 with nearly 4 \log_{10} reduction for parechovirus 1 to complete inactivation ($> 4 \log_{10}$ reduction) for all the other tested viruses (Figure 1c).

Viral nucleic acid reduction

The PCR or RT-PCR reduction after heating at 56°C for 30 min and 73°C for 3 min is shown in Table 3. Overall, the PCR reduction was less than 1 \log_{10} for all the viruses tested except for parechovirus 1 at 73°C. The results show that virus infectivity loss occurs much more rapidly than does loss of amplifiable viral RNA or DNA, for all viruses and all conditions tested, with viral RNA persisting even after complete loss of infectivity.

Table 3: \log_{10} PCR unit reduction of genetic material on heating at 56°C between 0 and 30 min and between 0 and 3 min at 73°C in DMEM and 1% stool

Virus	56°C		73°C	
	DMEM	1% Stool	DMEM	1% Stool
Poliovirus Sabin 1	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.2	0.0 ± 0.1
Adenovirus type 5	0.1 ± 0.2	0.1 ± 0.1	0.1 ± 0.1	0.0 ± 0.0
Parechovirus 1	0.1 ± 0.1	0.6 ± 0.2	1.1 ± 0.3	1.4 ± 0.1
Influenza A (H1N1) virus	0.4 ± 0.3	ND	0.0 ± 0.0	ND
MNV1	0.3 ± 0.3	0.1 ± 0.2	0.8 ± 0.2	0.4 ± 0.1
Human NoroGII.4	0.2 ± 0.0	0.1 ± 0.0	0.1 ± 0.1	0.0 ± 0.0

Bold font type indicate a significant reduction of RNA, n = 3, ± SD, ND = not done

Discussion

The aim of the study was to estimate time and temperature-dependent inactivation of structurally different groups of viruses at 56°C and 73°C. In general, it is assumed that small non-enveloped viruses are among the most stable viruses and that their sensitivity to heat (and other environmental stresses) increases with size and because of the presence of a viral envelope. However, few studies have compared the thermo-stability of structural different viruses under identical conditions.

In this study we did not find a relation between virus structure and thermal stability. We did find a wide range in thermo-stability when comparing the small non-enveloped positive stranded RNA viruses to one another. In our comparisons, we assumed that *TFL* reduction values represent the inactivation of the most sensitive virus fraction in the suspension, i.e. the monodispersed fraction. Based on *TFL* reduction values at 56°C, the two representatives of the *Picornaviridae* (poliovirus and parechovirus) showed very different thermo-stabilities, while the non-enveloped adenovirus was less thermo-stable than the enveloped and larger influenza virus. The relatively high thermal stability of highly pathogenic influenza virus has been previously reported for highly pathogenic avian influenza viruses (30) and may thus be an important characteristic of influenza A viruses in general. Additionally, a recent study (27) also showed that the reduction of poliovirus Sabin 1 by heat (dry) was faster than that of the enveloped bovine viral diarrhoea virus and vaccinia virus. These results suggest that the viral envelope may have less impact on thermal stability of viruses than previously thought (3, 25, 27). This suggests that some enveloped viruses may remain infectious throughout the food chain if only mild heat treatment is performed.

To the best of our knowledge, this is the first study reporting thermal stability of parechovirus. Parechovirus is a recently discovered virus of the family *Picornaviridae* and found to be associated with gastrointestinal and respiratory syndromes similar to human enteroviruses (28, 33). The monodispersed virus fraction showed the highest thermo-stability of the viruses tested at 56°C, a relatively rapid reduction of a large fraction of the virus at 73°C, but inactivation was still incomplete after 3 min at 73°C (Figure 1 a, c), indicating the potential presence of a highly stable fraction. For parechovirus we found that limited reductions of infectious viruses were concomitant with relatively high reductions of RT-PCR. This might suggest that the RNA of the large fraction of non-infectious viruses, as present in all virus stocks (19), is more sensitive to breakdown than the RNA of infectious viruses. Overall, parechovirus was the most thermo-stable of all viruses tested. While its transmission by foodborne routes has not yet been reported, the fact that they are shed in feces, replicate in the gastrointestinal tract, and are relatively heat stable warrants further investigation of their role in foodborne illness.

Except for influenza A virus, we conducted our experiments on virus suspensions with and without stool components. When enteric viruses are found on foods, it is usually the result of fecal contamination. This low level of contamination was chosen since we expected that by adding 1% stool the levels of virus aggregation would already increase significantly, thereby decreasing the magnitude of the most sensitive (monodispersed) virus fraction. Additionally, very low levels of interfering substances have been shown to have a profound impact on chemical inactivation of viruses in suspensions (24, 37). In most cases in our study, the *TFL*-values were not significantly affected by the presence of 1% stool, which is consistent with the assumption that *TFL*-values represent the inactivation of the most sensitive (monodispersed)

virus fraction. Apparently 1% stool in suspension is not enough to cause aggregation of such a large fraction (> 90%) of the viruses that the *TFL* is affected. Overall, we conclude that 1% stool is not a significant interfering substance for assessing the thermo stabilities of the viruses we tested.

We calculated the *TFL*-values from the inactivation data based on best fit statistics, considering the monophasic (log-linear), Weibull and biphasic models as candidates. At 56°C the time dependent reduction of the non-enveloped viruses was best modelled using the Weibull model. Most of the data for the 73°C showed monophasic inactivation kinetics, although two of the nine virus-condition combinations demonstrated non-monophasic kinetics. When inactivation occurs in two or more phases, as is suggested by the biphasic and Weibull models, virus infectivity loss is not constant over time, but rather changes under progressing treatment. At 56°C the shape of the inactivation curves for adenovirus, poliovirus and MNV1 were characterized by a rapid initial drop in the infectivity, followed by tailing caused by diminishing reduction rates as time increased (Figure 1 a and b; $p < 1$ Table 1). On the other hand, a shoulder was observed for parechovirus at 56°C and MNV at 73°C ($p > 1$). The presence of tails and shoulders (and hence biphasic inactivation kinetics) tends to indicate the presence of virus fractions having different native thermal stability. The most likely cause of this phenomenon would be that some of the virus suspension presents itself as single or monodispersed virions, while other parts of the suspension consist of aggregated viruses. The monodispersed fraction would be considered more heat labile and should theoretically show monophasic (log linear) reduction. On the other hand, the aggregated virus fraction will display a higher thermo-stability, depending upon the degree of aggregation, among other factors. This phenomenon has been described for MS2 bacteriophage (22). Interestingly, for the enveloped influenza A (H1N1) virus, a monophasic model provided the best fit at both 56°C and 73°C, indicating the constant rate of reduction over time and apparent limited heterogeneity of virus fractions.

TFL-values are generally calculated under the assumption of log linear inactivation kinetics (17, 30), even though the inactivation curves often do not follow this pattern. Values that are falsely calculated by linear extrapolation, thereby ignoring shoulders or tails, could lead to over- or underestimation of the time-temperature combinations needed to achieve a desired degree of log reduction.

Because of the large number of viruses tested, this study was performed with a limited number of time points at 56°C and 73°C. For those viruses demonstrating rapid \log_{10} reduction with a matter of seconds, or a wide 95% confidence interval, more frequent sampling and additional replicates would be warranted. Nonetheless even with these study limitations, the deviations from monophasic reduction are apparent in almost half the cases, especially at the lower temperature, thereby providing valuable insight for intervention by heat treatment.

In summary, heating at 73°C for 3 min was sufficient to inactivate (i.e. to less than 1 infectious virus per ml, > 4 log₁₀ reduction) all the tested viruses except parechovirus 1. Influenza A virus and parechovirus 1 showed thermal stability similar to, or greater than, other viruses that have been proven to be transmitted by foodborne routes, and hence their inactivation by common food processing methods should not simply be assumed. Caution should be taken in using and extrapolating *TFL*-values when analysing thermal inactivation, for example in food or blood products, since reductions in numbers of infective viruses often do not follow log linear (monophasic) kinetics and resistant fractions may remain infectious.

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Chapter 3

Residual viral and bacterial contamination of surfaces after cleaning and disinfection

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Abstract

Environmental surfaces contaminated with pathogens can be sources for indirect transmission, and cleaning and disinfection are common interventions focused on reducing contamination levels. We determined efficacy of cleaning and disinfection procedures for reducing contamination by noroviruses, rotavirus, poliovirus, parechovirus, adenovirus, influenza virus, *Staphylococcus aureus*, and *Salmonella enterica* from artificially contaminated stainless steel surfaces. After a single wipe with water, liquid soap, or 250 ppm free chlorine solution, the numbers of infective viruses and bacteria were reduced by 1 log₁₀ for poliovirus to close to 4 log₁₀ for influenza virus. There was no significant difference in residual contamination after wiping with water, liquid soap, or 250 ppm chlorine solution. When a single wipe with liquid soap was followed by a second wipe using 250 or 1000 ppm chlorine, an extra 1 to 3 log₁₀ reduction was achieved and, except for rotavirus and norovirus genogroup I, no significant additional effect of 1000 ppm compared to 250 ppm was found. A reduced correlation between reduction in PCRU and reduction in infectious particles suggests that at least part of the reduction achieved in the second step is due to inactivation instead of removal alone. We used data on infectious doses and transfer efficiencies to estimate a target level to which the residual contamination should be reduced and found that a single wipe with liquid soap followed by a wipe with 250 ppm free chlorine solution was sufficient to reduce the residual contamination to below the target level for most of the pathogens tested.

Introduction

Viruses are the most common cause of infectious diseases acquired in the indoor environment in hospitals, schools and households (4) causing considerable impact on human health. Transmission of enteric and respiratory viruses is assumed to occur predominantly directly from person to person followed by indirect transmission through contaminated surfaces (7, 40, 47, 53). The risk of infection resulting from transmission through contaminated surfaces depends on a number of factors, including the level of shedding of infective particles, their stability on surfaces and resistance to decontamination procedures and low dose required for infection. Among the enteric viruses, human noroviruses (NoVs) and rotaviruses are most notorious for causing outbreaks of gastroenteritis within hospitals, nursing homes and cruise ships and are significant cause of hospitalization (12, 36, 44). Human NoV outbreaks are often prolonged and re-occurring (5) due to the high levels of shedding of over 10^7 NoV particles/g in stool (45) or vomitus (43), and the low number of particles required for infection (52). Noroviruses are found on different types of surfaces (floors, tables, door knobs, handles, bed rails, carpets and curtains) in health care facilities, in food production facilities, schools and in the community (7, 25, 59). Moreover, the NoV, and many other enteric viruses, stay infectious for up to several weeks (14, 38, 56), which is considered another important factor in the environmental transmission.

Besides human NoV, other enteric viruses like poliovirus and rotavirus, and respiratory viruses like influenza and adenovirus may also be transmitted through contaminated surfaces (7). Influenza A virus was frequently associated with epidemics and occasional pandemics. Adenovirus type 5 is a recommended test organism for testing disinfectants (22) as well as an interesting virus since it can be detected in respiratory excretions and in feces (48). Parechovirus infections have commonly been associated with mild gastrointestinal symptoms in young children and are excreted in feces as well (6). The transmission of parechovirus through contaminated surfaces has not been reported yet but an indirect transmission route is likely to play a role in its spreading, given its similarities with enteroviruses.

Cleaning and disinfection of contaminated surfaces is one of the frequently implemented measures to control transmission of pathogens in indoor environments (16, 24, 31). The effectiveness of cleaning and disinfection practices is often monitored by determining reductions for bacteria such as Gram positive *Staphylococcus aureus* in hospital setting and Gram negative *Salmonella* Enteritidis in food preparation facilities (15, 17). Additionally, the importance of environmental cleaning to control NoV outbreaks in health care settings is widely accepted (5, 28, 33) and decontamination of food production facilities may reduce the number and size of food borne outbreaks (8, 18). However, the reduction levels achieved for bacterial contaminations do not necessarily correlate to reduction levels for viral contaminations and as recently reported by Greig and Lee (30) the scientific proof supporting

effectiveness of implemented intervention measures is limited. Therefore, effective science-based control measures to reduce environmental contamination are urgently needed to reduce the burden of disease of these viruses.

To be able to implement the most effective viral decontamination method, it is necessary to have quantitative data on residual contamination levels after commonly applied cleaning and disinfection practices for some of the most relevant viruses, and preferably, these data should be comparable to data for some bacteria. Thus in the present study, we assessed the effects of different cleaning and disinfection procedures on stainless steel carriers that were artificially contaminated with poliovirus Sabin 1, parechovirus 1, NoV GI.4, GII.4 and its cultivable surrogate MNV 1 (10), simian rotavirus SA 11, influenza A (H1N1) virus, adenovirus type 5 and the bacteria *St. aureus* and *S. Enteritidis*. The experiments were designed to reflect the order of magnitude of the levels of contamination that may result from common events such as toilet flushing (3), poor hygiene, or environmental dispersal of viral particles through droplets generated during a vomiting accidents (11) or remain after removal of visible contamination. The residual contamination was quantified by (cell) culture and PCR assays. As human NoV cannot be cultured (21), the residual contamination of these viruses was determined by quantitative PCR only.

Materials and methods

Test organisms and stocks

Viruses used for the test were poliovirus Sabin 1 (vaccine strain), simian rotavirus SA 11 (ATCC nr.VR-1565), adenovirus type 5 (Hu/adenovirus/type 5/6270/1988/Ethiopia), influenza A (H1N1) virus (Hu/influenza A/266/2008/Netherlands (H1N1) virus), parechovirus 1 (Hu/parechovirus/type1/147/2008/Netherlands), MNV1 (Mu/NoV/GV/MNV1/2002/USA), human NoV GI.4 (Hu/NoV/GI.4/946/2009/ Netherlands) and human NoV GII.4 (Hu/NoV/GII.4/1803/2008/Netherlands). The bacterial test organisms were *Staphylococcus aureus* (196E, toxin producer, human isolate) and *Salmonella enterica* serovar Enteritidis (phage type 4).

Virus stocks were prepared as described before (57) and stored at -80°C . The stocks used contained: poliovirus Sabin 1: 7.2×10^8 50% Tissue Culture Infective Dose / ml (TCID₅₀ / ml) and 5.3×10^{11} PCR units (PCRU) / ml, adenovirus type 5: 2.8×10^7 (TCID₅₀ / ml) and 6.7×10^9 PCRU / ml, parechovirus 1: 3.9×10^8 (6.7×10^{10} PCRU / ml), rotavirus 5: 1.4×10^8 (6.7×10^9 PCRU / ml), influenza A (H1N1) virus: 2.3×10^7 (2.0×10^9 PCRU / ml) and MNV 1: 4.9×10^6 50% Tissue Culture Infective Dose (TCID₅₀) / ml (1.2×10^9 PCRU / ml). The human NoVs GI.4 and GII.4 stocks were 6.6×10^8 and 1.1×10^8 PCRU / ml, respectively.

St. aureus and *S. Enteritidis* were cultured in Brain Heart Infusion broth (Difco, USA) and enumerated on Tryptone Soy Agar (Oxoid, England) as described before (37). Bacterial stocks contained *St. aureus*: 8.8×10^9 and *S. Enteritidis*; 4.2×10^8 colony forming units (CFU) / ml and the detection limit of both bacteria was 10 colony forming units per contaminated spot.

Preparation of sterile stool suspension

The sterile stool suspension from a healthy volunteer was prepared (57) and the suspension was free of rotaviruses, enteric adenoviruses, astroviruses and sapoviruses as determined by PCR (50).

Cleaning and disinfection experiments

The cleaning and disinfection experiments were performed on 2.2 cm × 2.2 cm stainless steel carriers (AISI type 304 standard, Netherlands). The carriers were degreased by dipping into acetone for 10 min, followed by five times rinsing under running tap water. Thereafter, the carriers were soaked in 70% alcohol and dried. The carriers were then sterilized by autoclaving (121°C for 15 min). The viscose wiping cloth was cut into pieces (approximately 4 cm × 3.5 cm) and sterilized by autoclaving.

One chlorine tablet (Suma tab D4, Germany) was dissolved in 1000 ml sterile water. From this solution, 250 and 1000 ppm chlorine solutions were freshly prepared and free chlorine concentrations were measured using a HATCH colorimeter kit (HANNA HI 96771, Romania).

Bovine serum albumin (BSA) (3% w/v in water) or sterile stool suspension (20% w/v) were added to the virus stock to perform the experiments in clean and dirty conditions. Final concentrations were 0.03% BSA and 1% stool respectively. Since human stool is not the natural matrix for influenza A virus, this experiment was performed in clean conditions only. The human NoVs were used as 10% (w/v) stool suspensions and no extra feces was added. Stainless steel carriers were contaminated by spreading 30 µl of each virus suspension in 0.03% w/v BSA or 1% w/v stool separately (contaminated spot) and thereafter dried inside a biosafety cabinet for 1 h at room temperature (22-25°C, 40-45% RH). Then the following cleaning and disinfection procedures were applied:

Single wiping: One thousand milliliters of each cleaning and disinfection solution was prepared. The cloth pieces were soaked into water, water with liquid soap or 250 ppm or 1000 ppm free chlorine solutions separately and excess liquid was squeezed out by hand. With this wet cloth the contaminated carriers were wiped once by hand and sampled 20 min after wiping.

Double wiping: The carriers contaminated with viruses and bacteria were wiped once with the cloth soaked in water with liquid soap as described in procedure single wiping and followed by wiping once again with clothes that were soaked in 250 or 1000 ppm free chlorine solution and wringed. The carriers were sampled after 20 min. Gloves were worn throughout the cleaning process and changed after each wiping.

For sampling, the carrier was kept in a sterile flat bottom tube (Sarstedt 60.597.001, Germany) with the wiped surface facing upwards and 2 ml cold DMEM (4-8°C) with 10% fetal bovine serum (DMEM-FBS) was added for neutralization. For the carriers that were wiped with chlorine solutions, 500 µl 7% w/v sodium thiosulphate solution in water was added for neutralization first and then 1500 µl DMEM-FBS was added. Thereafter the virus was extracted by vortexing at maximum speed for 30 s and flushing the carrier with the medium several times. The suspensions were then collected and infective viruses were enumerated by cell culture assays. Additionally, quantitative PCR assays were performed on samples obtained from wiping with water with liquid soap, 1000 ppm free chlorine solution (single wiping) and on samples obtained from wiping with water with liquid soap followed by wiping with 1000 ppm free chlorine solution (double wiping) to quantify the genomic copies left.

Spot disinfection: If infective virus could still be detected after wiping with water with liquid soap followed by wiping with 1000 ppm free chlorine solution, virus inactivation was further tested by spot disinfection in dirty conditions to determine if extra contact time with the chlorine solution would result in lower residual contamination levels. After wiping the contaminated carrier with water with liquid soap, 800 µl 1000 ppm free chlorine solution was added onto the carrier so that the carrier was completely covered with the chlorine solution for 5, 10 and 20 min. After the exposure time, the chlorine solution was neutralized with an equal volume of 7% w/v sodium thiosulphate solution in water and 400 µl DMEM-FBS was added to make a total volume of 2 ml.

Untreated carriers were kept as control. For neutralization control, compounds (liquid soap or chlorine solutions) were diluted with DMEM-FBS or neutralized with 7% sodium thiosulphate solution before addition to the virus. The experiments were also performed with *St. aureus* and *S. Enteritidis*. Neutralized bacteriological peptone water (Oxoid, England) was used instead of DMEM-FBS. As stool is not the natural matrix for *St. aureus*, the experiment was done only in clean conditions.

TCID₅₀ determination

The viruses were enumerated by titration in 96 well plates on sensitive cells as described before (57).

Real time PCR

To allow comparison of virus reduction between the cultivable viruses and the non-cultivable human NoVs (21), quantitative PCR assays were performed. Viral nucleic acid extraction was performed using Magna Pure total nucleic acid extraction kit as described before (50). Real time PCR assays were performed as described before for poliovirus Sabin 1 (19), adenovirus type 5 (34), rotavirus SA 11 (50), parechovirus 1 (55), MNV1 (2), human NoV GI.4 (50) and NoV GII.4 (57). Amplifiable PCRUs were determined by slopes of standard curves made for each virus. The standard curve was made by plotting cyclic threshold (Ct) values versus log PCRUs of 10 fold dilutions of the virus stocks. The highest dilution giving a positive result was assigned a value of 1 PCRUs.

Residual contamination

In order to provide data that will allow for risk assessments we present data on basis of residual contamination instead of pathogen reduction. The number of pathogens present on the carrier after cleaning or after cleaning and disinfection was considered the residual contamination. The reduction of the pathogens was calculated as: (\log_{10} pathogens on the control carrier) - (\log_{10} pathogens on wiped carrier). The control carriers were contaminated and dried but not subjected to the treatments. All the experiments were performed in triplicate and repeated for confirmation (n = 6).

Data analysis

Statistical analysis was performed by using the student's t-Test. The \log_{10} values of infectivity (x) and PCRUs (y) reduction for cleaning with liquid soap, 1000 ppm chlorine solution and wiping with liquid soap followed by wiping with 1000 ppm chlorine solution were plotted to compare with the line of equality $y = x$.

Results

Calculation of the residual contamination target level

The residual contamination on the carrier after cleaning and disinfection possess a risk when enough infectious microorganisms can be transferred to individuals to cause either infection or to continue transmission indirectly through handling. The data for transfer of microorganisms from contaminated surfaces to human hand (fingerpad) have been determined for rotavirus and hepatitis A virus (1, 42) and shown to be approximately 20% after 20 min drying (1). The number of viruses required for peroral infection is estimated as 10-100 infectious particles for rotavirus, norovirus, poliovirus, parechovirus, and influenza A virus (23, 32, 51, 60), and approximately 150 infectious particles for adenovirus virus (29). An estimated 10-100

cells are required for peroral *S. Enteritidis* infection (49) and *St. aureus*. If we assume 20% transfer from fomite to fingers for all microorganisms tested, then the risk of infection will be small if the residual contamination is less than 5 times the particles required for infection; this level may result in an infection only in the unlikely event that a contaminated finger is directly put in the mouth. We therefore assumed that at residual contamination levels of infective particles of less than 50 ($1.7 \log_{10}$) for rotavirus, MNV1, poliovirus, parechovirus and influenza A (H1N1) virus, *S. Enteritidis* and for *St. aureus* and less than 750 ($2.9 \log_{10}$) for adenovirus type 5, per contact spot, the probability of continued transmission or getting infected is low (but not zero). On the basis of this assumption, lines indicating the residual contamination target levels were drawn in figure 1.

Residual contamination after cleaning – single wiping

The recovery of the viruses and bacteria from the stainless steel carriers after drying for 1 hour ranged from 24 to 76%. After wiping the surfaces were visibly dry within 3 min. The residual contaminations of infective viruses and bacteria in clean and dirty conditions after single and double wiping are shown in figure 1. There was no significant difference in residual contamination after wiping with water or water with liquid soap. Only for poliovirus and rotavirus there was a minor but significantly higher residual contamination when feces were present compared to clean conditions. We found little or no effect of the use of 250 ppm chlorine solutions instead of liquid soap in the cleaning step; only for rotavirus under dirty conditions and influenza A virus (i.e. in only 2 out of 14 pathogen-matrix combinations tested), a lower residual contamination was seen when 250 ppm chlorine was used.

The residual contamination after wiping with 1000 ppm chlorine solution was significantly lower ($p < 0.05$) than wiping with water or liquid soap in 10 out of 14 pathogen-matrix combinations. Additionally, in 7 out 14 pathogen-matrix combinations the wipe with 1000 ppm chlorine solutions resulted in a significantly lower residual contamination when compared to wiping with 250 ppm chlorine solution.

Residual contamination after cleaning and disinfection – double wiping

The residual contamination after wiping with liquid soap followed by wiping with 250 ppm chlorine solution (double wiping) was significantly lower ($p < 0.05$) than after wiping with liquid soap alone (single wiping) for most of the viruses (except MNV1 and rotavirus) and bacteria tested (Figure 1). After the double wiping procedure there was no significant difference ($p > 0.05$) in residual contamination between 250 or 1000 ppm chlorine solution in 12 out of 14 pathogen-matrix combinations. Only for rotavirus the reduction achieved with 1000 ppm was better than the reduction achieved with 250 ppm chlorine solution, resulting in a residual contamination of less than 2 infectious particles per spot (detection limit; $> 6 \log_{10}$ reduction).

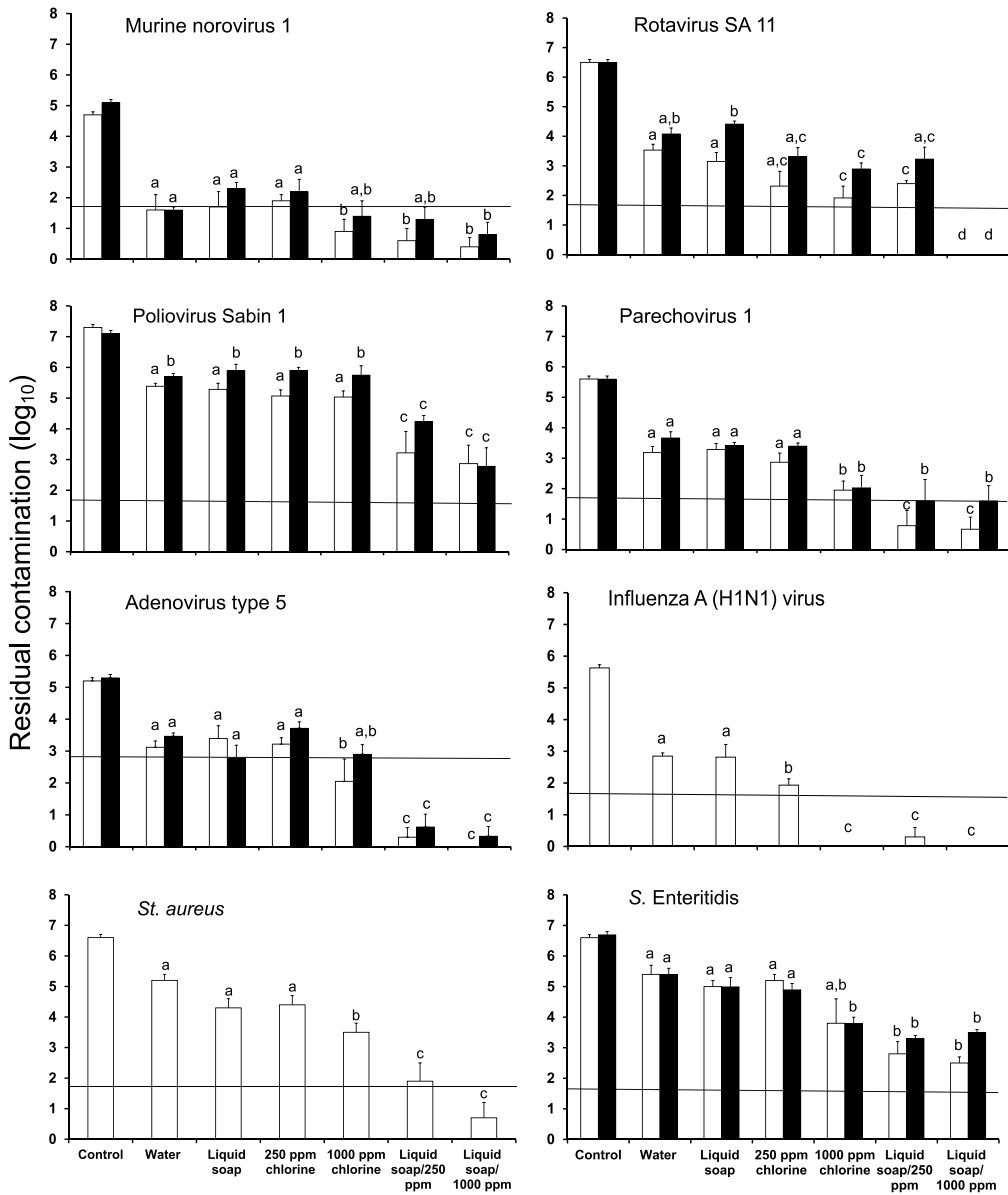


Figure 1: Residual contamination of different pathogens on stainless steel carrier in clean (white) and dirty (black) conditions after different cleaning and disinfection methods. Control is the recovery after 1 h of drying. Water, Liquid soap, 250 ppm chlorine, and 1000 ppm chlorine indicate the suspensions used to wet a wipe for the one-wipe (cleaning) procedure. Liquid soap/250 ppm and Liquid soap/1000 ppm indicate the consecutive suspensions used to wet wipes for the two-step (cleaning and disinfection) procedure. Error bars indicate standard deviation of the mean and the means with a different letter differ significantly ($p < 0.05$) ($n = 6$). The horizontal lines in the figures indicate the residual contamination target levels.

Reduction of genomic copies of norovirus after cleaning and disinfection

As human NoVs could not be cultured, the reductions in genomic copies were quantified by PCR assays. The reductions in genomic copies of NoV GI.4, GII.4 and MNV1 are shown in figure 2. For MNV1, all the treatments resulted in a comparable reduction while for NoV GI.4 and GII.4 we observed a significant higher reduction in PCRU with the double wiping protocol. In 5 out of 6 treatments the reduction in PCRU for NoV GI.4 and MNV1 differed, in 3 out of 6 they differed between NoV GI.4 and NoV GII.4 and in 2 out of 6 between NoV GII.4 and MNV1.

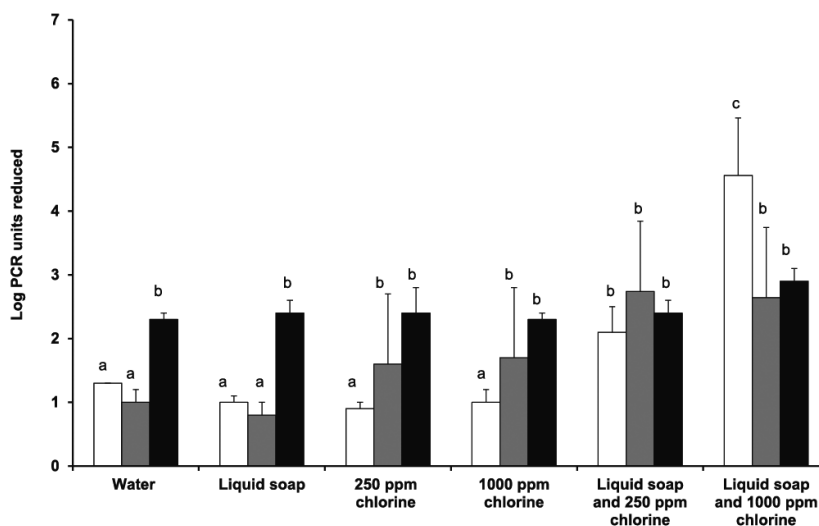


Figure 2: Reduction of genomic copies of human NoVs GI.4 (white), GII.4 (grey) and MNV1 (black) in dirty condition after different cleaning methods. Water, Liquid soap, 250 ppm chlorine, and 1000 ppm chlorine indicate the suspensions used to wet a wipe for the one-wipe (cleaning) procedure. Liquid soap/250 ppm and Liquid soap/1000 ppm indicate the consecutive suspensions used to wet wipes for the two-step (cleaning and disinfection) procedure. Error bars indicate standard deviation of the mean and the means with a different letter differ significantly ($p < 0.05$) ($n = 6$).

