FSA Project FS101120:

A critical review on the survival and elimination of norovirus in food and on food contact surfaces

A Report to the United Kingdom Food Standards Agency

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Executive Summary

Foodborne outbreaks of human norovirus (NoV) gastroenteritis demonstrate that NoV have the ability to survive within foods and the environment for extended periods. Several studies have been carried out to determine the survival characteristics of NoV, and have attempted to evaluate whether procedures used in the food industry for elimination of pathogens are effective against NoV. The lack of an established or effective *in vitro* system for determining NoV infectivity continues to hamper the acquisition of extensive and precise data on NoV survival characteristics. A few studies have used human volunteers to directly determine whether the virus is infectious after treatment, and such studies are useful in providing definitive information. Today, most studies use molecular methods (RT-qPCR) to detect portions of the viral RNA as indicators of NoV survival or elimination. When a decline in numbers of virus genome equivalents has been observed, a reliable inference is that the treatment had an effect on the numbers of infectious virus particles but no reliable inference can be made when the studies observe no decline in genome equivalents.

The principal conclusions which can be drawn from this critical review are that NoV is resistant to freezing and thawing and may persist in water for extended periods, possibly for several weeks, depending on the source of the water. The persistence of NoV in fruits and vegetables may exceed 7 days at refrigeration or room temperatures, Studies on bivalve shellfish showed that NoV can persist within shellfish subjected to depuration for 7 days. The NoV genome has been observed to persist in other food products such as cooked turkey (at least 10 d at 7^{o}C), processed foods (at least 2 days at 6^{o}C), and apples (at least 7 d at 11^{o}C), however the duration of persistence is likely dependent on the level of contamination. The NoV genome was resilient on Formica, stainless steel, PVC and ceramic surfaces, and on human finger pads.

Numerous studies were reviewed on the inactivation of NoV by various interventions. Studies involving heating as a means to degrade NoV RNA on surfaces or suspended in various solutions showed widely varying results, depending on the technique applied and the composition of the solutions. Autoclaving was effective in reducing NoV by at least 5 log. Heating of NoV at 63^{o}C and above in various suspensions reduced NoV RNA levels, although temperatures required to destroy the RNA depended greatly on the suspension solutions and the duration of application.

Chlorine appears to be a suitable disinfectant against NoV. In a volunteer study, treatment of water with 10 mg ml^{-1} of chlorine for 30 min (reportedly 5-6 mg ml^{-1} free chlorine) prevented NoV infection of volunteers. Other studies evaluated the effectiveness of chlorine/sodium hypochlorite to reduce NoV RNA with generally good results. Chlorine solutions at concentrations of 200-500 ppm were also effective as a surface disinfectant for steel, melamine, berries and herbs. Hypochlorous acid was demonstrated to be effective in reducing NoV RNA. Hydrogen peroxide, quaternary ammonium compounds, ethoxylated alcohol-based disinfectants and antiseptics were generally ineffective as disinfectants against NoV. Ethanol-based hand sanitizers at concentration ≥90% alcohol were also relatively ineffective in reducing NoV. Some commercial disinfectants containing alcohols formulated with other compounds were reportedly effective against NoV. Liquid soap containing 0.5 % triclosan and a water rinse alone reduced the levels of NoV on fingers of volunteers by 1-1.5 log.

Various processing techniques have shown some promise in reducing NoV loads from foods, namely the use of heat; high hydrostatic pressure to inactivate NoV in oysters, as determined by a volunteer study, and on berries; ozone in solutions containing NoV; and for moderate reductions of NoV on surfaces. High pH (8.0-10.0) appears to destabilize NoV capsids and could be useful as a rinse to disinfect the surface of some food products whereas neutral and low pH had no effect. Heating and marinating mussels in a pH 3.75 solution was ineffective in reducing NoV levels. Depuration has also been unsuccessful in totally eliminating NoV from shellfish. Freeze drying of some products (like blueberries and parsley) reduced NoV levels by up to 3.5 log; however, reductions are generally better for GII NoV. Gamma irradiation appeared to alter the NoV capsid structure but its effect on NoV RNA levels or infectivity has not been studied. Ultraviolet (UV) irradiation reduced RT-PCR products slightly in some studies and more extensively in others indicating that NoV is inactivated by UV. From an industrial standpoint, thorough washing of basil with water was effective in reducing NoV GI by up to 3.4 log and GII by 1.1 log; while, similar washing of blueberries reduced G1 NoV by <1 log and GII by ≤2.1 log.

Further research is needed on the development of cell culture propagation systems for NoV. Reports and data on successful attempts to propagate NoV should be reviewed and additional funding to support promising propagation studies should be considered to determine if the results are reproducible. Questions that will require further clarification about the

effectiveness of cell culture propagation studies include: is the proposed culture system limited to specific genogroups or genotypes, what is the apparent multiplicity of infection, and could the method be applied to environmental samples? In the future, culturing may well be the principle method to monitor NoV infectivity. With the advent of practical cell culture propagation methods potentially on the horizon, methods should be developed and evaluated to extract and concentrate intact NoV from foods and environmental samples for use in future cell culture analyses. Without sensitive and reproducible extraction protocols, comparisons of data from one laboratory to another or even within a laboratory may not be possible.

Progress on other potential methods to determine infectious versus inactivated NoV, such as the porcine mucin binding assay as a possible indicator of virus infectivity, should be closely monitored and support for validation studies of those methods deemed most promising should be explored. Volunteer studies are the only conclusive means currently available to identify NoV infectivity. Such studies offer the opportunity to determine the ability to inactivate NoV under various environmental conditions or processing scenarios. In the absence of other infectivity assays, volunteer studies can provide definitive information and should be considered for funding priority.

Research with NoV surrogates, like feline calicivirus and murine norovirus, have provided little to no insight into the inactivation of human NoV. Research and publications on NoV surrogates should be discontinued unless the inactivation kinetics of the surrogate is directly compared with the inactivation kinetics of NoV under the same well defined conditions. The literature on surrogate viruses was eliminated from this review, unless it was compared directly with NoV. Unfortunately, most surrogate studies do not involve any comparisons with NoV and are of little value.

Much progress has been made in recent years in developing harmonised methods for NoV extraction from various commodities but further research is needed. Current methods for NoV detection generally require the use of RT-qPCR, which requires that viral RNA be extracted for analysis; therefore, current extraction procedures for NoV are designed to extract viral RNA. The limitations in the use of RT-qPCR must be recognised and assay methods should be harmonised to the extent possible. There are many different NoV within several genogroups and all represent unique challenges for their detection and quantification. The use of RT-qPCR may well be discontinued as a means of NoV detection and

quantification once cell culture propagation methods are developed for NoV. Until that time, RT-qPCR should be standardised to the extent possible. Data on the occurrence and survival of NoV in the environment are limited by the very low levels of contamination present. This could be improved by the application of rapid and efficient separation and concentration methods. Collaborative research on NoV should be supported as it is useful to ensure repeatability of methods and it adds confidence to experimental results.

Keywords: Norovirus, survival, persistence, elimination, food, water, surfaces, heat, disinfection.

Glossary

Antigenicity. The capacity to stimulate the production of antibodies or the capacity to react with an antibody.

Capsid. The protein shell of a virus.

Cryo-electron microscopy. A form of transmission electron microscopy where the sample is studied at cryogenic temperatures.

Ct: **Cycle threshold value.** The relative concentration of a PCR product based on the fluorescence intensity reaching an arbitrarily chosen threshold level during real-time PCR

Electron beam irradiation. A process which involves using electrons, usually of high energy, to treat an object for a variety of purposes (e.g. sterilization).

Elimination. Removal of infectious virus by a deliberately applied procedure.

FCV: feline calicivirus. A commonly used surrogate for human norovirus

Fomite. Any object or substance capable of carrying infectious organisms, and hence transferring them from one individual to another.

GE: Genome equivalents. The approximated copy number of the RT-(q)PCR target sequence based on standards or the dilution of known positive samples to extinction.

HHP: High hydrostatic pressure. A food preservation technology based on the inactivation of microorganisms by applying extreme pressures.

Inactivation. The decline in virus infectivity effected by a deliberately applied procedure or through natural processes.

kGy: Kilogray. A measure of radioactivity.

MPa: MegaPascals. A measure of force per unit area, defined as 1,000,000 newton per square metre.

MNV: Murine norovirus. A commonly used surrogate for human noroviruses

NoV: Human norovirus. A member of the *Caliciviridae* family and a frequent cause of gastroenteritis.

ORF: Open reading frame. The part of a gene that contains no stop codons.

Picornavirus. A virus belonging to the family *Picornaviridae*. They are non-enveloped, positive-stranded RNA viruses with an icosahedral capsid.

PFU: Plaque-forming units. Number of infectious virus units which can be quantified by the formation of discrete zones of infection on cell culture monolayers.

Positive-sense RNA virus. A virus whose genetic information consists on a single strand of RNA that is the positive (or sense) strand which encodes mRNA and protein.

RT-PCR: Reverse transcription polymerase chain reaction. A molecular technique to amplify and subsequently detect the amount of viral or other RNA in a sample.

RT-qPCR. RT-PCR used to estimate the number of original target RNA copies in the reaction, based on calibration using known standards.

Stability. Persistence of the intact virus capsid.

Survival. The persistence of virus infectivity after exposure to natural or man-made conditions.

T90. Time taken for the first 90% reduction in virus infectivity, where the decay rate is linear.

$TCID_{50}$: Tissue culture infectious $dose_{50}$. The dilution of a virus suspension which can infect 50% of cultured cell sheets exposed to it (analogous to a most probable number test in bacteriology). $TCID_{50}$ can be related to infectious virus units.

TFL: The time required to reduce the first log_{10} of infectivity. Log_{10} reduction in virus infectivity, where the decay rate is non-linear.

Virion. A complete virus particle that consists of an RNA or DNA core with a protein coat sometimes with an external envelope and that is the extracellular infective form of a virus.

VLP: Virus-like proteins. An assembly of virus proteins that usually self-form to produce capsid-like structures that do not contain nucleic acids and which are therefore non-infectious.

1. Introduction

1.1 Background to the report

Human enteric viruses are known to be a major cause of infectious intestinal disease in the UK, with foodborne transmission an important route of infection. The Food Standards Agency (FSA) funded a second study of infectious intestinal disease in the community (IID2 Study) which provides extensive information on the enteric virus-associated burden of infection (http://www.foodbase.org.uk/results.php?f_report_id=711). Foodborne transmission of NoV is a concern, however there are significant gaps in our knowledge regarding the survival of NoV in foods and the environment (including food contact surfaces), and also regarding the effect of elimination procedures used in food supply chain settings.

In April 2015, the FSA launched its Strategic Plan for 2015-20[1] which focuses on 6 key areas one of which is to continue to make sure food is safe and that people are not exposed to unacceptable risk. As part of this strategy, the FSA will evaluate the effect of interventions designed to reduce foodborne disease in the UK, including illness caused by emerging pathogens and agents not specifically addressed by individual risk management programmes. To assist the FSA with this evaluation, it is timely to review the available information on survival and elimination of NoV, to inform risk analysis and to assist in identifying effective control measures to reduce the occurrence of foodborne transmission of these pathogens.

A recent systematic review of the available scientific information (FS241043)[2] indicated that NoV is more robust than surrogate studies have suggested. The critical review presented here aims to build on the systematic findings by an in-depth examination of published studies and other relevant information to determine the survival characteristics of NoV in foods and on food contact surfaces, whether the food matrix or surface structure affects NoV survival or inactivation, and whether chemical or physical treatments are effective in reducing or eliminating NoV in the food chain. The review will also identify the key knowledge gaps, and make recommendations on how to fill them where it is practical to do so given the limitations of available technology. The review will discuss what may be inferred regarding the effectiveness of current disinfection procedures, and whether these are appropriate for

[1] The FSA Strategic Plan 2015-20 is available at:
http://fsahome/aboutus/whatwedo/Pages/fsastrategyto2020.aspx
[2] FS241043 is available at:
http://www.food.gov.uk/science/research/foodborneillness/b14programme/b14projlist/fs241043

control of NoV contamination of foods, and in food production and preparation environments. This information will be useful to inform specific hazard characterisation and risk managers in the control and reduction of human NoV contamination of foodstuffs. The knowledge gaps, which will be identified by the project, will form a foundation for consideration of future research needs to advance food safety and enhance public health by reducing virus transmission, which should ultimately contribute to a decrease in the burden of foodborne NoV illness.