The reduction of infective load and genomic copies

The PCRU reductions of poliovirus Sabin1, adenovirus type 5, parechovirus 1, MNV1, rotavirus SA 11 and influenza A (H1N1) virus by wiping with water with liquid soap, with 1000 ppm chlorine solution and wiping with water with liquid soap followed by wiping with 1000 ppm chlorine solution were also determined. The equality between reduction of genomic copies and reduction of infectivity of the tested viruses in clean condition is shown in figure 3. After wiping with water with liquid soap there was a correlation between the infectivity

and PCRU reduction except for rotavirus SA11 and influenza A (H1N1) virus. The infectivity reduction was higher than the PCRU reduction (i.e. deviating from the equality line) on wiping with 1000 ppm chlorine solution and with liquid soap followed by wiping with 1000 ppm chlorine solution in case of parechovirus 1, rotavirus SA 11, MNV1, adenovirus type 5 and influenza A (H1N1) virus.

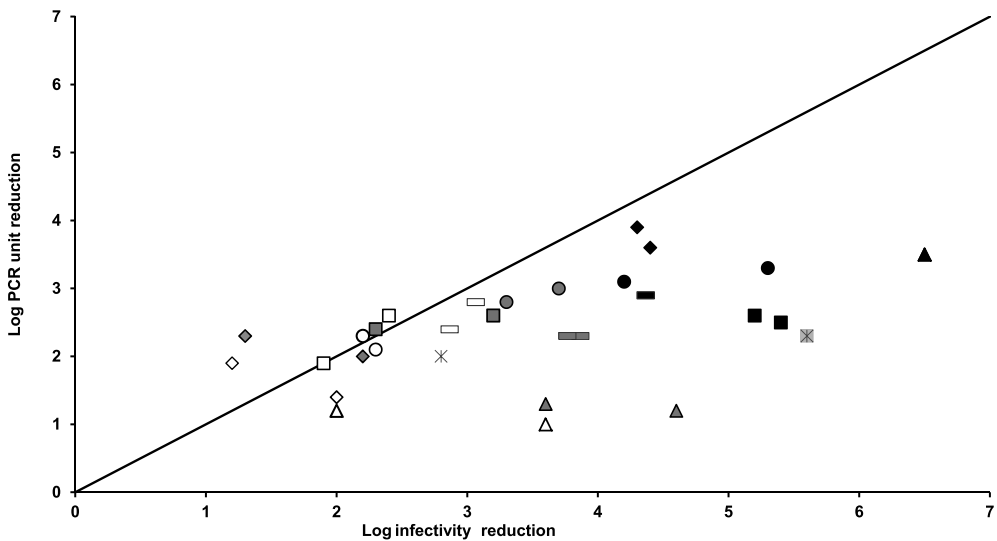


Figure 3: Correlation between PCR units and infectivity reduction by different cleaning methods in clean and dirty conditions of poliovirus Sabin 1 (\diamond), adenovirus type 5 (\square), parechovirus 1 (O), rotavirus SA 11(Δ), MNV1 (\square) and influenza A (H1N1) virus (\times) (n = 6). White, grey, and black symbols represent the reduction by wiping with liquid soap, 1000 ppm chlorine solution and wiping with liquid soap followed by wiping with 1000 ppm chlorine solution, respectively. In some cases only one data point is visible due to overlap of data points.

Residual contamination after spot disinfection

Since there was residual contamination of MNV1, poliovirus Sabin 1, adenovirus type 5, parechovirus 1 and *S. Enteritidis* after the double wiping procedure using 1000 ppm chlorine, spot disinfection of the bacteria and viruses in dirty conditions by 1000 ppm chlorine solution after cleaning with water with liquid soap was tested to determine if this treatment would result in a residual contamination that is below the detection limit. The residual contamination was reduced to below the detection limit of 10 particles of MNV1 in 5 min (a reduction of $5 \log_{10}$), poliovirus Sabin 1 ($6.9 \log_{10}$) and adenovirus type 5 ($5.3 \log_{10}$) in 10 min. The infective loads of parechovirus 1 and *S. Enteritidis* were reduced with 3.2 ± 0.1 and $4.9 \pm 0.4 \log_{10}$ respectively within 20 min of disinfection. Genomic copies of NoVs GI.4 ($6.7 \log_{10}$ PCRU) and GII.4 ($5.2 \log_{10}$ PCRU) were reduced to below the detection limit of 60 PCRU/spot after 10 and 5 min respectively. MNV1 was reduced with $6.9 \pm 0.7 \log_{10}$ PCRU within 20 min of disinfection with 1000 ppm free chlorine solution.

Discussion

Our data indicate that in case of an outbreak of gastroenteritis, by either NoV, rotavirus or *Salmonella*, a cleaning step with liquid soap followed by a wipe using a 1000 ppm chlorine solution most consistently results in the lowest residual contamination level of all treatments tested. However, if we assume that an equivalent of 1 in 2 NoV PCRUs is infectious (data for NoV GI.1 (52)), the residual infectivity of NoV GI.4 and GII.4 will be approximately 5×10^2 or 5×10^3 infectious particles (approximately 1×10^3 or 1×10^4 PCRUs), respectively, per contaminated spot, which is well above the level we defined as target level. Increasing the contact time between pathogen and the 1000 ppm chlorine solution to at least 5 min (as studied by spot disinfection) did result in residual contamination below the target levels of NoV and rotavirus and may be considered to be an effective intervention strategy in controlling gastroenteric pathogens transmission via hard surfaces, although it may be impractical. Our data suggest that *S. Enteritidis* may still be present at loads above our target levels, however, the low prevalence of *S. Enteritidis* in non-food and health-care related outbreaks (58) suggests that transmission via hard surfaces is not a main route of transmission for this pathogen. We did not find clear differences in the reduction in infective enteric viruses or viable bacteria in our experiments, indicating that the apparent greater outbreak potential of NoV and rotavirus is not due to a higher resistance to cleaning and disinfection, but more likely due to the extremely high infectivity of NoV and the high levels of shedding for rotavirus.

Due to the inability to cultivate the human NoVs in vitro, several cultivable viruses such as feline calicivirus (FCV), canine calicivirus (CaCV), MS2 bacteriophage and MNV1 have been used as surrogates to study NoV inactivation (20, 46). However, NoV GI and GII viruses differ in binding properties to for example shellfish tissues and lettuce surfaces (41, 54), but also in resistance to freeze-drying and heat treatment (9, 35), making it unlikely that one model virus will be a valuable surrogate for NoV GI and NoV GII. This was confirmed in our studies that showed inconsistencies in the level of correlation of MNV results with those for NoV GII.4 and GI.4 in complex situations such as this study where removal and disinfection were combined. In the absence of cultivation method for the human NoV we postulate that especially for quantitative risk assessment purposes, the use of any model virus should be accompanied by a PCR based method to allow comparison.

The two picornaviruses tested (poliovirus and parechovirus) showed remarkable differences in residual contamination and thus risk of infection remaining after cleaning, however this was mainly caused by a $2 \log_{10}$ difference in starting contamination level. Since differences in levels of shedding do occur (13, 39), these data may reflect real variation in levels of contamination after cleaning and disinfection. Spot disinfection showed a remarkable resistant parechovirus fraction as some could still be cultured after 20 min exposure to 1000 ppm chlorine solution. Such a very resistant virus fraction, representing 0.01% of the stock

suspensions used, was also shown to exist during thermal inactivation at 73°C (55). Due to the low doses able to cause infection, these resistant fractions may represent a risk when present in foods or on surfaces when very high levels are shed.

In this study we confirmed the higher sensitivity of the enveloped respiratory influenza A virus to chlorine disinfection, compared to sensitivity of the non-enveloped enteric viruses (56) and the complete removal of infectious influenza virus after a single wipe, with a 1000 ppm confirms a recent study that showed complete inactivation of human influenza A viruses by wipes containing 1% bleach (sodium hypochlorite and sodium hydroxide) (27). The two step procedure consisting of a single wipe with liquid soap followed by a disinfection step using 250 ppm chlorine solution is likely to be a good intervention strategy in case of viral respiratory disease outbreaks since it reduced the infectivity of both respiratory viruses tested to well below the target level.

Efficacy of cleaning and disinfection is not only determined by the intrinsic effectiveness of the method applied but also by the appropriateness of the surfaces treated. Cleaning and disinfecting should be focused on the critical spots, i.e. the surfaces really involved in transmission. Reducing the infective load on critical spots such as door knobs, handles, light switches and other frequently touched surfaces is more likely to have a profound impact on transmission than treating rarely touched surfaces. Interestingly, a recent study on the removal of viruses from hard surfaces found a comparable reduction of infective MNV1 after wiping the surfaces 6 times (26) as we found after a single wipe, indicating that surface cleaning and disinfection can be performed quite efficiently. Nonetheless, manual cleaning and disinfection procedures will always be more labor intensive than for example room disinfection using hydrogen peroxide vapor (57) and for the control of outbreaks a combination of both methods is most likely needed.

In this study we performed cleaning and disinfection by wiping as it may be carried out in health care settings. Since these procedures will be carried out by different individuals, variability in residual contamination levels is likely. Additional variation will occur due to differences in level of shedding, differences in temperature and humidity and types of contaminated surfaces. However, tests like these, even if just describing one scenario, provide the scientific background for evidence based cleaning and disinfection guidelines or protocols.

In health care facilities cleaning may be performed according to different protocols: general cleaning performed on a day to day basis and more stringent cleaning, often in combination with disinfection procedures, during outbreaks. Our findings show that in all cases a single wipe with a wet cloth with either water or liquid soap resulted in a significant reduction ($> 1 \log_{10}$) of the infective load of all pathogens tested, but the residual contaminations

indicate that further transmission may still occur. Adding a wiping step with 250 or 1000 ppm chlorine solution resulted in an additional reduction of the infective load, most likely through inactivation of the pathogens rather than by particle removal, as indicated by the discrepancy between infectivity and PCRU reduction. Pre-cleaning before disinfection of the contaminated surfaces is recommended and the removal and disinfection together will often result in residual contamination levels below the target levels of residual contamination.

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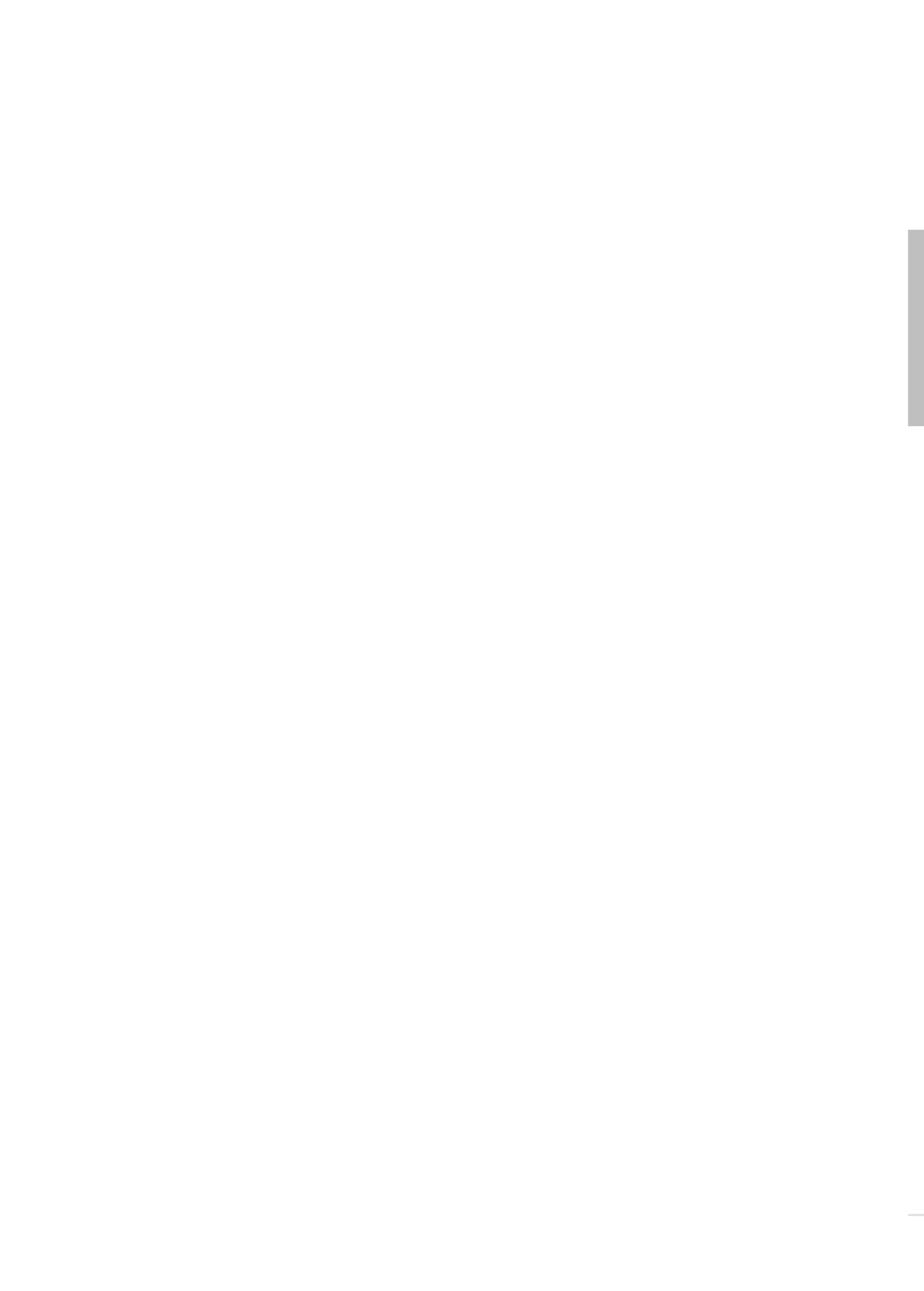
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Chapter 4

Virucidal efficacy of vapourized hydrogen peroxide disinfection

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Abstract

Contamination of surfaces by viruses is common in individual households, hospitals, health care settings and believed to play an important role in their transmission. Chemical disinfection can be an effective means of transmission intervention. Therefore, we measured the antiviral efficacy of vapourized hydrogen peroxide (VHP) disinfection against different human viruses applied to stainless steel, framing panel or gauze. VHP disinfection at 127 ppm for 1 h at room temperature resulted in complete inactivation of all viruses tested. Complete inactivation was characterized as $> 4 \log_{10}$ reduction of the number of infectious particles for poliovirus Sabin 1, rotavirus SA 11, adenovirus type 5, and murine norovirus 1 on stainless steel and framing panel, and more than $2 \log_{10}$ reduction of all viruses tested on gauze. For influenza A (H1N1) virus complete inactivation on stainless steel and framing panel was characterized as more than $2 \log_{10}$ reduction. Complete inactivation was confirmed under dirty conditions at several locations in a room for poliovirus Sabin 1. Reductions of viral genomes measured in PCR units were minimal on framing panel and gauze, but significant on stainless steel. Comparison of reductions of PCR units suggests that the RNA of human NoV GII.4 was more resistant to VHP disinfection than the other viruses tested. Thus the VHP disinfection at 127 ppm for 1 h, as approved disinfection in the Netherlands for hospitals and health care centers, is effective against poliovirus Sabin 1, rotavirus SA11, adenovirus type 5 and MNV1 on stainless steel, framing panel and gauze.

Introduction

Contamination of surfaces is common in individual households, food processing facilities, hospitals and health care settings and may lead to long-term presence of viruses on such surfaces (5, 6). Epidemiological evidence of transmission of respiratory and enteric viruses through fomites at home and in institutions has been documented (5). Relevant factors for the success rate of this indirect transmission route are high level of shedding, low minimal infectious dose, and resistance to inactivation or removal by environmental factors or cleaning and disinfection procedures. Enteric viruses like human norovirus (NoV) and rotaviruses are fecal oral pathogens that can cause large scale outbreaks and often cause nosocomial infections and outbreaks (4, 27). These viruses are often shed at over 10^7 particles/g in stool or vomit (18), indicating that even in case of a contamination of only 1 mg, a reduction of $4 \log_{10}$ of the number of infective viruses is not always enough to prevent transmission through environmental spread (26). Virucidal efficacy is defined as $> 4 \log_{10}$ reduction of the number of infective viruses and this level is often not accomplished by commonly used cleaning and disinfection methods (3, 25,18).

Environmental decontamination by manual chemical disinfection is very labor-consuming and may lead to occupational and environmental risks (8). Moreover, norovirus could still be detected in a hospital setting after cleaning (18). The use of vapourized hydrogen peroxide (VHP) has been shown to be effective against bacteria and its spores (12, 23). Moreover, a treatment of VHP at > 100 ppm for 1 h is a legally approved procedure for decontamination of hard surfaces in rooms for humans in the Netherlands. The procedure is approved as application against bacteria (excluding myco-bacteria), spores of bacteria and yeasts and is applied regularly in several hospitals when dealing with *Clostridium difficile* or Methicillin-resistant *Staphylococcus aureus* (MRSA) contamination problems (Dr. Bijlmer, Bronovo Hospital, Den Hague, the Netherlands, personal communication). Due to the absence of personnel in the room during VHP treatment, there is little occupational risk and due to complete decomposition of the peroxide there is little environmental risk.

The objective of the study was to measure the virucidal efficacy of the approved VHP disinfection protocol in a quantitative carrier test against structurally different respiratory and enteric viruses such as poliovirus (enteric, non-enveloped, model for enterovirus), human NoV genogroup II.4 (NoV GII.4; enteric, non-enveloped) and murine NoV 1 (MNV1), a surrogate for human NoV (7, 29), rotavirus (enteric, non-enveloped), adenovirus (respiratory and enteric, non-enveloped) and influenza A (H1N1) virus (respiratory, enveloped). The effect of stool as an interfering substance was determined by comparing the reduction of MNV1 in cell culture medium and in a 1% stool suspension. The tests were performed on viruses applied to stainless steel, framing panel and gauze carriers. The framing panel is one of the commonly used materials for external cladding in institutes, laboratories, industries, homes and hospitals. Stainless steel is used for door handles, hand and grab rails in toilets and on

cruise ships, surfaces in food production areas and is the material of choice for work surfaces and in sanitary environments. Gauze was included as a model for textile fabrics. The different viruses dried on the different carriers were exposed to VHP in an isolator and poliovirus was additionally exposed on different locations in a room to mimic a real life situation.

Materials and methods

Test organisms

Viruses used for the test were poliovirus Sabin1 (vaccine strain), simian rotavirus SA11 (ATCC nr.VR-1565), adenovirus type 5 (reference strain, Hu/adenovirus/ type 5/6270/1988/ Ethiopia), influenza A (H1N1) virus (clinical isolate, Hu/influenza A/266/2008/ Netherlands (H1N1) virus), MNV1 (Mu/NoV/GV/MNV1/2002/USA) and human NoV GII.4 (stool sample, Hu/NoV/GII.4/1803/2008/Netherlands). MNV1 was tested as cell suspension and after mixing with human NoV suspension to get the suspension in 1% stool (human).

Virus stocks were prepared by infecting monolayers of respective host cells essentially as described before (11). Shortly, poliovirus Sabin 1 and adenovirus type 5 were cultivated on Hep-2 cells (11), rotavirus SA11 on MA-104 cell (11), MNV1 on Raw-264.7 cells (29) (cells and virus suspension were kindly provided by Christiane Wobus) and influenza A (H1N1) virus was cultivated on MDCK-1 cells (11). The respective monolayers of the cells were infected at a multiplicity of 0.1 infective particles per cell and incubated at 37°C at 5% CO₂. The viruses were harvested after 2 to 3 days when complete cytopathic effect was apparent followed by two cycles of freezing, thawing, and centrifugation at 1512 g for 15 min at 4°C. The virus stocks were as follows: poliovirus 2.5×10^9 , rotavirus 1.3×10^7 , adenovirus 6.3×10^7 and influenza A (H1N1) virus 3.2×10^6 TCID₅₀/ ml, MNV1 was 1.6×10^7 PFU/ ml.

Human NoV GII.4 was used as 10% stool suspension in cell culture medium prepared as described before (28) and filtered through 0.2 µm pore size filter. The suspension is free of all other enteric viruses tested (28) (human NoVs GI, rotaviruses, enteric adenoviruses, astroviruses and sapoviruses). The PCR units were determined by using the slopes of standard curves of semi quantitative PCR assays (9). The suspension used contained human NoV GII.4 at 7.9×10^7 PCR units/ml.

Carrier Preparation and inoculation

Framing panel (Trespa®, a solid fiber board which consists of wood fibers with a phenolic resin-based binder, Trespa International BV, Weert, the Netherlands) and stainless steel (AISI-specification 304, Wageningen, Netherlands) carriers used in the experiment were 2.2 cm × 2.2 cm in size and 2.5 mm and 1.5 mm thick for stainless steel and framing panel respectively. Gauze pads (Klinion NW compress extra, Biotrading Benelux bv, Mijdrecht,

the Netherlands, 67% viscose and 33% polyester) were 2.2 cm × 1.9 cm in size with thickness approximately 1.5 mm. All these carriers were sterilized by autoclaving at 121°C for 15 min and transferred to individual wells of a six well plate. Twenty µl of virus suspension was dispensed and spread at the centre of each sterile carrier separately and allowed to dry inside a biohazard cabinet for 1 h at room temperature.

VHP Disinfection

VHP disinfection in the room and in the isolator was performed according to the procedure that has been authorized by the Board for the Authorization of Plant Protection Products and Biocides (Ctgb), the Netherlands. VHP disinfection was performed in two experiments, using triplicate samples per virus per carrier per experiment. In the first experiment we determined the virucidal activity of VHP treatment at an average of 127 ± 7 ppm for 1 h against poliovirus, human NoV GII.4 and MNV1, rotavirus, adenovirus, and influenza A (H1N1) virus in an isolator of 1.5 m³. In the second experiment the inactivation of poliovirus was studied at different locations and was performed in a room (7m × 5m × 2.7m) using a similar VHP treatment of an average of 126 ± 15 ppm for 1 h. In the room, the VHP machine was installed in one corner and the carriers with poliovirus were positioned on a table in the middle of the room at 90 cm height, on a closet along a wall at 2 m height, at the corner near the window, in an open and closed closet as shown in figure 1.

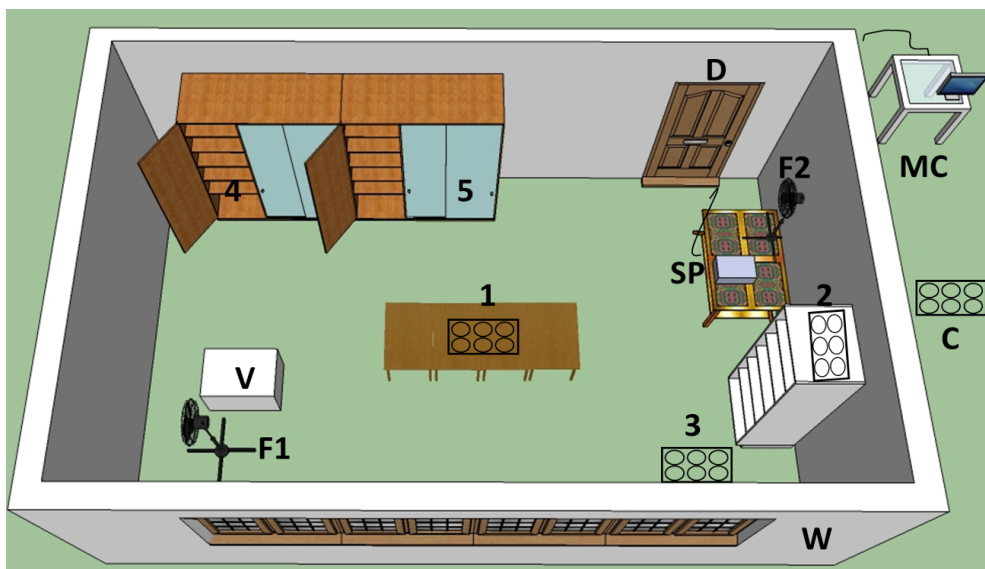


Figure 1: Schematic diagram of sample setting in room (7 m × 5 m × 2.7 m) for hydrogen peroxide disinfection. D door; W window; V disinfection machine; F fan; SP sensor probes; MC control panel; C control plate kept outside the room. Sample locations are indicated by numbers: 1 on a table in the middle of the room, height 90 cm; 2, on a closet, height 2 m; 3, in a corner by a window, height 70 cm; 4, inside an open closet, height 130 cm; 5, inside a closet, height 130 cm.

The room had been used as an office, contained fitted carpet, and was not cleaned prior to the experiment to mimic the real life situation with respect to the peroxide demand of the environment. VHP in a room was generated using Liquid Verne Veiling equipment and Alpha-Bac 12 F (12% hydrogen peroxide) (Alpheios, Heerlen, the Netherlands) at a flow rate of 2.3 l/h.

In the isolator, carriers were positioned such that replicates were not adjacent to each other and not directly in front of the exhaust of the VHP machine. The VHP machine (Boneco 7131, Plastron AG., Widnau, Switzerland) was placed at one corner such that the VHP concentration can be easily recorded. Control carriers were treated similar to the exposed carriers except for VHP disinfection. The peroxide concentration was measured in both experiments by a sensor (Hydrogen peroxide sensor Drager HC 68090705, Germany). The peroxide level was controlled remotely and maintained at an average of 120 ppm during the experiments. In the room, a uniform distribution of air was maintained by using two propeller fans. Disinfection was performed in the room and isolator with relative humidity between 65% and 80% and the average peroxide level at 120 ppm. The temperature of the room and isolator were 16-18°C and 20-21°C respectively. After 1 h exposure, the aeration phase lasted approximately 45 min in the room and 70 min in the isolator.

Extraction of viruses from carriers

The viruses were extracted from the stainless steel and framing panel by swabbing (swab sticks-COPAN CE0344-rayon swab material) with a swab moist with Dulbecco's modified Eagles medium (DMEM). The swab stick was then transferred into a tube with 2 ml DMEM and vortexed at maximum speed for 1 min. The gauze was transferred to a tube with 2 ml DMEM medium and vortexed at maximum speed for 1 min. The gauze was pressed against the wall and the suspension was collected. All viruses were suspended in DMEM within 1 h after the aeration phase. During aeration, hydrogen peroxide is converted into water and oxygen (12, 21).

Enumeration of viruses

Undiluted and serial 10 fold dilutions of the samples were prepared and analyzed immediately. MNV1 was quantified by plaque assay in six well plates as described before (29). The other viruses were enumerated by titration in 96 well plates on sensitive cells. The TCID₅₀ values were calculated by the Spearman-Kärber method (15). Log₁₀ reduction was calculated by subtracting log₁₀ virus of VHP treated sample from log₁₀ virus of the control (log₁₀ reduction = log₁₀ virus from control – log₁₀ virus from fumigated sample). Since undiluted suspensions were used for enumeration, the detection limit is 1 infectious virus retrieved from the exposed carriers. Even though the complete absence of infective viruses on the carriers after exposure is thus not proven, we define this as complete inactivation.

Real time RT-PCR

To allow comparison of virus reduction between the cultivable pathogens and the non-cultivable human NoV GII.4, semi-quantitative PCR assays were performed (28). A comparable fragment length between 130-207 bp was amplified for the different viruses. Real time PCR for poliovirus (9), MNV1 (1), rotavirus (17), adenovirus (16) were done as described before. For human NoV GII.4 forward primer 5'GATGGGTCCACAGCCAAC3', reverse primer 5'GGGCGCGCTCCATAGTA3' (5118-5324) and amplicon specific probe 5'CAGTRTCCCCTAGAAAYG CTCC3' labeled with Texas Red /BHQ-2® was used for PCR. For influenza A (H1N1) virus forward primer 5' GCCGACTATGAGGARC 3', reverse primer 5'TCAGCCATAGCAAATTTYTG 3' (337-486) and amplicon specific probe 5'AGCTCATGGCCCAACCACA3' labeled with FAM/BHQ-1® was used. The PCR for human NoV GII.4 and influenza A (H1N1) virus was performed at an annealing temperature of 43°C for 20 s and 50°C for 20 s respectively. PCR units were determined by standard curves.

Results

Recovery of viruses from control carriers

In these experiments different viruses, different carriers, a drying step, and an extraction method of several steps were used. The recovery of infective viruses from the controls on stainless steel and framing panel varied from 1.9 to 37.5%. On gauze the recovery was 0.1% to 1%. The recovery of PCR units from the control carriers ranged from 22.2% to 80.1% for stainless steel and framing panel and from 2.1% to 69.0% for gauze.

Virucidal effect of the VHP treatment

In the first experiment (VHP at 127 ± 7 ppm, 1 h, isolator) complete inactivation was shown for all viruses tested. This meant, more than $4 \log_{10}$ infectivity reduction of all viruses tested, except influenza A virus, were shown on stainless steel and framing panel and $> 2 \log_{10}$ infectivity reduction on gauze after VHP treatment (Figure 2). Due to relatively low-titer virus stock and over $1 \log_{10}$ infectivity reduction by drying on the carriers, we were unable to show $> 4 \log_{10}$ reduction for influenza A virus due to VHP treatment. In the second experiments (VHP at 126 ± 15 ppm, 1 h, different locations in a room) inactivation of poliovirus was complete ($> 5 \log_{10}$) at all locations, except in the closed closet, as shown in figure 3.

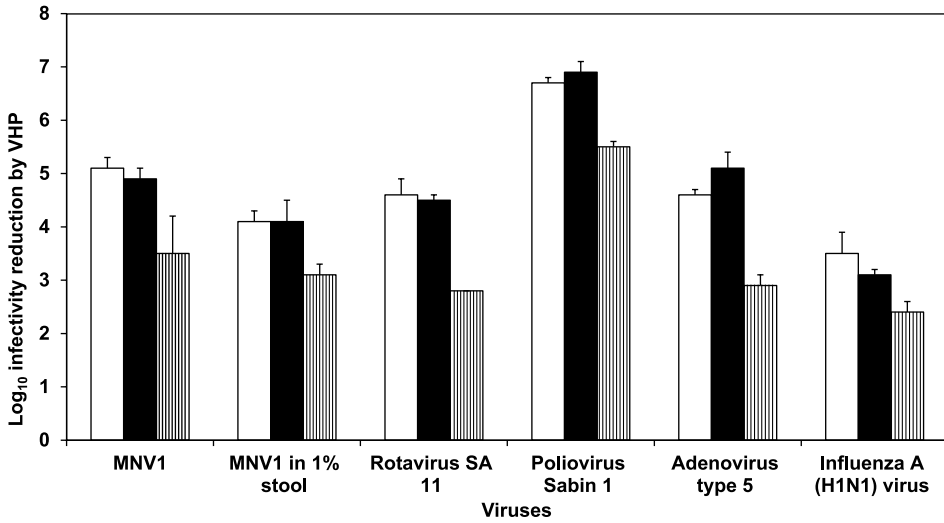


Figure 2: Infectivity reduction of viruses following hydrogen peroxide vapour (VHP) disinfection of 127 ppm for 1 h in an isolator. Results for MNV1 are expressed as plaque forming units, and for those other viruses are expressed as 50% Tissue Culture Infections Dose (TCID₅₀). Data are expressed as mean (standard deviation) for n = 3. Open bars, stainless steel; solid bars, framing panel; striped bars, gauze.