1.2 Norovirus

Noroviruses are known to be the most important causative agents of acute gastroenteritis worldwide (Belliot *et al.*, 2014) in all age groups. Norovirus infection is typically acquired via the faecal-oral route by person-to-person contact, or the indirect routes, such as ingestion via contaminated food or from surfaces (Duizer & Koopmans, 2014). Noroviruses (previously known as Norwalk virus) belong to the family *Caliciviridae*, genus *Norovirus*, and are non-enveloped, single stranded, RNA viruses. They are small in size (27-38 nm in diameter) and have an amorphous surface structure. They contain a positive sense RNA genome of approximately 7.5 kb encoding a major structural protein (VP1) and a minor capsid protein (VP2). The genus can be subdivided into five genogroups (GI-GV), with GI, GII, and GIV being the most common to infect humans.

NoV infections can be difficult to prevent and control due to their low infectious dose, high environmental stability and high titre shedding post infection (Barclay *et al.*, 2014). It has been estimated (Teunis *et al.*, 2008) that ingestion of approximately 18 NoV particles can result in a 50% probability of infection. The majority of outbreaks occur during the winter season, however sporadic cases can occur at any time throughout the year (Lopman *et al.*, 2003). Those in community health and social care settings are at particular risk, due to the close proximity of high numbers of vulnerable patients. In order to meet these challenges, there must be robust surveillance studies of NoV activity in place both in organisations and the wider community (MacCannell *et al.*, 2011). One such study involved NoV shedding within a population of 102 persons, including those who were asymptomatic. A quantitative, dynamic model was used to calculate the shedding level. The results show a similarity of levels of shedding between symptomatic and asymptomatic subjects who were infected with NoV, showing a high variability in levels (average 10^5–10^9 g^{-1} faeces) over a duration of 8-60 days (Teunis *et al.*, 2014). With this information, it is understandable why NoV is so efficient

at infecting large numbers of people in close proximity in settings such as cruise ships, where isolation of affected persons and major clean-up procedures are the only measures which can be taken. These types of outbreaks occur on a relatively common basis, due to the ability of the virus to quickly spread amongst populations in close contact, as in nursing homes, hospitals, and on cruise ships. Problematic areas are those which are regularly in contact with the hands, such as door handles, water closet facilities and any other surface which is handled by multiple persons (Barker *et al.*, 2004; Repp & Keene, 2012). Foodstuffs most commonly associated with norovirus contamination tend to be raw or minimally processed, such as berry fruits and leafy green vegetables (Rodriguez-Lazaro *et al.*, 2012), and bivalve molluscan shellfish (Campos & Lees, 2014).

Due to the inability to culture NoVs reliably *in vitro*, currently the only way to estimate the infectious dose of NoV is by human volunteer studies. It is important to be able to calculate the infectious dose of NoVs for the purpose of risk assessment studies. One study carried out in the United States enrolled 57 persons in a randomized, double-blind, placebo-controlled evaluation of different dosages of NoV. The 50% human infectious dose was calculated to be approximately 1320 genome equivalents for secretor-positive blood group O or A persons and approximately 2800 genome equivalents for all secretor-positive persons (Atmar *et al.*, 2014).

NoVs are not yet culturable; therefore, the only practical way to detect them for research and monitoring purposes is by real-time reverse transcription PCR (RT-PCR). This detects the RNA genetic material of the virus. This can be used for a variety of matrices including stool, environmental and food samples. The test can be used to further distinguish between genogroups I and II (GI and GII), with GII being the most common genotypes associated with human illness.

1.3 Efforts to propagate human norovirus in cell or tissue cultures

Research on human NoV persistence in foods, the environment and in clinical samples has been stymied by the inability to propagate infectious human NoV in cell lines, tissue cultures, or in small animal models. As a result, methods for determining the ability of chemical and physical treatments and processing technologies to reduce NoV have remained elusive. For over 40 years, many laboratories worldwide have attempted, without success, to replicate NoV in cell culture systems. Most of these efforts remain unpublished. Consequently, the

same research approaches may have been duplicated repeatedly by laboratories around the world, wasting valuable research funds and scientific efforts. A few reports describing unsuccessful attempts to propagate human NoV in cell culture have been published (Duizer *et al.*, 2004; Malik *et al.*, 2005; Richards, 2009) and offer some appreciation for the difficulties faced in this quest. In propagation studies, virus infectivity is based on the appearance of cytopathic effects in the host cells, plaque formation in monolayer cultures, and/or direct assay for viral proteins or RNA.

Early studies by Richards and colleagues were conducted before the discovery of RT-PCR and employed ELISA assays to monitor for increases in NoV proteins in cell lines and tissue culture cells that had been subjected to filtrates of stool-derived human NoV GI.1 (8F2a strain) (Richards, 2009). No positive outcomes were detected, perhaps as the result of the insensitivity of the ELISA assay. With the advent of RT-PCR, additional testing was performed on a variety of monkey and human cell lines and on primary monkey kidney cells, all of which can be infected by other virus types. Assays were performed over extended periods, with some cells and viruses pre-treated with trypsin or human bile, and others subjected to various pHs. The successful propagation of human NoV could not be detected by cytopathic effects (CPE) on the cells, plaque assay or by molecular techniques. Tests were performed to evaluate the effects of various treatments on virus replication, including different salt concentrations, media and buffer types, and the presence or absence of serum in the media of several cell lines. No virus replication was detected by RT-PCR (Richards, 2009).

A long history of efforts to propagate NoV was also published by Duizer and colleagues (Duizer *et al.*, 2004) describing research in the US and the Netherlands to propagate NoV in a wide range of human, monkey, and other animal cell lines. Cells were challenged with a variety of GI and GII strains under a wide variety of conditions including the use of modifying chemicals, like DMSO, butyric acid, insulin, dexamethasone, saliva, trypsin, pancreatin, bile, and a monkey intestinal content preparation on cell cultures or viral suspensions. None of the assays clearly demonstrated any replication of NoV (Duizer *et al.*, 2004).

Malik *et al.* (2005) also published a report on their attempts to propagate NoV on seven primary cultures and 13 continuous cell lines derived from 11 different animal species. Cells

were challenged with filtered NoV stool followed by adsorption for 90 min with constant shaking of the inoculum to promote adsorption, but neither CPE nor positive RT-PCR amplicon was detected after five passages of the cells (Malik *et al.*, 2005).

Excitement was generated in 2007 when it appeared that human NoV was successfully propagated in cell cultures using a 3-dimensional human small intestinal epithelium organoid model (Straub *et al.*, 2007; 2011). Although promising, those results could not be replicated in follow-up studies in Int-407 cells (Herbst-Kralovetz *et al.*, 2013; Papafragkou *et al.*, 2014) or in Int-407 and CaCo-2 cells (Papafragkou *et al.*, 2014; Takanashi *et al.*, 2014).

Recently, Jones *et al.* (2014) published a study reporting the successful propagation of GII.4 NoV in a human lymphoblastoid B cell line (BJAB) in the presence of enteric bacteria that express histo blood group antigens. Viral genome copies increased 10- and 25-fold at 3 and 5 days post infection, respectively. Increases were also reported in the levels of viral structural and non-structural proteins. In a co-culture of HT-29 intestinal epithelial cells and BJAB cells, there was a nearly 600-fold increase in viral genomes in 3 days. These data look promising, but will need to be validated in other laboratories. If successful, a cell culture-based assay for practical use in the evaluation of NoV infectivity may become available soon.

1.4. Norovirus surrogates

The inability to propagate human NoV has led to the use of a variety of potential NoV surrogates. Ideally a surrogate is a virus that responds in a manner that is the same or very similar to that of the virus it represents. Among the surrogates used for human NoV are murine norovirus (MNV), feline calicivirus (FCV), and Tulane virus, all of which can be propagated in cell culture. Over the years, much research has been performed with MNV and FCV; however their track record as valid surrogates has been disappointing. A critical review of the surrogates shows that most do not mimic their human NoV host in regard to survival, or persistence under various environmental conditions or processing procedures (Richards 2012). Consequently, it was recommended that the use of surrogates be abandoned unless their use is directly tied with studies where their inactivation may be directly linked with the inactivation of human NoV, in an effort to validate the use of the surrogate under well-defined conditions (Richards 2012). In place of surrogate research, human clinical trials (volunteer studies) were recommended to provide definitive information on NoV persistence and inactivation (Richards 2012).

This report does not review studies performed exclusively with surrogate data. In some of the studies reviewed, the survival of surrogate viruses or the effect of elimination procedures upon them, was determined simultaneously with the persistence or elimination of NoV genome equivalents (GE). In such cases, the response of the surrogates to the treatments is briefly mentioned, however extrapolations to the possible response of infectious NoV should be made with caution.

1.5. Volunteer studies

The infectivity of NoV can be determined in clinical trials designed to minimise risk of undesirable effects to the volunteers. Clinical trials are expensive and time consuming; however, they can provide definitive evidence of NoV infectivity in humans. To date, four NoV challenge studies have been identified involving infectivity determinations based on NoV consumption (Grohmann *et al.*, 1981; Keswick *et al.*, 1985; Seitz *et al.*, 2011; Leon *et al.*, 2011). Grohmann *et al.* (1981) evaluated the effectiveness of oyster depuration on NoV infectivity in volunteers and found that properly depurated oysters still contained sufficient levels of NoV to cause infection in the volunteers. Keswick *et al.* (1985) used volunteers to evaluate the effectiveness of chlorine to inactivate NoV in water, while Seitz *et al.* (2011) studied the persistence of NoV in ground water over time. Leon *et al.* (2011) demonstrated the effectiveness of high pressure processing to eliminate NoV in oysters. Studies conducted by Liu *et al.* (2009, 2010, 2011) evaluated the effectiveness of rinsing and the use of liquid soap and various hand sanitizers to remove NoV GI and GII from the fingers of volunteers; however, virus presence was determined by RT-PCR and infection of volunteers was not the means of determining infectivity. These studies will be discussed in more detail below.

1.6. Molecular detection methods for noroviruses and their limitations

In most of the studies reviewed for this report, quantification of NoV has been performed using RT-PCR. Quantification by RT-PCR can be performed using conventional (i.e. detection of amplicons by gel electrophoresis) or a real-time quantitative format. In the former approach, dilutions of a viral RNA solution are analysed by RT-PCR, and the highest dilution where a positive signal is obtained is interpreted as the reciprocal of the number of RNA targets present. In real-time quantitative RT-PCR (RT-qPCR), standard solutions containing known copy numbers of target RNA are used to calibrate the RT-PCR, so that the copy number in a nucleic acid extract derived from a sample can be estimated by comparing

the signal with the calibration curve. In this review any such data will be expressed as "genome equivalents" (GE) on the supposition that the lowest detectable unit or copy number may represent amplification of one target RNA molecule, and to facilitate a harmonised comparison of findings of the different studies. It should be noted that due to the lack of culturable NoV (and consequently well-established reference materials), detection and quantification limits may differ depending upon the exact experimental conditions used in the cited works.

There are serious limitations in the use of RT-PCR-based methods alone in determining the infectivity of NoV in foods (Richards, 1999) and by extension, in environmental and clinical samples. This stems from the fact that viral RNA that is enclosed within an intact capsid would be protected from destruction by exogenous RNases even though the surfaces of the viral capsid may have been degraded sufficiently to prevent it from binding to cellular receptors. Thus NoV within intact capsids would give positive RT-PCR results, even though the virus may have been inactivated by damage to surface structures which are key to the subsequent binding and penetration of the virus into susceptible host cells, without which natural infection cannot occur. Thus the detection of NoV RNA *per se* does not by itself confirm that infectious virus particles are present[3]. In contrast, the absence of NoV RT-PCR amplicon suggests that RNA is absent or has been totally inactivated, but could also indicate that there are inhibitors interfering with RT-PCR. The method of extraction and concentration of NoV from foods, water and contact surfaces may also affect RT-PCR results since virus yields may vary greatly depending on the method uses. Likewise considerable variability in RT-PCR results may occur depending on the target, primers and probes, cycling conditions, age and brand of the reagents and the PCR platform employed. In order for RT-PCR data to be meaningful, it is essential to include adequate positive and negative controls in the analyses.

Different inactivation treatments cause inactivation of NoV either by acting on the capsid, the genome or both. Transfection studies and PCR data have shown that the genome is not degraded by heat and that heat treatment does not significantly impact the RT-PCR data unless an enzymatic pre-treatment is applied to preferentially degrade exposed RNA

[3] Also, it is possible that free RNA may be able to persist in the environment under some conditions. Purified NoV RNA could be detected in tap water and filtered groundwater samples for at least 14 d (Seitz *et al.*, 2011), and Dancer *et al.* (2010) could detect short fragments of NoV GI RNA spiked into seawater samples for up to 14 d.