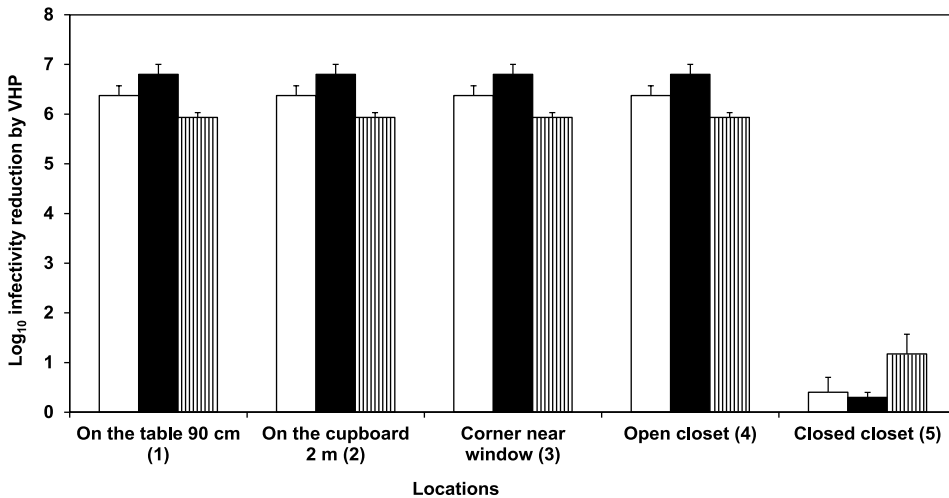


Figure 3: Infectivity reduction of poliovirus Sabin1 following hydrogen peroxide vapour (VHP) disinfection of 126 ppm for 1 h in different location in a room. The numbers in brackets refer to the locations shown in Figure 1. Data are expressed as mean (standard deviation) for n = 3. Open bars, stainless steel; solid bars, framing panel; striped bars, gauze.

Reduction of detectable PCR units by VHP treatment

The reduction in detectable PCR units after the VHP treatment is expressed in \log_{10} PCR unit reduction in figure 4. Overall, loss of RNA/DNA was less pronounced than the infectivity loss and higher on stainless steel carrier than on framing panel and gauze. The PCR unit reduction of poliovirus by the VHP treatment on stainless steel was $> 3 \log_{10}$ on all locations, except in the closed closet. The PCR unit reduction of all the viruses tested on stainless steel was significantly higher ($P < 0.05$) than on framing panel and gauze. Human NoV GII.4 RNA was reduced by $0.5 \log_{10}$ PCR units on stainless steel, which is less than for all other viruses ($P < 0.05$). The highest PCR unit reduction on stainless steel and framing panel was found for influenza A (H1N1) virus ($P < 0.05$).

Discussion

In this study we show a clear virucidal effect of VHP disinfection at 127 ppm against poliovirus, rotavirus, adenovirus, MNV1, and influenza A virus. The complete inactivation of poliovirus at all different locations tested in a room, except in the closed closet, suggests that good peroxide vapour distribution throughout a room can be achieved but relevant peroxide levels are not reached in closed compartments. The minimum level required to claim effective virucidal activity is $4 \log_{10}$ infectivity reduction (26) and this was demonstrated for poliovirus, rotavirus, adenovirus and MNV1 on framing panel and stainless steel. The maximum inactivation that could be shown for influenza A virus was $3.5 \log_{10}$ on stainless

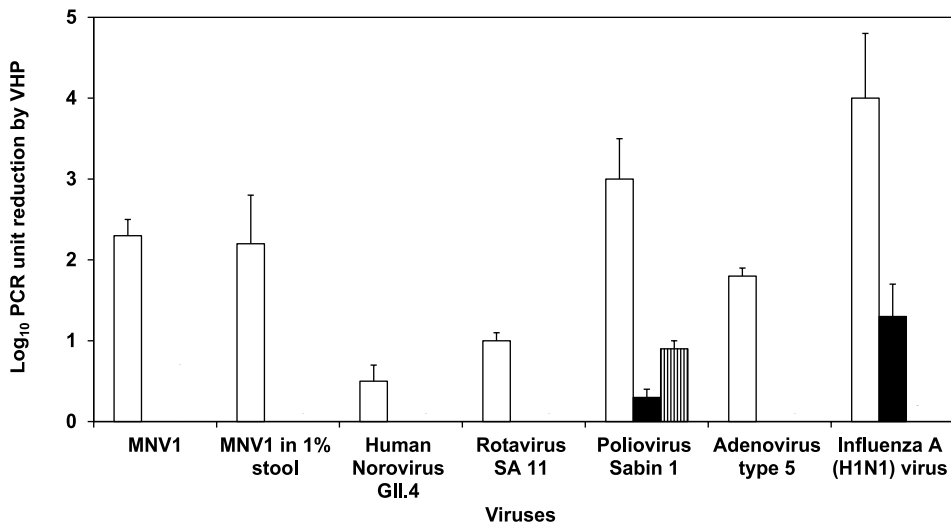


Figure 4: Polymerase chain reaction (PCR) unit reduction of viruses following hydrogen peroxide vapour (VHP) disinfection of 127 ppm for 1 h in an isolator. Data are expressed as mean (standard deviation) for $n = 3$. MNV1, murine norovirus 1; NoV GII.4, human norovirus genogroup. Open bars, stainless steel; solid bars, framing panel; striped bars, gauze.

steel, 3.1 log₁₀ on framing panel and 2.4 log₁₀ on gauze. However, a previous study (24) already showed > 4 log₁₀ reduction of influenza A virus after 15 min VHP exposure at 90 ppm.

The comparative analysis of the reduced number of infective viruses and the reduction in PCR units shows that for this chemical treatment, PCR unit reduction is a poor indicator for the virucidal effect on all non-enveloped viruses. Overall, a complete inactivation of all viruses (often > 4 log₁₀) was concomitant with a much smaller, or even undetectable, reduction in PCR units.

The virucidal effect of VHP treatment towards MNV1 was studied in the presence and absence of stool as interfering substance. This comparison showed that the VHP decontamination resulted in complete inactivation of this murine NoV in the presence and absence of stool. These results indicate that low levels of fecal matter, that may for example result from aerosols after toilet flushing or after hand-contact after applying suboptimal hand hygiene, do not significantly hamper the VHP decontamination as tested in this study. This may imply that with respect to VHP decontamination, blood is a worse interfering substance than stool, since for MS2 a significant protective effects of blood was shown (17).

To evaluate efficacy of VHP disinfection against human NoV, we studied infectivity and PCR unit reduction for MNV1 and PCR unit reduction for NoV GII.4. The comparative analysis showed that the amount of RNA of human NoV GII.4 was reduced less during the VHP treatment than the RNA of MNV1. The limited reduction of human NoV RNA relative to the RNA reduction of several model viruses such as MNV1 and feline calicivirus was shown before for treatment with sodium hypochlorite solution, ethoxylated alcohol (13), aldehydes, peroxides (20), and UV radiation (10).

The reduction of human NoV GII.4 RNA on stainless steel was significantly lower than the reduction of MNV1 RNA; however it was not significantly different from the reduction of rotavirus RNA. Whether or how much the infectivity of human NoV GII.4 was reduced could not be shown due to its resistance to culture (11). However, the complete inactivation of all other viruses tested, including other non-enveloped enteric viruses such as another member of the norovirus genus and rotavirus, suggests virucidal activity towards the human NoV is likely.

In conclusion, the VHP disinfection at 127 ppm for 1 h, as approved and applied in the Netherlands for decontamination of hospitals and health care centers, is effective against poliovirus Sabin 1, rotavirus SA11, adenovirus type 5 and MNV1 on stainless steel, framing panel and gauze. We show virucidal activity of VHP against a range of enteric and respiratory viruses. Similar effectiveness has been shown against *Bacillus anthracis* (22), *Mycobacterium tuberculosis* (14), MRSA (19) and *Clostridium difficile* spores (2). The

continued gastrointestinal and respiratory illness outbreaks at homes and institutions increase the need for effective virucidal treatments and the VHP treatment can serve as one of the useful means for decontamination of in-house environments after contamination with enteric or respiratory pathogens.

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Chapter 5

Different virucidal activities of hyperbranched quaternary ammonium coatings on poliovirus and influenza virus

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Abstract

Virucidal activity of immobilized quaternary ammonium compounds (IQACs) coated onto glass and plastic surfaces was tested against enveloped influenza A (H1N1) virus and non-enveloped poliovirus Sabin 1. The IQACs tested were virucidal against the influenza virus within 2 min but no virucidal effect against poliovirus was found in 6 h.

Introduction, materials and methods and results and discussion

Enteric and respiratory viruses can be transmitted directly from person to person but also indirectly via contaminated surfaces (1, 3, 4). Key factors in successful transmission are the quantity of infectious virus particles shed, the minimal infectious dose, the stability in the environment and the resistance to disinfection (2). Consequently, cleaning and disinfection are means to control outbreaks and transmission of viruses, however, proper cleaning and disinfection can be laborious and for non-enveloped enteric viruses such as noroviruses, the effectivity of currently practiced cleaning and disinfection procedures are not clear (5, 7). Therefore, self-decontaminating surfaces may be helpful in preventing transmission in health care settings, food production areas and in general from frequently touched and contaminated fomites. Applying antimicrobial coatings to produce self-decontaminating surfaces could help to interrupt the indirect transmission of pathogens and reduce the labor and time required for adequate cleaning and disinfection.

In this study we coated plastic and glass surfaces with quaternary ammonium compounds. The immobilized quaternary ammonium compounds (IQACs) are comprised of hyperbranched polymers functionalized with tertiary amines, which are quaternized by alkylation with an alkylhalide. Various types of Hybrane® hyperbranched polymers (HA1690, HA5290, DA17395, and DA33295, all from DSM, Heerlen, the Netherlands) were quaternized with heptylbromide ($C_7H_{15}Br$) to obtain various IQACs using the following procedure: A solution consisting of 10 g polymer; 7.1 g K_2CO_3 ; 70 ml *tert*-amylalcohol and 12 ml heptylbromide was mixed and stirred for 24 hours at 96°C. After removing the solids by filtration, the polymer was precipitated by pouring the solution in a large excess of hexane or diethylether. After washing the polymer with hexane or ether, the polymer was dried under vacuum. The polymer was dissolved in dry acetone and the resulting solutions were used to form transparent films on glass slides (76 × 26 × 1 mm) and plastic wells (24 well plate, Corning, Germany) by applying a thin layer of fluid on the surfaces followed by drying under vacuum for 24h. The polymers are very poorly water-soluble and can only be extracted from the surface using organic solvents. These positively charged IQAC's interact with the negatively charged outer surface of bacteria and change the permeability of the bacterial membrane. In this exploratory study, we determined the virucidal properties of several IQACs against the enveloped (lipophilic) respiratory influenza virus and non-enveloped (hydrophilic) enteric poliovirus.

Viruses used for the test were poliovirus Sabin1 (vaccine strain) and influenza A (H1N1) virus (clinical isolate, Hu/influenza A/266/2008/Netherlands (H1N1)). Virus stocks were prepared as described before (10). The poliovirus stock contained 1.6×10^7 TCID₅₀/ml, the influenza A (H1N1) virus stock contained 4.0×10^6 TCID₅₀/ml. Fifty µl of poliovirus or influenza virus were dispensed and spread at the center of each surface. Environmental persistence of both viruses was studied for 10 days on uncoated plastic and glass. The poliovirus was left on the

coated surfaces for 1 and 6 h and influenza virus for 2 min and 1 h. The viruses were removed from the glass slides by swabbing (swab sticks-COPAN CE0344-rayon swab material) as described before and from the plastic wells by rinsing with 1 ml cell culture medium (10). Visual inspection of the coatings after virus removal did not show any damage to the coat. Enumeration of the viruses and quantitative real time RT-PCR was performed as described (10). Data for environmental resistance are presented as total infective viruses recovered from the surfaces (at $t = 0$ h, the suspension is not yet dried) and data for virucidal activity of the IQACs are presented as reduction relative to uncoated glass and plastic carriers. Recoveries of infective viruses after 1 h from the control carriers were $1.3 \pm 0.0\%$ and $45.0 \pm 7.3\%$ for influenza virus from glass and plastic respectively and $56.6 \pm 9.2\%$ and $89.7 \pm 14.5\%$ for poliovirus from glass and plastic respectively. All experiments were performed at room temperature in duplicate and the outcome is from two experiments. Statistical analysis was done using the t-test.

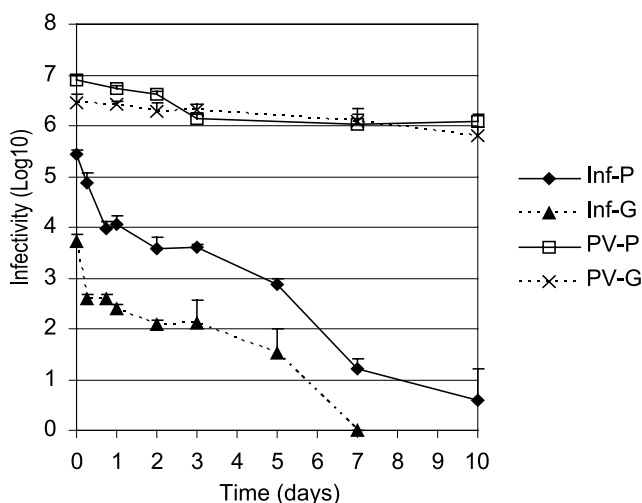


Figure 1: Environmental decay of poliovirus and influenza virus at room temperature. PV-P is poliovirus on plastic, PV-G is poliovirus on glass, Inf-P is influenza virus on plastic, Inf-G is influenza virus on glass. Data are from 2 experiments with duplicate samples per experiment, error bars indicate SD.

The persistence of poliovirus was high with less than 1 \log_{10} decay in 10 days (Figure 1). Influenza virus showed fast decay the first 24 h, probably mostly due to drying, followed by slower decay up to day 5 and again faster decay after day 5 (Figure 1). The decay in infective virus that could be retrieved from the coated carriers differed widely for poliovirus and influenza virus (Figure 2). After 1 h, no significant reduction of infectious poliovirus was measured for any of the coatings, while for influenza virus the reduction of infective virus was complete: no infective influenza viruses could be retrieved from the coated surfaces, except from the non-quaternized HA5290 polymer, for which no reduction was found. Identical

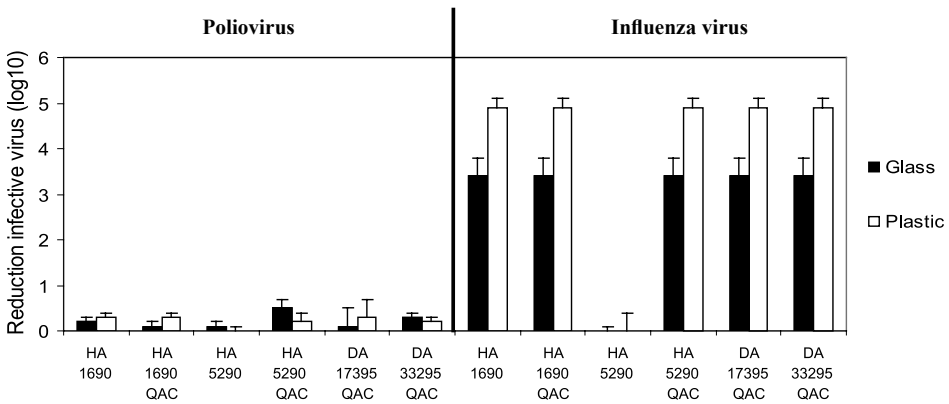


Figure 2: Reduction of infective poliovirus and influenza virus by different IQACs after 1 h exposure at room temperature. Data are from 2 experiments with duplicate samples per experiment, error bars indicate SD.

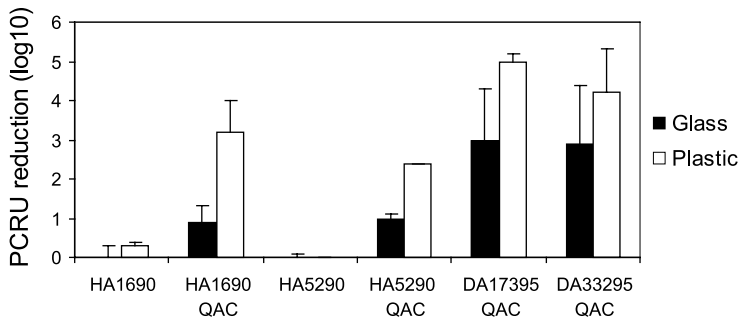


Figure 3: Reduction of PCRU of influenza virus by different IQACs after 1 h exposure at room temperature. Data are from 2 experiments with duplicate samples per experiment, error bars indicate SD.

levels of inactivation of influenza virus were already achieved after 2 min, while even after 6 h no significant reduction of poliovirus could be detected (data not shown). Remarkable was the complete inactivation of influenza virus by the quaternized and the non-quaternized HA1690 polymer. The reduction in infective influenza virus by quaternized HA1690 was concomitant with a significant reduction in PCR units while this was not true for the non-quaternized HA1690 (Figure 3).

Even though quantitative data are missing; transmission of enteric and respiratory viruses via hard surfaces is a concern in health care settings, individual houses, and food production facilities. In this study we found that no infective influenza viruses could be retrieved from the coated surfaces, indicating that the environmental survival of these enveloped respiratory viruses was reduced from over 5 or 10 days for glass and plastic respectively, to less than 2 min on IQAC coated surfaces. The virucidal mechanism of QACs has been described for the lipophilic enveloped viruses to involve disruption or detachment of the viral envelope and

enveloped viruses such as herpes simplex virus have been found to be very sensitive (8, 9). However, data on influenza viruses and QAC resistance are less unambiguous. In suspension tests, avian influenza H5N1 strains were not completely inactivated by 0.02% benzylalkonium chloride (manufacturers' recommended concentration) after 10 min (11) while in general QACs are considered good disinfection agents for influenza viruses (http://www.cdc.gov/h1n1flu/guidelines_labworkers.htm; accessed November 23, 2011). We confirm virucidal effects of the IQACs tested against influenza viruses in our carrier tests. Further studies will be needed to determine how many times the coated surfaces can be cleaned and maintain this activity and if activity remains when surfaces are filthy or dusty.

The finding that no infective influenza viruses could be retrieved from the HA1690 base-polymer coated slides might be due to the length of the lipophilic tails and the high density of functional end-groups (tertiary amines) which are available to be quaternized in the HA1690 base-polymer (both, tail length and density are higher for the HA1690 than for HA5290). The full recovery of influenza virus RNA in the HA1690 base-polymer shows that the infectivity reduction is not the result of a profound effect on the viral genome but indicates a primary effect on the virus envelope.

QACs have been reported to show low virucidal activity against hydrophilic non-enveloped viruses such as picornaviruses. Infective poliovirus was reduced by 1.1-2.3 \log_{10} by a QAC (alkyldimethylbenzyl ammonium chloride plus ethylenediaminetetraacetic acid) in a suspension test (450 ppm, 10 min, 20°C) (6) while less than 1 \log_{10} reduction was reported for a similar QAC in a comparable exposure in the presence of 20% blood but over 2.4 \log_{10} in the absence of interfering substances (12). We report the lack of significant virucidal activity of the IQACs tested against poliovirus after up to 6 h exposure. To the best of our knowledge this is the first report on virucidal activity of IQACs against poliovirus and influenza virus and we show that hyperbranched QACs coating is effective against influenza A (H1N1) virus but not effective against poliovirus Sabin 1.

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Chapter 6

Transfer of noroviruses between fingers and fomites and food products

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Abstract

Human norovirus contaminated hands are important routes for transmission. Quantitative data on transfer during contact with surfaces and food are scarce but necessary for a quantitative risk assessment. Therefore, transfer of MNV1 and human NoVs GI.4 and GII.4 was studied by artificially contaminating human finger pads, followed by pressing on stainless steel and Trespas[®] surfaces and also on whole tomatoes and cucumber slices. In addition, clean finger pads were pressed on artificially contaminated stainless steel and Trespas[®] surfaces. The transfers were performed at a pressure of 0.8-1.9 kg / cm² for approximately 2 s up to 7 sequential transfers either to carriers or to food products. MNV1 infectivity transfer from finger pads to stainless steel ranged from 13 ± 16% on the first to 0.003 ± 0.009% on the sixth transfer on immediate transfer. After 10 min of drying, transfer was reduced to 0.1 ± 0.2% on the first transfer to 0.013 ± 0.023% on the fifth transfer. MNV1 infectivity transfer from stainless steel and Trespas[®] to finger pads after 40 min of drying was 2.0 ± 2.0% and 4.0 ± 5.0% respectively. MNV1 infectivity was transferred 7 ± 8% to cucumber slices and 0.3 ± 0.5% to tomatoes after 10 min of drying, where the higher transfer to cucumber was probably due to the higher moisture content of the cucumber slices. Similar results were found for NoVs GI.4 and GII.4 transfers measured in PCR units. The results indicate that transfer of the virus is possible even after the virus is dried on the surface of hands or carriers. Furthermore, the role of fingers in transmission of NoVs was quantified and these data can be useful in risk assessment models and to establish target levels for efficacy of transmission intervention methods.

Introduction

Human noroviruses (NoVs) are the leading cause of gastroenteritis affecting people of all age groups (27). The virus is a major threat to public health due to numbers of outbreaks in closed settings like hospitals, nursing homes, cruise ships, and long term care facilities (20, 21, 27, 29). Though the NoV infection is usually self-limiting in otherwise healthy persons, due to the high incidence of disease and closing of wards in hospitals and nursing homes, and due to absence from work the burden of disease is high (19, 24). Transmission of the human NoV occurs directly through person to person contact or indirectly via consumption of contaminated food and water and contaminated surfaces (28, 38). The person to person transfer has been reported as a major means of transmission in NoV outbreaks in closed settings (30) and hands are thought to be the main vehicle for the transmission (44).

Virus transfer between hands and surfaces or food can be quantified by determination of the fraction of the virus on artificially contaminated hands that is transferred to the receiving fomites or food surfaces. Previous studies have shown that many bacteria (22, 39, 51) and bacteriophages MS2 and ϕ X174 are readily transferred via hand contact (25). Similar results have been found for pathogenic viruses like rotavirus (1), hepatitis A virus (HAV) (36), human parainfluenza virus-3 and rhinovirus (2). Transfer of NoV has been estimated by studying Feline Calicivirus (FeCV), which belongs to the same family *Caliciviridae*, and the infectivity transfer was estimated as 13% from finger pad to stainless steel when pressed for 10 s after air drying (6).

This study was conducted with a NoV GI.4 strain and an epidemic NoV GII.4 strain (40). As human NoV cannot be cultured (15), their transfer was determined by quantitative PCR. Additionally, cultivable murine NoV (MNV1) was used as a model virus (50) to study transfer of infectious viruses. MNV1 has been described as a more suitable model virus than FeCV, as MNV belongs to the same genus as human NoV, is an enteric virus and resistant to low pH (12). We studied transfer of the virus from fingers to stainless steel or Trespa® and vice versa and to whole tomato and cucumber slices to better understand hands as a vehicle for transmission of human NoV. Quantitative data on transfer of the virus from fingers to different fomites and food products were collected, since this information will be helpful for quantitative risk assessment and to develop effective transmission intervention methods.

Materials and methods

Viruses, cell line, and propagation of virus

Viruses used for the tests were MNV 1 (Mu/NoV/GV/MNV1/2002/USA), human NoV GI.4 (Hu/NoV/GI.4/946/2009/ Netherlands), and human NoV GII.4 (Hu/NoV/GII.4/1803/2008/ Netherlands).

MNV1 stock was prepared by infecting monolayers of murine macrophage cell line Raw-264.7 as described before (47). Briefly, monolayers of the cells were infected at a multiplicity of 0.1 infective particles per cell and incubated at 37°C at 5% CO₂ atmosphere. After 2 to 3 days, cytopathic effect was visible and the virus was harvested by freeze-thawing twice, followed by removal of cell debris by centrifugation at 1512 × g for 15 min at 4°C.

Human NoV suspensions were prepared as 10% w/v stool homogenates in Dulbecco's Modified Eagle Medium (DMEM, Gibco, Germany) as described before (42) and filtered through 0.2 µm pore size filter. The suspensions were free of all other enteric viruses tested (rotaviruses, enteric adenoviruses, astroviruses and sapoviruses) as determined before by PCR assays (42). The virus stocks were then stored at -80°C. The standard curve was made by plotting cyclic threshold (Ct) value versus log PCRU of 10 fold dilutions of the virus stock (14).

Preparation of sterile stool suspension

Stool suspension from a healthy volunteer was tested for presence of viral RNA or DNA as described before (42) and found free of all the enteric pathogens tested (human NoVs GI and GII, rotaviruses, enteric adenoviruses, astroviruses and sapoviruses) by PCR. A 20% (w/v, wet weight) stool suspension was prepared in phosphate buffer (0.01M, pH 7.2) and sterilized by autoclaving at 121°C for 15 min. The suspension was vortexed, centrifuged at 1512 × g for 20 min, and then the supernatant recovered was aliquoted and stored at -20°C.

Cleaning and sterilization of carriers

Stainless steel (AISI type 304 standard, Netherlands) and Trespa® (Framing panel, a solid fiber board which consists of wood fibers with a phenolic resin-based binder) carriers used were 2.2 cm × 2.2 cm in size and 1.5 mm and 2.5 mm thick for stainless steel and framing panel respectively. Trespa is used for bench surfacing in kitchens, offices and laboratories. The carriers were cleaned with soap and water, then soaked in 70% (v/v) ethanol for 30 min and dried, then sterilized by autoclaving at 121°C for 15 min and transferred to individual wells of a six well plate.

Washing food materials (tomato and cucumber)

Tomatoes (Roma, Holland) and cucumbers were purchased from a local market (The Netherlands). Both products were washed twice under running tap water (15°C) and dried for 30 min at room temperature. The cucumbers were cut into slices with a diameter of 4.8 ± 0.2 cm and thickness of 0.9 ± 0.2 cm. The small size tomatoes were about 4 cm in width and about 2.5 cm in height and were used without cutting. Each product was then marked with a circle of about 2 cm diameter to mark the contamination site.

Volunteers

Permission

Test persons participating in the transfer experiments were previously informed about the procedure and the risks before signing the informed consent form. The study protocol was reviewed and approved by the medical ethics review committee of Wageningen university (METC number: 12/01; NL number: 39407.081).

Inspection and preparation of hands for the test

One male and two females participated in this experiment. Prior to every test, both hands of each panelist were inspected carefully to make sure that they were free from any apparent cuts, scratches, or damages. Each panelist then washed their hands thoroughly using nonmedicated soap (Hegron Cosmetics, Netherlands) and running tap water (15°C) for 40 sec and dried the hands with sheets of paper towel. Approximately 2 ml 70% (v/v) ethanol was dispensed onto the palm of panelist hands, which was rubbed over the entire surface of hands and fingers until dry to disinfect the hands. The test procedure was then initiated by dropping 10 µl of virus suspension on a finger pad.

Survival of virus on fingers

To determine the number of infective virus particles on finger pads at 0 min, 10 µl of the test virus was placed on each finger pad and eluted immediately by placing the inoculated area over the open mouth of a cryovial (3.6 ml capacity: Nunc, Denmark) containing 1 ml DMEM. The vial was inverted 20 times with the finger pad still pressed to it, so that the skin was rinsed with the medium. The vial was then turned upright and the finger pad was scraped in downward position against the inside rim of the vial to recover as much of the remaining fluid as possible into the vial as described before (36). For the dried controls, finger pads were contaminated with 10 µl test virus and dried for 10 min (visibly dry) inside a biosafety cabinet at room temperature (25-26°C, 40-45% RH). The dried inoculum was then eluted as described above. Three finger pads from each individual (n = 9) were tested for both baseline controls (0 and 10 min).

Decontamination of finger pads

After the tests, the finger pads were immediately decontaminated by pressing for 1 min onto tissue paper soaked in 5000 ppm free chlorine solution in a petridish. Thereafter the hands were washed thoroughly with water and soap as described before.

Transfer experiment

The experiment with MNV1 was performed in presence of 1% stool, as enteric viruses always contaminate along with fecal material or vomit. Human NoVs GI.4 and GII.4 were mixed in equal volumes and used in the same experiments as 10% feces suspension. As human NoVs were prepared as stool homogenate, no extra stool was added. All the experiments were performed inside a biosafety cabinet at room temperature (25-26°C, 40-45% RH). The experiments were repeated three times with three individuals at different times (n = 9). Three fingers of each individual were tested per experiment. The titer of the virus stock used was MNV 1: 5.3×10^6 50% Tissue Culture Infective Dose (TCID₅₀) / ml and 7.1×10^8 PCR units (PCRU) / ml. The titers of NoVs GI.4 and GII.4 in the suspension used were 5.6×10^8 and 7.5×10^6 PCRU / ml, respectively. Transfer experiments were performed by applying a pressure varying between 0.8-1.9 kg / cm² for 2 s per finger-carrier contact.

Transfer of viruses from fingers to carrier

Transfer of virus from finger pads to surfaces (stainless steel and Trespac®) was performed directly (0 min) and after drying for 10 min after virus application. Sterile stainless steel and Trespac® carriers were kept in wells of a 6 well plate inside a biosafety cabinet. The plate was placed on a top loading balance (Sartorius, Germany) to measure the weight equal to the pressure applied during the transfer. Finger pads were contaminated with 10 µl virus suspension and pressed for 2 s on the sterile carrier sequentially up to 7 carriers (approximate time 14 s in total). These experiments were repeated with other finger pads up to 3 fingers from each individual. For the transfer experiments with dried virus suspension, the virus was applied on the finger pads and then dried for 10 min inside the biosafety cabinet (25-26°C, 40-45% RH) and pressed onto the sterile carriers sequentially as explained above. Then 1 ml DMEM was added onto the carrier immediately and the virus was recovered by flushing the carrier with the medium 20 times by pipetting the medium up and down. The virus suspension was collected and stored at -20°C until analysis.

Transfer of viruses from fingers to food product

Marked whole tomatoes and cucumber slices were kept in an individual sterile boat (50 ml reagent reservoir, Corning USA) inside a biosafety cabinet. As described above, the contaminated finger pads with 10 µl virus at 0 min and 10 min were successively pressed on the marked area of 7 food products: on skin of whole tomatoes and on cut face of cucumber slices. The pressure applied was measured as described above. The transferred virus was recovered immediately by flushing with 2 ml DMEM on the marked area for 20 times by pipetting. MNV1 suspensions were then filtered through a 0.2 µm pore size filter (Whatman, Germany) to prevent contamination in the cell culture assay and the sterile filtrate was

collected for further analysis. The samples were then stored at -20°C until analysis. There was no difference in PCR signals observed from filtered and non-filtered stocks.

Transfer of viruses from carriers to finger

Stainless steel and Trespa® carriers were kept in wells of 6 well plates separately. All carriers were individually contaminated with 10 µl of virus suspension and spread by a pipette tip over a round area of approximately 1 cm diameter. Thereafter, the carriers were dried for 40 min inside a biosafety cabinet (25-26°C, 40-45% RH) until visibly dry. Test persons pressed their clean and dry finger pads (cleaned and disinfected with ethanol 70% as explained above) onto the contaminated spot sequentially. Sequential pressing was performed with five fingerpads of each person. The virus on the finger pads was eluted by inverting the open mouth of a cryovial with 1 ml DMEM on the contaminated area on the finger pad as described above. The virus remaining on the carrier was eluted by flushing the carrier with 1 ml DMEM as described above. The collected samples were then stored at -20°C until analysis. After the experiment, the volunteers decontaminated and then washed their finger pads as explained above.

Infectivity determination

The number of infective MNV1 was enumerated by titration on Raw-264.7 cells as described before (47). Briefly, 1×10^5 cells / ml cell suspension was freshly prepared and 100 µl of the suspension was loaded on each well of a 96 well plate separately. 10 fold serial dilutions of the virus were titrated on the cells and the cytopathic effect was observed after 5 or 7 days of incubation at 37°C in 5% CO₂. Virus titers were then calculated by the Spearman-Kärber method (26).

Reverse Transcription PCR (RT-PCR)

To allow comparison between the cultivable MNV1 and the non-cultivable human NoVs, quantitative RT-PCR assays were performed for all three viruses. The virus RNA extraction was performed using a Magna Pure Light cycler total nucleic acid extraction kit as described before (42). Amplification of MNV1 (4), NoV GI.4 (42) and GII.4 (47) was performed as previously described. RT-PCR amplifiable units were determined from slopes of standard curves made for each virus. The standard curve was made by plotting cyclic threshold (Ct) value versus log RT-PCR U of 10 fold dilutions of the virus stock. The highest dilution giving a positive result was assigned a value of 1 amplifiable unit. Samples of transfer 1,3 and 5 of all the experiments were analyzed by RT-PCR assay.

Calculations and statistical analysis

The MNV1 infectivity and PCRU recovery from the finger pads (0 and 10 min), carriers and food products were calculated as (virus recovered [infectivity or PCRU] after time (t) from source / virus applied) \times 100%. Student's t-Test was performed to analyze and compare results.

Results

Recoveries of viruses from human finger pads

The MNV1 titer loaded on finger pads was $5.0 \pm 0.2 \times 10^4$ TCID₅₀ per 10 μ l. The MNV1 infectivity recoveries at 0 and after 10 min drying were 57 ± 28 (n = 9) and $20 \pm 18\%$ (n = 9), respectively. Although approximately 80% of loaded infectious viruses were lost; still over 10,000 infectious MNV1 was retrieved from the finger pad after 10 min drying. The MNV1 PCRU recoveries at 0 and after 10 min drying were 63 ± 9 (n = 9) and $28 \pm 3\%$ (n = 9), respectively. For NoVs GI.4 and GII.4, 2.8×10^6 and 3.8×10^4 PCRU were loaded on the finger pads (n = 9), respectively. The PCRU recoveries of NoV GI.4 from finger pads at 0 and 10 min were $80 \pm 25\%$ and $31 \pm 13\%$, respectively and for NoV GII.4 recoveries were $28 \pm 12\%$ and $9 \pm 6\%$, respectively. The PCRU recovery of NoV GI.4 was significantly higher ($P < 0.05$) than NoV GII.4.

Viruses transfer from finger pads to stainless steel and Trespa® carriers

The sequential MNV1 infectivity transfers from finger pads to stainless steel at 0 and 10 min are shown in figure 1A. The infectivity transfer of MNV1 at immediate transfer (t = 0 min) on stainless steel and Trespa® were $13 \pm 16\%$ and $13 \pm 3\%$ respectively. The infectivity transfer from the finger pad to stainless steel and Trespa® after 10 min drying were reduced to 0.1 ± 0.2 and $0.1 \pm 0.1\%$ respectively, and were significantly lower ($P < 0.05$) than at 0 min, however the transfers were similar for stainless steel and Trespa® (data for Trespa® not shown). Infectious virus was detectable up to 5-6 out of 7 consecutive transfers to both types of carriers at 0 and 10 min of drying.

The PCRU transfers of MNV1, NoVs GI.4 and GII.4 from finger pads at time 0 and 10 min to stainless steel are shown in figure 1B. The PCRU transfers of MNV1 and the NoVs GI.4 and GII.4 in the first transfer to stainless steel (t = 0) was $50 \pm 18\%$, $69 \pm 31\%$ and $19 \pm 10\%$ respectively, and after 10 min when the finger pads were dry, the transfer reduced to $1.1 \pm 0.9\%$, $2.2 \pm 3.1\%$ and $0.6 \pm 1.6\%$, respectively. The PCRU transfers of MNV1 and the NoVs GI.4 and GII.4 in the first transfer to Trespa® (t = 0) were $54 \pm 23\%$, $54 \pm 32\%$ and $13 \pm 14\%$ respectively. After 10 min when the finger pads were dry, the transfers were reduced to $3.7 \pm 2.7\%$, $1.7 \pm 2.2\%$ and $1.1 \pm 3.2\%$ respectively. There was a significant difference

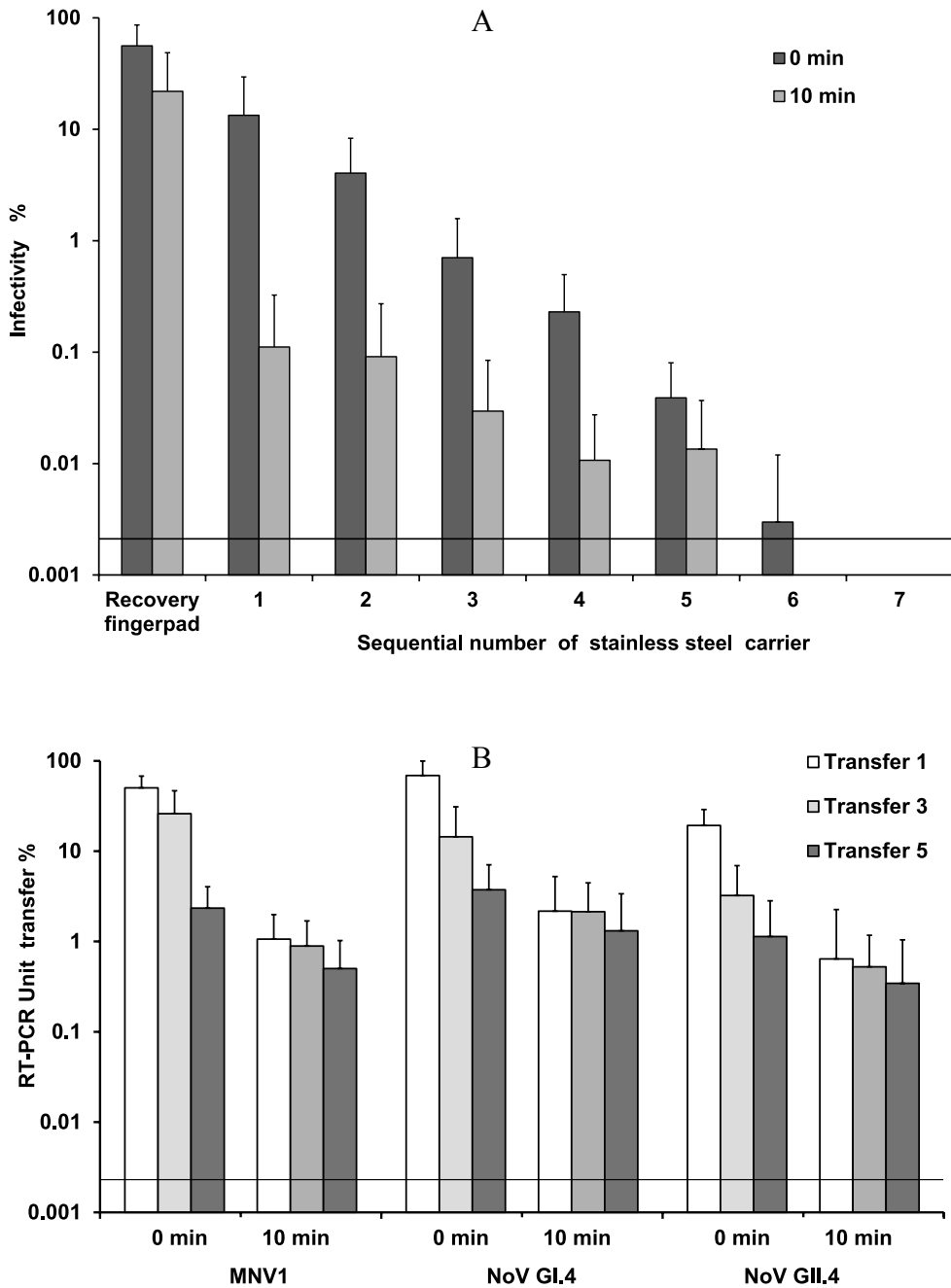


Figure 1: MNV1 infectivity transfer (A) and PCRU transfer of MNV1, human NoV GI.4, and GII.4 (B) from finger pads to stainless steel after 0 and 10 min of drying on sequential transfer. Horizontal lines show detection limit. Error bars show standard deviation of series (n = 9). The transfer percentage is calculated as fraction of the number of viruses applied.

($P < 0.05$) in PCRU transfers at 0 and 10 min for both stainless steel and Trespa® but the transfers were not significantly different ($P > 0.05$) between the two carriers (data not shown). PCRU transfers of MNV1 and NoV GI.4 is similar ($P > 0.05$), but the transfer of NoV GII.4 to stainless steel and Trespa® at immediate transfer ($t = 0$ min) was significantly lower ($P < 0.05$) than MNV1 and NoV GI.4.

However, there was no significant difference ($P > 0.05$) in transfer among the NoVs after drying for 10 min. Comparing the MNV1 infectivity and PCRU transfers, a significant difference ($P < 0.05$) was observed at both immediate transfer and after drying at 1, 3 and 5 transfers. MNV1 PCRU transfers were higher than infectivity transfers.

Virus transfer from contaminated stainless steel and Trespa® to human finger pads

The sequential transfer of MNV1 infectivity from contaminated stainless steel and Trespa® carriers to the finger pads after 40 min drying are shown in figure 2A. The MNV1 infectivity at first transfer to finger pad from stainless steel and Trespa® carrier was $1.8 \pm 1.8\%$ and $3.8 \pm 4.8\%$ respectively. There was no significant difference ($P > 0.05$) in infectivity transfer from the two carriers to the finger pads.

The PCRU transfers from contaminated stainless steel and Trespa® to finger pads are shown in figure 2 B. For NoV GI.4, transfer from stainless steel and Trespa® to the finger pads was $4.2 \pm 6.6\%$ and $3.2 \pm 3.2\%$ respectively. The transfer of NoV GII.4 was 3.5 ± 3.4 and $2.4 \pm 3.8\%$ respectively and for MNV1 was 1.8 ± 1.2 and $2.4 \pm 1.3\%$ respectively. The PCRU transfer of NoVs GI.4 and GII.4 was not significantly ($P > 0.05$) different from PCRU transfer of MNV1 for both carriers. There was no significant difference ($P > 0.05$) in MNV1 infectivity and PCRU transfer from both the carriers to finger pads. Average of $3.5 \log_{10}$ infective MNV1 still remained on the carriers after 5 sequential transfers.

Transfer from fingers to food products (whole tomatoes and cucumber slices)

Infectivity transfer of MNV1 from finger pads to whole tomatoes and cucumber slices at 0 and 10 min are shown in figure 3A and 4A respectively. The virus was transferred to all the seven food products touched sequentially. The MNV1 infectivity transferred to whole tomatoes and cucumber pieces at immediate transfer ($t = 0$) were similar: $14 \pm 6\%$ and $12 \pm 11\%$ respectively. However, after 10 min drying, transfer to cucumber slices was $7 \pm 8\%$ which was significantly higher ($P < 0.05$) than the transfer of $0.3 \pm 0.5\%$ to tomatoes at 10 min. For tomatoes, there was a significant difference ($P < 0.05$) for infectivity transfer at 0 and 10 min but not for cucumber slices. On both the food products, infectivity was detected even on the 7th transfer as shown in figures 3A and 4A. Dried finger pads became wet on pressing the cucumber slices resulting in higher percentage of transfer than on tomatoes.

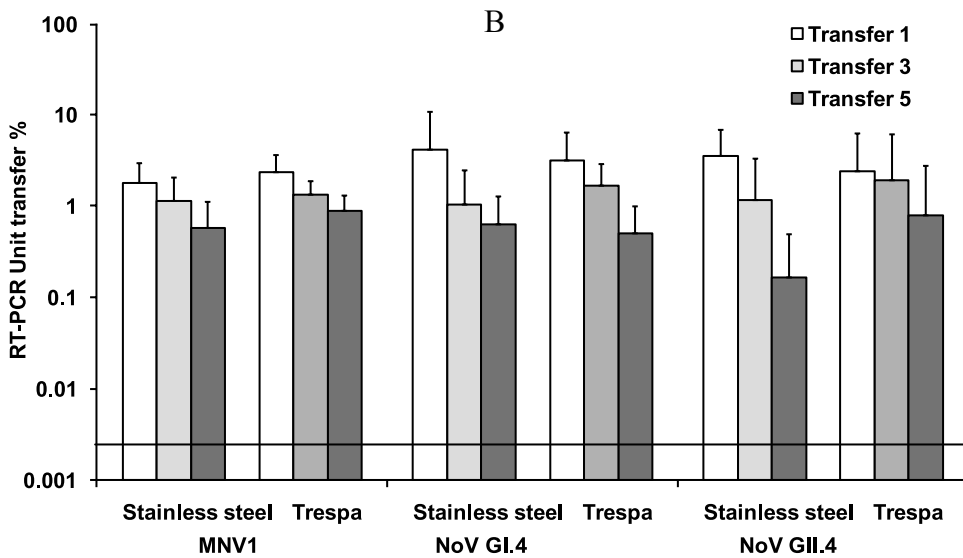
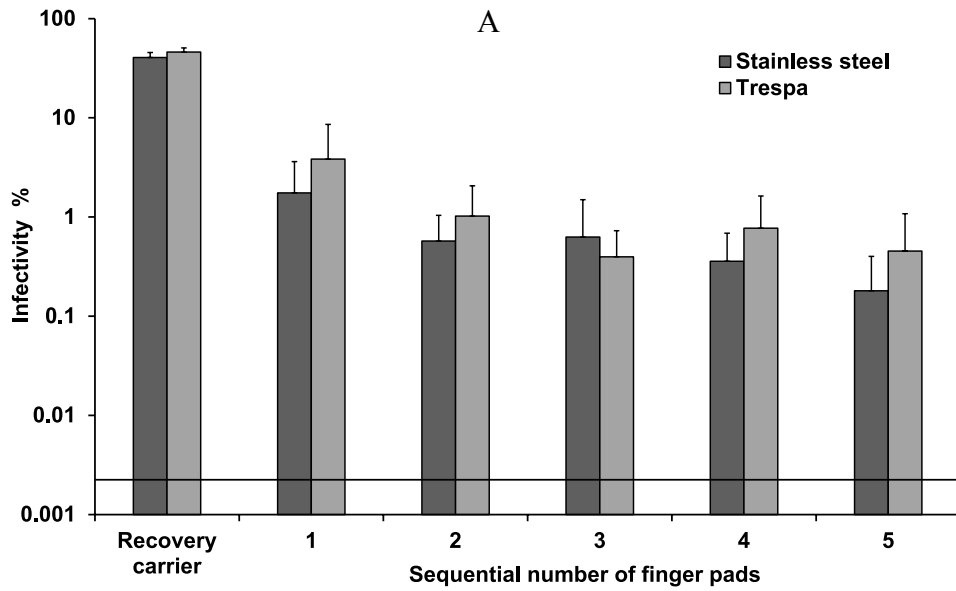


Figure 2: MNV1 infectivity transfer (A) and PCRU transfer of MNV1, human NoV GI.4, and GII.4 (B) from stainless steel and Tresa® to finger pads by sequential transfer after 40 min drying on the carriers. Horizontal lines show detection limit. Error bars show standard deviation of series (n = 9). The transfer percentage is calculated as fraction of the number of viruses applied.

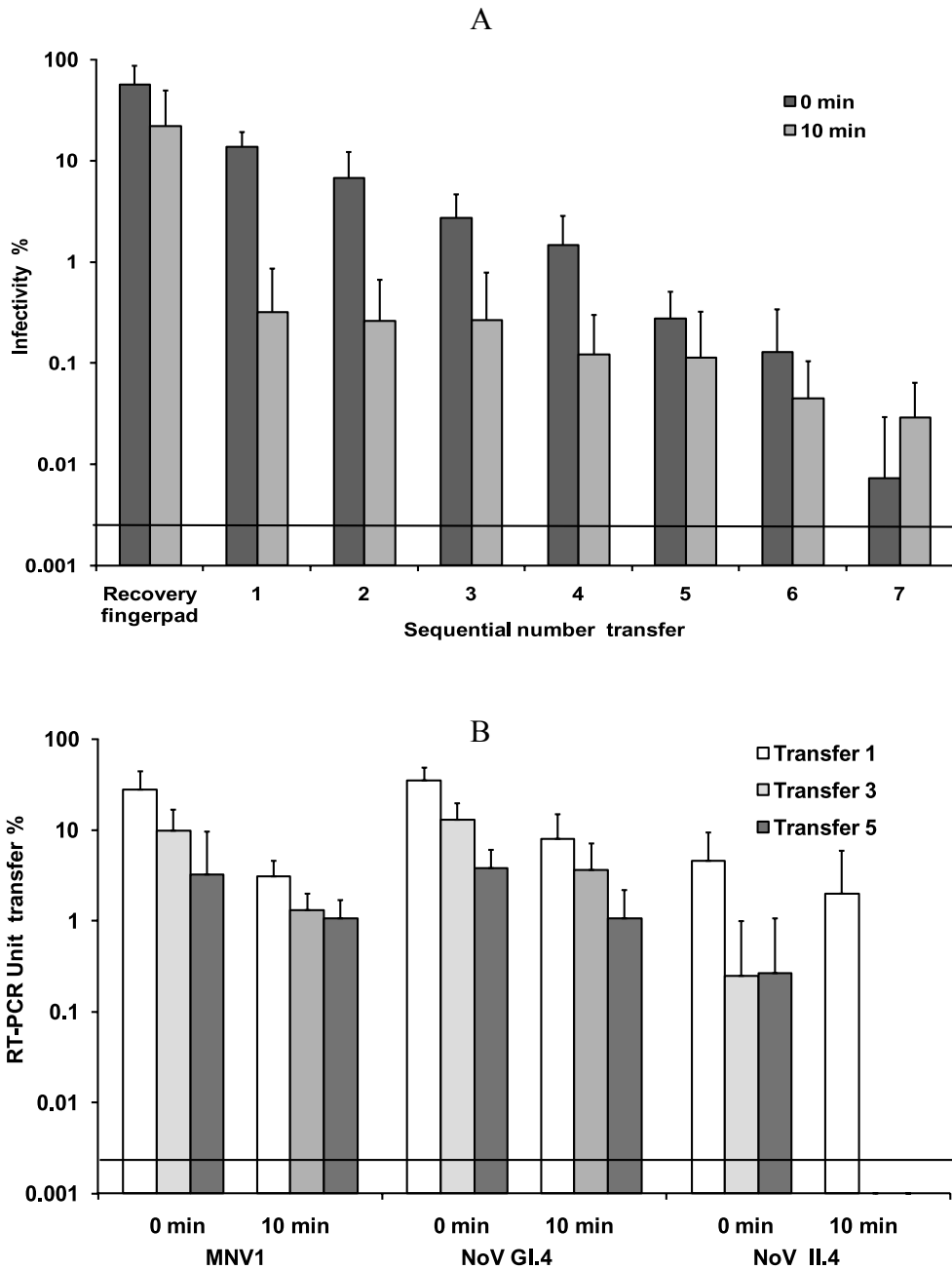


Figure 3: MNV1 infectivity transfer (A) and PCRU transfer of MNV1, human NoV GI.4, and GII.4 (B) from finger pads to whole tomato at 0 min and 10 min on sequential transfer. Horizontal lines show detection limit. Error bars show standard deviation of series ($n = 9$). The transfer percentage is calculated as fraction of the number of viruses applied.

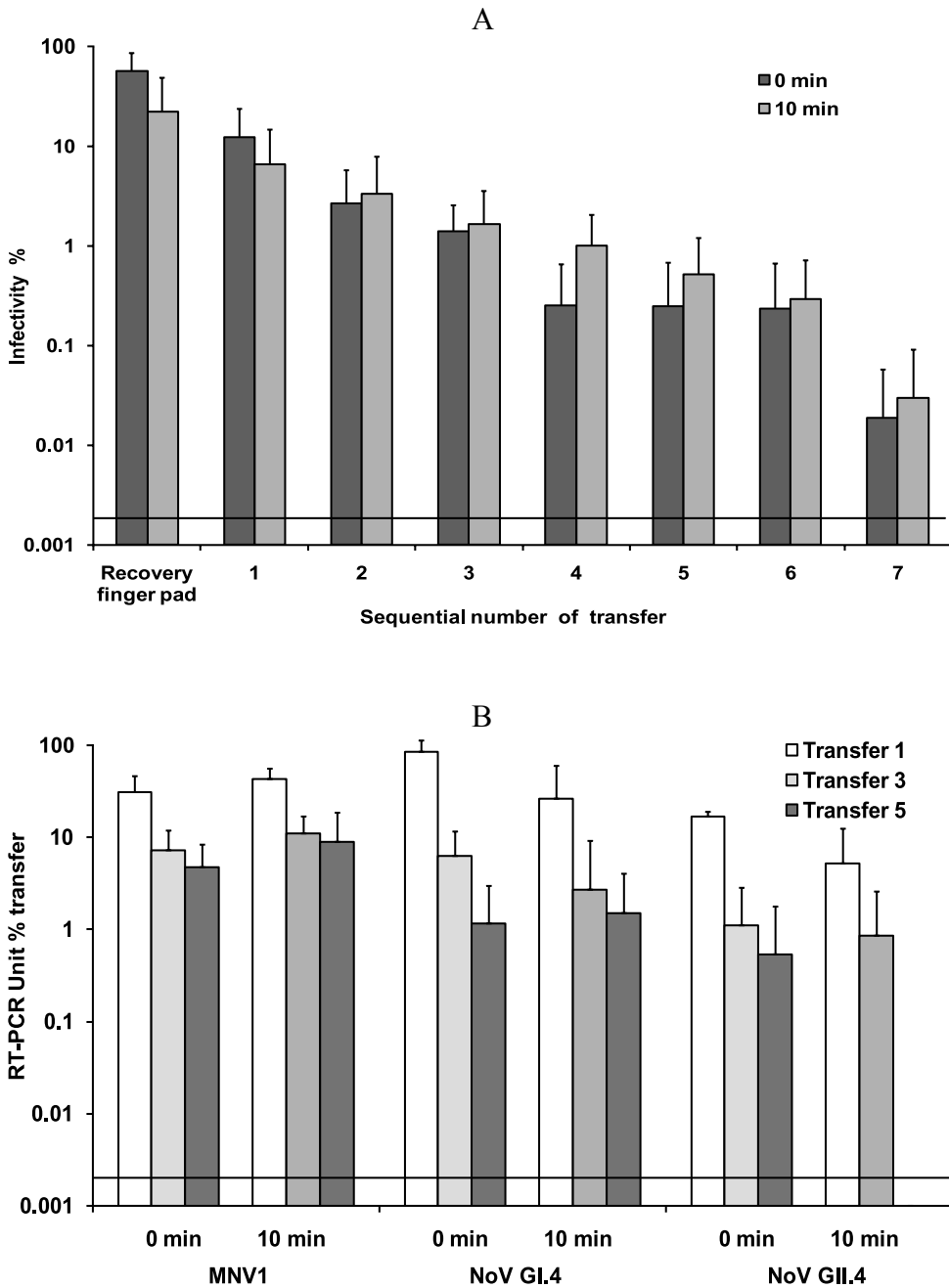


Figure 4: MNV1 infectivity transfer (A) and PCRU transfer of MNV1, human NoV GI.4, and GII.4 (B) from finger pads to cucumber slices at 0 min and 10 min on sequential transfer. Horizontal lines show detection limit. Error bars show standard deviation of series (n = 9). The transfer percentage is calculated as fraction of the number of viruses applied.

The PCRU transfers of MNV1, NoVs GI.4, and GII.4 from finger pads to whole tomatoes at 0 and 10 min are shown in figure 3 B. The PCRU transfer of MNV1 and NoV GI.4 at immediate transfer ($t = 0$) were significantly higher ($P < 0.05$) than at 10 min. There was no significant difference ($P > 0.05$) in the case of NoV GII.4 for the first transfer. On 3rd and 5th consecutive transfers, NoV GII.4 was below the detection limit. The PCRU transfers of MNV1 and NoV GI.4 to tomatoes at immediate transfer ($t = 0$ min) were comparable ($P > 0.05$), whereas the transfer of NoV GII.4 to tomatoes at immediate transfer ($t = 0$ min) is significantly lower ($P < 0.05$) than MNV1 and NoV GI.4. The PCRU transfers from finger pads to cucumber slices are shown in figure 4B. On cucumber slices, PCRU transfers of MNV1, NoV GI.4, and GII.4 at 0 and 10 min were significantly different ($P < 0.05$).

Discussion

In the majority of human NoV outbreaks, person to person transmission has been described as one of the most important routes. Cross contamination from contaminated fingers to food and fomites has been described as significant means of transmission (45, 49). The main aim of this study was to quantify the transfer of NoVs from fingers to fomites and foods to gain a better understanding of the spread of the viruses. We have chosen stainless steel and Trespa® as carriers representative for nonporous inanimate surfaces which can be found in kitchens, hospitals, and health care settings as for example food contact surfaces or other surfaces often touched by humans and found to be contaminated by NoV (9-11). Norovirus has been shown to be persistent on environmental surfaces like soil, stainless steel (12, 18) formica, ceramic (31), and also capable of attaching and surviving on food materials like lettuce (44) and strawberry (35).

Fingers are the most active part of the hands that come in contact with different surfaces and foods. In this study, the infectivity transfer of MNV1 to stainless steel, Trespa® and to tomatoes was high (10 to 100 times the infectious dose up to 5 or 6 transfer) when transferred immediately and significantly decreased as the virus was dried on the fingers for 10 min. This is consistent with data on transfer of HAV which also showed a decrease on transfer when drying time increased (36). The MNV1 infectivity transfer was approximately 0.1% on both the carriers after 10 min drying on the finger pad. The transfers are lower than transfer of rotavirus (16 %; (1)), HAV (27%; (36)) and FeCV (13%; (6)) to stainless steel carriers after 20 min drying on the finger pads. The higher HAV transfer might be explained by different transfer technique (rotating the finger in half circles for ten times over 10 s contact (36)), whereas the high rotavirus transfer might be due to its higher recovery on the finger pads anyway, namely $> 60\%$ in 20 min for rotavirus compared to 20% of the MNV1 in 10 min. Similarly, higher FeCV transfer might also be due to the higher survival of the virus (71 % in 20 min; (6)); than MNV1. Overall, the transfer is lower after drying than on immediate transfer. This might be explained by the fact that moisture facilitates the transfer (8, 36). As a lower percentage of the viruses are transferred after drying, and the number of

infective viruses transferred was lower than the number required for the infection in some of the transfers, perhaps risk is associated with higher level of shedding than the transfer after drying. Nevertheless, the observed transfer rates showed considerable contribution of hand in spreading the virus.

Fresh products such as tomatoes, cucumber, and raspberries contaminated with NoVs via food handlers are implicated in a number of outbreaks (5). These products are frequently eaten raw individually or combined with salads. Visually observed higher moisture level on the cucumber slices compared to tomatoes may account for observed higher transfer percentage of MNV1 infectivity from the dried finger pads. This shows that transfer from hand to moist and wet vegetables could be higher than to dried surfaces and thus moisture might increase the risk of infection. A similarly high transfer was also reported for FeCV (46%) to moist ham (6). A previous study indicated nearly 66% transfer of porcine enterovirus type 3 by a fecally contaminated human finger to whole tomato (13), which is higher than we showed in this study for MNV1. This difference in transfer may be due to a number of different factors including differences in virus recovery method and binding affinity of the virus to the product which was also reported for FeCV and echovirus 11 on butter head lettuce (48). Thus different binding affinity of the viruses might have a role in different transfer capabilities. In addition, transfer capabilities might differ per individual due to variability in moisture, soil and skin composition of the hands.

The amount of the NoV excreted in feces from infected individuals ranges from 10^6 to 10^9 particles per g (37). The amount of fecal material that can be present on human hands due to unhygienic practices is unknown. Nevertheless assuming the amount of fecal material possibly present on hand as 1 mg, the amount of virus would be 10^3 to 10^6 virus particles. Since not all virus particles may be infectious, if we assume that half of the particles are infectious as estimated by Teunis et al., (43) for NoV GI.1, then 1 mg of fecal material would contain between 5×10^2 and 5×10^5 infectious viruses. If the upper limit is used and in the case of approximately 14% transfer to tomatoes and 12% to cucumber slices, approximately 6.8×10^4 and 6.2×10^4 infectious virus particles would be transferred respectively, which would be largely sufficient to initiate infection in susceptible individuals on consumption.

Surfaces contaminated with NoV can be a source of transmission (4, 16, 31). Indirect transmission of the virus from contaminated surfaces by touching with hands has been thought to be a potent source of transmission (17, 32). In the possible case of 13% infectivity transfer in wet conditions, transfer would be 6.5×10^4 infectious virus from a contaminated finger pad to surfaces and from a dried finger pad (10 min) approximately 5.0×10^2 infectious virus (0.1%) transfer to the surfaces. Such contaminated surface might remain a potent source of transmission if not cleaned and disinfected. When approximately 6.5×10^4 infectious virus would be present on the surfaces transferred from a wet finger pad, after 40 min drying about

40% of the virus (data from this experiment) would still be infectious (2.6×10^4). In the case of approximately 4% transfer from the contaminated surface to finger pad, an approximate 1×10^3 infectious virus particles will be transferred to the finger pad, which would be sufficient to initiate infection on direct ingestion or indirectly by further transmission from the hand. Continuing this calculation, a level of contamination on toilet surfaces after leaving the toilet needs to be reduced to as low as 200 infectious particles. Then the risk of infection after touching the contaminated surfaces will be minimal, as the transfer percentage is approximately 4% (8 infectious particles) and the number of the infective virus required for infection has been described to be low as 8 particles (43). With this scenario it seems that the risk of infection is associated with the high level of shedding ($> 10^8$) (37). The high level of shedding of NoV has been found both in symptomatic and asymptomatic patients (3, 41). However, symptomatic shedders are more often involved in transmission events of the virus than rare involvement of asymptomatic shedders in hospital setting.