(Nuanualsuwan and Cliver, 2002 & 2003). For poliovirus, low temperature virus inactivation at 37°C for 30 min results in a loss of receptor binding to HeLa host cells and an altered cell tropism, resulting in transient infection of CHOs and murine L-cells without RNA exposure. In contrast available chlorine and chlorine dioxide are oxidising agents that primarily act upon the genome (O'Brien and Newman, 1979; Alvarez and O'Brien, 1982). Other treatments such as UV or ionising radiation also predominantly act upon the genome resulting in strand scission or dimerization of bases (typically pyrimidine dimers). Owing to the difference in inactivation mechanisms, it is difficult to assess the significance of RT-qPCR signals. Pre-treatment of viruses with RNase to eliminate the genomic material from damaged (and therefore non-infectious) capsids has been proposed as a useful step in estimating the infectivity of NoV (Nuanualsuwan and Cliver, 2002; Pecson *et al.*, 2009; Topping *et al.*, 2009; Nowak *et al.*, 2011a & 2011b). Unfortunately even following RNase treatment RT-qPCR signals can persist as ribonucleoprotein (RNP) when infectivity has been abolished leading to an underestimation of loss of infectivity (Knight *et al.*, 2014). In general RT-qPCR signals significantly underestimate loss of infectivity owing both to the presence or formation of RNP and the fact that the RT-qPCR target sequence typically represents a small fraction of the genome (Knight *et al.*, 2014). Small RT-PCR targets although allowing efficient amplification cannot detect loss of genomic integrity elsewhere in the genome. Different models have been proposed to estimate overall loss of infectivity using RT-qPCR (Topping *et al.*, 2009; Pecson *et al.*, 2011). Alternatively researchers have sought methods for predicting infectivity that include ligand "receptor" binding combined with RT-qPCR (Dancho *et al.*, 2011) and "long PCR" for genomic integrity (Li *et al.*, 2014; Wolf *et al.*, 2009). Methods to predict the infectivity of NoV detected by RT-PCR have been critically reviewed by Knight *et al.* (2014).

In this report, virus survival or inactivation is often expressed as a decrease of the initial viral load as a function of time. The kinetics may follow a log linear function, and in such cases the T90 (the time to reduce 90 percent of initial load) inactivation coefficient is a convenient way to compare decay between different viruses for different situations. If the function expresses a non-linear pathway, T90 cannot be used considering its initial definition. Another denomination should then be used to express a reduction for a given time. Time to reduce the first $\log_{10}$ (TFL) of infectivity has, therefore, been introduced to compare decay rate of different viruses expressing a non-linear decay. In this report, where the reviewed data are

clearly linear, T90 has been used to describe the initial decrease in infectious viral load; where the data are not explicitly linear, TFL has been used.

1.7 Structure of the Report

In this report, information on NoV survival is presented, followed by information on elimination by chemical and physical procedures, in an encyclopaedic format, i.e. by topic (matrix, procedure). Elimination procedures are listed alphabetically. Some procedures (such as heat, disinfection by chlorine-based substances) are in widespread use within the food industry, whereas others (such as ultrasound or ozonisation) are not widely applied or are currently only being examined in academic proof-of-concept studies.

Details of how the review was performed are given in Annex 1.

2. Survival of Norovirus

2.1 In water

Seitz *et al.* (2011) spiked 9 ml samples of filtered groundwater with 1 ml of NoV GI.1 suspension to a final concentration of ~6.5 × 10^7 GE ml^{-1}, then stored the sample at room temperature in the dark for up to 61 d. At various time points, the samples were added to 90 ml distilled water, and the 100 ml water samples were ingested by human volunteers. Two volunteers ingested the samples which had been stored for 61 d and both became symptomatically infected. This indicates that NoV may remain infectious for at least 61 d in groundwater; however the composition of the spiking medium may have had a protective effect and the removal of the natural microflora may have encouraged virus persistence. The nature of these tests precluded determination of any reduction in infectivity. The authors examined the persistence of the NoV genome by RT-qPCR, and found no decline in GE after 622 d of storage, and a 1.79 log reduction after 1266 d of storage. Ingestion of the samples by volunteers was not done at these time points however, so it is not possible to state whether the NoV remained infectious.

Ngazoa *et al.* (2007) spiked 0.3 ml samples of mineral water and tap water with 100 GE NoV GII, and incubated the samples for up to80 d at -20, 4 and 25°C. They quantified the number of GE remaining in the samples at each time point by RT-qPCR. The data is shown in Table 2.1, expressed as % GE remaining at each time point. In all samples, the number of detectable GE declined, but viral RNA was still present in most samples even at 80 d.

Table 2.1: Survival of NoV in water (from Ngazoa *et al.*, 2007)

Water type	**Temperature (°C)**	**% NoV GE remaining after**				
		20 d	**40 d**	**60 d**	**80 d**	**100 d**
Mineral	-20	92	88	54	1	0
Tap	-20	85	75	59	0	0
Mineral	4	90	70	24	13	0
Tap	4	54	13	3	<1	<1
Mineral	25	91	36	17	5	<1
Tap	25	68	33	29	16	<1

Bae and Schwab (2008) spiked samples of surface water (pH 6.9-7.7), groundwater (pH 5.7-6.5), and sterile reagent grade water (pH not given, possibly 7.0) with a suspension of NoV containing ~10^5 RT-PCR GE ml^{-1}, and incubated the samples for 3-5 week at 4°C and 25°C in the dark with continuous mixing. They quantified the number of NoV GE at 6-8 time

points (not detailed) over the incubation period, and calculated rates of NoV decline as GE reduction rates in $\log_{10}$ d^{-1}. Table 2.2 shows the results obtained. The rates of decline were low, indicating that NoV could persist at least as intact particles in ground- and surface waters under the conditions tested. In a previous report, Bae and Schwab (2005) had stated that RT-qPCR cycle threshold values increased (indicating fewer GE present) when the samples were tested at the end of the incubation period. The increase was greater in surface water than ground water, which may reflect a lower persistence of NoV in surface water, possibly due to the presence of endogenous microorganisms.

Table 2.2: Survival of NoV in water (from Bae and Schwab, 2005)

Water type	Temperature (^{o}C)	Rate of NoV decline*
Reagent grade	4^{o}C	ND
	25^{o}C	0.00
Groundwater	4^{o}C	0.01
	25^{o}C	0.01
Surface water	4^{o}C	0.04
	25^{o}C	0.08

* mean GE reduction rate ($\log_{10}$ d^{-1}).
ND: not done

Charles *et al.* (2009) reported that NoV GII could be detected by conventional RT-PCR after 728 d in artificially contaminated samples of groundwater stored at 12^{o}C, whereas NoV GI could only be detected up to 651 d and in 50% of samples was undetectable at 140 d. No quantification was performed; therefore, it is not possible to estimate the rate of RNA decline. Other enteric viruses (adenovirus, Coxsackievirus B1, poliovirus) tested simultaneously remained infectious for at least 364 d, and the authors speculated that NoV could likewise survive.

Some information on virus survival in water can be extracted from a study that examined the potential for attachment of NoV to the internal surfaces of bottles containing mineral water (Butot *et al.*, 2007). NoV GI (~2×10^6 GE) was spiked into ~333 ml of bottled water, and then stored in the dark at room temperature for up to 62 d. A 1 log reduction was reported in NoV GE after 20 d. NoV GE was detected in the samples up to the end of the experiment, but the reduction was only reported for filtered samples, in which GE numbers were reduced by less than 1 log, suggesting an effect of the indigenous microflora on NoV.

When NoV GI was suspended in PBS for 6 weeks, Liu *et al.* (2012) observed log GE declines of <1 and ~1 at 4^{o}C and room temperature, respectively. At 37^{o}C, a >7 GE decline was observed after 5 weeks.

2.2 In soil and organic wastes

Meschke *et al.* (2000) spiked samples of 5% clay, sand, and "organic muck" suspended in groundwater, with ~10^6 GE NoV (genogroup not stated), and incubated the samples at 14°C for 6 months. Detectable GE declined by less than 1 log in that time. Concurrently, the infectivity of poliovirus and bacteriophage MS2 spiked into the samples declined by 2-4 log in the same period.

2.3 On fomites

In a report of two cases of gastroenteritis, Cheesbrough *et al.* (1997) presented intriguing circumstantial evidence to suggest that prolonged survival of NoV may occur on fomites. Twelve days after an outbreak of NoV infection in a hospital room, two carpet fitters removed a carpet from an adjacent room. Both men subsequently presented with the symptoms of NoV infection and their only common exposure appeared to be to the carpet, which they had to handle extensively and vigorously in order to remove it. No patients with symptoms of NoV infection had been in the room since the outbreak. The carpet had been vacuumed daily since the ward outbreak, and if it was indeed the source of the two fitters' illnesses, it would demonstrate considerable robustness of the virus.

To examine the potential persistence of NoV on materials which can constitute food preparation surfaces, D'Souza *et al.* (2006) contaminated 5 cm^2 Formica, stainless steel and ceramic coupons with ~10^6 NoV GI GE and stored the samples at 22±2^{o}C for 7 d (168 h). The presence of NoV RNA was determined qualitatively by conventional RT-PCR, so any reduction in GE number could not be precisely determined. Free NoV GI RNA was also added to stainless steel coupons and incubated with the virus-contaminated samples. NoV RNA could be detected at all-time points on all the coupons. As free NoV RNA could only be detected to 24 h, the implication was that the virus capsids remained intact and therefore potentially infectious. The survival of infectious FCV on the coupons was also examined simultaneously and a significant (~8 log) reduction was observed. The experiments were repeated by Liu *et al.* (2007; 2009), who also tested NoV GII and used RT-qPCR to determine the overall reduction of virus GE. NoV GII was significantly more stable on the surfaces than GI (the 2009 full report indicated identical results for NoV GI as in the 2007

conference abstract, but the results reported for NoV GII were slightly different, although probably not significantly so). The authors postulated that this greater stability was a cause for the higher number of outbreaks seen with NoV GII than GI. Liu *et al.* (2013) extended their studies using NoV GI to examine persistence on stainless steel at 4^{o}C, RT and 37^{o}C. After 4 week, GE titres were reduced by 0.9 log at 4^{o}C, 1.5 log at RT and 2.4 log at 37^{o}C.

Park *et al.* (2007) reported that NoV GII.4 could persist on ceramic and stainless steel tiles for ~1 d with no reduction in detectable GE. After 50 h a 1 log reduction in GE was observed. Bacteriophage MS2 tested simultaneously displayed identical persistence of GE, but a 3 log reduction in infectivity. Escudero *et al.* (2012) observed a linear decline in NoV GI and GII GE on ceramic, Formica and stainless steel coupons, and calculated T90 values. Similar NoV GE persistence was observed as in previous studies; MNV infectivity declined by 4-6 log, but no decline in GE was observed under the same conditions.

Lamhoujeb *et al.* (2008) used a tandem enzymatic pre-treatment approach to eliminate damaged virus particles prior to amplification of virus genome sequences by nucleic acid sequence-based amplification. They then employed this approach to determine persistence of NoV GII GE inoculated at 10^2 GE on stainless steel and PVC disks which were then stored for 56 d at 7^{o}C and 20^{o}C under high (86%) and low (30%) relative humidities (RH). The authors considered that low humidity at 7^{o}C is rarely encountered, so they did not test this condition. At 20^{o}C and high RH, NoV could not be detected after 28 d on either surface; at low RH, virus was undetectable after 7 d. At 7^{o}C under high humidity, NoV persisted on stainless steel for 49 d and on PVC for 56 d. The authors postulated that NoV persistence on the materials tested was inversely proportional to temperature and proportional to RH, and that in high RH the virus could remain infectious for several weeks on surfaces composed of PVC or stainless steel.

Using a capsid integrity assay based on elimination of exposed RNA in damaged capsids by RNase, Mormann *et al.* (2015) studied the persistence of NoV GII.3 on stainless steel and polyethylene surfaces. They inoculated a 20% (in PBS) faecal suspension (3 x 10^7 GE) of the virus onto each surface, and monitored the GE titre at 6 h, and 1, 7, 14, 21 and 70 d. Tests were carried out at RT and 7^{o}C. MNV and FCV were also inoculated onto the surfaces, and their infectivity monitored over the same time period together with their capsid integrity. In their report, only the data on virus persistence on stainless steel was given; the authors stated

that there was no difference in virus reduction between the two surfaces. At 7°C, no significant reduction in NoV GE was observed over the 70 d. FCV GE titre was similarly unaffected, although infectivity declined by 3 log over the 70 d. MNV GE and infectivity declined by less than 1 log. At room temperature there was an initial GE reduction of ~1 log within 6 h to 1 day for all viruses, followed by a linear decline over 70 d, with a final reduction of 3 log. It is possible that the initial decline in GE was due to an effect of desiccation. The infectivity of both FCV and MNV was reduced by 1 to 3 log, respectively, within 6 h to 1 d, and by over 4.5 log after 7 d, below the detection limit of the assay for each virus. The authors concluded that NoV could remain infectious on contaminated surfaces for prolonged periods, which is highly relevant to the food industry and the consumer, and that refrigerated storage could encourage the virus' persistence.

Table 2.3 shows data obtained from several studies on NoV survival on fomites.

Table 2.3: Survival of NoV on various fomites and material surfaces

Surface	Temperature (°C)	RH (%)	NoV genogroup	Initial decrease in GE (d)	Approximate overall log decline of GE	Reference
Ceramic	22	NG	I	ND	3 in 28 d	Liu *et al.* (2009)
Ceramic	22	NG	II	ND	0.4 in 42 d	Liu *et al.* (2009)
Ceramic	25	NG	I	ND	1 in 50 h[a]	Park *et al.* (2007)
Ceramic	RT	NG	I	34[b]	1.5 in 42 d[a]	Escudero *et al.* (2012)
Ceramic	RT*	NG	II	ND	1.2 in 42 d	Liu *et al.* (2007)
Ceramic	RT	NG	II	33[b]	<1 in 42 d[a]	Escudero *et al.* (2012)
Formica	22	NG	I	ND	1.6 in 28 d	Liu *et al.* (2009)
Formica	22	NG	II	ND	0.6 in 42 d	Liu *et al.* (2009)
Formica	RT*	NG	II	ND	0.8 in 42 d	Liu *et al.* (2007)
Formica	RT	NG	I	29[b]	1.5 in 42 d[a]	Escudero *et al.* (2012)
Formica	RT	NG	II	33[b]	1.5 in 42 d[a]	Escudero *et al.* (2012)
PVC	7	86	II	ND	<1 in 56 d	Lamhoujeb *et al.* (2009)

PVC	20	30	II	ND	2 in 14 d	Lamhoujeb *et al.* (2009)
PVC	20	86	II	ND	2 in 35 d	Lamhoujeb *et al.* (2009)
Stainless steel	4	NG	I	> 28[b]	0.9 in 4 week	Liu *et al.* (2012)
Stainless steel	7	86	II	ND	2 in 56 d	Lamhoujeb *et al.* (2009)
Stainless steel	7	50	II	>70	<1 in 70 d	Mormann *et al.* (2015)
Stainless steel	20	30	II	ND	2 in 14 d	Lamhoujeb *et al.* (2009)
Stainless steel	20	86	II	ND	2 in 35 d	Lamhoujeb *et al.* (2009)
Stainless steel	22	NG	I	ND	1.5 in 28 d	Liu *et al.* (2009)
Stainless steel	22	NG	II	ND	0.5 in 42 d	Liu *et al.* (2009)
Stainless steel	25	NG	I	ND	1 in 50 h[a]	Park *et al.* (2007)
Stainless steel	RT	NG	I	34[b]	1.5 in 42 d[a]	Escudero *et al.* (2012)
Stainless steel	RT	NG	I	21	1.5 in 28 d	Liu *et al.* (2012)
Stainless steel	RT	NG	II	43[b]	<1 in 42 d[a]	Escudero *et al.* (2012)
Stainless steel	RT	50	II	30[b]	3 in 70 d	Mormann *et al.* (2015)
Stainless steel	RT*	NG	II	ND	1.1 in 42 d	Liu *et al.* (2007)
Stainless steel	37	NG	I	7	2.4 in 28 d	Liu *et al.* (2012)

[a] values estimated from graphical display of data
[b] T90
GE: genome equivalents
ND: not determined
NG: not given
RH: relative humidity
RT: room temperature
RT*: not given; presumably RT

De la Noue *et al.* (2014) observed the binding of NoV GII.4 virus-like proteins (VLPs) to human blood group antigens following exposure to various levels of humidity. They hypothesised from the data obtained that the absolute humidity (AH), i.e. the quantity of moisture in the air, was critical to the persistence of NoV infectivity and that AH below 0.007 kg water / kg air, typical of winter conditions in temperate climates, is favourable to NoV survival on surfaces. Lopman *et al.* (2009) had previously hypothesised that cool and dry

weather was one of the major driving factors of NoV epidemiology, where increased numbers of infections are seen during the winter months. Such infectivity data seems at odds with studies showing that NoV RNA is more persistent at high RH (Lamhoujeb *et al.*, 2009) suggesting that infectivity and RNA levels may not correlate.

2.4 On hands

Liu *et al.* (2009) also examined the persistence of NoV GI (6×10^6 GE) and GII (9×10^8 GE) on volunteers' hands. They reported that for both virus types the decline in GE was ~0.15-0.2 log at 15 min after inoculation, but then no decline was observed for the remaining 2 h of the experiments.

2.5 On berry fruit

To evaluate the persistence of NoV on fresh berries under commonly applied storage temperatures, Verhaelen *et al.* (2012) monitored the decline in virus GE following artificially contaminating samples of raspberries and strawberries with 8×10^6 NoV GI.4 and 2×10^6 NoV GII.4 and storing at 4°C and 10°C for 7 d, and at 21°C for 3 d. At 4°C, no decline in GE was observed (Table 2.4) in any sample. At 10°C, GE decline was less than 1 log and at 21°C, no GE decline greater than 1.1 log was observed (Table 2.4). The authors stated that a higher decline in NoV GE was seen on strawberries than on raspberries, and that NoV GI appeared more stable than GII at 21°C, although the differences do not appear significant. The infectivity of human adenovirus and MNV also declined more significantly on strawberries than raspberries.

Table 2.4: Persistence of NoV GE on berry fruit at various storage temperatures (from Verhaelen *et al.*, 2012)

Temperature (°C) / time (d)	NoV strain	Log reduction in GE	
		On raspberries	On strawberries
4 / 7	GI.4	0	0
4 / 7	GII.4	0	0
10 / 7	GI.4	0.3	0.4
10 / 7	GII.4	0.4	0.5
21 / 3	GI.4	0.2	0.5
21 / 3	GII.4	0.3	1.2

2.6 On vegetables

Escudero *et al.* (2012) inoculated ~ 4 log NoV GI and GII on pieces of lettuce and stored them for up to 14 d at 4°C and 22°C, taking samples for analysis at various time points. They observed a linear decline in NoV GI and GII GE on lettuce pieces, and calculated T90 values (Table 2.5). The rates of decline of NoV GI stored at the two temperatures were significantly

different, but the rates of decline of NoV GII were not. MNV infectivity declined by 1.5-3 log under the same conditions, with no significant difference between the rates.

Lamhoujeb *et al.* (2008) used a tandem enzymatic pre-treatment approach to eliminate damaged virus particles prior to amplification of virus genome sequences by nucleic acid sequence-based amplification. They then employed this approach to determine persistence of NoV GII GE on lettuce pieces stored at 7^{o}C over 7 d with sampling every 2 d. They reported a linear decline in detected GE, with an ~1.5 log loss after 7 d.

Table 2.5 summarises the data obtained from the above studies.

Table 2.5: Survival of NoV on vegetables

Vegetable	**Temperature (^{o}C)**	**NoV genotype**	**Initial decrease in GE**	**Approximate overall log decline in GE**	**Reference**
Lettuce	4	I	17 d[a]	<1 in 14 d[b]	Escudero *et al.* (2012)
Lettuce	4	II	25 d[a]	<1 in 14 d[b]	Escudero *et al.* (2012)
Lettuce	7	II	6[ab]	1.5 in 7 d[b]	Lamhoujeb *et al.* (2008)
Lettuce	22	I	9 d[a]	<1 in 14 d[b]	Escudero *et al.* (2012)
Lettuce	22	II	17 d[a]	2 in 14 d[b]	Escudero *et al.* (2012)

[a] T90
[b] values estimated from graphical display of data.

2.7 In shellfish

To investigate whether NoV could survive in marinated mussels, Hewitt and Greening (2004) performed a study mimicking commercial marination. The process was simulated by immersing mussels (artificially contaminated with 10^{9} NoV GII.3 GE) in boiling water for 37 sec, steaming for ~3 min, then shucking them and immersing them in an acidic marinade (pH 3.75) before storage at 4^{o}C for up to 4 week. GE were quantified at time 0 and weekly for 4 week. There was no significant reduction in GE titre at any time. Hepatitis A virus simultaneously contaminating the shellfish also showed no reduction in GE titre, although infectivity did decline (by 1.7 log after 4 weeks). In studies with volunteers, Grohmann *et al.* (1981) showed the persistence of NoV in naturally contaminated oysters that were subjected to depuration for 7 d. The persistence of enteric viruses in bivalve shellfish appears to be

related to the ability of the viruses to become sequestered within shellfish haemocytes (Provost *et al.*, 2011). The duration of virus persistence within shellfish appears to be related to the acid stability of the particular virus within the acidic environment of the haemocytes (Provost *et al.*, 2011).

2.8 In other foods

Lamhoujeb *et al.* (2008) used a tandem enzymatic pre-treatment approach to eliminate damaged virus particles prior to amplification of virus genome sequences by nucleic acid sequence-based amplification. They then employed this approach to determine persistence of NoV GII GE on pieces of cooked turkey stored at 7^{o}C over 10 d with sampling approximately every 2 d. No significant decline in detected GE was observed over the 10 d period, which the authors speculated may have been due to the protective effect of protein and fat in the food matrix.

Mormann *et al.* (2010) inoculated NoV GII.3 into various foodstuffs and stored the samples under refrigeration or frozen. They quantified NoV GE using RT-qPCR. Table 2.6 shows the data obtained. Little reduction if any was observed in GE titre in most foods / storage conditions, except for potato salad at 6^{o}C, where a 1.7 log GE reduction was observed after 24 d.

Table 2.6: Persistence of Norovirus on foodstuffs stored frozen or refrigerated (from Mormann *et al.*, 2010)

Foodstuff	Storage temperature (^{o}C) / pH	Time (d)	Approximate overall log decline in GE
Tomato ketchup	6 / 4.5	58	0.5
Potato salad	6 / 5.0-5.5	24	1.7
Noodle salad	6 / 5.0-5.5	24	0.5
Minced meat	-18	8	0
Minced meat	6 / NG	2	0
Apple	11 / NG	7	0.2
Iceberg lettuce	11 / NG	5	0

NG: not given.

2.9 The effect of pH

Ausar *et al.* (2006) constructed NoV VLPs by cloning the gene encoding the VP1 protein into a baculovirus vector, then expressing the protein in an insect cell line. They suspended the VLPs in citrate/phosphate buffer at various pHs and observed the effects on the structure of the particles using a combination of spectroscopic, microscopic and calorimetric analyses and observed that the particles were highly stable from pH 3-7. At pH 8 however, significant

capsid disruption was observed. They postulated that virus disassembly at near-alkaline conditions may be a mechanism for release of NoV RNA when the virus has penetrated into the cytoplasm of target cells in the intestinal epithelia. Similar findings were obtained by Shoemaker *et al.* (2010), who found complete dissociation of NoV VP1 VLPs at pH 9. However the use of an alkaline buffer at pH 9.0–9.5 in the widely-used method for detection of NoV in foodstuffs (International Standards Organisation, 2012), to mediate the separation of virus particles from food surfaces, indicates that NoV can remain intact under alkaline conditions >pH 8.0 (although whether the extracted particles remain infectious has not yet been ascertained). Yao *et al.* (2014) reported that VLPs containing NoV structural protein VP2 as well as VP1 were more stable at pH 8.0 than VLPs containing VP1 alone. Using nanoindentation, in which pressure by extremely small mechanical tips is applied to the virus capsid and the amount of compression measured, Cuellar *et al.* (2010) observed that NoV VLPs were much less rigid at pH 10.0 than at lower pH (tested down to pH 2.0); it is possible therefore that NoV infectivity is affected by highly alkaline conditions.

Duizer *et al.* (2004) incubated NoV GII.4 in buffers or cell culture media at various pHs for 30 min at 37^{o}C, and then performed RT-PCR. They expressed the results as the change in Ct values obtained. Assuming that a 1 Ct value increase signifies a 50% reduction in starting GE number, the results they obtained indicate log GE reductions of <1 at pH 2, and 3, and ~2.5 at pH 10.

3. Norovirus elimination by chemical and physical means

3.1 Antimicrobial surfaces

To examine whether light-activated fluorinated titanium oxide (TiO_2) coatings could have an antiviral effect, Park *et al.* (2014) inoculated NoV GI.1 and GII.4 (starting GE titre not given) on coated surfaces and exposed them to a commercial fluorescent lamp which emitted light at a wavelength of 365 nm. After 120 min exposure NoV GI was reduced by 1.1 log while NoV GII.4 was reduced by less than 1 log. Oxidation of virus capsid proteins was observed indicating potential loss of infectivity. Bacteriophage MS2 exposed to the same treatment lost 3.6 log infectivity. When NoV GI.4 and GII.4 were spot-inoculated onto fiber-based wipes coated with chemicals which produced singlet oxygen, GE reductions of <1 log were observed after 1 h, and ~2 log after 6 and 24 h (Verhaelen *et al.*, 2014); reductions in MNV infectivity were <1, ~1, and ~2 log, respectively, after these exposure periods.