We presented RT-PCR data on transfer potential of NoVs. The amount of viral RNA estimated by RT-PCR does not necessarily correlate with the number of infectious viruses quantified by cell culture assays (46). It is unlikely that all the genomic copies detected by RT-PCR were associated with infectious viruses as a larger fraction of noninfectious viruses is present in all virus stocks (23). However, the data on PCRU transfer provided an estimate of transfer potential of the NoVs since an amount of infectious particles higher than the number of PCRU is rather unlikely. Both NoVs GI and GII have been implicated in NoV outbreaks, however, GII strains are more often associated with outbreaks (7). In our study, transfer percentage of MNV1 and NoV GI.4 was comparable but NoV GII.4 transfer percentage was significantly lower when transferred from hand to stainless steel and Trespas® at immediate transfer. The reason of proportionally higher number of outbreaks by GII strains was attributed to possible environmental stability or host susceptibility (31), and higher levels of shedding (37). In addition, it might also be due to a lower transfer percentage and different binding affinity of different genogroups of noroviruses as shown for shellfish (33) and lettuce (44). However, more in-depth studies are needed to confirm lower transfer percentages of NoV GII.4 than NoV GI.4 since the lower cannot be shown after drying. The lower transfer might also be due to lower initial concentrations of NoV GII.4 than NoV GI.4 in the stock suspensions.

The transfer of the virus is possible even after the virus is dried on the surface of hands or carriers. Control and prevention of human NoV should focus on interruption of the virus transmission through hand hygiene practices (34) and disinfection of the contaminated surfaces (47) giving a special focus on high risk areas in health care settings and food preparations facilities. The quantitative data on transfer of the viruses will help to determine efficacy of implementing prevention methods and can be useful in risk assessment models and to establish target levels for efficacy of transmission intervention methods.

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Chapter 7

Reducing viral contamination from finger pads: hand washing is more secure than alcohol based hand disinfectants

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Abstract

Hand hygiene is important for interrupting the transmission of viruses through hands. The effectiveness of alcohol based hand disinfectant has been shown for bacteria but their effectiveness in reducing transmission of viruses is ambiguous. Efficacies of a propanol and an ethanol based hand disinfectant against human enteric and respiratory viruses were tested in carrier tests. Efficacy of an alcohol based hand disinfectant and hand washing with soap and water against norovirus GI.4, GII.4, and MNV1 were tested using finger pad tests. The alcohol based hand disinfectant reduced the infectivity of rotavirus and influenza A virus completely within 30 s whereas poliovirus Sabin 1, adenovirus type 5, parechovirus 1, and MNV1 infectivity were reduced $< 3 \log_{10}$ within 3 min. MNV1 reduction by washing hands with soap and water for 30 s ($> 3.0 \pm 0.4 \log_{10}$) was significantly higher than treating hands with alcohol ($2.8 \pm 1.5 \log_{10}$). Washing with soap and water for 30 s removed genomic copies of MNV1 ($> 5 \log_{10}$), NoVs GI.4 ($> 6 \log_{10}$) and GII.4 ($4 \log_{10}$) completely from all finger pads. Treating hands with propanol-based hand disinfectant showed little or no reduction to complete reduction with mean genomic copy reduction of NoVs GI.4, GII.4, and MNV1 being > 2.6 , > 3.3 and $> 1.2 \log_{10}$ PCRU respectively. Washing hands with soap and water is better than using alcohol based hand disinfectants in removing noroviruses from hands.

Introduction

Human norovirus (NoV) is the leading cause of acute gastroenteritis affecting people of all age groups. Virus transmission takes place directly by person to person contact and / or indirectly via contaminated food, water and environmental fomites (12). The majority of NoV outbreaks are associated with person to person transmission (13) and hands are thought to be a principal vehicle for transmission of viruses, directly or indirectly. Besides human NoV, other pathogenic viruses like hepatitis A virus and poliovirus (21) can remain infectious on human hands for hours. Transfer via hands was assessed in experimental settings and found to be approximately 10% for hepatitis A virus from fingers to lettuce and $7 \pm 8\%$ for NoV from fingers to food (cucumber slices) (1, 25). The enteric viruses poliovirus, parechovirus, rotaviruses, and human NoV are shed in feces and transmitted through the fecal oral route, therefore hands are likely vehicles for transmission of the viruses, directly or indirectly (14, 27). The respiratory influenza virus and adenovirus are both secreted in respiratory secretions and may also be transmitted by contaminated hands (23). Influenza virus is implicated in a number of epidemics and occasional in pandemics, which makes it crucial to decrease or stop transmission via hands. Adenovirus type 5 is an interesting virus, as the virus is secreted both in feces and in respiratory fluid (18).

Hand hygiene is important for interrupting the transmission chain of viruses through hands. Normally different types of non-medicated or antimicrobial soap or alcohol based hand disinfectants are used. The latter are widely used in hospitals and health care facilities, due to convenience, rapidity, and broad acceptance by health care personnel (9). The effectiveness of alcohol based hand disinfectant has been shown for bacteria but their effectiveness in reducing transmission of viruses is less certain, therefore, it is important to know their effectiveness against a range of viruses.

Human NoV outbreaks are frequently occurring in nursing homes, hospitals and long term healthcare centers, so in particular in places where people live closely together (11). In some studies, alcohol-based hand disinfectants have been shown to be effective against NoV (15), in other studies its effect has been disputed in other studies (19) and in some NoVs outbreaks in health care facilities the use of alcohol hand disinfectants by health care personnel was identified as a possible risk factor for these outbreaks (3).

The purpose of the study was to test antiviral efficacy of two alcohol based hand disinfectants against enteric and respiratory viruses that are likely to be spread by hands i.e., the enteric viruses poliovirus Sabin 1, parechovirus 1, simian rotavirus SA11 (model virus for human enteric rotavirus), human NoVs GI.4 and GII.4, murine NoV (MNV1), which is a cultivable model virus for human NoV (4) and respiratory viruses influenza A (H1N1) virus and adenovirus type 5 by quantitative carrier tests. In addition, we evaluated the effectiveness of

an alcohol based hand disinfectant and traditional washing with water and soap in reducing human NoVs and MNV1 from artificially contaminated finger pads of human volunteers. The removal of, and virucidal efficacy against all the viruses was tested by cell culture assays, however, as human NoVs could not be cultured (6), the effectiveness against human NoVs was tested only by quantitative PCR.

Materials and methods

Viruses and cells

Viruses used for the test were poliovirus Sabin 1 (vaccine strain), simian rotavirus SA 11 (ATCC nr. VR-1565), adenovirus type 5 (Hu/adenovirus/type 5/6270/1988/Ethiopia), influenza A (H1N1) virus (Hu/influenza A /266/2008/Netherlands (H1N1) virus), parechovirus 1 (Hu/parechovirus/type 1/147/2008/Netherlands), MNV1 (Mu/NoV/GV/MNV1/2002/USA), human NoV GI.4 (Hu/NoV/GI.4/946/2009/ Netherlands) and human NoV GII.4 (Hu/NoV/GII.4/1803/2008/Netherlands).

The virus stocks were prepared by infecting monolayers of the respective cells as described before (24). Poliovirus and adenovirus were cultivated on Hep-2 cells, rotavirus on MA-104 cells, MNV1 on Raw-264.7 cells, parechovirus 1 on HT-29 cells and influenza A virus was cultivated on MDCK-1 cells. Human NoVs stocks were prepared as 10% w/v stool suspensions in Dulbecco's Modified Eagle Medium (DMEM) as described before (24) and filtered through 0.2 μm pore size filters. The NoV suspensions were free of rotaviruses, enteric adenoviruses, astroviruses, and sapoviruses as determined by PCR (24). The virus stocks were stored at -80°C . The titer of the virus stocks used were as follows: poliovirus Sabin 1: 5.9×10^8 , adenovirus type 5: 2.1×10^8 , parechovirus 1: 2.1×10^9 , rotavirus SA 11: 1.1×10^8 , influenza A (H1N1) virus: 8.4×10^6 and MNV1: 5.3×10^6 50% Tissue Culture Infective Dose (TCID₅₀)/ ml. The human NoVs GI.4 and GII.4 stocks were 5.6×10^8 and 7.5×10^6 PCR units (PCRU)/ ml respectively.

Preparation of bovine serum albumin solution and sterile stool suspension

Bovine serum albumin fraction V (BSA) was prepared as described before (24) and sterile stool suspension from a healthy volunteer was prepared (24) and the suspension was free of noroviruses, rotaviruses, enteric adenoviruses, astroviruses and sapoviruses as determined by PCR (24).

Alcohol based hand disinfectants and hand washing soap

Sterillium (Propan-2-ol 45% (w/w), propan-1-ol 30% (w/w) and Mecetroniumetilsulfate 0.2% (w/w), Bode Chemicals, Germany) and Sterillium Viruguard hand disinfectants (ethanol 95% w/w, Bode Chemicals, Germany; further referred to as Viruguard) were tested. Unicura hand soap (Colgate Palmolive, active components: pareth sulfate, sodium benzoate, sodium salicylate, benzyl salicylate) was tested in finger pad tests.

Cell toxicity testing of alcohol based hand disinfectants

The alcohol based hand disinfectants were diluted in Dulbecco's Modified Eagle Medium (DMEM) (Gibco, Germany) at the ratio of 1:10 and up to 1:500. The dilutions were then inoculated into 2×10^5 cell suspension of Hep-2, MA-104, Raw-264.7, MDCK-1 and HT-29 cells in a 96 well plate separately and incubated at 37°C. Morphological changes in the cells were studied until 24 h.

Quantitative carrier test of alcohol based hand disinfectants

Stainless steel carriers (AISI-type 304 standard, Wageningen, Netherlands) 1.4 cm × 1.5 cm in size were degreased by being dipped into acetone for 10 min, followed by being rinsed five times under running tap water and then soaked in 70% (v/v) ethanol for 30 min and dried. The carriers were then sterilized by autoclaving at 121°C for 15 min. The quantitative carrier test was performed in clean conditions in the presence of 0.03% (w/v) BSA according to European standard NEN-EN 14476 (7) and in dirty conditions in the presence of 1% (w/v) stool suspension. Since human stool is not the natural matrix for influenza A virus, experiments with the influenza A (H1N1) virus were performed in clean conditions only. The human NoVs were used as 10% (w/v) stool suspensions and no extra feces was added.

Thirty µl of the virus suspension in 1% stool or 0.03% BSA was dispensed and spread at the center of each sterile carrier separately and allowed to dry inside a biohazard cabinet for 1 h at room temperature (22-25°C, 40-45% RH). The visibly dried carriers were kept inside sterile flat bottom tubes (Sarstedt 60.9922.115, Germany) with the inoculated surface facing upwards. Then 60 µl Sterillium or Viruguard were loaded onto the contaminated carriers for 10, 30, 60 s and 2 and 3 min. After the exposure time, the reaction was stopped by adding 2940 µl cold DMEM (4-8°C) with 10% fetal bovine serum (DMEM-FBS). Thereafter, the virus was extracted by vortexing at maximum speed for 30 s and the carrier was subsequently flushed with the medium 10 times by pipetting. The suspension was then collected and the appropriate cell culture assay was performed to enumerate the number of infective virus particles. The samples were also tested for PCRU reduction.

Contaminated dried carriers were kept as controls. For neutralization controls, 60 μ l disinfectant were mixed with 2940 μ l DMEM-FBS and then added to the virus dried on the carrier. No reduction in virus titer was observed (data not shown). All the experiments were performed in triplicate and repeated for confirmation (n = 6). Log₁₀ reduction was calculated by subtracting log₁₀ virus of treated samples from log₁₀ virus of the dried controls.

Volunteers

The study protocol was reviewed and approved by the medical ethics review committee of Wageningen university (METC number: 12/01; NL number: 39407.081). Viral decontamination efficacy of washing hands with soap and water or by rubbing with Sterillium (that showed the highest infectivity reduction of MNV1 in the carrier test of the 2 alcohol based hand disinfectants tested) was tested against human NoVs GI.4, GII.4 and MNV1 in dirty conditions (1% stool) by a finger pad test. Before the start of the experiment signed consent forms from the participants were obtained.

Inspection and preparation of hands before the tests

One male and two females participated in the finger pad test. Prior to every test, both hands of each panelist were inspected carefully to make sure that they were free from any apparent cuts, scratches, or damages. Each panelist then washed their hands thoroughly using non medicated soap (Hegron Cosmetics, Netherlands) and running tap water (15°C) for 40 s and dried the hands with paper towels. Approximately 2 ml of 70% (v/v) ethanol was dispensed onto the palm of panelist hands, which was rubbed over the entire surface of both hands until hands were dry. The test procedure was then initiated by dropping 10 μ l virus suspension on a finger pad.

Finger pad test

To determine the number of virus particles on finger pads directly after contamination (t = 0 minute), the applied virus was eluted immediately by placing the inoculated area over the open mouth of a cryovial (3.6 ml capacity: Nunc, Denmark) containing 1 ml DMEM medium. The vial was inverted with the finger pad still pressed to it, so that the medium was brought in contact with the skin and 20 inversions were carried out. The vial was then turned upright and the finger pad was scraped downwards over the inside rim of the vial to recover as much of the remaining fluid as possible into the vial. For the dried controls, finger pads were contaminated with 10 μ l test virus and dried for 10 min (visibly dry) inside a biohazard cabinet at room temperature (22-25°C, 40-45% RH). The dried inoculum was eluted as described above. Three finger pads from each of the three individuals were tested for both baseline 0 and 10 min drying controls. Finger pads of left and right hands of the 3 individuals were used for the tests. One individual was tested twice (n = 12).

Decontamination of finger pads

After the tests, the finger pads were immediately decontaminated by pressing for 1 minute onto tissue paper soaked with 5000 ppm free chlorine solution in a petridish. Thereafter hands were washed thoroughly with water and soap as described before.

Finger pad test for testing hand washing with soap and water

The efficacy of removing the test viruses by washing hands with soap and water was tested as in a real life situation. Hands of the panelists were inspected and disinfected as described above. The finger pads were contaminated with 10 µl test virus and dried for 10 min (visibly dry) at room temperature (22-25°C, 40-45% RH) inside a biohazard cabinet. One ml soap (Unicura Hand soap, Colgate Palmolive) and 3 ml tap water (15°C) was dispensed onto the palm of one hand and the panelist rubbed both hands to distribute the soap over the whole surface. Thereafter, hands were washed for 15 s under running tap water (15°C). Then hands were dried with tissue paper. The remaining virus on the finger pads was eluted as described above. For the MNV1 infectivity test, the samples were filtered through 0.22 µm pore size filters (Whatman, Germany) and used for a cell culture assay.

Test of alcohol based hand disinfectant on fingers

Among the two alcohol based hand disinfectants, Sterillium showed the highest infectivity reduction of MNV1 in the carrier test. Therefore, this disinfectant was further tested with the finger tests. One ml of Sterillium was rubbed on the hands for 30 s and the extract was collected from the finger pads as described for the finger pad test. The extract was then inoculated into Raw 264.7 cell suspension (2×10^5 cells / ml) and incubated at 37°C for 24 h. The finger pads were contaminated with 10 µl test virus and dried for 10 min (visibly dry) in the same conditions as explained before (*Inspection and preparation of hands before the tests*) at room temperature (22-25°C, 40-45% RH) inside a biohazard cabinet. One ml of Sterillium was dispensed onto the palm of one hand and panelists rubbed both hands for 30 s. The remaining virus on the finger pads was eluted by inverting a cryovial with the medium as described above. All samples were frozen at -20°C until further analysis. Reduction of the viruses on the finger pads was determined with reference to the dried controls at 10 min. To check if virus transfer from finger pad to hand palm occurred, after the test with alcohol and washing hands with soap and water, the palm was swabbed with swab sticks (COPAN CEO344- rayon swab material) moistened with DMEM. The swab stick was then transferred into a tube with 500 µl DMEM and vortexed at maximum speed for 1 minute and the suspension was frozen until analysis.

Decontamination of hands

After the tests were performed, participants rubbed their hands with 2 ml 5000 ppm free chlorine solution for 1 minute, followed by traditional hand washing with water and soap.

Enumeration of infective viruses

The infective viruses were enumerated by titration of 10 fold serial dilutions in 96 well plates on sensitive cells using the TCID₅₀ approach and virus titers were calculated by the Spearman-Kärber method (24).

Viral nucleic acid extraction and real time PCR

To allow comparison of virus reduction between cultivable viruses and non-cultivable human NoVs, quantitative PCR assays were performed. Viral nucleic acid extraction was performed using Magna Pure total nucleic acid extraction kit (24). Real time PCR assays for poliovirus Sabin 1, adenovirus type 5, rotavirus SA 11, parechovirus 1, MNV1, human NoV GI.4 and NoV GII.4 were performed as described before (24). PCRU were determined by slopes of standard curves made for each virus. The standard curve was made by plotting cyclic threshold (Ct) values versus log PCRU of 10 fold dilutions of the virus stocks. The highest dilution giving a positive result was assigned a value of 1 PCRU. To screen for PCR inhibition, 60 µl hand disinfectant was mixed with 2940 µl DMEM–FBS and then added to 30 µl virus with 1% stool dried on the carrier and tested for PCR. The reduction of genomic copies from 0 to 3 min treatment was calculated for the quantitative carrier tests.

Results

Both alcohol based hand disinfectants were non-toxic to cells tested at 1:200 dilutions. No PCR inhibition was detected with and without stool samples with hand disinfectants from the carrier tests and with stool samples from the finger pad tests.

Infectivity reduction of viruses in carrier tests

The recovery of infective viruses from the carriers ranged from 20 ± 11% to 79 ± 14% for parechovirus 1 and MNV1, respectively. The virucidal efficacy of the two alcohol based hand disinfectants tested against the viruses in clean and dirty conditions is shown in Figure 1. The enveloped influenza A (H1N1) virus was inactivated completely (> 5 log₁₀) within 30 s by both Sterillium and Viruguard under clean conditions. Similarly, rotavirus SA 11 was inactivated completely (> 6 log₁₀) at 30 s in both clean and dirty conditions by Sterillium, however for Viruguard, a contact period of 2 min was needed for complete inactivation (> 6 log₁₀). Both disinfectants reduced the infectivity of poliovirus Sabin 1, adenovirus

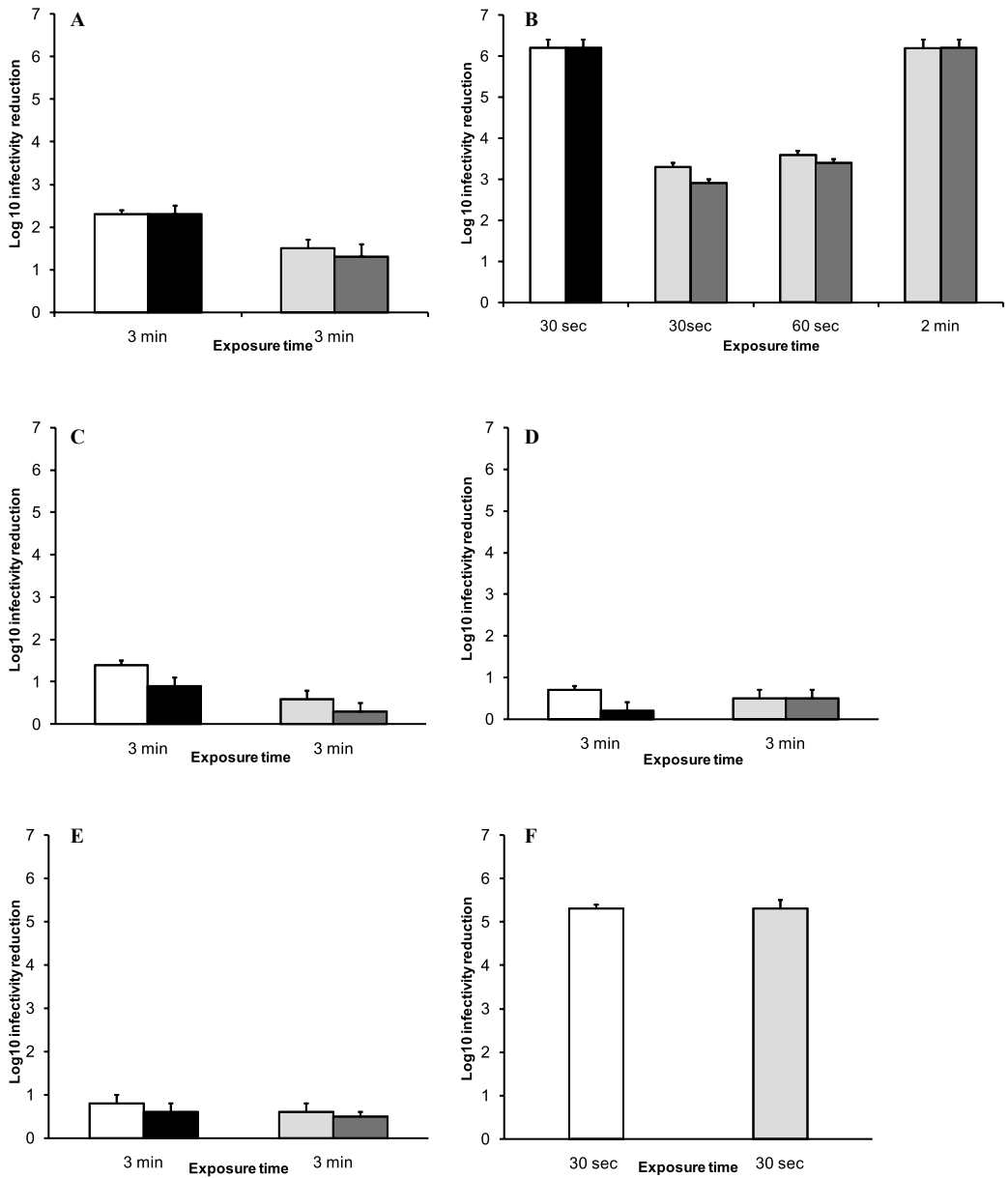


Figure 1: Effect (expressed as log₁₀ infection reduction) of Sterillium in clean (white) and dirty (black) conditions and Viruguard in clean (grey) and dirty (dark grey) conditions against MNV1 (A), rotavirus SA 11 (B), poliovirus Sabin 1 (C), parechovirus 1(D), adenovirus type 5(E) and influenza A (H1N1) virus (F) at different exposure time in carrier tests (n = 6). Error bars indicate standard deviation.

type 5, and parechovirus 1 with less than $1.5 \log_{10}$, even after 3 min of exposure time. There was no significant difference ($P > 0.05$) in infectivity reduction of poliovirus Sabin 1, adenovirus type 5 and parechovirus 1 at 30 s and 3 min both under clean and dirty conditions. The infectivity of MNV1 was reduced with about $2 \log_{10}$ units by Sterillium and $1.3 \log_{10}$ by Viruguard after contact times of 2 and 3 min. There was no significant difference ($P > 0.05$) in infectivity reduction of MNV1 in clean and dirty conditions after the contact times of 1, 2 and 3 min.

Reduction in genomic copies in carrier tests

No significant ($P > 0.05$) reduction of genomic copies of the tested viruses by Sterillium and Viruguard was observed for any of the tested viruses, including the enveloped influenza A (H1N1) virus and human NoVs GI.4 and GII.4 (data not shown).

NoV reduction from finger pads by alcohol based hand disinfectant and virus removal by washing with soap and water

The extracts from finger pads after rubbing with Sterillium and washing with soap and water were not toxic to Raw 264.7 cells and did not show PCR inhibition. The infectivity recovery of MNV1 from finger pads at 0 and 10 min were $57 \pm 28\%$ and $20 \pm 18\%$ respectively. The PCRU recovery of MNV1 from finger pads at 0 and 10 min was $63 \pm 9\%$ and $28 \pm 3\%$ respectively. PCRU recoveries of human NoV GI.4 from finger pad at 0 and 10 min were $79 \pm 25\%$ and $31 \pm 13\%$ respectively and for human NoV GII.4 $28.1 \pm 11.7\%$ and $9.3 \pm 5.6\%$, respectively.

Washing with soap and water (30 s) removed infectious MNV1 $> 3.0 \pm 0 \log_{10}$ to below the detection limit of 10 infective particles from all finger pads (infectious virus retrieved from 0/12), whereas rubbing with Sterillium reduced the infectivity with a mean value of $2.8 \pm 1.5 \log_{10}$, with the infective virus detected on 5 out of 12 finger pads (Figure 2). The average remaining infective MNV1 load on the 5 positive finger pads was $2.8 \pm 0.5 \log_{10}$.

Table 1: In vivo efficacy of Sterillium against MNV1 (1% stool) infectivity compared to that of soap and water

Treatment	Exposure time (sec)	No. of finger pads examined	Mean infectivity reduction ($\log_{10} \pm SD$) [No. of positive samples]	P value
Sterillium	30	12	2.8 ± 1.5 [5]	0.004
Soap and water	30	12	$>3.0 \pm 0.4^*$ [0]	

*Complete reduction

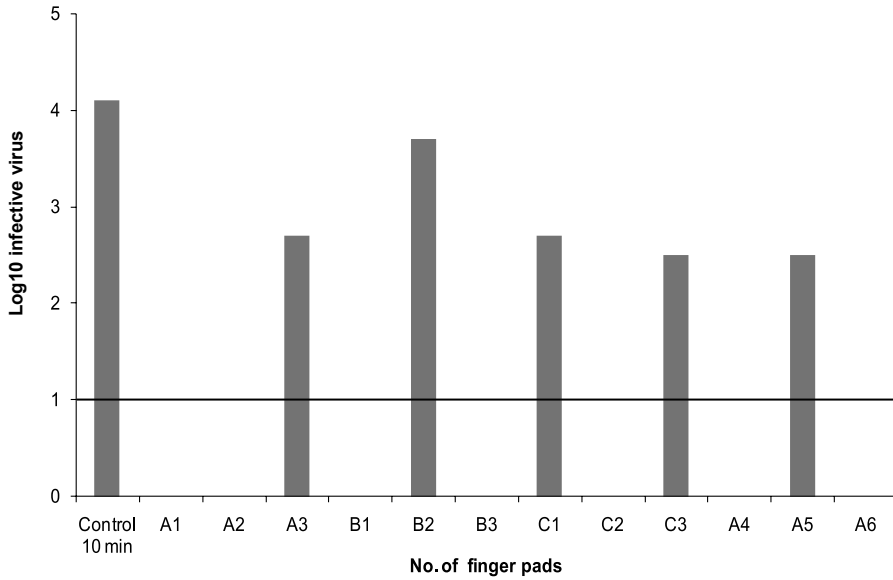


Figure 2: Effect of Sterillium against infective MNV1 (1% stool) after 30 s rubbing of the hands (n = 12). The infective virus was detected on 5/12 finger pads. Control is the recovery after 10 min drying on the finger pad. Horizontal line shows detection limit.

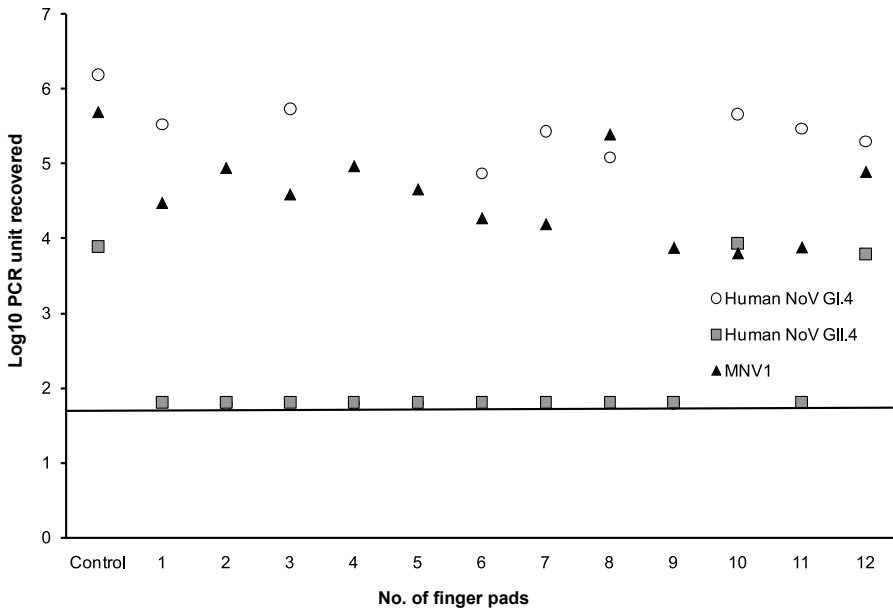


Figure 3: Effect of Sterillium on genomic copies of human NoV GI.4, GII.4 and MNV1 (1% stool) on finger pads (n = 12) after 30 s. Genomic copies of NoV GI.4, GII.4 and MNV1 were detected on respectively 8/12, 2/12, 12/12 finger pads. Control is the recovery after 10 min drying on the finger pad. Horizontal line shows detection limit.

The reduction of infectious virus is significantly higher ($P = 0.004$) for soap and water than for Sterillium as shown in Table 1.

The genomic copies of human NoVs GI.4 ($6.2 \pm 0.2 \log_{10}$ PCRU), GII.4 ($3.9 \pm 0.0 \log_{10}$ PCRU), and MNV1 ($5.7 \pm 0.2 \log_{10}$ PCRU), were removed completely from the finger pads (infective virus retrieved from 0/12 finger pads) by washing with soap and water. Genomic copies of NoVs GI.4, GII.4 and MNV1 were detected on 8/12, 2/12 and 12/12 finger pads respectively (Figure 3) after treatment with Sterillium. The mean genomic copy reduction of human NoVs GI.4, GII.4, and MNV1 by Sterillium was > 2.6 , > 3.3 and $> 1.2 \log_{10}$ PCRU respectively. The average remaining load of genomic copies on the finger pad, when calculated for positives only, was 5.3 ± 0.3 , 3.9 ± 0.1 and $4.5 \pm 0.5 \log_{10}$ PCRU respectively. PCRU reductions of human NoV GI.4 and MNV1 were significantly higher ($P < 0.05$) in washing with soap and water than by Sterillium treatment. We could not detect transferred NoV contamination on the palm after washing with soap and water or treating with Sterillium (detection limit of 60 PCRU).