3.2 Autoclaving

To evaluate the effect of this common sterilisation procedure on NoV, Choi *et al.* (2014) autoclaved samples of NoV in liquid suspension and in a dried state. Fifty µl of a faecal sample containing NoV GII (~4×10^6 GE) was added to 1 ml of sterile water in a glass test tube. The sample was then autoclaved for 18 min (chamber pressure 18-20 psi; temperature not stated), the nucleic acids were extracted and RT-qPCR performed to try to amplify NoV sequences. No NoV GE were detected. When 20 µl of the faecal sample was dried onto the surface of the glass and autoclaved for 18 min, 1.1% NoV GE was still detectable (~2 log reduction) by the standard RT-qPCR system used. After a second round of autoclaving no NoV GE could be detected, initially indicating that this treatment might be necessary for dried contaminated material. However, the authors then used a long-range reverse transcription approach to try to evaluate any genome damage more effectively. In this technique, the site of PCR amplification is set several kb along the genome from the site of reverse transcription priming. The hypothesis is that the further away the primer binding sites are from each other, the more likely it is that nucleic acid strand breaks will occur between them, and this will prevent amplification from occurring. When they repeated the test on the dried sample which had been autoclaved for 18 min, no NoV GE could be detected using the long range reverse transcription. The results of this experiment indicate that autoclaving is effective at eliminating at least 5 log infectious NoV. Small (2 log reductions) in NoV GE observed using small amplicon PCR can be indicative of more extensive genomic damage.

3.3 Chlorine

In an early study to determine the effect of chlorine on NoV, Keswick *et al.* (1985) performed a human challenge study using Norwalk virus GI. A 2% faecal extract containing NoV (titre not determined) was added to 2 l sterile distilled water. Diluted household bleach was added to the suspension. Two hundred and fifty ml was then immediately (T_0) given to a volunteer to drink. The suspension was held at room temperature for 30 min then sodium thiosulfate was added to neutralise the available chlorine. Another volunteer was then given 250 ml of the suspension. The residual chlorine was measured at T_0 and at T_{30}. Serum specimens were collected from each volunteer up to 3 weeks from ingestion of the samples. Table 3.1 shows the results obtained. With an initial dose of 3.75 mg ml^{-1} chlorine, 14 out of 16 volunteers became infected after drinking the water immediately (1-1.5 mg ml^{-1} free chlorine), 5 out of 8 volunteers became infected by drinking water after 30 min incubation (free chlorine was undetectable). With a dose of 10 mg ml^{-1}, no volunteer became infected by drinking water after 30 min incubation (5-6 mg ml^{-1} free chlorine), however 14 out of 16 volunteers became infected after drinking the water immediately after treatment (5-6 mg ml^{-1} free chlorine).

Table 3.1: Inactivation of NoV in water by chlorine (from Keswick *et al.*, 1985)

Chlorine dose (mg l^{-1})	**T_0**		**T_{30}**	
	Free chlorine (mg l^{-1})	**Infections[a]**	**Free chlorine (mg l^{-1})**	**Infections[a]**
3.75	1-1.5	14/16	0	5/8
10	5-6	14/16	5-6	0/8

[a] Number of volunteers with serological evidence of infection / number tested.

Duizer *et al.* (2004) incubated NoV GII.4 in sodium hypochlorite solutions at various concentrations, and then performed RT-PCR. They expressed the results as change in Ct values obtained. Assuming that a 1 Ct value increase signifies a 50% reduction in starting GE number, the results they obtained indicate log GE reductions of <1 when the virus was treated at room temperature with 3,000 ppm for 10 min, ~2 when treated with 6,000 ppm for 2 min, and >2.5 when treated with 6,000 ppm for 10 min. Shin and Sobsey (2008) observed that 1 mg l^{-1} free chlorine at pH 6-10 at 5^{o}C reduced detectable NoV GI GE by 3 log over 60 min. They concluded that NoV is not highly resistant to free chlorine disinfection. In support of this conclusion, NoV GII.4 GE titre was reduced by ~2 and ~2.3 log after exposure to 1 mg l^{-1} free chlorine for 5 and 15 min, respectively (Sano *et al.*, 2010); oxidative damage to the virus capsid was also observed. Used against NoV in suspension, sodium hypochlorite at concentrations of 0.5 ppm were able to reduce NoV GII.4 GE by ~1 log after 5 min, ~2 log after 10 min and ~3.6 log after 30 min exposure (Kitajama *et al.*, 2010) and at 160 ppm and

above were able to reduce GI GE titre by 5 log (Liu *et al.*, 2010). Tung *et al.* (2013) reported that NoV GII.4 lost <0.5 GE after 30 sec exposure to hypochlorite concentrations up to and including 1,000 ppm; GII.2 GE was similarly reduced by <0.5 log at concentrations of 500 ppm and below, but was reduced by >4 log by 1,000 ppm.

Using RT-qPCR of NoV GI.1 particles which could bind to porcine gastric mucin (which contains glycoproteins similar to the virus receptors in the human gut) linked to magnetised beads, Kingsley *et al.* (2014) observed GE reductions of 1.5, 3.5 and 4 log after 1 min exposure in suspension to 33, 173 and 189 ppm free chlorine.

Barker *et al.* (2004) contaminated melamine surfaces with ~10^3 GE NoV GII in 1:10 and 1:80 faecal suspensions, then wiped the surfaces with cloths containing detergent or by applying a hypochlorite-based disinfectant containing 5,000 ppm available chlorine for 1 or 5 min followed by 10 sec wiping with a detergent-soaked cloth. Then they determined the presence of NoV by eluting the virus from the surfaces, followed by nucleic acid extraction and conventional RT-PCR (thus, only presence / absence results were obtained). Wiping with detergent-soaked cloths alone could not eliminate NoV from the surfaces. Application of 5,000 ppm chlorine removed NoV in 1:10 faecal suspension from ~75% of surfaces, and totally removed NoV in 1:80 faecal suspension. The conclusion was that applying this level of chlorine followed by wiping a surface with a cloth would be effective in removing NoV, as long as the surface was not highly soiled. Using pre-treatment with RNase to eliminate RNA from damaged virus particles subsequent to RT-PCR, Girard *et al.* (2010) tested the efficacy of sodium hypochlorite against NoV on stainless steel surfaces. They inoculated stainless steel discs with 10^4 GE NoV (genogroup not given), then applied 3% sodium hypochlorite for 5 and 10 min at RT. After 5 min, a 2-log reduction in detectable NoV GE was observed, but no GE was detected after 10 min. Park and Sobsey (2011) applied a 10% faecal suspension containing $10^{6.6}$ GE ml^{-1} NoV GII.4, and spiked with FCV ($10^{6.7}$ PFU ml^{-1}), MNV ($10^{7.4}$ PFU ml^{-1}) and MS2 phage ($10^{8.3}$ PFU ml^{-1}), onto the surface of stainless steel disks, and air-dried the inocula for 1-1.5 h. The disks were then placed in solutions of sodium hypochlorite containing 500 and 5,000 ppm. Table 3.2 shows the effects on human pathogen (measured as GE reduction) and on the surrogates (measured as reduction of infectivity). They reported that the treatments affected reductions in the infectious titre of each surrogate, particularly 5,000 ppm sodium hypochlorite with 4 min contact. The reduction of NoV GE was less pronounced – approx. 1 log or less, but this was similar to the reduction of GE for each

surrogate (not shown). This indicates that if the sodium hypochlorite did effect any reduction in NoV infectivity, it would not have exerted that effect primarily through capsid disruption. The authors postulated that using 5,000 ppm hypochlorite may be reasonably effective against NoV on surfaces, but contaminated surfaces should be pre-cleaned to reduce any organic load, and the disinfectant applied for longer than 3 min to try to obtain a 3 log inactivation of the virus.

Table 3.2: Effect of sodium hypochlorite on NoV GE and virus surrogate infectivity (from Park and Sobsey, 2011)

Na hypochlorite concentration (ppm)	Contact time (min)	Log reduction in NoV GE[a]	Log reduction in infectious MNV[a]	Log reduction in infectious FCV[a]	Log reduction in infectious MS2 phage[a]
500	10	<1	1	2	1
5,000	4	1	3.5	4.5	2.5

[a] values estimated from graphical display of data.

Nowak *et al.* (2011a) examined reductions in GE for NoV and surrogate FCV following exposure to available chlorine under standardised light soil disinfection conditions. Both RT-qPCR data and infectivity data for FCV in PBS showed similar log-linear reductions resulting in a 4 log reduction at 50 ppm available chlorine. In contrast similar GE reductions for GII.4 NoVs required 500-600 ppm available chlorine. Reductions in GE observed for FCV-spiked into GII.4 isolates were significantly less than that observed in PBS suggesting that this was owing to a protective effect of the faecal matrix.

Tuladhar *et al.* (2012b) performed a similar series of experiments, using NoV GI.4 and GII.4 (~10^7 GE each) on stainless steel discs. They used RT-qPCR to quantify the number of GE detected. Wiping with cloths containing 250 or 1,000 ppm chlorine removed ~1-1.5 log NoV, whereas wiping with cloths soaked in detergent followed by wiping with the disinfectant-containing cloths produced NoV GE reductions of ~2 log (for 250 ppm chlorine-containing cloths) and 2-4 log (for 1,000 ppm chlorine-containing cloths).

Wang and Kniel (2014) examined the effect of calcium hypochlorite ($Ca(OCl_2)$) on various pathogens including NoV. To evaluate the industrial relevance of using this disinfectant, they inoculated 1 g alfalfa (*Medicago sativa*) seeds with $10^{7.7}$ NoV, dried the seeds for 1 h at 20^{o}C, then added $Ca(OCl_2)$ at 2,000 and 20,000 ppm. Foetal bovine serum was added at various concentrations to mimic an organic load. Virus was then extracted from the sample

(apparently from both seeds and suspending liquid) and quantified by RT-qPCR. An initial decrease of 1.4 log GE was observed after the drying of the inoculum. After each $Ca(OCl_2)$ treatment, a reduction in NoV GE number was observed (Table 3.3). Increasing the organic load appeared to have a protective effect when $Ca(OCl_2)$ was used at 2,000 ppm but not at 20,000 ppm. As NoV will not be infectious without an intact genome, treatment of seeds with $Ca(OCl_2)$, especially at 20,000 ppm appears to be an effective control measure that may have application in disinfecting processing equipment.

Table 3.3: Effect of calcium hypochlorite treatment of alfalfa seeds on NoV GE (from Wang and Kniel, 2014)

$Ca(OCl_2)$ concentration (ppm)	**Organic load**	**Log reduction in GE**
2,000	-	5.2
	10% FBS	4.5
	20% FBS	3.1
	30% FBS	2.8
20,000	-	4.6
	10% FBS	4.5
	20% FBS	4.4
	30% FBS	4.0

In a series of experiments, Butot and colleagues (2008) examined the effectiveness of commercial processes for the elimination of NoV from berries and other produce. To simulate post-harvest conditions, fresh berries were artificially contaminated with 1.2×10^5 NoV GI GE and 2×10^6 NoV GII GE, and then washed with 200 ml chlorinated water, or with a chlorine dioxide (ClO_2) solution followed by rinsing with 200 ml tap water as recommended by the US FDA. Treatment with chlorine at an available concentration of 200 ppm for 30 sec reduced NoV GE in parsley and raspberry samples by less than 2 log; the reduction of GI NoV in basil, blueberry and strawberry samples was much greater (≥3.1 log), while GII NoV reductions were considerably lower in basil and strawberries (reductions ≤1.6 log). Table 3.4 summarises the data from these studies.

Table 3.4: Elimination of NoV on berry fruit and fresh herbs by chlorine (from Butot *et al.*, 2008)

Product	Chlorine Treatment	Time	Temperature (^{o}C)	Approximate overall log decline in GE
Basil	200 ppm	30 sec	18	>3.4 (GI) 1.6 (GII)
Blueberries	200 ppm	30 sec	18	3.4 (GI) 3.0 (GII)
Parsley	200 ppm	30 sec	18	<1 (GI) 1.8 (GII)
Raspberries	200 ppm	30 sec	18	0.0 (GI) 0.9 (GII)
Strawberries	200 ppm	30 sec	18	>3.1 (GI) 1.4 (GII)

3.4 Chlorine dioxide

Shin and Sobsey (1997) reported that ClO_2 at concentrations of 0.5 to 1 mg l^{-1} could effect NoV GE reductions of 0.5 to 1 log at pH 6 and 1 to 3 logs at pH 10. The temperature of the tests was 5^{o}C, but the contact time was not reported. Butot and colleagues (2008; 2009) examined the effectiveness of ClO_2 as used in commercial processes for the elimination of NoV from raspberries and parsley (Table 3.5). To simulate post-harvest conditions, fresh berries were artificially contaminated with 1.2×10^5 NoV GI GE and 2×10^6 NoV GII GE, and then washed with a chlorine dioxide solution followed by rinsing with 200 ml tap water as recommended by the US FDA. Immersing samples in ClO_2 at 5 or 10 ppm for 10 min had a limited effect (1.2 log GE reduction or less).

Table 3.5: Elimination of NoV on berry fruit and fresh herbs by chlorine dioxide (from Butot *et al.*, 2008)

Product	Treatment	Time	Temperature (^{o}C)	Approximate overall log decline in GE
Parsley	ClO_2 (5 ppm)	10 min	18	<1(GI/GII)
Parsley	ClO_2 (10 ppm)	10 min	18	<1(GI) 1.2 (GII)
Raspberries	ClO_2 (5 ppm)	10 min	18	<1(GI/GII)
Raspberries	ClO_2 (10 ppm)	10 min	18	<1(GI/GII)

Using RT-qPCR of NoV GI.1 particles which bound to porcine gastric mucin, Kingsley *et al.* (2014) observed GE reductions of <1, ~1.5 and ~3 log after 10 min, 30 min and 60 min exposure to chlorine dioxide, respectively.

3.5 Commercial disinfectants

Poschetto *et al.* (2005) reported that a sodium hypochlorite-based disinfectant generating ≥5,500 ppm free chlorine could reduce detectable NoV (genogroup not reported) by ~1 log after 15 min contact time. FCV infectivity was reduced by ~5 log. Poschetto *et al.* (2005) also reported that an aldehyde-based disinfectant at 1% concentration could reduce detectable NoV (genogroup not reported) by ~1 log after 15 min contact time. At a 0.1% concentration FCV infectivity was reduced by >5 log, whereas no effect on NoV GE was seen.

Park and Sobsey (2007) tested the efficacy of a hypochlorous acid generated by a commercial disinfection system against NoV GII.4, in suspension and dried onto the surfaces of stainless steel and ceramic tiles. In suspension, NoV GE titre could be reduced by 3 log in 20 sec. The GE titre of NoV dried onto surfaces could be reduced by 3 log in 1 to 10 min depending on the concentration of the disinfectant. Applied as a fog and allowed to settle for 1 h, the disinfectant had a greater effect, reducing the GE titre by 4.5 to >6 log. Such substantial reductions in GE likely reflect a reduction in virus infectivity. Poschetto *et al.* (2005) reported that an organic acid-based disinfectant at 4% concentration could reduce detectable NoV (genogroup not reported) by ~1 log after 15 min contact time. FCV infectivity was reduced by ~5 log with this treatment.

To evaluate the effectiveness of germicidal formulations used in hospital settings, Park *et al.* (2007) tested disinfectants containing hydrogen peroxide (3%), quaternary ammonium compound (0.0625%), or antiseptics (4% chlorhexidine gluconate and 1% chloroxylenol) against NoV inoculated onto 1 cm stainless steel discs. Neither the genogroup of NoV tested nor the initial GE used was stated. None of the formulations reduced NoV GE by more than 1 log.

Park *et al.* (2010) tested seven hand sanitisers based on chlorhexidine, ethanol, or triclosan for efficacy against NoV GII.4 in suspension. Two ethanol-based sanitisers effected small reductions (0.7 log and below after 5 min exposure) in GE titre; the others five sanitisers had little or no effect (0.1 log GE reductions at most).

Using pre-treatment with RNase to eliminate RNA from damaged virus particles subsequent to RT-PCR, Girard *et al.* (2010) tested the efficacy of an ethoxylated alcohol-based disinfectant against NoV on stainless steel surfaces. They inoculated stainless steel discs with 10^4 GE NoV (genogroup not given), then applied the disinfectant for 5 and 10 min at room temperature. No reduction in GE was observed. Using a similar RNase approach Nowak *et al.* (2011b) measured reductions in GE for GII.4 NoVs using a wide range of commercial disinfectants and sanitisers including quaternary ammonium compounds, chlorine dioxide, ethanol, isopropanol and alkali. Only hot (50^{o}C) alkali resulted in significant (2 log) reductions in RT-qPCR signals.

Liu *et al*. (2011) evaluated six ethanol-based hand rub formulations for effect against NoV strains. They inoculated finger pads with ~10^6 NoV GI and ~10^8 NoV GII, then applied the formulations for a contact time of 15 sec. Viruses were then recovered from the finger pads by precipitation and the remaining GE titre determined. Certain alcohol based sanitisers were considered effective resulting in up to 3.75 GE reductions. However these studies are difficult to undertake and require multiple controls. Although possible effects of RT-qPCR inhibition were considered, the effect of the sanitiser itself on the precipitation recovery step and the influence of RNase activity were not investigated.

To evaluate procedures commonly used to clean textiles, Lemm *et al.* (2014) contaminated 2 cm^2 pieces of cotton fabric with a faecal suspension of NoV GI (titre not reported) and washed them in a standard front-loading washing machine with 2 kg of clean clothes, using the cycle settings provided on the machine. After each treatment, the textile pieces were treated to extract RNA, RT-qPCR was then performed to try to detect NoV sequences. Table 3.6 summarises the information from this study. The RT-qPCR was performed qualitatively, i.e. results were reported as cycle threshold (Ct) values and only those below Ct 60 were considered positive. This cut-off would appear to be rather high, but all positive Ct values reported were below 40, they are likely to represent true detection of NoV (Cook *et al.*, unpublished observations). Only wash cycles at 60^{o}C (actual temperature 50.4^{o}C) resulted in removal of NoV in all tests, therefore the conclusion from the study was that when washing of NoV-contaminated fabrics, the temperature should reach at least 50^{o}C for effective virus removal. This information may be useful to the restaurateur in the laundering of table cloths

and cloth napkins as well as to the food processing industries, since it provides minimum conditions for the disinfection of work apparel (aprons, smocks, etc.).

Table 3.6: The removal of NoV on cotton textile by commonly used washing procedures (from Lemm *et al.*, 2014)

Washing conditions[a]	**NoV RNA detected on textile after washing**[b]
30°C (28.8°C) with liquid detergent	3/3
40°C (38.5°C) with liquid detergent	2/3
60°C (50.4°C) with liquid detergent	0/3
30°C (28.8°C) with powder detergent	1/3
40°C (38.5°C) with powder detergent	1/3
60°C (50.4°C) with powder detergent	0/3

[a] temperature according to machine settings and temperature reached during the wash cycle (in parentheses); time of wash not reported.
[b] number positive/total number tested.

Snyder *et al.* (2013) patented a formulation containing alcohol in combination with a polyquaternium compound and a zinc or copper compound, which they claimed can reduce NoV GE on finger pads by >4 log after 30 sec contact.

3.6 Depuration

Grohmann *et al.* (1981) examined the outcome of a volunteer study where 40-129 volunteers test consumed oysters each week over a 9-month period. The shellfish had been depurated in pollution-free water for 7 d in tanks irradiated with UV or disinfected with ozone, according to the standards then current, for 48 h. Fifty two volunteers subsequently became ill, with the virus being detected in 32% (8/25) of the stool samples examined. No details were given of the UV dose or the level of ozone applied. Ueki *et al.* (2007) bioaccumulated NoV GII in oysters to an average level of 1.7×10^3 GE, then depurated the shellfish for 10 d. No decrease in GE titre was observed. Savini *et al.* (2009) found one out of 29 samples of mussels, which had undergone depuration with UV-disinfected water, positive for NoV (genogroup not given). They estimated that there was no significant difference between the probabilities of NoV contamination of depurated and non-depurated shellfish. McLeod *et al.* (2009) found no significant decrease of NoV GII.4 bioaccumulated in Pacific oysters after 23 h of depuration in virus-free seawater. NoV GI.1 RNA could be qualitatively detected after 29 d of depuration (at 8, 12 and 20 ppt salinity) in the oyster species *Crassostrea ariakensis* and up to between 22 d (12 ppt salinity) and 25 d (20 ppt salinity) in *Crassostrea virginica* (Nappier *et al.*, 2008). Nappier *et al.* (2010) in a further test of depuration at 12 ppt salinity again observed NoV GI.1 RNA in *Crassostrea ariakensis* after 29 d but only up to 15 d in

Crassostrea virginica. NoV GI and GII could be detected in clams after 7 d depuration, whereas NoV GI could be detected in mussels only up to 4 d (Polo *et al.*, 2014). From the data available, no significant reduction in GE titre was apparent although only some shellfish appeared to bioaccumulate the virus prior to depuration.

3.7 Ethanol

Panek *et al.* (2008) inferred from a study of hospital NoV outbreaks and the infection control procedures used to contain them, that outbreaks could be contained by following disinfection procedures which stipulated the use of alcohol hand rubs with a 1 min exposure time, but no precise details were supplied. However, NoV is non-enveloped and non-enveloped viruses are generally more resistant to the effects of alcohols than enveloped viruses (Rotter, 2001, Eterpi *et al.*, 2009). Liu *et al.* (2010) observed reductions in NoV GI GE titre of less than 0.5 log, after exposure of virus in suspension to concentrations of ethanol between 3% and 100% for 30 sec. Ethanol concentrations of 50%, 70% and 90% likewise reduced NoV GII.2 and GII.4 GE titre by 0.5 log or less after 30 sec contact in suspension (Tung *et al.*, 2013). Liu *et al.* (2011) evaluated six ethanol-based hand rub formulations for effect against NoV GI and GII strains, by measuring reductions in GE titre after 15 sec contact on finger pads. The most effective formulations (effecting GE reductions of ~2-4 GE) contained additional ingredients such as citric acid and copper gluconate. Li *et al.* (2014) used a combination of binding to Caco-2 cells with subsequent long-range RT-qPCR. The latter technique employs priming of the nucleic acid amplification by oligonucleotides which bind to widely separated sequences on the virus genome, the hypothesis being that genome damage between the binding sites will prevent efficient amplification. Following addition of a NoV GII.4 suspension to ethanol to a concentration of 70% for 1 min, a ~1 log decline in detectable GE was observed. Wang and Tian (2014) used a method in which NoV was captured by histo-blood group antigens prior to RT-qPCR, to determine the effect of ethanol on NoV GII.4. Exposure to 70% ethanol reduced detectable GE by ~2.5 log. This result is surprising considering the lack of effectiveness of ethanol against non-enveloped viruses, and it may be that the residual alcohol (~7% after the suspension was diluted with PBS before the antigen capture assay) affected the antigen receptor rather than the virus.

3.8 Freezing

Butot *et al.* (2008) reported that freezing of berries and other produce at -20^{o}C reduced NoV GE by less than 1 log (Table 3.7). Freeze-drying, such as is commonly performed to dry berry fruit for subsequent incorporation into confectionaries and cereals, reduced the titre of

NoV GE by less than 2.7 log on berry fruits (Butot, *et al.*, 2009), the process involved freezing at -20^{o}C followed by a heating cycle during which the berries reached an internal temperature of 55^{o}C. The reduction was greater in samples of blueberries than in blackberries, raspberries and strawberries possibly due to a protective effect of the more textured surfaces of the latter berries

Table 3.7: Effect of freezing and freeze drying on NoV GE on produce (from Butot *et al.*, 2008 and 2009)

Product	NoV genotype	Treatment	Approximate overall log decline in GE
Basil	I	Freezing	<1
Basil	II	Freezing	<1
Blackberries	I	freeze drying	<1
Blackberries	II	freeze drying	1.7
Blueberries	I	Freezing	<1
Blueberries	II	Freezing	<1
Blueberries	I	freeze drying	1.3
Blueberries	II	freeze drying	2.7
Blueberries	II	frozen storage	2.3 after 90 days
Parsley	I	freezing	<1
Parsley	II	freezing	<1
Parsley	I	freeze drying	2.1
Parsley	II	freeze drying	3.5
Raspberries	I	freezing	<1
Raspberries	II	freezing	<1
Raspberries	I	freeze drying	<1
Raspberries	II	freeze drying	1.2
Strawberries	I	freezing	<1
Strawberries	II	freezing	<1
Strawberries	I	freeze drying	<1
Strawberries	II	freeze drying	1.5

A demonstration of the robustness of NoV particles was given by Richards *et al.* (2012). Ten percent stool suspensions containing 1 × 10^{8} GE ml^{-1} NoV GII.4 were clarified by centrifugation and filtration and stored at -80^{o}C for up to 120 d. Samples were taken periodically over this period and no significant decline in GE titre was observed. The effect of repeated freeze-thaw cycles was also evaluated. A second set of samples was frozen at -80^{o}C then slowly thawed at room temperature at weekly intervals over the course of 14 weeks and refrozen (1-14 freeze/thaw cycles). Again, no significant effect on GE titre was observed, even after 14 cycles of freezing and thawing. Binding of NoV to porcine gastric mucin (which contains glycoproteins similar to the virus receptors in the human gut) was also

unaffected after 14 freeze-thaw cycles, indicating that the virus capsid remained intact after the treatment and may have been competent to bind to host cell receptors to initiate infection (Richards *et al*., 2012). These findings were taken as confirmation that freezing and thawing are unsuitable as a food processing measure against NoV.