Discussion

In this study, the virucidal efficacy of two commonly used alcohol based hand disinfectants against enteric and respiratory viruses was determined in quantitative carrier tests. Sterillium is a propanol based hand disinfectant and Viruguard is ethanol based. The European standard for testing EN 14476 requires at least $4 \log_{10}$ virus infectivity reduction within a defined exposure time of 30 s or 1 min (obligatory) (7). A 30 s exposure time for hand disinfectant would be the most applied practice.

Both hand disinfectants meet the EN 14476 criteria for influenza A (H1N1) virus. The fast inactivation of the virus is probably due to the destruction of the lipid layer of the envelope which is susceptible to chemicals (20). The EN 14476 criteria were also met with Sterillium for rotavirus, in both clean and dirty conditions. It has been described that rotavirus is sensitive to alcohol as the outer capsid layer is quickly removed rendering the double layered non-infectious particle (17).

For other enteric viruses poliovirus Sabin 1, parechovirus 1, MNV1 and respiratory adenovirus type 5, the alcohol based hand disinfectants showed insufficient infectivity reduction of even less than $1 \log_{10}$ within 30 s. Similar low infectivity reductions were shown before for enteric hepatitis A virus and poliovirus type 1 on human skin by 70% ethanol within 10 s (16), MNV1 by 80% ethanol at a 30 s exposure time (19) and human enterovirus 71 by 75% ethanol at 30 s exposure (5). Thus the tested hand disinfectants showed relatively poor efficacy against non-enveloped human enteric viruses except for rotavirus. However a contrasting result has been reported before, where a gel based on 85% ethanol was shown to be effective with a reduction factor > 4 against poliovirus within 3 min and adenovirus within 2 min (10).

Similar activity of isopropanol during extended exposure time against Feline Calicivirus (50 to 70% isopropanol for > 3 min) has also been shown before (8).

Human NoV excreted in feces range from 10^6 to 10^9 virus particles per g whereas doses of the virus required to cause disease are low (22). The amount of fecal material that might be present on soiled human hands may vary from individual to individual. However, considering that 1 mg of the fecal material might be present on the hand due to unhygienic practices, the amount of viruses present will range from 10^3 to 10^6 virus particles. A study by Liu et al. (14), described an average of about 10^4 virus genomic particles per hand from NoV infected volunteers. In a scenario, when the transfer rate from hands to fomites (stainless steel) is about 10% (2), the amount of virus transferred from the hand would be up to 10^3 virus particles. Further transmission of the virus from contaminated hands is possible to fomites and food surfaces (25).

The removal of human NoVs and MNV1 by soap and water and the reduction by Sterillium was tested on artificially contaminated finger pads and the application was tested as in a real life situation. The infectivity reduction of MNV1 by alcohol in the carrier tests was significantly less ($P < 0.05$) than by the finger pads test at 30 s. The higher infectivity reduction might be due to the rubbing effect in the finger pad tests in case of Sterillium compared to the stationary setup studied in the carrier test. However rubbing hands with Sterillium showed variable reduction of NoVs GI.4 and MNV1 particles: from little or no reduction to complete reduction from the finger pads. On the other hand, in most cases NoV GII.4 particles could not be detected after treatment which might be due to the lower recovery of human NoV GII.4 from the dried finger pads and lower starting titer of the virus stock.

We showed complete removal of MNV1 and human NoVs from all fingers by soap and water which is significantly higher than inactivation by the alcohol based hand disinfectant tested for MNV1 and human NoV GI.4. The higher removal by soap and water might be due to the rubbing step and the rinsing effect of water and/or drying with the tissue paper. The physical removal of the viruses showed a significant role in efficacy of the hand hygiene.

In this study we showed that washing with soap and water is sufficient to remove $> 5\log_{10}$ virus particles of human NoV GI and MNV1 from the finger pads. But when contaminated hands will be treated with the alcohol based hand disinfectant, about 42% (5/12 finger pads) of the finger pads will still contain infectious viruses which might be further transferred. Though we have tested the use of an alcohol based hand disinfectant and washing with soap and water in a controlled environment, the observed effect might explain a number of human NoV outbreaks in health care settings, where health care personnel preferably used alcohol hand disinfectants routinely and during outbreaks (26). Thus use of alcohol based hand disinfectants might be a risk factor for NoV outbreaks as previously described for long term

care facilities (3). Therefore, the recommendation of using alcohol based hand disinfectants in health care facilities needs to be reconsidered. However, alcohol-based hand disinfectants will have some applications particularly when running water is not accessible.

The alcohol based hand disinfectants tested were virucidal for influenza A (H1N1) virus and rotavirus, but less effective against poliovirus, parechovirus, adenovirus, and MNV1. The low virucidal activity of the tested alcohol based hand disinfectants against epidemiologically important enteroviruses and NoVs and the incomplete reduction of infectious viral contaminations from finger pads will have important implications for infection control in health care settings and food production areas where these products are used. Our findings indicate that hand washing with soap and water is most effective in reducing viral contamination from finger pads and thus is the preferred intervention method to prevent transmission through hands in health care settings and food preparation facilities.

Acknowledgements

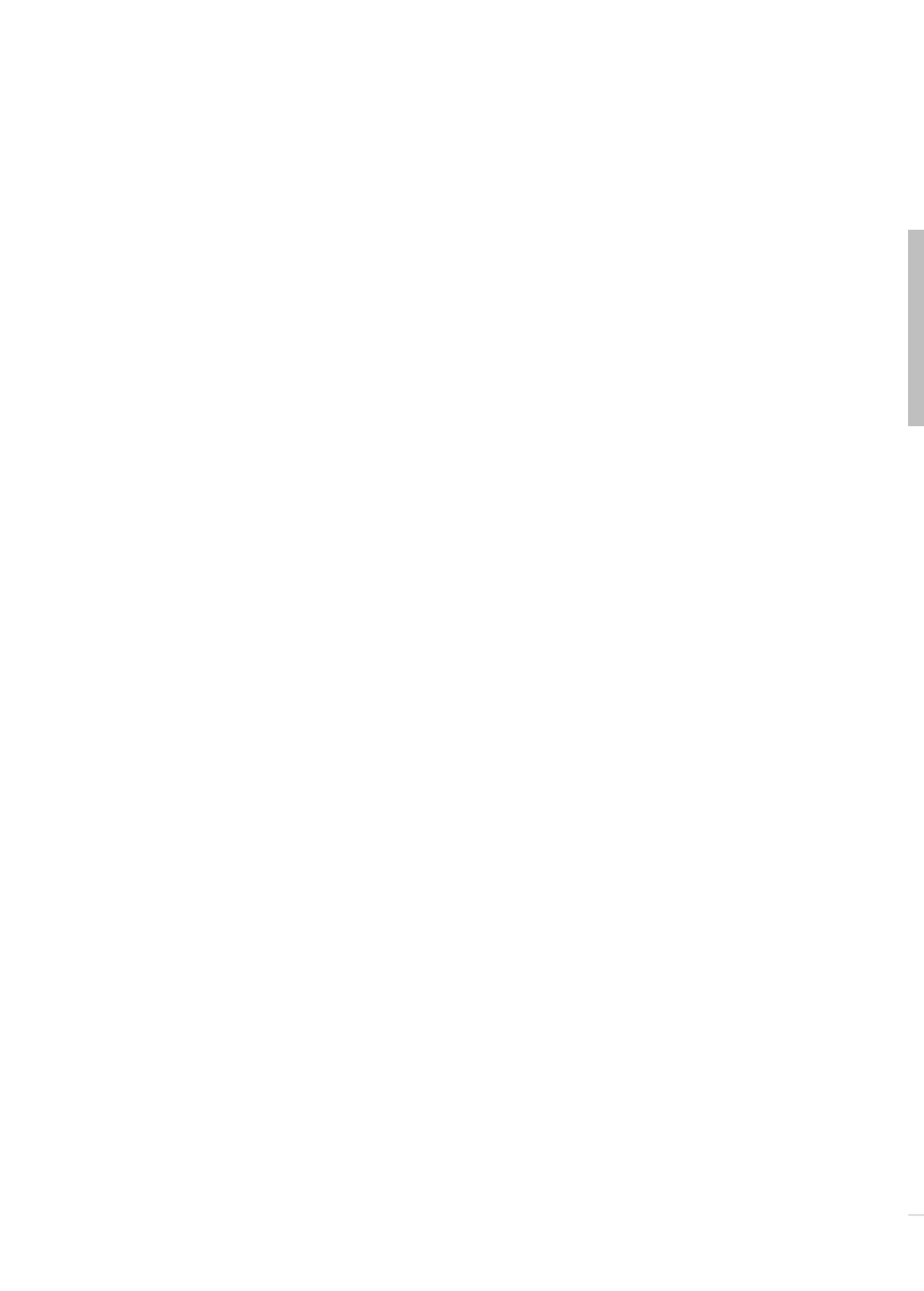
We would like to thank all volunteers participating in the finger pad tests.

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Chapter 8

General discussion

Viral gastroenteritis is one of the common illnesses that affect people in both developed and developing countries. For infectious gastroenteritis, human norovirus is the most common cause. Outbreaks of gastroenteritis affect people of all age groups (11, 45, 46, 76). Due to high numbers of outbreaks in health care facilities, individual homes and communities, the estimates for burden of disease are high (26, 34, 70). Norovirus and other enteric viruses are shed in feces and vomit and are often transmitted directly by person to person contact and indirectly via exposure to contaminated surfaces (13, 38, 44, 74) or foods. Besides enteric viruses, respiratory viruses can also be transmitted through direct and indirect routes.

This thesis focuses on methods to reduce the transmission of human noroviruses, other enteric viruses, and some respiratory viruses. The enteric viruses included in the study are human norovirus GI.4, GII.4, murine norovirus 1 (MNV1), poliovirus Sabin 1, parechovirus 1 and rotavirus SA 11. The respiratory viruses influenza A (H1N1) virus and adenovirus type 5 were also included in the study. Contaminated hands and surfaces are the main sources of transmission, and this thesis discusses interventions to reduce the transmission of the viruses via these sources. To estimate infectivity of viruses, culture methods are needed. As human norovirus cannot be cultured yet, our efforts to cultivate the virus are also presented. In this chapter, results described in the thesis and possible applications and implications are discussed. Furthermore, suggestions for future study are included.

Efforts to cultivate human norovirus

Since the discovery of the human norovirus, many efforts have been made to cultivate the virus. Duizer et al. (21) tried to replicate the virus and evaluated a number of different cell lines and laboratory methods. This study was conducted with an *in-vitro* cell-culture system that mimicks intestinal epithelium cells, gastric cells, duodenal cells and small intestinal cells. The cell-culture system was not successful in replicating the virus. Straub et al. (64)

described an *in-vitro* cell-culture system for human noroviruses, using an intestinal cell line INT-407 on collagen I coated porous micro-carrier beads in rotating-wall vessel (RWV) bioreactors. The authors suggested that human norovirus genogroups I and II could infect three dimensional organoid models of the cells. They described that a cytopathic effect was found and human norovirus genomic copies were detected at each passage of the infected cells. However, the level of virus replication and the quantity of newly synthesized virus particles are unclear as virus infectivity and the virus cell entry were detected but the level of replication has not been estimated (17). Leung et al. (39) reported a culture system using freshly collected duodenal tissue that closely resembles the primary site of infection *in-vivo* for human norovirus. They reported an increased viral RNA concentration in cell-free culture supernatants which kept increasing in time, as measured by quantitative real time PCR. This indicates increase in genomic copies (RNA strands) of the virus, however, complete virus particles or their infectivity were not confirmed. Thus for quantification of infectious viruses a culture method is still needed.

We have cultured INT-407 cells, and other human intestinal cell lines CaCo-2 (epithelial colorectal adenocarcinoma cells), HuTu-80 (human duodenum adenocarcinoma cell line) and HT-29 cells (human colorectal adenocarcinoma) in three dimensional structure on cytodex beads in a rotary cell-culture system (RCCS) (Figure 1) and on basal membrane extract (BME) containing laminin and collagen (Figure 2). Chang et al. (18) showed that inhibition of cholesterol biosynthesis using statins significantly increase the levels of norovirus protein and RNA in replicon bearing cells. Hydroxymethylglutaryl-coenzyme A (HMG-CoA) reductase is one of the important enzymes in cholesterol synthesis and statins are inhibitors of this enzyme. When norovirus replicon bearing cells were cultured in the presence of Simvastatin (a HMG-CoA reductase inhibiting statin), a significant increase in the level of norovirus proteins and RNA was shown (18). In our study, we infected three-dimensional cells with human norovirus GII.4 alone and in presence of Simvastatin. However, neither visible cytopathic effect on the cells, nor replication of the virus could be detected. Our attempts of culturing norovirus according to the method described by Straub et al. (64) and in presence of Simvastatin, on three-dimensional cells were not successful. Other attempts of culturing norovirus according to Straub et al. (64) have also been proven unsuccessful by several other labs (17, 39). However, further testing of both human and non-human cell lines with addition of infectivity enhancers and cell infection with other human norovirus genogroups, is still relevant. Moreover, efforts to culture on human biopsy cells from different parts of the intestinal tract may also be worthwhile, as the native organoid cells of a biopt might support the growth of the virus. As the cultivation method is yet not developed, other methods for estimating the infectivity can also be explored. Culture independent tests for infectivity screening, like using the intercalating dye propidium monoazide in quantitative reverse transcriptase PCR to distinguish between infectious and non-infectious enteric viruses (57) can also be an option. The treatment with proteinase K, RNase (6, 52) and other method

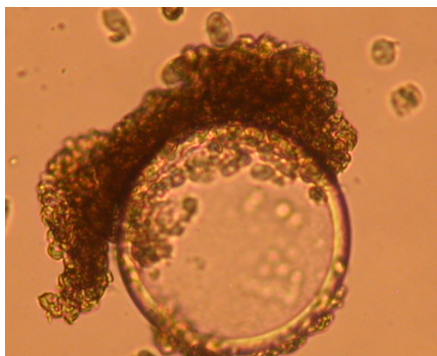


Figure 1: Three dimensional structure of HuTu-80 cells on cytodex bead in rotary cell culture system.

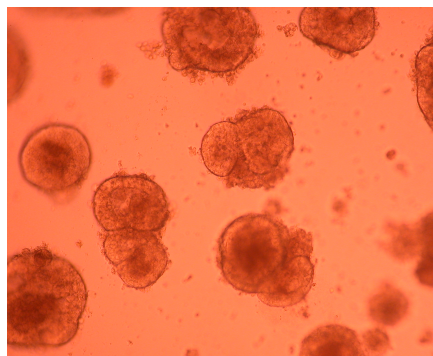


Figure 2: Three dimensional structure of Caco-2 cells on basal membrane extract system.

(40), were not robust and did not distinguish between infectious and non-infectious viruses. Since no culture methods could be developed and no culture independent test was reliable, this project was continued with MNV1 as a model to test norovirus infectivity.

Murine norovirus as a model virus for human norovirus

As human norovirus still cannot be cultured reliably, all the infectivity reduction and removal of this virus has been investigated using infectivity reduction data of a model virus and quantitative RT-PCR for the model virus and human norovirus. Murine norovirus (MNV1) is a better model virus for norovirus than Feline Calicivirus (FeCV), due to its genetic similarity to norovirus; it belongs to the same genus *Norovirus*, and MNV1 stability data correlates better to stability data of norovirus like particles and norovirus GII.4 (24, 41, 56) than FeCV (2, 14). MNV1 has the characteristic features of enteric viruses, capable of withstanding low and high pH (3-8) values. In this thesis, norovirus infectivity was assessed using MNV1 as a model virus. QRT-PCR was used as an alternative method for detection and evaluation of efficacy of disinfections for both MNV1 and human norovirus. Thus, the culture and quantitative PCR of MNV1 were compared with quantitative PCR of human norovirus.

MNV1 genomic copy and infectivity reduction were tested in physicochemical treatments with heat, alcohol, hydrogen peroxide vapour and chlorine solutions. In all these treatments, except for heat (56°C) and alcohol, MNV1 did not show similar reduction of genomic copies when compared with that of human norovirus (Table 1). Similar results have been shown by Wang et al. (72) with porcine sapovirus, an enteropathogenic calicivirus compared to human norovirus, where the porcine sapovirus was shown to have a similar stability as human norovirus during heat treatment (56°C for 30 min and 2 h), pH (3-8), chlorine (2.5 to 200 ppm for 30 min) and alcohol (60% and 70% for 5 min).

Table 1: MNV1 infectivity (\log_{10} TCID₅₀) reduction and RNA reduction (\log_{10}) of MNV1 and human norovirus GII after physiochemical treatments

Treatment	Parameter	Infectivity reduction	RNA reduction		RNA reduction
		MNV1	MNV1	HNv GII	P values
Heat	56°C for 30 min	1.8 ± 0.1*	0.3 ± 0.3	0.2 ± 0.0	> 0.05
Alcohol	Ethanol (95% w/w) for 3 min	1.3 ± 0.3	< 0.5	< 0.5	> 0.05
	Propanol (45% w/w) for 3 min	2.3 ± 0.2	< 0.5	< 0.5	> 0.05
H ₂ O ₂ vapor	127 ppm for 1 h (stainless steel)	4.1 ± 0.2	2.2 ± 0.2	0.5 ± 0.2	< 0.05
Chlorine solution	1000 ppm for 10 min	5.0 ± 0.0	6.3 ± 0.8	5.2 ± 0.0	< 0.05

*Infectivity in plaque forming units (the number of infectious virus per volume of sample); ± is the standard deviation of the mean

For those treatments where human norovirus reacts differently from MNV1, due to a difference in genomic stability, a different model virus might be needed. A model virus for human norovirus must (at least) show similar genomic stability. The porcine sapovirus may be an improved model virus; however, more information on stability and susceptibility towards other chemicals like hydrogen peroxide will be needed. In addition, stability of the model virus in food processing technologies like gamma radiation, high pressure processing, boiling and high temperature treatments (4) needs to be investigated.

Among enteric viruses, hepatitis A virus has been described to be a highly stable virus able to persist for extended time in the environment and it is stable in a number of treatments. As hepatitis A virus has been described to be the most stable enteric virus, the data for the stability of the hepatitis A virus can be used for other enteric viruses but the virus grows only slowly in cell culture systems and no or poorly visible cytopathic effect is observed. As norovirus was thought to be more stable than other enteric viruses, biphasic or non-log linear heat inactivation was expected for MNV1. But in this study we have shown that parechovirus is highly stable and showed a biphasic inactivation curve when heated 56°C for 30 min. Furthermore, the virus was not completely inactivated when heated at 73°C for 3 min in suspension. High stability of the parechovirus might be due to a resistant fraction of the virus. Due to the high stability, parechovirus can also be used as model for stable enteric viruses.

From Table 1 it is clear that results for MNV1 infectivity and genomic copies are not correlating. This phenomenon has also been published for other enteric and respiratory viruses such as poliovirus, hepatitis A virus (31, 53) and influenza A virus (35). Infectivity and genomic copy reduction do not correlate if treatments do not damage the capsid protein of a virus. In heat and alcohol treatments, viruses lose infectivity without damage of their

capsids (1, 37) and the genetic material is well protected within the capsid. The correlation exists in case of damage of the capsid protein. In higher temperature treatments above 80°C non intact capsid has been described (32). Throughout the experiments described in this thesis, reduction of the virus was compared with respect to the infectivity data of MNV1 and quantitative PCR data of both human norovirus and MNV1. The approach worked only when RNA damage could be measured and the reduction of human norovirus and MNV1 was correlated in those cases only. Detecting genomic copies of human norovirus by PCR might be associated with infectious virus, non-infectious inactivated virus or from degraded RNA from inactivated viruses. Thus detecting only genomic copies is not a reliable method.

Reduction of viral contamination from surfaces

Different surfaces can get contaminated with viruses and the viruses may be transferred between hands and surfaces (8) (chapter 6). Contaminated hands are one of the main sources of transmission of noroviruses and other enteric viruses. These viruses are most often transmitted directly by person to person contact. Norovirus contaminated hands are a good vehicle to transfer the virus to different surfaces and foods (chapter 6). Contaminated hands can transfer about 13% of virus infectivity on immediate transfer to about 0.1% after drying for 10 min from finger pads to surfaces. Furthermore, contaminated hands are able to transfer the virus to multiple surfaces by sequential touching (chapter 6). Though the numbers of infective viruses transferred to surfaces are low, contamination via hands is still a risk, as low numbers of the virus particles already have a high probability to cause infection. Transmission of the viruses from contaminated surfaces to individuals has proven to be responsible for a number of outbreaks (48, 58, 74).

Noroviruses transferred to surfaces can remain infective for a considerable amount of time. On stainless steel (14) and ceramic (23) norovirus has been reported to remain infectious for over 5 days. Noroviruses also have been found on floors, tables, door knobs, handles, bed rails, carpets and curtains both in health care facilities and individual homes (12, 27, 75). Besides noroviruses, other enteric and respiratory viruses have been found to be persistent on surfaces in hospitals (13, 74). PCR data on persistence of human norovirus on surfaces often do not reveal information on infectivity. The genomic copies of the virus might be persistent on surfaces for considerable time however infectivity might have been lost already. In this way, the PCR data may largely overestimate infectivity of the virus (chapter 2, 4).

Cleaning and disinfection in combination are effective in reducing the load of contaminants to a level where the risk of infection and continued transmission is minimal. Cleaning and disinfection are meant to reduce contamination but not meant for sterility. Reduction of infective load can be achieved by removal and inactivation (chapter 3). Removal of the microorganism indicates the physical removal, and may or may not be combined with a loss

of infectivity or viability. Inactivation indicates loss of infectivity, which can be achieved for example by using chemicals (chlorine, hydrogen peroxide, and quaternary ammonium compounds) and physical treatments (heat, UV and high pressure). The use of chemical disinfection is a key approach to interrupt transmission through contaminated surfaces. In tests, removal is characterized by a very good correlation between reduction in infective units and reduction in PCR units. However, for most inactivation processes, there is a very poor correlation between infectivity reduction and PCR unit reduction, since infectivity will be largely reduced while the reduction in PCR units is mostly very limited. The infectivity can be completely destroyed while all PCR units remain detectable. The other way round is not possible, if all PCR units are gone, then infectivity is gone too.

The removal or reduction, of virus load can be achieved by cleaning by wiping with water and soap or disinfection (chapter 3, 4 and 5). Wiping once with water and detergents (liquid soap) and low concentration of chlorine solution (about 250 ppm) removes the enteric and respiratory viruses about $1 \log_{10}$ whereas only wiping with 1000 ppm chlorine solution can reduce the enteric viruses $> 2 \log_{10}$ through a combination of removal and inactivation. In contrast, cleaning with a combination of wiping and disinfection reduces the infectivity of the viruses $> 3 \log_{10}$. However, different enteric and respiratory viruses showed varying degrees of susceptibility towards cleaning and disinfection (chapter 3). Besides the combination of cleaning and disinfection, chemical disinfection alone by hydrogen peroxide vaporization inactivated all the tested enteric viruses $> 4 \log_{10}$ from contaminated surfaces (chapter 4). The reduction was also tested by real time PCR and the relationship between the number of infectious viruses and the number of viral genome copies detected by quantitative PCR is not constant, therefore the risk-of-infection-estimates based on detected positives by PCR, results in overestimation.

The process of cleaning by wiping is performed as a daily cleaning, whereas cleaning and disinfection in combination are performed to reduce the load of contamination during outbreaks in health care facilities. Spot disinfection is applied for disinfection of visible spills. The loads of surface contamination can be reduced by wiping with liquid soap, followed by wiping with 1000 ppm chlorine solution. The process of cleaning and disinfection together can reduce residual contamination to about $1 \log_{10}$ (chapter 3) (initial contamination level ranged from 5 to $7 \log_{10}$), this level gives minimal risk of infection for the enteric viruses and respiratory adenovirus type 5. For respiratory enveloped influenza A (H1N1) virus wiping with 1000 ppm chlorine solution is sufficient to reduce the virus infectivity completely ($> 5 \log_{10}$), whereas norovirus infectivity (MNVI) can be reduced more than $3 \log_{10}$ by wiping with liquid soap, followed by wiping with 1000 ppm chlorine solution (chapter 3). A surface contaminated with human norovirus GI.4 and GII.4 can be decontaminated by wiping with liquid soap followed by spot disinfection with 1000 ppm chlorine solution for 10 min to reduce the genomic copies completely ($> 5 \log_{10}$ PCRU). Cleaning and disinfection

are not meant for complete reduction of micro-organisms: since the transmission via the contaminated surfaces to an individual is an indirect method of transmission and therefore reducing the residual contamination to a minimal risk level will be sufficient. However, more strict rules may be applied for intensive units of hospitals and nursing homes. We have introduced a minimal target level for each of the tested viruses that give the minimal risk of infection in case of indirect transmission from the contaminated surfaces. The minimum target level assists in quick risk assessment for the viruses in case of contamination.

Antimicrobial surfaces

Enteric and respiratory viruses are often transmitted through sites that are touched frequently. Use of surfaces coated with antimicrobials might be another way to reduce transmission of viruses and it could be used for disinfection of often touched surfaces such as doorknobs, handles, and bed rails. Application of antimicrobial coatings of TiO₂ with copper ion, calcium phosphate and zeolite against methicillin resistant *Staphylococcus aureus* and *Listeria monocytogenes* has been described (16, 30, 49). The viability of bacteria decreased on application of the coating materials. However, the application against viruses has not been studied extensively. Materials coated with immobilized quaternary ammonium compounds (IQACs) are effective in inactivating influenza A (H1N1) virus completely ($> 4 \log_{10}$) within 2 min but are not effective against poliovirus Sabin 1 (chapter 5). The IQACs cause disruption or detachment of viral envelope. As IQACs are not effective against non-enveloped poliovirus, they probably are not effective against other non-enveloped enteric viruses (22, 73). Nevertheless, it might be possible to use other immobilized antivirals to inactivate the virus and to prevent further transmission from the locations that are often contaminated and are difficult to clean. Chemical compounds that are shown to be effective against enteric viruses might be used as immobilized coating material as shown before for TiO₂ and CuO coated material against bacteriophage T4 (20). However, the effects of such antimicrobial compounds need to be studied, in presence of bodily fluids like nasal excretion, feces and vomits which might inhibit the efficacy of the compounds. The inactivation by immobilized IQACs showed again poor correlation between infectivity and PCR unit reduction.

Room decontamination

Manual cleaning and disinfection of surfaces to decrease the level of contamination to below detection limit or target level is difficult and, depending on the compound, could be hazardous to personnel involved. Instead of manual cleaning, room decontamination with gaseous products like ozone (69) and hypochlorous acid gas (55) has shown to be effective against viruses. Hydrogen peroxide vaporization of a room at 127 ppm for 1 hour is sufficient to inactivate infectivity of enteric and respiratory viruses completely ($> 4 \log_{10}$) from contaminated surfaces (chapter 4). Vaporized hydrogen peroxide has the advantage

of having non-toxic end products of water and oxygen. This type of vaporization has been approved in The Netherlands for disinfection of rooms. Thus it is an efficient method of disinfection of contaminated surfaces in health care facilities in case of outbreaks. It can also be used in food production facilities. The possible limitation of the method is the long time required for aeration after the disinfection cycle. Different enteric and respiratory viruses have been shown to have varying susceptibilities to chemicals. It would be worthwhile to test lower concentrations of hydrogen peroxide vaporization for respiratory enveloped influenza A (H1N1) virus, as enveloped viruses are more susceptible to chemicals than non-enveloped enteric viruses (59, 62). A lower concentration of hydrogen peroxide vapor might be sufficient to inactivate influenza A virus (59) and could be used in case of outbreaks. For good sanitation programmes, transmission intervention methods should not be specific but general and applicable for most of the pathogens. Thus the vaporization at 127 ppm can be an efficient method for sanitation. The vaporization is effective against number of enteric and respiratory viruses for decontamination. The vaporized hydrogen peroxide caused genome destruction by oxidation on the stainless steel carrier. The overall correlation between infectivity and PCR unit reduction by the vaporization was again poor.

Reduction of viral load from hands

Hands are used for many activities, and there are many possibilities for contamination. Fecal contamination of hands is an important source of transmission of viruses. Contaminated hands can transfer viruses to surfaces and food products by touching (chapter 6) and food handlers often are the cause of foodborne infections (9). In our experiments with hands contaminated with noroviruses, approximately 14% and 0.3% of the initial contamination of infective norovirus (MNV1) are transferred to whole tomatoes on immediate transfer and after 10 min of drying on the finger pads respectively. Similarly 12% and 7% of the infective virus can be transferred to cucumber slices on touching (chapter 6). The viruses that are present in food products can be inactivated completely ($\geq 4 \log_{10}$ infectivity) by heating at 73°C for 3 min (chapter 2) to prevent infection after consumption. However, in the presence of food matrix inactivation might decrease due to protection of the virus by components of the food matrix (4). Furthermore, the risk of infection remains in those products that are eaten raw such as tomatoes, cucumbers, and strawberries. Such products have been implicated in a number of food borne outbreaks (5). The level of contamination from such products can be reduced by washing with ozone (33) or high pressure processing (47).

Human norovirus shedding in stool ranges from 10^6 to 10^{11} particles per g (28, 54). In a scenario, assuming that 1 mg of stool on hands is left by unhygienic practices, the average number of human norovirus particles on hands be approximately 1×10^6 . If 50% of the particles are infectious (67) then the number of infective virus on hand would be approximately 5×10^5 . Similar number of the virus particle has been described from hands

of norovirus infected volunteers (43). The contaminated hand can then transfer viruses to a number of surfaces. In wet conditions, hands transfer approximately 13% of infective virus and about 0.1% is transferred after the viruses are dried for 10 min on the hands (chapter 6). In case of a scenario of contamination of hands with 1 mg stool (starting point approximate 5×10^5 infective viruses, 13% transfer per contact moment), approximately 65 transfers can result into infection (≥ 8 infective viruses) due to transfer of the virus from hand to hard surfaces. If hands come into contact with surfaces contaminated with noroviruses after 40 min of application of the virus, about 4% of the infective viruses can be transferred to hands (chapter 6). Even after 5 times pressing on the contaminated carriers about the same number of viruses are transferred to the finger pads and an approximately $3 \log_{10}$ infective viruses still remains on the surfaces. Approximately up to 200 times pressing, sufficient number of viruses for infection (≥ 8 infective viruses) can be transferred when approximate 5×10^5 infective viruses are initially present on contaminated surfaces. This is probably one of the features of norovirus that helps explaining the success of this virus in causing outbreaks and points to the need of stringent hygiene measures to render contaminated surfaces and hands.