3.9 Gamma irradiation

Using the VP1 gene from NoV GII.4 to construct VLPs, Feng *et al.* (2011) studied the effect of gamma irradiation on the virus capsid. They examined the appearance of the VLPs by electron microscopy after irradiation. At 2.8 kGy VLPs were clumped together and their morphology was altered, appearing much less structured. At 5.6 kGy the effect on the VLP structure was greater and there were large amounts of protein debris. At 22.5 kGy complete disruption of the VLPs had occurred. As similar effects were observed on MNV and this virus retained some infectivity at the two lower doses (40% after 2.8 kGy and 25% after 5.6 kGy), the authors suggested that NoV might display similar resistance to gamma irradiation at these doses.

3.10 Hand washing

Liu *et al.* (2010) inoculated ~6 log NoV GI onto finger pads of human volunteers, who then placed the finger pads over vials containing tap water, or liquid soap containing triclosan. The vials were inverted and held in place for 10 sec, then rapidly inverted 20 times in succession. Virus remaining on the finger pads was then eluted with a buffer. The water rinse effected GE reductions of only ~1.1-1.5 log, with no statistical difference between the two treatments.

3.11 Heat

Ausar *et al.* (2006) subjected NoV VLPs suspended in buffers at different pHs (3-8) to temperature gradients from 10^{o}C to 90^{o}C and observed the effects on the structure of the particles using a combination of spectroscopic, microscopic and calorimetric analyses. Above 60°C at all pHs, the VLPs underwent distinct changes at the secondary, tertiary, and quaternary protein level. Kissmann *et al.* (2008), examining the effect of potential vaccine excipients (chitosan, dextrose, glycerol, lactose, mannitol, sorbitol, sucrose and trehalose) on NoV VLPs, found that all could stabilise the secondary and tertiary structure of the virus proteins as evidenced by increased Tm values (from 58.7-61.0^{o}C in untreated controls to 61.5-70.0°C in the presence of excipients). Twenty percent sucrose appeared to effect the most pronounced change in VLP stability by stabilising both the VP1 protein P and S domains. Li *et al.* (2012a), examining the receptor-binding capacities of heat-treated VLPs representing several GI and GII strains, reported that overall the VLPs were relatively

resistant to heating at 70°C for 2 min but binding was greatly reduced by heating at 85°C for 2 min.

Duizer *et al.* (2004) incubated NoV GII.4 suspensions at 37°C and 100°C, and then performed RT-PCR. They expressed the results as change on Ct values obtained. Assuming that a 1 Ct value increase signifies a 50% reduction in starting GE number, the results they obtained indicate log GE reductions of <1 when the suspension was heated to either 37°C for 120 h or 100°C for 1 min, ~1 log at 37°C for 168 h, and >2.5 log at 100°C for 3 min.

Dancho *et al.* (2012), using RT-qPCR of NoV particles which could bind to porcine gastric mucin linked to magnetised beads, observed that after a 60 sec treatment, during which temperature was increased from 0°C to ~64°C, there was a <1 log reduction of RT-PCR-detectable NoV GI which could bind to the beads and after 120 sec, where the temperature increased from 0°C to ~73°C, a ~3 log reduction in binding was observed. Croci *et al.* (2012) heated suspensions of NoV GII.4 at 60°C and 80°C, obtaining a <1 log reduction by 15 min and a 3 log reduction by 6 min, respectively. In contrast, FCV in suspension lost ~4.5 log by 3 min at each temperature.

Topping *et al.* (2009), using RNase treatment prior to RT-PCR to eliminate NoV RNA from damaged capsids, found that a >4 log reduction in detectable GE was achieved by heating to 77°C for 2 min. Examining the response of FCV they observed a 2 log reduction in GE with a >4.5 log reduction in infectivity following heating to 63°C for 2 min.

Using two methods to estimate capsid integrity (propidium monoazide [PMA] intercalation and RNase treatment), Escudero-Abarca *et al.* (2014) produced thermal inactivation profiles using NoV GII.2. They purified the virus from a faecal suspension and then exposed aliquots of the purified suspension to various time-temperature combinations. Heat-treated samples were immediately treated with PMA or RNase, and RT-qPCR was performed. The data obtained on GE decline using the two methods were in general agreement. The authors reported the data as D-values (T90). However in the graphical display they provided (only for heating at 72°C and 90°C) the inactivation profile for heating NoV at 90°C shows an initial rapid decline of ~4 log by 1 min, followed by a less pronounced decline to apparent nondetectability by 15 min. Since the profile was not linear, a TFL value has been given in Table 3.8. The results of this study may indicate that the NoV capsid can retain considerable integrity at temperatures up to around 70°C, and that NoV can be rapidly inactivated at

temperatures of 85°C and higher. Using a combination of RNase pre-treatment and binding to cultured mammalian cells (Caco-2), where the hypothesis is that only undamaged virus particles will bind to the cells, Li *et al.* (2012a) observed a ~1.5 log reduction in detectable NoV GII.4 GE at 70°C for 2 min and a 2.3 log reduction at 85°C for 2 min. Li *et al.* (2014) extended their study using a combination of binding to Caco-2 cells with subsequent long-range RT-qPCR. The latter technique employs priming of the nucleic acid amplification by oligonucleotides which bind to widely separated sequences on the virus genome. The hypothesis is that genome damage between the binding sites will prevent efficient amplification. Following heat treatment at 60°C for both 2 min and 30 min they observed ~3 log reductions in detectable NoV GII.4 GE. Wang and Tian (2014) used a method in which NoV was captured by histo-blood group antigens prior to RT-qPCR, to determine the effect of heat on NoV GII.4. From the time / temperature combinations tested, <1 log reductions in detectable GE were only observed after heating virus suspensions at 60°C for 15 min, and 72°C for 3 min (starting GE titre ~ 3 log). After 100°C for 2 min no detectable GE remained. Table 3.8 summarises the data obtained from the above studies.

Table 3.8: Effect of heat on NoV

Genogroup	Assay	Temperature (°C)	Initial decrease in GE	Approximate overall log decline in GE	Reference
GII	Receptor binding / RT-PCR	22	ND	0 in 2 min	Wang and Tian (2014)
GI	RT-PCR	37	ND	1 in 2 week	Parshionikar *et al.* (2010)
GII	RT-PCR	37	ND	<1 in 3 min	Tuladhar *et al.* (2012a)
GII	Receptor binding / RT-PCR	56	ND	0 in 2 min	Wang and Tian (2014)
GI	RT-PCR	60	ND	<1	Croci *et al.* (2012)
GII	Cell binding / long-range RT-PCR	60	ND	<1	Li *et al.* (2014)
GI	Receptor binding / RT-PCR	63	ND	<1 in 2 min	Wang and Tian (2014)
GII	Receptor binding / RT-PCR	63	ND	1.4 in 60 min	Wang and Tian (2014)

GII	Cell binding / RT-PCR	70	ND	1.5 in 2 min	Li *et al.* (2012a)
GI	Capsid integrity/ RT-PCR	72	ND	<1 in 15 min	Escudero-Abarca *et al.* (2014)
GII	Receptor binding / RT-PCR	72	2 min[a]	>3 in 4 min	Wang and Tian (2014)
GII	Capsid integrity/ RT-PCR	77	ND	>4 in 2 min	Topping *et al.* (2009)
GII	Capsid integrity/ RT-PCR	77	16.4 min[b]	NG	Escudero-Abarca *et al.* (2014)
GI	Capsid integrity/ RT-PCR	80	3.86 min[b]	NG	Escudero-Abarca *et al.* (2014)
GII	RT-PCR	80	~3 min[a]	3 in 6 min	Croci *et al.* (2012)
GII	Capsid integrity/ RT-PCR	82	0.94 min[b]	NG	Escudero-Abarca *et al.* (2014)
GI	Capsid integrity/ RT-PCR	85	0.12 min[b]	NG	Escudero-Abarca *et al.* (2014)
GII	Cell binding / RT-PCR	85	ND	2.3 in 2 min	Li *et al.* (2012)
GII	Capsid integrity/ RT-PCR	90	~15 sec[bc]	>4.5 in 15 min	Escudero-Abarca *et al.* (2014)

[a] TFL
[b] T90
[c] values estimated from graphical display of data
GE: genome equivalents
ND: not determined
NG: not given

Croci *et al.* (2012) spiked homogenates of blue mussel (*Mytulis edulis*) digestive gland with NoV GII.4 (~10^7 GE) and heated them to 80°C for 15 min. Less than 1 log reduction in GE titre was obtained. Infectious FCV homogenates spiked simultaneously and subjected to the same treatment declined by ~2 log. To evaluate the effect of cooking practices used in domestic settings on NoV, Flannery *et al.* (2014) bioaccumulated NoV GI and GII in blue mussels by holding them 24 h in a tank of seawater spiked with NoV contaminated faeces, then placed them in a domestic cooking pan and subjected them to various cooking regimes. Mussels were removed for analysis before and after cooking, and NoV GE numbers in the shellfish digestive tissue were measured by real-time RT-PCR. The cooking regimes included

lightly simmering (water temperature ~70^{o}C) with the starting water temperature at ambient temperature or preheated to 70^{o}C for 10 and 6 min, respectively. From the graphical display of the results the log NoV GI and GII GE reduction appeared to be <1 in each case. The two other regimes tested were boiling, with the starting water temperature either at ambient temperature or pre-heated to >90^{o}C. When the water started at ambient temperature, both NoV GI and GII were reduced by > 2 log after a total cooking time of 8 min. When the water was pre-heated, NoV GI and GII were reduced by 1-2 log after a total cooking time of 5 min. F-specific bacteriophage were included in each test. Simmering produced no significant reduction in infectious bacteriophage titre whereas boiling reduced infectivity by >6 log after 8 min in water starting at ambient and after 3 min in water which had been pre-heated. F-specific FCV GE titres were concomitantly reduced by ~2–2.5 log after the boiling treatments. The authors recommended that consumers immerse mussels in boiling water for at least 3 min, to reduce the risk of acquiring viral illness. Alfano-Sobsey *et al.* (2012), reviewing the information from an outbreak of NoV gastroenteritis in which consumption of steamed mussels was implicated, recommended that temperatures above 63^{o}C for 15 sec be employed to inactivate NoV in shellfish, parameters considerably different from those recommended by Croci *et al*., (2012).

Hewitt and Greening (2006) examined a series of cooking scenarios likely to be found in restaurants or domestic settings. When New Zealand greenshell mussels (*Perna canalicus*) were boiled for 3 min, steamed for 3 min, or boiled for 37 sec then steamed for 3 min, no reductions in NoV GII.3 GE titre were observed. However it may be that capsid disruption did not occur even when infectivity was reduced as significant reductions (>1, 1.5 and 2 log, respectively) in infectious hepatitis A virus titre were observed under these conditions but no effect was seen on RT-PCR-detectable units. Hewitt and Greening (2006) recommended that immersion for at least 3 min in boiling water rather than steaming should be performed to reduce the risk of viral illness from contaminated shellfish.

Butot *et al.* (2009) investigated the effect of steam blanching on NoV GE on herbs. They artificially contaminated samples of basil, chive, mint and parsley with NoV GI.4 (1×10^5 GE) and GII.4 (2×10^6 GE), then blanched them at 75^{o}C or 95^{o}C for 2 min. The results were variable, with reductions between <1 and <3 log varying within treatment and herb type. For comparison, HAV treated simultaneously lost between 1.7 and >3 log at 75^{o}C and between 2.4 and >3 log at 95^{o}C.

Mormann *et al.* (2010) examined the effect of common cooking procedures on NoV GII.3 inoculated onto various food products. They quantified NoV GE using RT-qPCR. Table 3.9 shows the data obtained. Pasteurisation of spiced tomato sauce effected less than 1 log GE reduction. Roasting of minced meat was less effective at reducing NoV GE than boiling; the authors speculated whether this might have been due to different heat distribution, although it is possible that a protective effect of fat may have been responsible.