A hand hygiene procedure is needed to reduce the number of infectious microorganisms on hands to a level that is considered safe, or acceptable for its intended use. This can be achieved by a process of removal of microbes or by inactivation using physical or chemical treatments or a combination of both. If we assume transfer of the norovirus is similar to transfer of other viruses (phages) as shown before (60), then the target level for residual contamination on hands should be below three times the infectious dose of the virus (67), since the contaminated hand has been shown to transfer approximately 30% of the phage from hand to mouth (lip) (60). Washing hands with soap and water and rubbing hands with alcohol hand disinfectant are common hand hygiene practices where use of alcohol hand hygiene is more popular among health care personnel (36, 66). Hand washing with soap and water for 30 s can be used to remove norovirus from finger pads completely ($> 5 \log_{10}$ PCRU), whereas rubbing hands with alcohol-based hand disinfectant can reduce the virus by approximately $3 \log_{10}$ PCRU (chapter 7). A similar high efficiency of soap and water in removing rhinovirus (29) and influenza A virus (61) has been described previously as well. Washing hands with soap and water properly removes the virus completely ($>5 \log_{10}$) and from all fingers and is therefore better in preventing transmission of the norovirus than wiping with alcohol-based hand disinfectant alone. However, a risk factor remains due to non-compliance with the recommended hand hygiene practices.

The alcohol can be used for disinfection of hands. In case of low level of contamination ($3 \log_{10}$ PCRU or less) use of alcohol hand disinfectant may be sufficient. However, the risk of infection still remains due to variation in alcohol treatment. The alcohol disinfection may give a limited reduction on some fingers and completely inactivate viruses on others. Thus for health care settings, alcohol hand disinfectant may be sufficient only when applied after

washing hands with soap and water. But in both the cases, personnel applying hand hygiene treatments must have good compliance (e.g. procedure, time).

Recommendations

Guidelines for prevention and control of human norovirus in health care settings have been issued. Human noroviruses and other tested enteric and respiratory viruses can be transmitted via contaminated hands and surfaces. For preventing transmission through contaminated surfaces, spot disinfection by 5000 ppm chlorine solution for decontamination of human norovirus contaminated surfaces has been suggested (15). The exposure to high concentration of chlorine solution is generally not preferred by working personnel. This decontamination by 5000 ppm chlorine solution can be replaced by wiping with liquid soap followed by spot disinfection with 1000 ppm chlorine solution for 10 min to reduce human norovirus genomic copies to $> 5 \log_{10}$ PCRU. In addition, wiping contaminated surfaces with liquid soap followed by wiping once with 1000 ppm chlorine solution is sufficient to reduce norovirus and other tested viruses to the target levels of minimal risk of the infection. Thus the combination treatments are recommended as a prevention and control method for the enteric and respiratory viruses. These methods are recommended to be included in guidelines for prevention of human norovirus and other tested enteric and respiratory viruses.

Use of hydrogen peroxide vapour for disinfection has not been included in the guidelines for disinfection of rooms but has been mentioned as an option for room disinfection (15). With the outcomes in this thesis, inclusion of hydrogen peroxide vapour treatment (127 ppm, 1 hour) for disinfection of surfaces in closed rooms is recommended to be included in the guidelines to inactivate enteric and respiratory viruses. However, the hydrogen peroxide vapour disinfection in a room might not be effective to those materials kept inside closed cabinets where there is no access of the vapour. In addition, the vaporization might be safe to use only on those materials in rooms that are compatible with the vaporized hydrogen peroxide.

For prevention of transmission of human noroviruses through hands in health care settings, washing hands with soap and water for 30 sec is recommended. The recommendation of using only alcohol-based hand disinfectants for prevention of transmission of human noroviruses through hands in health care settings should be amended. Wearing disposable gloves while handling soiled items to minimize likelihood of cross-contamination has also been recommended (15).

Hygiene measures are important for prevention of transmission of human norovirus and other enteric and respiratory viruses. The measures are important for individuals working in health care institutions and at individual homes. Contamination can take place by lack of

personnel hygiene, and it is most likely to cause infection. Pathogens for which transmission is well understood may be controlled. The measures like washing hands frequently with soap and water particularly after visiting toilet, changing diapers, before preparing food and after handling the patient will help to lower the incidence of outbreaks in closed areas. Other measures includes avoiding contamination of stool on surfaces will also prevent the transmission of viruses. In addition, awareness and knowledge on implementation of guidelines for prevention of outbreaks are necessary. Moreover, realization of personal ability to reduce the risk of infection also holds importance (7). Persistence of individual habits and attitudes may influence the compliance to the prevention procedures.

In addition to intervention methods and hygienic practices, approaches for improving comparability of infectivity and PCR unit reductions are recommended. For quantitative analysis of all the tested viruses with real time PCR, an amplicon of about 300 bp was used. During disinfection, the capsid might disintegrate, which allows damage of the genetic material. Due to the rather small target size of the genetic material, it can still be amplified, which results in underestimation of the disinfection efficiency. Probably a bigger amplicon and/or multiple short sections across different areas of the genome would give a better estimation of infectivity. Such an approach, a longer amplified region, showed a decrease in PCR detectable units for hepatitis A virus and poliovirus (10, 42, 63). In an inactivation study with ClO_2 , authors suggested preferential targets associated with secondary structure mainly located at 5' UTR and 3' UTR region with improved relation between infectious particles and genomic copy detection (37). Smart selection of the amplification region might improve the comparability of genetic copies and infectivity.

In this project, experiments were performed under standardized clean and dirty conditions, the latter in presence of 1% stool. In most of the experiments, no significant difference between clean and dirty conditions was observed. The dirty condition tested in low concentration of stool did not affect the decontaminations applied. Therefore, it is recommended to perform future experiments with enteric viruses with higher stool concentrations up to 10 to 20% w/v. This concentration is likely to have a protein content between 0.2 to 0.5% (77), which is about the same concentration of protein (0.3% w/v bovine serum albumin) that is added in sheep blood erythrocytes for testing antimicrobials in dirty conditions. However, the protein content and organic material in stool might differ per individual in time. Thus it might be more informative to have more data on a wider range of stool concentrations to have a better comparison to the situation in practice. Besides that, information on actual real life contamination levels in quantities of feces and pathogen load might be needed. So quantified wipes analysis for stool need to be launched. The present guidelines include bovine serum albumin, not stool as interfering substance for testing antimicrobials. Thus, a comparison in reduction of virucidal activity of antimicrobials between bovine serum albumin and feces needs to be carried out.

Future Experiments

Detection of human norovirus by PCR or quantitative PCR is widely used though it does not distinguish between infectious and non-infectious viruses. Novel methodologies should be developed which can distinguish infectious and non-infectious viruses. Several authors reported about quantitative PCR with the selective nucleic acid intercalating dye propidium monoazide (PMA), which makes it possible to differentiate between viable and non-viable bacteria (3, 50) and fungi (71). This also has been demonstrated for infectious and non-infectious bacteriophage T4 (25) and the enteric viruses poliovirus Sabin 1 and is compared with human norovirus (57) for heat and sodium hypochlorite inactivation. In case of viruses, PMA enters into the capsid-compromised particles and inside the particles it binds covalently to RNA/DNA by photo activation (57). In this state RNA cannot be amplified by PCR (51). As intercalation is required for the covalent binding, a secondary structure may play a role in successful treatment. The sets of primers used for MNV1 (2) did not distinguish infectious and non-infectious MNV1 (unpublished data). It will be fruitful to investigate further, looking at new sets of primers for MNV1 at different locations, preferably in the region of stable secondary structures that may facilitate covalent binding of PMA to the viral RNA so that bound RNA will not be amplified and compare these results with culture analysis. However, the technique may be not useful for those disinfectants that may cause loss of infectivity without damaging the protective capsid. Such methodologies might also be used for other enteric and respiratory viruses to determine infectious and non-infectious viruses and hence estimate the risk for public health in case of contamination.

Human noroviruses have been shown to bind porcine gastric mucin (68). This binding is negatively affected by UV irradiation, hypochlorite, and high pressure treatments. The lack of binding has been described to be due to loss of receptor binding sites after the treatments (65). The loss of binding capacity with porcine mucin has been described as a possible method to exclude non-infectious norovirus particles from subsequent RT-PCR detection (19). However from public health point of view, precaution should be taken when such method is used for detection and diagnostic studies, as the porcine gastric mucin has been shown to interact with 100% of norovirus GI but only 85% of GII strains tested (68). Nevertheless, the method can be used for inactivation studies. To overcome the mentioned limitations, development of a culture method for norovirus still holds importance.

Concluding remarks

Human noroviruses and other tested enteric and respiratory viruses can be transmitted via contaminated hands and surfaces. The human norovirus contaminated surfaces can be disinfected by wiping contaminated surfaces with liquid soap followed by wiping once with 1000 ppm chlorine solution which is sufficient to reduce norovirus and other tested viruses

to the target levels of minimal risk of the infection. The combination treatments will avoid exposure to the currently recommended high concentration of the chlorine solution without compromising the reduction of the virus. The target levels described in this study is based on transfer rates and the number of viruses required to cause infection, and will help in risk assessment of these viruses. Hydrogen peroxide vapor treatments of 127 ppm for an hour can be sufficient for disinfection of surfaces in closed rooms against enteric and respiratory viruses. The recommendation of using alcohol-based hand disinfectants for prevention of transmission of human noroviruses through hands in health care settings should be amended and replaced by the advice to wash hands with soap and water for 30 seconds.

Human norovirus is presumed to be one of the highly resistant viruses among enteric viruses. However, we showed that norovirus is as stable as other enteric viruses in the heat, hydrogen peroxide and chlorine treatments tested in this study. The major problem associated with norovirus might instead be due to high levels of shedding in stool and vomit and low infectious dose. The research in this thesis adds to our knowledge on methods for reducing transmission of human norovirus and gives comparative data on other enteric and respiratory viruses. It also contributes to a better understanding of human norovirus transfer efficiency. The results will contribute to improve methods for prevention of human norovirus infections. Nevertheless, future research for an efficient culture method for human norovirus still holds importance, although the results of this study have clarified many intervention methods.

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Summary

Human noroviruses are the leading cause of acute and outbreak associated gastroenteritis worldwide. The outbreaks occur commonly in health care settings including hospitals, nursing homes, health care centers as well as in individual homes. Due to the high numbers of outbreaks in health care facilities and outbreaks in individual homes, the burden of disease is high. The virus transmission takes place commonly through person to person directly through the fecal oral route and indirectly through contaminated surfaces. This thesis work was performed with as aim to evaluate methods to reduce the transmission of human norovirus, other enteric and respiratory viruses. The study especially focused on reducing the transmission through hands and contaminated surfaces. The introduction of this thesis (chapter 1), presents an overview of human norovirus, the disease (gastroenteritis) it causes, burden of the disease and transmission and prevention and control methods. In addition to that, other enteric and respiratory viruses that were included in the study are also described in brief. Enteric viruses included in the study were human norovirus GI.4 and GII.4, poliovirus Sabin1, rotavirus SA11, parechovirus 1 and murine norovirus 1 (MNV1). The respiratory viruses were adenovirus type 5 and influenza A (H1N1) virus.

The enteric and respiratory viruses are shed in feces or respiratory secretions and can contaminate foods through food handler-related contamination. Heating is commonly used in households and industries for inactivation of microorganisms in food. Thermal stability of these viruses were tested (chapter 2) at two commonly used temperatures of 56°C and 73°C in suspension test to have comparative data. Time to first \log_{10} reduction (TFL-value) was calculated based on best fit using the monophasic, biphasic, or Weibull models. The TFL at 56°C varied between a high value of 27 min (parechovirus) to a low value of 10 s (adenovirus) and ranked parechovirus > influenza > MNV1 > poliovirus > adenovirus. The monophasic model best described the behavior of the viruses at 73°C, in which case the TFL values obtained ranked MNV1 (62 s) > influenza > adenovirus > parechovirus > poliovirus (14 s). The study showed that heating at 73°C for 3 min is sufficient to reduce the infectivity of the tested viruses to > 4 \log_{10} and the viruses do not always follow log-linear thermal inactivation kinetics and the thermo-stability of parechovirus and influenza virus is similar to that of proven foodborne viruses.

In addition to food handler related contamination, contaminated hands can directly or indirectly contaminate environmental surfaces. Contaminated surfaces can be sources for indirect transmission of the viruses and cleaning and disinfection are common intervention methods used to reduce the contamination levels. In chapter 3 residual contamination after cleaning and commonly used disinfection were assessed by quantitative carrier tests against human noroviruses, different human enteric and respiratory viruses, and some bacteria for comparison. After a single wipe with water or liquid soap, the numbers of infective viruses and bacteria were reduced by 1 \log_{10} for poliovirus to close to 4 \log_{10} for influenza A (H1N1)

virus. There was no significant difference in residual contamination after wiping with water, liquid soap, or 250 ppm chlorine solution. An extra 1 to 3 \log_{10} reduction was achieved when a single wipe with liquid soap was followed by a second wipe using 250 or 1000 ppm chlorine, and no significant additional effect of 1000 ppm compared to 250 ppm was found, except for rotavirus and norovirus genogroup I. A reduced correlation between reduction in PCRU and reduction in infectious particles suggests that at least part of the reduction achieved in the second step is due to inactivation instead of removal alone. Data on infectious doses and transfer efficiencies was used to estimate a target level to which the residual contamination should be reduced and it was found that a single wipe with liquid soap followed by a wipe with 250 ppm free chlorine solution was sufficient to reduce the residual contamination to below the target level for most of the pathogens tested. Spot disinfection by 1000 ppm chlorine solution after wiping with liquid soap reduced infectivity of the poliovirus ($> 5.9 \log_{10}$), adenovirus ($> 4.3 \log_{10}$), MNV1 ($> 4 \log_{10}$) and viability of *St. aureus* ($> 5.5 \log_{10}$) completely and genomic copies of human norovirus GI.4 and GII.4 were also reduced ($> 5 \log_{10}$ PCRU) completely within 10 min. On the other hand parechovirus 1 infectivity and *S. Enteritidis* viability was not reduced completely within 20 min.

In addition to manual cleaning and disinfection, non-touch disinfection of contaminated surfaces in critical areas of health care facilities by vaporized hydrogen peroxide (VHP) has been approved for decontamination of hard surfaces in rooms for humans in the Netherlands. In chapter 4, antiviral efficacy of vaporized hydrogen peroxide disinfection against different human viruses applied to stainless steel, framing panel or gauze is described. VHP disinfection at 127 ppm for 1 h at room temperature resulted in complete inactivation of all viruses tested. On stainless steel and framing panel the reduction was $> 4 \log_{10}$ (complete inactivation) per carrier of infectious particles for poliovirus Sabin 1, rotavirus SA 11, adenovirus type 5, and MNV1, and more than $2 \log_{10}$ reduction (complete inactivation) of all viruses tested on gauze. Complete inactivation was confirmed under dirty conditions at several locations in a room for poliovirus Sabin 1. Reductions of viral genomes measured in PCR units were minimal on framing panel and gauze, but significant on stainless steel. Comparison of reductions of PCR units suggests that the RNA of human norovirus GII.4 was more resistant to VHP disinfection than the other viruses tested. In addition to VHP disinfection, self-decontaminating surfaces against bacteria and viruses can be helpful in preventing transmission in health care settings, food production areas and in general from frequently touched and contaminated surfaces. Such antimicrobial coatings have been recently tested and in chapter 5 efficacies of immobilized quaternary ammonium compounds (IQACs) coated onto glass and plastic surfaces were tested against enveloped influenza A (H1N1) virus and non-enveloped poliovirus Sabin1. The IQACs tested were virucidal against the influenza virus within 2 min but no virucidal effect against poliovirus was found in 6 h. Thus the coating can be used against the influenza A virus during outbreaks but is not promising to control the transmission of naked enteric viruses.

Contaminated hands are thought to be a major vehicle for transmission of enteric viruses. However, limited data is available on transfer of for example noroviruses, though norovirus is thought to be transmitted mainly through contaminated hands. Since quantitative data on transfer during contact with surfaces and food are scarce, transfer of MNV1, and human noroviruses GI.4 and GII.4 was studied by artificially contaminating human finger pads, followed by pressing on stainless steel and Trespa® surfaces and also to tomatoes and cucumber slices were studied in chapter 6. The transfers were performed at a pressure of 0.8-1.9 kg/cm² for approximately 2 s in sequential transfer up to 7 carriers or food products. MNV1 infectivity transfer from finger pads to stainless steel and Trespa® was approximately 13% on first transfer on immediate transfer. After 10 min of drying transfer was reduced to approximately 0.1%. MNV1 infectivity transfer from stainless steel and Trespa® to finger pads after 40 min of drying was approximately 4%. Infectious MNV1 was transferred more onto cucumber slices than to whole tomatoes, probably due to the higher moisture content on the surface of the sliced cucumber. Similar results were found for noroviruses GI.4 and GII.4 transfer measured in PCR units. The results show that transfer of the virus is possible even after the virus is dried on the surfaces.

Hand hygiene is one of the most important measures to prevent transmission of noroviruses through contaminated hands. Alcohol based hand disinfectants are more frequently used in health care facilities for hand hygiene than hand washing, therefore efficacy of alcohol based hand disinfectants against a number of human enteric and respiratory viruses was tested by quantitative carrier tests and finger pad tests and compared to washing with soap and water against human norovirus GI.4 and GII.4 and its cultivable model virus MNV1 in chapter 7. The alcohol based hand disinfectants reduced the infectivity of rotavirus and influenza A virus completely within 30 s in carrier tests. In contrast, poliovirus Sabin 1, adenovirus type 5, parechovirus 1, and MNV1 infectivity were reduced with $< 3 \log_{10}$ within 3 min exposure time. The MNV1 infectivity reduction achieved by washing hands with soap and water for 30 s ($> 3.0 \pm 0.4 \log_{10}$) was significantly higher than by treating hands with alcohol hand disinfectant ($2.8 \pm 1.5 \log_{10}$). Washing with soap and water removed the genomic copies of MNV1 ($> 5 \log_{10}$), noroviruses GI.4 ($> 6 \log_{10}$) and GII.4 ($4 \log_{10}$) completely. Thus we showed that washing hands with soap and water is better than using alcohol based hand disinfectants in removing noroviruses from hands.

In conclusion, the transmission of human norovirus and other tested enteric and respiratory viruses from hands and environmental surfaces can be reduced. Guidelines for prevention and control of the viruses have been issued. With the outcome of this thesis, it is recommended to include hydrogen peroxide vaporization of 127 ppm for 1 hour for disinfection of surfaces in closed rooms during outbreaks. Spot disinfection by 5000 ppm chlorine solution for decontamination of human norovirus contaminated surfaces has been suggested in case of localised contamination. The decontamination can be replaced by wiping with liquid soap followed by spot disinfection with 1000 ppm chlorine solution for 10 min to reduce human

norovirus genomic copies completely ($> 5 \log_{10}$ PCRU). Use of alcohol hand disinfectants for prevention of transmission of human noroviruses through hands in health care settings should be amended and replaced by washing hands with soap and water for 30 sec when reduction of $> 6 \log_{10}$ PCRU of the genomic copies are needed. However, alcohol based hand disinfectants will have some applications particularly when running water is not accessible and lower reduction ($2 \log_{10}$ PCRU) of the genomic copies is enough. The research in this thesis adds to the knowledge on methods to reduce transmission of human norovirus and gives comparative data on other enteric and respiratory viruses. In addition to that it also contributes in better understanding of human norovirus transfer efficiency. The results may contribute in implementation of prevention and control methods for human norovirus and the other enteric and respiratory viruses tested.

Samenvatting

Humane norovirussen zijn wereldwijd de belangrijkste veroorzakers van acute en uitbraakgerelateerde gastro-enteritidis. Regelmatig vinden uitbraken plaats in medische instellingen zoals ziekenhuizen, verpleegtehuizen alsook in individuele huishoudens. Door het grote aantal uitbraken in medische instellingen en in huishoudens, is de volksgezondheidslast hoog. Virustransmissie vindt gewoonlijk plaats direct van mens naar mens, via de fecaal-orale route en indirect via besmette oppervlakken. Dit onderzoek is uitgevoerd met als doel om methoden te evalueren om de transmissie te reduceren van humaan norovirus en andere enterische en respiratoire virussen. Specifiek aandacht is besteed aan het reduceren van de transmissie door handen en besmette oppervlakken. De introductie (hoofdstuk 1) geeft een overzicht van humaan norovirus, de ziekteverschijnselen (gastro-enteritidis), de volksgezondheidslast en transmissie en preventie en beheersmethoden. Andere enterische en respiratoire virussen die in het onderzoek gebruikt zijn worden daar ook kort besproken. Enterische virussen die onderzocht zijn, zijn humaan norovirus GI.4 en GII.4, poliovirus Sabin1, rotavirus SA11, parechovirus 1 en het murine norovirus 1 (MNV1). De respiratoire virussen zijn adenovirus type 5 en het influenza A (H1N1) virus.

Enterische en respiratoire virussen worden uitgescheiden in feces of respiratoire uitscheidingen en voedselbewerkers kunnen hierdoor levensmiddelen besmetten. Verhitting is veel gebruikt in huishoudens en de levensmiddelenindustrie voor de inactivering van micro-organismen in levensmiddelen. De thermische stabiliteit van de virussen is getest (hoofdstuk 2) bij twee gebruikelijke temperaturen van 56°C en 73°C in suspensietesten. De tijd tot de eerste log₁₀ reductie (TFL-waarde) is berekend, gebaseerd op de beste fit van het monofasische, bifasische, of Weibull-model. De TFL-waarde bij 56°C varieerde tussen een hoge waarde van 27 min (parechovirus) tot een lage waarden van 10 s (adenovirus) en had als volgorde parechovirus > influenza > MNV1 > poliovirus > adenovirus. Het monofasische model beschreef het gedrag van de virussen bij 73°C goed, waarbij de verkregen TFL waarden afliepen van MNV1 (62 s) > influenza > adenovirus > parechovirus > poliovirus (14 s). De studie laat zien dat verhitting bij 73°C gedurende 3 min voldoende is om de infectiviteit van de geteste virussen tot meer dan 4 log₁₀ te reduceren en dat de virussen niet altijd een log-lineaire thermische inactiveringskinetiek laten zien. De thermostabiliteit van parechovirus en het influenzavirus blijkt vergelijkbaar met de bewezen voedselgerelateerde virussen.

Behalve directe voedselbewerkergerelateerde besmetting, kunnen ook besmette handen direct of indirect omgevingsoppervlakken besmetten. Besmette oppervlakken kunnen een bron zijn voor indirecte virustransmissie en schoonmaken en desinfecteren zijn de aangewezen interventiemethoden om besmettingsniveaus te verlagen. In hoofdstuk 3 is de residuele besmetting na schoonmaken en gebruikelijke desinfectie bepaald met kwantitatieve carriertesten voor humane norovirussen, verschillende humane enterische en respiratoire virussen, en enkele bacteriën ter vergelijking. Na een enkele veegactie met water of vloeibare

zeep, werden de aantallen infectieuze virussen en bacteriën gereduceerd met 1 log₁₀ voor poliovirus tot bijna 4 log₁₀ voor influenza A (H1N1) virus. Er was geen significant verschil in residuele besmetting na vegen met water, vloeibare zeep, of 250 ppm chlooroplossing. Een extra 1 tot 3 log₁₀ reductie kon bereikt worden wanneer de enkele veeg met vloeibare zeep gevolgd werd met een tweede met 250 of 1000 ppm chloor, waarbij er geen significant additioneel effect van 1000 ppm vergeleken met 250 ppm werd gevonden, behalve voor rotavirus en norovirus genogroup I. Een verlaagde correlatie tussen de reductie in PCRU en reductie in infectieuze deeltjes suggereert dat ten minste een deel van de bereikte reductie in de tweede stap door inactivering komt in plaats van door alleen verwijdering. Data van infectieuze doses en overdrachtsefficiënties werden gebruikt om doelniveaus te schatten tot waar de residuele besmetting in ieder geval tot gereduceerd zou moeten worden en hieruit volgde dat een enkele veeg met vloeibare zeep gevolgd door een veeg met 250 ppm vrije chlooroplossing voldoende was om de residuele besmetting te reduceren tot beneden het doelniveau voor de meeste van de geteste pathogenen. Spotdesinfectie met 1000 ppm chlooroplossing na vegen met vloeibare zeep reduceerde de infectiviteit van poliovirus (>5.9 log₁₀), adenovirus (>4.3 log₁₀), MNV1 (>4 log₁₀) en de levensvatbaarheid van *St. aureus* (>5.5 log₁₀) volledig en ook genomische kopieën van humaan norovirus GI.4 en GII.4 werden volledig gereduceerd (> 5 log₁₀ PCRU) binnen 10 min. Aan de andere kant werd de parechovirus 1 infectiviteit en *S. Enteritidis* levensvatbaarheid niet volledig gereduceerd binnen 20 min.

Naast handmatig schoonmaken en desinfectie, is desinfectie van besmette oppervlakken in kritieke ruimtes in gezondheidszorgvoorzieningen door waterstofperoxidedamp (VHP vaporized hydrogen peroxide) toegestaan voor desinfectie van harde oppervlakken in ruimtes in Nederland. In hoofdstuk 4 is de antivirale efficiëntie van waterstofperoxide-desinfectie beschreven tegen verschillende humane virussen toegepast op roestvast staal, Trespa en gaas. VHP desinfectie bij 127 ppm gedurende 1 uur bij kamertemperatuur resulteerde in volledige inactivering van alle geteste virussen. Op roestvast staal en Trespa was de reductie > 4 log₁₀ (complete inactivering) per carrier van infectieuze deeltjes voor poliovirus Sabin 1, rotavirus SA 11, adenovirus type 5, en MNV1, en meer dan 2 log₁₀ reductie (complete inactivering) van alle geteste virussen op gaas. Complete inactivering van poliovirus Sabin 1 werd bereikt onder niet-schone condities op verschillende locaties in een ruimte. Reducties van virale genomen, gemeten als PCR units, waren minimaal op Trespa en gaas, maar significant op roestvast staal. Vergelijking van de reductie van PCR units suggereert dat het RNA van humaan norovirus GII.4 resistenter was tegen VHP-desinfectie dan dat van de andere geteste virussen. Naast VHP-desinfectie, kunnen zelf-decontaminerende oppervlakken tegen bacteriën en virussen nuttig zijn in het voorkomen van transmissie in gezondheidszorginstellingen, voedselbereidingsruimten en in zijn algemeenheid van veel aangeraakte oppervlakken. Zulke antimicrobiële coatings zijn recentelijk veel getest en in hoofdstuk 5 zijn de efficiëntie van geïmmobiliseerde quaternary ammonium compounds (IQACs) gecoat op glas- en plasticoppervlakken getest tegen enveloped (membraankapsel)

influenza A (H1N1) virus en non-enveloped poliovirus Sabin1. De geteste IQACs waren virus-inactiverend tegen het influenza-virus binnen 2 minuten maar er werd geen inactivering gevonden tegen poliovirus na 6 uur. De coating kan dus goed gebruikt worden tegen het influenza A virus gedurende uitbraken maar is niet erg veelbelovend voor het beheersen van de overdracht van non-enveloped enterische virussen.

Besmette handen worden gezien als de belangrijkste bron van transmissie van enterische virussen. Er zijn echter weinig data beschikbaar over de overdracht van bijvoorbeeld norovirussen, alhoewel aangenomen wordt dat norovirus vooral overgedragen wordt via besmette handen. Aangezien kwantitatieve data over overdracht bij contact met oppervlakken en levensmiddelen schaars zijn, werd overdracht van MNV1, en humaan norovirussen GI.4 en GII.4 onderzocht, van kunstmatig besmette humane vingertoppen, gevolgd door afdrukken op roestvast staal en Trespa® oppervlakken, en ook overdracht naar tomaten en komkommerschijfjes werd onderzocht in hoofdstuk 6. De overdracht werd uitgevoerd bij een druk van 0.8-1.9 kg/cm² gedurende ongeveer 2 seconden in opeenvolgende afdrukken tot 7 oppervlakken of voedingsmiddelen. MNV1 infectiviteitsoverdracht van vingertoppen naar roestvast staal en Trespa® bedroeg ongeveer 13% bij een eerste directe afdruk. Na eerst gedurende 10 min drogen reduceerde de overdracht tot ongeveer 0.1%. MNV1 infectiviteitsoverdracht van roestvast staal en Trespa® naar vingertoppen na 40 minuten drogen bedroeg ongeveer 4%. Infectiviteit van MNV1- werd beter overgedragen naar komkommerschijfjes dan naar hele tomaten waarschijnlijk door hogere vochtigheid op het oppervlak van de schijfjes komkommer. Vergelijkbare resultaten werden gevonden voor overdracht van norovirussen GI.4 en GII.4 gemeten in PCR units. De resultaten laten zien dat de overdracht van het virus zelfs na drogen op oppervlakken mogelijk is.

Handhygiëne is een van de belangrijkste maatregelen om overdracht van norovirussen door besmette handen te voorkomen. Alcoholgebaseerde handdesinfectanten worden frequenter gebruikt in gezondheidszorginstellingen voor handhygiëne dan handenwassen, daarom werd de efficiëntie van alcoholgebaseerde handdesinfectanten tegen een aantal humane enterische en respiratoire virussen getest met kwantitatieve dragertesten en vingertop-testen en vergeleken met wassen met zeep en water tegen humaan norovirus GI.4 en GII.4 en het kweekbare modelvirus MNV1 in hoofdstuk 7. De alcoholgebaseerde handdesinfectanten reduceerden de infectiviteit van rotavirus en influenza A virus volledig binnen 30 seconden in dragertesten. Poliovirus Sabin 1, adenovirus type 5, parechovirus 1, en MNV1 infectiviteit werden echter slechts gereduceerd met $< 3 \log_{10}$ binnen 3 min blootstellingstijd. De MNV1-infectiviteitsreductie die bereikt werd door handwassen met zeep en water gedurende 30 s ($> 3.0 \pm 0.4 \log_{10}$) was significant hoger dan de behandeling met alcohol handdesinfectanten ($2.8 \pm 1.5 \log_{10}$). Wassen met zeep en water verwijderde genomische kopieën van MNV1 ($> 5 \log_{10}$), norovirussen GI.4 ($> 6 \log_{10}$) en GII.4 ($4 \log_{10}$) volledig. Hiermee wordt dan aangetoond dat handwassen met zeep en water beter is dan het gebruik van alcoholgebaseerde handdesinfectanten in het verwijderen van norovirussen van handen.

Concluderend kan gesteld worden dat de transmissie van humaan norovirus en andere geteste enterische en respiratoire virussen van handen en omgevingsoppervlakken verlaagd kan worden. Er bestaan al verschillende richtlijnen voor preventie en beheersing van virussen. Met de uitkomsten van dit proefschrift, is waterstofperoxidedamp van 127 ppm gedurende 1 uur voor desinfectie van oppervlakken in gesloten ruimtes gedurende uitbraken, aan te bevelen. Spotdesinfectie met 5000 ppm chlooroplossing voor ontsmetting van met humaan norovirus besmette oppervlakken wordt soms aanbevolen in geval van een lokale besmetting. De ontsmetting kan ook vervangen worden door vegen met vloeibare zeep gevolgd door spotdesinfectie met 1000 ppm chlooroplossing gedurende 10 minuten om het aantal humane genomische kopieën van norovirus volledig te reduceren ($> 5 \log_{10}$ PCRU). Het gebruik van alcohol handdesinfectanten voor preventie van de transmissie van humane norovirussen door handen in gezondheidszorginstellingen zou beter aangepast kunnen worden en vervangen door de aanbeveling handen met zeep en water te wassen gedurende 30 seconden, wanneer reductie van $> 6 \log_{10}$ PCRU van genomische kopieën nodig zijn. Alcohol gebaseerde handdesinfectanten kunnen echter nuttig zijn vooral daar waar geen kraanwater aanwezig is en wanneer een lagere reductie ($2 \log_{10}$ PCRU) van genomische kopieën voldoende is.

Het onderzoek in dit proefschrift heeft de kennis vergroot over methoden om transmissie van humaan norovirus te reduceren en geeft vergelijkbare gegevens van andere enterische en respiratoire virussen. Daarnaast draagt het ook bij aan een beter begrip van de overdrachtsefficiëntie van humaan norovirus. De resultaten kunnen bijdragen in de implementatie van preventie- en beheersmethoden tegen humaan norovirus en andere geteste enterische en respiratoire virussen.

मूढ

दलितं चक्रं याकनं वलनीगु यकाय (जाउट वक्र) नायं यूनीगु ब्रयु माना (थासुटा व्हेन्टननाव्हेरिस) या मू
 द्नी मन्तूयाग कळेंगु नांना राव्हेन्स रव४ । थूडा४गु यकाय जय४ याना४ साम्बुय कद्र, जयगाल, नरिस्त्रि हाम लिंसं
 मन्तूयाग व्हेन्टननाव्हेरिस रव४ । साम्बुय कद्र व व्हेन्टननाव्हेरिस जय४ ब्यारव्यु यनावनीगु थ्र यकायया द्नीं थ्र ब्रयुया
 धाम जायालं दळें । गथं क ६ मन्तूवं मन्तूयाग, मन्तूया रिव व द्नीगुया लैयू (दिकल व डानल नूट) जया४ थ्र राव्हेन्स
 यनावनीगु (ट्रासिमिसन व्हेन्टननाव्हेरिस) रव४ । नायं थ्र ब्रवं क४यिं याखं मचायक ६ यनावनीगु नं ड । थुगु साधयप्र जाया
 मू जाड्जा धयागु ६ मानव नांना राव्हेन्स व भगु रिव व सास४ (व्हेन्टननिक व भयिभटनी) याखं यूनीगु राव्हेन्स
 यनीगु विधिगु क्वालंगु रव४ । थ्र जथयन खास याना४ द्वा४ व दूषिगु थायू (कन्ट्रामिन्ट उ सद्स) याखं यनावनीगु
 याग क्वा यायगु जायू व४वीगु रव४ । थुगु साधयप्रया क्वासीका व्रयु नांना राव्हेन्स व थुं वलंकीगु ब्रयु, ब्रयुया
 धाम, यनावनीगु जा, यभगु जा व यभगु विधि क्वात्रयागु दू । थुकिया नाय नायं भगु रिव व सास४ याखं यनावनीगु
 राव्हेन्सया रव्यं व४चारुकयक क्वाथनागु दू । जथयनय दूथाकागु व्हेन्टनीक राव्हेन्सगु धयागु मानव नांना राव्हेन्स
 (GI.4 व GII.4), थालिया राव्हेन्स सविन १, भाटा राव्हेन्स एस १ ११, याभका राव्हेन्स १, यनाव्हेन
 नांना राव्हेन्स (एम एन टी १) रव४ । सास४ याखं यनावनीगु राव्हेन्सगु जाठिना राव्हेन्स टाव्हेय ५ व
 व्हेन्टननाव्हेरिस १ (एव वान एन वान) राव्हेन्स रव४ ।

रिव व सास४याखं यूनीगु थुयिं राव्हेन्सगु ब्रवं क४क व्रिगिया रिव, द्वि व र्वे (भयिभटनी सिक्वीसन)
 जादिलय दळें व थूडा४क मन्तूवं थुगु नसायाग नं दूषिगु याना वी । नथगु नसायाग जाया४ याना४ दयका४,
 व्का४ उकी दगु खनमदुयिं कीगु थायगु याळें । थ्र विधि व्हेन्टननायनायं कानखानायं नं थलंगु याळें । थुयिं
 राव्हेन्सगुयागु का४ज४ सह याथ र्वेगु उमगा वा भिन्ना (थर्मल स्ट्रुक्चरि) या गुलनाभक उटाया निर्गिं
 सथसन टष्टयाखं जाया४ याना४ थलावलायु दळेंगु ५६ व १३ तिथी सभियस का४जलय यनिज्ज यायगु जा
 जल (व्र २) । द्वायांगु \log_{10} क्वा (टि. १६. १ल. यालू या व्हेल्यु भाभाशुजिक भाउल, वाव्हेन्टनिक वा वाव्हेन्टन
 भाउल थला४ दकल यायुक्ति खनदगु भाउलया लिधंसायु जल । ५६ तिथी सभियसया निर्गिं टि. १६. १ल. यालू
 दकल जय४ २१ मिन्ट (याभका राव्हेन्स) व दकल क्वा १० सकंभ (जाठिना राव्हेन्स) दथळें व्रन व थुयिं
 टि. १६. १ल. यालूया लिधंसायु राव्हेन्सगुयागु व्र४व्हेन्टन व्रलय क्वासीकथरं याभका राव्हेन्स > व्हेन्टननाव्हेरिस
 राव्हेन्स > एम. एन. टी १ > थालिया राव्हेन्स > जाठिना राव्हेन्स जवळें । १३°C यु थुयिं राव्हेन्सगुयागु
 यद४वद४ क्वाथनगु निर्गिं भाभाशुजिक भाउल दकल यायुक्ति खनदगु व थुयिं टि. १६. १ल. यालूया लिधंसायु
 राव्हेन्सया व्र४ थुयिं व्रन - एम. एन. टी. १ (६२ सकंभ) व्हेन्टननाव्हेरिस राव्हेन्स > जाठिना राव्हेन्स >
 याभका राव्हेन्स > थालिया राव्हेन्स (१४ सकंभ) 73°C का४जलय ३ मिन्ट काका४ यनिज्ज यानाव्र४गु थुयिं
 राव्हेन्सगुयागु यंकागु उमगा (व्हेन्टननाव्हेरिस) ४ \log_{10} तक क्वा यायदळेंगु र्वं थुगु जथयनयाखं सीदुगु दू ।
 जथ ६ थुयिं राव्हेन्सगुयागु थनमल व्हेन्टननाव्हेरिसन काव्हेन्टननाव्हेरिसयाखं क्वावलं \log लिनियन गनिकायाखं
 उक क्वा४ वनीमरवु धयागु र्वं नं सीदुगु दू । थुकिया नायनायं याभका राव्हेन्स व व्हेन्टननाव्हेरिस राव्हेन्सया
 का४ज४ व्रथासं व्रनीगु यद४ नं भभगु नथगु नसायु दळेंगु (दू वनं राव्हेन्स) थं ६ रव४ धयागु र्वं नं क्वा ।

नथु नसा थीवल मनूयसं यूकीगु (दू हानुलन निक्षुत) कन्ट्रानामिनसन) लिस लिसं ब्रवं कथियं मनूयसु द्वाःगं नं वाथकं व मवाथकं लकसयाग दूषिग याना वी यः। दूषिग लकसयाखं यूनीगु आ क्का याथग जायाः यानाः थलीगु गनिकाग (कमन व्हेन्टरनरसन मथउ) सूवूयिव् व खनमदूयिं की याथगु (उिस्व्हेन्डरसन) खः। थ साधयपया व ३ य सूवूयिव् व खनमदूयिं की याथगु आ धूकाः बंधनिगु यनावनीगु (निसिगुवल कन्ट्रामिनशन) या कवाक्षका याव्हेगु यनिमाधासक यनिज्ज विधि (कान्तिरटिरेर आनिगुन रुथ) याखं ज्जथयन याथगु आ इल। थुगु ज्जथयनय मानव नांजा राव्हेनस, मनूयसु थीथी खि व सासःयाखं यूनीगु राव्हेनसग नाय नायं व्हे व्हे आकुनिया लिसं गुलनासक यनिज्ज याथगु आ इल। व्हेकः लः व सावूनं (लिकितु सायं) व्हेधूकाः यूनीगु राव्हेनस व आकुनिया यागु यनावनीगु जमगा $1 \log_{10}$ थालिया राव्हेनस निसं थंमथं $4 \log_{10}$ व्हेन्डरना राव्हेनस तक क्का इल। लः, द्वाल्गु साधां वा २५० यी.यी.एम. क्कानिन दगु लखं व्हेधूकाः बंधु यनावनीगु जमगाय व्हे क्कथनवरुःगु हिउयाः (सिगिनिडिकस उिडरस) खनमदू।

व्हेकः साधां जल २५० वा १००० यी.यी.एम. क्कानिन लखं व्हेधूवल गँसा १ निसं $3 \log_{10}$ तक यनावनीगु जमगा क्का इगु सीदग। लिसं भाटा राव्हेनस व नाभाराव्हेनस जिनाधय १ य २५० यी.यी.एम. क्कानिनया ब्यारख्य १००० यी.यी.एम. क्कानिन लखं मगु गँसा लिद्वः लकगु सिधं मगु राव्हेनसगयक व्हे क्कथनवरुःगु हिउयाः खनमन। यिसिगान यूनिरया क्कागि व यनावनीगु कध (व्हेन्डरिरेर यारिकस) या क्कागि दथव्हे क्का ६ जक यू स्यायू (निउउउ कानिखसन) या दूनं यानाः निगुगु वय खनदगु यनावनीगु जमगाया क्कागिया व्हे व व्हेनजाकिरसनया दूनं इगु व थ व्हेथासं मथाय क्काव्हेगु (निमूरल) या दूनं जक मज्जगु क्कां। यनावनीगु माःगु खनाक (व्हेन्डरिरेर उज) व थाय द्दिलीगु जमगा (ट्रासदन व्हेदिसियासि) या उटा जाइ (टानगट खरल) धूकमां याथग थःगु खः, जल उगु जाइ तक बंधं दनिगु यनावनीगु (निसिगुवल कन्ट्रामिनशन वीक माःगु इल। द्वायां द्वाल्गु साधां जल निक्कःगु यालय २५० यी.यी.एम. क्कानिन लखं व्हेधू धूकाः यनिज्ज याःगु खनमदूयिं जायाः यानाः कीतयुगु बंधनिगु यूकगु जमगा जाइ स्यां कय थंकग गाःलाःगु इल। द्वाल् सावूनं व्हेधूकाः १००० यी.यी.एम. क्कानिन लखं यानासयागु थर उिस्व्हेन्डरसन याखं १० गू मिन्टर दून ६ थूयिं खनमदूयिं कीग - थालिया राव्हेनस ($3.5 \log_{10}$), जठिना राव्हेनस ($4.3 \log_{10}$), एम एन री १ ($> 4 \log_{10}$) व सराद जनिथास ($> 3.5 \log_{10}$) या यूकगु जमगा यूवंक क्का याःगु लिसलिसं मानव नांजा राव्हेनस GI.4 व GII.4 या जनामिक कयिज नं १० गू मिन्टरया दून ६ यूवंक क्का याथरुग ($3 \log_{10}$)। मखं यापका राव्हेनस १ व एस व्हेन्टरनिरिउिसया यूकगु जमगा धःसा २० गू मिन्टर लिया तक नं यूवंक क्का इगु मखू।

यून धूकगु (दूषिग) कःग सगर (हार्थ सड्स) या निगिं मनूयसं थक्कं याव्हेगु सूवूयिगु (थानूयल क्किनिड) व खनमदूयिं की याथगु नायं द्वागं मथूस खनमदूयिं की याथगु (नर. रुव. उिस्व्हेन्डरसन) सधयनगील सभूय सूविधाय याव्हेगु काःगु हा दगु हाव्हे ग्राजन थनाक्काव्हेउ (री.एव.यी) मधनव्याभय मनूयसु निगिं थलीगु काःगया यून धूकगु सगरया यनिसाधन (उिकन्ट्रामिनसन) याग उजं वियागःगु इल। व ४ य सुटनखस स्टिल, इमिन थानल वा गजय गयाः मनूयसु व्हेधूगु राव्हेनसग लिस री.एव.यी.या एन्टि राव्हेनल

यस्यवकानिगाया खँ द्धथनागु जल । काऒाया काऒऒा १ घट्टाया निर्गि १२१ यीयी उम री उवयी ३ खनमदूयिं की स्याथगु यनिज्ध याना स्यावले दूकू राळ्ळनसग यूवंक ६ सीगु (कश्चित् व्ळनउक्कीटिरससना) जल । स्टनलस सिटल व झूमिन थानल याखया थालिया राळ्ळनस सविन १, नारा राळ्ळनस उस १ ११, जाठिना राळ्ळनस राळ्ळय १ व उम उन री १ या संक्रासक कध (व्ळन्क्षिकल यारिक्कस) $> ४ \log_{10}$ च्याखं क्का जल व गजय यनिज्ध यानागु दूकू राळ्ळनस $२ \log_{10}$ स्यां जयऒ क्का जगु खऒ सूवूयिव् मझगु काऒाया थीथी थसं थालिया राळ्ळनस सविन १ यूवंक ६ मदुगु यृष्टि जल । थूयिं राळ्ळनसगयगु जिनाभासय् वऒगु क्कागि यीसिज्जानयूलय् मायन यानावले झूमिद्व थानल व गजय क्का जक खनदग । तन स्टनलस सिटलय् धाऒसा अर्थयूई जल । यीसिज्जान यूनिरया क्कागिळ् तुलनासककथं सथवल नाभा राळ्ळनस G II.4 या जान उन. १. मगु यनिज्ध यानागु राळ्ळनसग स्याऒ री. उव. यी. खनमदूयिं की स्याथगु ज्ञाया जयऒ यगिनाध (नसिस्टान्ट) दगु खनदग । री. उव. यी. खनमदूयिं की मदयकगु नायं द्याङ्गुनिया व राळ्ळनस लिस याळ्ळगु जथं६ यनावनीगु सगदया यनिसाधन (सलद उिकन्ट्रामिनंथन सनदस) नं उसाँयया विवाऒ याळ्ळगु थाय्, नथगु नसा दयकीगु थाय् व ज्ञायाऒ यानाऒ द्वागं थियाऒ व यन्धकूगु थसं यनावनीगुयाग यनग शालि जळ्ळदू । थूजाऒगु लय्या जध् यगिनाध (गनिरमाळ् क्काविगल) काटिद्व (लय) नकागिन ६ यनिज्ध यानासयागु द् । व १ य् खाऒ व ग्लासिरकय् तुनागळ्ळगु व्ळनमाविलाळ् उ काटननीन जमानियम कथाउधस (जळ्ळ्कुऒसी उस) व्ळन्सलय् उ व्ळन्दूजा १ (उव १ उन १) राळ्ळनस व नन व्ळन्सलय् उ राळ्ळनसगय् दथळ्ळ यनिज्ध यानासऒगु खऒ । यनिज्ध जगु जळ्ळ्कुऒ उसी उस २ मिभरया दून व्ळन्दूजा १ (उव १ उन १) राळ्ळनस दथळ्ळ राळ्ळसिउल जगु, तन थालिया राळ्ळनस लिस ६ धौ तक नं व्ळं नं लिब्रऒ मलाऒगु खऒ । थूकथं थूजाऒगु लय व्ळन्दूजा १ राळ्ळनसयाखं यनावनीगु मदयकगु थलदळ्ळ । तन थ लय नाकउ व्ळन्टनिक राळ्ळनसयाखं यनावनीगु यनग धाऒसा थलमाऒगु जळ्ळमव् ।

व्ळन्टनिक राळ्ळनस यनीगुया जगु मू दूनिं दूषिग द्वाऒ खऒ । जथसां थ ज्जथासं मथाय् यनावंगुया उरा क्का जक द् । दसूया निर्गि नाभा राळ्ळनस ज्जथासं मथाय् यना वनीगुया मू दूनिं ६ राळ्ळनस यनावंगु द्वाऒ खऒ । नथगु नसानाय द्वाऒया स्याय् जळ्ळवल यनावनीवलयागु माक उरा मदुगुलिं उम उन री १ नाभा राळ्ळनस GI.4 व GII.4 या यनावनीगुया ज्जथयन हकगंया लय् यंगु यगिंयाग स्टनलस सिटल व दूयाय् व ज्जथलं गोलयाना व कूवाकूवा यानागऒगु गुसि द्यऒनं ललाऒ यनिज्ध याथगु ज्ञा जल (व द्) । थाय् हिलीगु ०.८ निसं १.९ / सन्टिमिटरं सकंभया द्वाव २ सकंभया निर्गि व्ळसीकथं (सिक्कनसियल) १ गू वादक वा नथगु नसाय् क्कवथगु ज्ञा जल । उमउनरी १ या यंगुया थाय् हिलाऒ, यगिंयाखं सिटल व दूयाया सगदलय् थंमथं १३ % उवल ६ थाय् हिलाया द्वायांगु थाय् हिला जल । १० गू मिभर गंके धूकाऒ थाय् हिलाऒ क्का ज्ञायाऒ थंमथं ०.१०% थन । ४० गू मिभर गंके धूकाऒ उमउनरी १ या यंगु थाय् हिलाऒ सिटल व दूयाया यगिंवाय् थंमथं ४% जवल । उम उन री १ यंगु ज्जथलं गोलयाना स्यां कूवाकूवा गुसिळ् जयऒ थाय् हिला जल । गानागऒगु गुसिळ् जयऒ खाउँजऒ (भाळ्ळसुवन) दगुलिं नं थथ जगु जळ्ळदू । थूजाऒगु ६ लिब्रऒ नाभाराळ्ळनस GI.4 व GII.4 या थाय् हिलाय् नं खनदग, गगु यीसिज्जान यूनिरय् मायन याथगु ज्ञा जगु खऒ । थूयिं लिब्रऒयाखं थाय् गन धूकाऒ नं राळ्ळनस यनावनदू धयागु सी द् ।

भनूगयगु द्वाऒ याखया नाभा राळ्ळनस यनावनीगु यनगु दकल याय्जिगु उयाय् ६ द्वाऒ यव्कगु

(दायुक्त दाहजिनिद्ध) रवः । जायाः यानाः उसायुं धूताः गळंगु थासयुं सावां लखं द्वाः सिलगु स्यां जयुलारवं द्वाः यवकगु (जकाहल दायुक्त तिसुव्वन्नुक्कटन) गनिका थलगु याव्वं । जथ जयाः ६ मानव व्वन्नुक्कटनक राव्वन्स व सासः याखं यनीगु राव्वन्स नाय माप्रासक वारुक यनिज्ज (कानियन टट्ट) व दिद्धन थाउत्सु याखं यनिज्ज याना स्यागु जल । दिद्धन थाउत्सु टट्टयाखं सावां लखं द्वाः सिलगु व जयुलारवं द्वाः द्दुव्वंगु (जकाहल दायुक्त तिसुव्वन्नुक्कटन) विधिया गुलना याना स्यागु जल । थुगु ज्जयनयुं मानव नाभारव्वन्स GI.4 व GII.4 व उकिया उपायन मूलक भाउल (कलिट्टरुवल भाउल) एम एन सी १ लिस गुलना याथगु का जल (व १) । जयुलारवं द्वाः द्दुव्वंगु यनिज्जयुं भाटा राव्वन्स, व्वन्नुक्कटन राव्वन्सया युकीगु जमगा ३० सकंभुयुं युवकं कयाल दग । थुकिया ज्जखः थालिया राव्वन्स सविन १, जाठिना राव्वन्स टाव्वय १, यानका राव्वन्स १, एम एन सी १ या युकीगु जमगा ३ मिन्ट दूत $< ३ \log_{10}$ का जल । सावां लखं ३० सकंभु गक द्वाः सिलावल एम एन सी १ या युकीगु जमगायुं का जगु ($> ३ \pm ०.४ \log_{10}$), जयुलारवं द्वाः सिलवल युकीगु जमगायुं का जगु ($> २.८ \pm १.१ \log_{10}$) स्यां सीपयक जायाः जल । सावां लखं द्वाः सिलावल एम एन सी १ ($\pm \log_{10}$), या ज्जनामिक कयिज युवकं ६ चीकरग । उकं मीगु द्वाः भाभा राव्वन्स चीकर जयुलारवं द्वाः सिलगु स्यां सावां लखं द्वाः सिलगु जयः यायुक्क रवः धयागु सीकरग ।

पकल लिया, मानव नाभा राव्वन्स व मगु यनिज्ज जगु व्वन्नुक्कटनक व सासः याखं यनीगु राव्वन्सयाग द्वाः व दूषिग थासं घनावनीगुयाग का याथ द्दुव्वंगु जल । थुगु साध यपया लिब्रः कथं राव्वन्स यनावनाव्वंगु व्वलयुं गिनागः काऽया सगहया खनमदूयिं की मययकगु गिगिं १ घयायाग १२१ यी.यी.एम. दाह्वं ज्ञान यनाकाव्वं वा (वार) थनग थागा वियागु जल । दूषिग सगहलयुं मानव नाभारव्वन्स मययकगु १००० यी.यी.एम. कानिन लः थलगु थागा वियागु जल । द्वायां सावां लखं द्दुव्वंगु जल १००० यी.यी.एम. कानिन लखं १० गू मिन्टया गिगिं थट्ट तिसुव्वन्नुक्कटन विधियाखं यगिम्हायना यानाः मानव नाभा राव्वन्सया ज्जनामिक कयिज युवकं ६ ($> १ \log_{10}$) का याथ द्दुव्वं । उसायुं रिंकीगु थासयुं नाभा राव्वन्स घनावनीगु यनग थलीगु जकाहल दायुक्त तिसुव्वन्नुक्कटनयाग ३० सकंभु गक सावां लखं द्वाः सिलगु विधि थलाः हिउयाः हयमाः गगु दू उवल ६ \log_{10} यीसीजानयुं ज्जनामिक कयिज का याथमाः गगु जल । जथ रवःसां ज्जयुक लः मयव्वंगु व्वलयुं व ज्जनामिक कयिज का जक याः गगानां गाः गगु ($२ \log_{10}$ यीसीजानयुं व्वलयुं जयुलारवं द्वाः द्दुव्वंगु विधि (जकाहलिक तिसुव्वन्नुक्कटन) जक थःसां गाः ।

थुगु साधयपया गिगिं याना स्यागु जन्सधनयाखं नाभा राव्वन्स घनावनीगुयाग का याथगु विधिगयुं गैसा ज्ञान थुगु जल । लिस लिसं थुकिं व्वन्नुक्कटनक व सासः याखं यनीगु राव्वन्सया गुलनासक जटा मूकदूगु जल । जल मगु, मानव नाभा राव्वन्सया थायुं हिलीगु जमगाया ज्ञान नं थुगु जल । उकं थुकिया लिब्रः याखं नाभा राव्वन्स व यनिज्ज याना स्यागु मगु व्वन्नुक्कटनक व सासः याखं यनीगु राव्वन्स चीकरग व यनगु विधिया थलाव्वायुं नं शाहलि व गिवः वीरुव्वंगु जल ।

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Era Tuladhar

Curriculum Vitae

Era Tuladhar was born on August 22, 1972 in Kathmandu, Nepal. She followed high school at Ananda Kuti Vidya Peeth, Swyambhu, Kathmandu and later joined Amrit Science campus for a certificate level study in Science. She completed her Bachelor study from Trichandra Multiple College, Tin Dhara, Kathmandu. After that, she did her Masters in Microbiology from Tribhuvan University, Kirtipur. For her thesis she studied 'Microbiological quality of cheese sold in Kathmandu market'. She was awarded with the 'Mahendra Bidya Bhusan Award' for securing the highest mark in Microbiology for the year 1996. After her Masters, in 1997 she joined Nepal Drugs Limited, a semi-government pharmaceutical company, where she worked as a senior quality control officer in the Quality Control Division.

In 2003 she got a Nuffic Scholarship for perusing a second Masters in Biotechnology at Wageningen University with specialization in cellular and molecular biotechnology. During her Master she did her thesis in the Department of Virology and an internship in the Department of Immunology. After completion of her Masters in 2005, she joined the company back in Nepal. She was also involved in teaching Microbiology and Biotechnology at Bachelor and Master levels in various institutions.

In 2008 she started her PhD program entitled 'Intervention methods to control the transmission of noroviruses and other enteric and respiratory viruses.' For the research she worked at the National Institute for Public Health and the Environment, Laboratory for Infectious Disease, Centre for Disease Control for The Netherlands (RIVM), for more than 2 years and later continued her research in the Laboratory of Food Microbiology, Wageningen University. In 2011 she received a poster prize award for the poster entitled 'Virucidal efficacy of vaporized hydrogen peroxide disinfection' at the European Symposium on Food Safety, which was held in Ede, The Netherlands. After completing her PhD degree, she will go back to the home country Nepal and continue to serve the nation.

List of publications

1. **Tuladhar, E., M. Bouwknegt, M. H. Zwietering, M. Koopmans, and E. Duizer.** 2012. Thermal stability of structurally different viruses with proven or potential relevance to food safety. *J Appl Microbiol* **112**:1050-1057.
2. **Tuladhar, E., M. C. de Koning, I. Fundeanu, R. Beumer, and E. Duizer.** 2012. Different virucidal activities of hyperbranched quaternary ammonium coatings on poliovirus and influenza virus. *Appl Environ Microbiol* **78**:2456-2458.
3. **Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, R. R. Beumer, and E. Duizer.** 2012. Residual viral and bacterial contamination of surfaces after cleaning and disinfection. *Appl Environ Microbiol* **78**:7769-7775.
4. **Tuladhar, E., P. Terpstra, M. Koopmans, and E. Duizer.** 2012. Virucidal efficacy of hydrogen peroxide vapour disinfection. *J Hosp Infect* **80**:110-115.
5. **Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, E. Duizer, and R. R. Beumer.** 2013. Transfer of noroviruses between fingers and fomites and food products. *Int J Food Microbiol* **167**:346-352.
6. **Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, E. Duizer, and R. R. Beumer.** 2014. Residual viral contamination from finger pads: hand washing is more secure than alcohol based hand disinfectants (Submitted for publication).
7. **Wang, M., E. Tuladhar, S. Shen, H. Wang, M. M. van Oers, J. M. Vlak, and M. Westenberg.** 2010. Specificity of baculovirus P6.9 basic DNA-binding proteins and critical role of the C terminus in virion formation. *J Virol* **84**:8821-8828.
8. **Tuladhar, E. and A. Sharma.** 1997. Bacteriological study of Cheese of Kathmandu City, *Journal of Nepal Medical Association* **35**:26-29.

Other scientific output

1. **Tuladhar, E., M. Koopmans, E. Duizer.** 2009. Thermal inactivation of confirmed and possible food-borne viruses. Future challenges in Food and Environmental Virology, 2nd Cost 929 symposium Istanbul Turkey, 7-9 October 2010.
2. **Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, R. R. Beumer, and E. Duizer.** 2011. Effects of wiping in reducing the load of several viruses on stainless steel. In Program Book 14th Annual Meeting of the European Society of Clinical Virology, Funchal, Madeira, 21-24 September 2011 (p. P0140).
3. **Tuladhar, E., P. Terpstra, M. Koopmans, and E. Duizer.** 2011. Virucidal efficacy of vaporized hydrogen peroxide disinfection. In IAFP's European Symposium on Food Safety, Ede, The Netherlands, 18-20 May 2011 (p. P1-05). IAFP.
4. **Tuladhar, E., R. R. Beumer, W. C. Hazeleger and E. Duizer.** 2011. The virucidal efficacy of hand disinfectants. *Nederlands Tijdschrift voor Medische Microbiologie*, 19 (Supplement), S78. (NVvM 100 Years Symposium, The Netherlands).
5. **Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, R. R. Beumer, and E. Duizer.** 2012. Effects of wiping and spot disinfection in reducing the load of enteric viruses. In Annual Meeting of European Society of Pediatric Infectious Disease ESPID, Thessaloniki, Greece, 8-12 May 2012.
6. **Tuladhar, E., W. C. Hazeleger, M. Koopmans, M. H. Zwietering, E. Duizer and R. R. Beumer.** 2012. Transfer of noroviruses from hands to tomatoes, cucumbers and surfaces. In 23rd International Symposium of the International Committee on Food Microbiology and Hygiene of the IUMS (ICFMH) Symposium- Food Micro 2012, Turkey, 3-7 Sep 2012.

Overview of completed training activities

Discipline specific activities

Courses

Management of Microbiological Hazards in Foods, VLAG, Wageningen (2008)
Laboratory Training on MNV1 Culture, University of Ghent, Belgium (2008)
Workshop Bioinformatics Analysis, Tools and Services, RIVM, Bilthoven (2008)
Handling Genetic Modified Organism, RIVM, Bilthoven (2009)
Advanced Virology Course, Erasmus MC, Rotterdam (2010)
Genetics and Physiology of Food-Associated Microorganisms, VLAG, Wageningen (2010)

Meetings

European Society for Clinical Virology Winter Meeting, The Netherlands (2009)
Cost Virology Meeting, Turkey (2010, oral presentation)
NVvM 100 Years Symposium, The Netherlands (2011, oral presentation)
IAFP European Symposium, The Netherlands (2011, poster presentation)
14th Annual Meeting of European Society for Clinical Virology, Portugal (2011, poster presentation)
Annual Meeting of European Society of Pediatric Infectious Disease, Greece (2012, poster presentation)
23rd International ICFMH Symposium-Food Microbiology, Turkey (2012, oral presentation)

General courses

Techniques for Writing and Presenting Scientific Papers, Wageningen (2009)
Basic Statistics PE & RC, Wageningen (2009)
Project and Time Management, WSG, Wageningen (2010)
Science, Media and General Public, Wageningen (2011)
Writing for Academic Publication by Linda McPhee, Wageningen (2011)
Endnote and Information Literacy, Wageningen (2011)

Other activities

Preparation of Research Proposal, Bilthoven (2008)
VLAG PhD Week, Bilthoven (2009)
Master Class CDC Atlanta, Bilthoven (2009)
Poster Presentation at Internal Audit at RIVM (2009)
PhD Trip, Switzerland (2010)
Scientific Meetings at LIS-VIR Department, RIVM (2008-2010)
Scientific Meetings at Gastrointestinal group, VIR at RIVM (2008-2010)
PhD days and PhD sessions (2008-2010)
Attending Proneri days (2008-2011)
Supervising BSc and MSc Students for Theses and Practical Courses (2008-2012)
Poster Presentation at SOR Project, RIVM (2008-2012)
Work Discussion Meetings-Laboratory of Food Microbiology (2010-2012)

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- Cover design : Era Tuladhar
- Front cover : Microscopic picture of Hep-2 cells infected with adenovirus type 5 with appearance of cytopathic effect (CPE)
- Back cover : Microscopic picture of MA-104 cells infected with rotavirus SA 11 (left)
Visible MNV1 plaques on Raw 264.7 cells (middle)
Microscopic picture of HT-29 cells infected with parechovirus 1 (right)