Table 3.9: Effect of common cooking procedures on NoV in foodstuffs (from Mormann *et al.*, 2010)

Foodstuff	Cooking procedure	Log reduction in GE
Frozen pizza	Baking at 200^{o}C for 12 min	>4
Spiced tomato sauce	74^{o}C 1 min	0.4
Minced meat	Roasting at 200^{o}C for 30 min	1.6
Minced meat	Boiling at 100^{o}C for 30 min	7

3.12 High hydrostatic pressure

A human volunteer challenge study to investigate the effect of HHP on infectious NoV was conducted by Leon *et al.* (2011). 10^4 GE NoV GI.1 were injected into the digestive tract of shucked oysters (previously treated with 400 MPa to kill potentially pathogenic bacteria). The oysters were then subjected to a range of HHP regimes and fed to the volunteers; contaminated (positive) controls were left untreated and fed to the volunteers. Table 3.10 shows the outcomes. It was observed that after a treatment of 600 MPa at 6^{o}C for 5 min, none of the volunteers became infected with NoV. An illness classification was based on the RT-PCR detection of NoV in stool or vomit rather than on clinical symptoms, since some patients who actively shed NoV showed no clinical manifestations of illness.. Consumption of oysters treated with 400 MPa at 6°C or 25°C for 5 min resulted in infection in 21% and 60% of the volunteers, respectively. It should be noted that 53% of the positive control volunteers who consumed contaminated but non-pressure-treated oysters failed to develop NoV infection.

Table 3.10: Effect of HHP on NoV in oysters, as determined by human volunteer challenge study (from Leon *et al.*, 2012)

Treatment	Number of infected volunteers / total
Untreated control	7/15
400 MPa, 6^{o}C, 5 min	3/14
400 MPa, 25^{o}C, 5 min	3/5
600 MPa, 6^{o}C, 5 min	0/10

Sanchez *et al.* (2011) subjected 1×10^7 GE ml^{-1} NoV GII.4 suspended in cell culture media (Dulbecco's minimal essential medium; DMEM) containing 10% foetal bovine serum (FBS) to HHP treatments from 300 to 500 MPa for 15 min at 25°C and 45°C. Concurrently, they subjected ~6.5 log $TCID_{50}$ MNV to the same treatments. The treatments were repeated, with the addition of $CaCl_2$ to the suspending medium, to determine whether calcium could have a protective effect as seen with bacteria subjected to HHP. Table 3.11 summarises the results from this study. At 400 MPa and above, a 5-6 log reduction in MNV infectivity was obtained; however with NoV, reductions were <0.5 log. The presence of calcium appeared to have a protective effect, particularly when HHP was applied at 25°C except in treatment at 500 MPa. No significant effect on the NoV GE titre was observed, indicating the HHP treatments, even if they affected the infectivity of NoV, did not disrupt the virus capsid. Some treatments did reduce the MNV titre by up to 6.5 log. This indicates that NoV and MNV are possibly affected differently by HHP, as is often the case with surrogate viruses (Richards, 2012).

Table 3.11: Effect of HHP on human NoV GE and MNV infectivity (from Sanchez *et al.*, 2011)

HHP Treatment (MPa)[a]	Suspending medium	Temperature (^oC)	Reduction in human NoV GE titre	Reduction in infectious MNV titre (log_{10}) [b]
300	DMEM + 10% FBS	25	0.01	-0.3
	DMEM + 10% FBS + 10 mM $CaCl_2$	25	0.11	0.3
	DMEM + 10% FBS	45	-0.03[c]	-0.3
	DMEM + 10% FBS + 10 mM $CaCl_2$	45	0.01	0.1
350	DMEM + 10% FBS	25	-0.36	3.0
	DMEM + 10% FBS + 10 mM $CaCl_2$	25	0.34	-0.1
	DMEM + 10% FBS	45	-0.43	0.8
	DMEM + 10% FBS + 10 mM $CaCl_2$	45	0.12	0.0
400	DMEM + 10% FBS	25	-0.15	6.5
	DMEM + 10% FBS + 10 mM $CaCl_2$	25	0.05	1.0
	DMEM + 10% FBS	45	0.21	5.2
	DMEM + 10% FBS + 10 mM $CaCl_2$	45	ND[d]	0.5
450	DMEM + 10% FBS	25	0.34	6.5
	DMEM + 10% FBS + 10 mM $CaCl_2$	25	0.10	4.5
	DMEM + 10% FBS	45	0.02	6.5
	DMEM + 10% FBS + 10 mM $CaCl_2$	45	-0.01	2.2
500	DMEM + 10% FBS	25	0.26	6.5
	DMEM + 10% FBS + 10 mM $CaCl_2$	25	-025	6.5
	DMEM + 10% FBS	45	0.46	6.5
	DMEM + 10% FBS + 10 mM $CaCl_2$	45	-0.26	3.0

[a] all treatments for 15 min
[b] values estimated from graphical display of data
[c] negative values indicate that a slight increase in titre was obtained
[d] Not done

Dancho *et al.* (2012), using RT-qPCR of NoV particles which could bind to porcine gastric mucin linked to magnetised beads, observed that treatment with 500 and 600 MPa reduced RT-PCR-detectable NoV GI by ≥4.7 log. This is in agreement with the findings of Leon *et al.* (2011) and indicates that the porcine gastric mucin technique may provide information on the potential infectivity of RT-PCR-detectable NoV. Ye *et al.* (2014) refined this detection

system by incorporating treatment with RNase prior to binding to the mucin, to remove RNA from damaged virus capsids. They observed that GI.1 appeared to be more resistant than GII.4 to inactivation at HHP of 300 and 400 MPa (Table 3.12). Reducing the temperature of the sample to 1^{o}C prior to application of the pressure treatment increased the GE reduction. From the results of this study, and taking into account the information derived from the use of volunteers (Leon *et al.*, 2011), Ye *et al.* (2014) speculated whether the pressure regimes typically used in industrial processing are sufficient to inactivate NoVs in oysters.

Table 3.12: Log reduction of NoV GE in oyster homogenates after HHP (from Ye *et al.*, 2014)

MPa*	**Initial sample temperature and log reduction in GE**			
	25^{o}C		**6^{o}C**	
	GI.1	**GII.4**	**GI.1**	**GII.4**
300	0.4	1.7	0.7	2.9
400	1.0	3.6	1.3	3.6
600	ND	ND	>4.1	>4.0

* treatment was performed for 5 min.
ND: not done.

The effect of HHP treatment on NoV on blueberries was evaluated by Li *et al.* (2013). NoV GI.1 was spot-inoculated onto berries and then subjected to HHP at 600 MPa either dry or immersed in water. In the dry state, the log GE reduction was 0.9, while when the berries were immersed, the log GE reduction was at least 3.0.

Lou *et al.* (2012) constructed NoV GII.4 VLPs and subjected them to various pressure / time combinations. They reported that the capsid structure, or the ability to bind to receptors, was not disrupted even at treatments of 600 MPa for 60 min, but that treatments were effective at 700, 800 and 900 MPa for 45, 15 and 2 min, respectively. They postulated that NoV was more resistant to HHP than volunteer studies indicated. However, this is not entirely sound, as it is possible that the virus capsid could remain intact but be damaged sufficiently to eliminate infectivity. The observed low reduction in NoV GE titre after HHP treatment by Sanchez *et al.* (2011) support this.

3.13 Hydrogen peroxide

After 1 h exposure to 127 ppm H_2O_2 vapour, NoV GII.4 GE was reduced by 0.5 log (Tuladhar *et al.* 2012c). The authors simultaneously tested a range of other virus types, including adenovirus, MNV, poliovirus, and rotavirus, and observed complete elimination of infectivity. They speculated that NoV would be likewise inactivated by the treatment.

However, Kingsley *et al.* (2014), using RT-qPCR of NoV GI.1 particles which could bind to porcine gastric mucin observed GE reductions of only ~0.1 log after 60 min exposure to 4% H_2O_2 in suspension.

3.14 Ozone

Shin and Sobsey (2003) observed a reduction of NoV GI.1 GE of >3 log after 10 sec contact with 0.37 mg l^{-1} ozone at pH 7 for 10 sec in suspension. Poliovirus and MS2 phage treated identically exhibited reductions of 3 log and 3.5 log respectively. Using a method to quantify remaining NoV RNA after exposure of virus in stool suspension to ozone generated by a commercial system, Hudson *et al.* (2007) observed a 1-2 log reduction in NoV (genogroup not stated) RNA following exposure to 20-25 ppm ozone on plastic and fabric surfaces. 10^3 log reductions were observed in the infectivity of FCV treated simultaneously. Ozone may thus potentially be effective against NoV, both in suspension and on surfaces.

3.15 Peroxyacetic acid

Using RT-qPCR of NoV GI.1 particles which could bind to porcine gastric mucin, Kingsley *et al.* (2014) observed GE reductions of <1 log after 1 min exposure in suspension to 150 ppm peroxyacetic acid.

3.16 Pesticides

Postulating that the use of virus-contaminated water for pesticide application to crops could be a source of virus contamination of fresh produce, Verhaelen *et al.* (2013) studied the persistence of NoV GI.4, GII.4 and the surrogate MNV in water samples containing fungicides or insecticides. In one of the insecticides, NoV GI.4 GE was reduced by 1.5–2 log immediately upon exposure (0 h), while NoV GII.4 was unaffected. MNV infectivity was reduced by 2 log at 0 h by this insecticide. The other 7 pesticides had no or minimal (<0.5 log) reduction in NoV GE or MNV infectivity. The authors concluded that if contaminated water was used for pesticide application during crop production, it would constitute a microbial risk to the food supply chain.

3.17 Plant extracts

A 1 log reduction in NoV GII.4 GE was produced by an extract of persimmon fruit containing 0.96 mg ml^{-1} tannin (Kamimoto *et al.*, 2014). The composition has been patented (Shimamoto *et al.*, 2014). Information available in the patent indicates that the persimmon extract in a hand-washing foam formulation could reduce NoV GE in suspension by at least 2 log after 120 sec. Li *et al.* (2012b) evaluated grape seed extract (GSE) for antiviral efficacy

by exposing NoV GII.4 to 0.2 and 2 mg ml^{-1} GSE in PBS or a homogenised lettuce extract. The suspensions were analysed by binding to Caco-2 cells followed by RT-qPCR. In PBS, 0.2 mg ml^{-1} GSE effected a 1 log reduction in detectable GE, while 2 mg ml^{-1} effected a 2 log reduction. In lettuce extract, 0.2 mg ml^{-1} GSE effected a 0.5 log reduction in detectable GE, while 2 mg ml^{-1} effected a 1.5 log reduction. The GSE had a disruptive effect on NoV GII.4 VLPs at both concentrations.

3.18 Quaternary ammonium compound (QAC)

Using pre-treatment with RNase to eliminate RNA from damaged virus particles subsequent to RT-PCR, Girard *et al.* (2010) tested the efficacy of QAC against NoV on stainless steel surfaces. They inoculated stainless steel discs with 10^4 GE NoV (genogroup not given), then applied a commercial QAC-based disinfectant for 5 and 10 min at room temperature. No reduction of GE was observed after any treatment. Tung *et al.* (2013) reported similar findings, with NoV GII.2 and GII.4 GE being reduced by <0.5 log after exposure to a commercial QAC-based disinfectant for 30 sec in suspension. When surrogate viruses (MNV and FCV) were tested simultaneously, similar minimal fluctuations in infectivity were observed.

3.19 Solar disinfection

To examine the effect of sunlight on NoV in seawater, Fagan *et al.* (2008) suspended ~10^6 NoV in transparent bags in a freshwater river at the surface and at 1 m depth. Samples were taken for RT-qPCR analysis at various time points up to 8 d. GE decline rates were <0.51 log d^{-1}. Flannery *et al.* (2013) suspended NoV GI and GII (each at 10^3 GE ml^{-1}) in 100 ml filtered seawater (depth 18 mm) and exposed them to simulated solar irradiance. As controls the suspensions were covered in aluminum foil. The average summer (mean daily irradiance of 235 W m^{-2}, maximal temperature 17^{o}C) and winter (mean daily irradiance of 56 W m^{-2}, maximal temperature 10^{o}C) conditions prevailing on the coast of Ireland were modelled; 10^9 PFU bacteriophage GA were also added to the test samples. They calculated the T90 values for each virus (Table 3.13; only NoV results displayed). Under summer conditions NoV GE declined more rapidly than under winter conditions. The decline in bacteriophage GE infectivity also followed this pattern, with T90 values for GE decline being similar but higher for reduction in infectivity (0.25 and 4.0 for summer and winter irradiated conditions, respectively). The fluence (irradiation) necessary to effect a 90% reduction in GE number was calculated and found not to be significantly different for each virus. This was postulated to indicate that the temperatures used in the tests did not have a significant effect on viral

inactivation; however as reductions in GE numbers were observed in the summer control tests (particularly for NoV GII) the inference can be drawn that infectious NoV in seawater at 17^{o}C will decline over time regardless of sunlight conditions.

Table 3.13: Effect of solar irradiance on NoV GE (from Flannery *et al.*, 2013)

Virus	Condition	T90 (h)	Approximate overall log decline in GE
NoV GI	Summer (irradiated)	21.5	4 in 80 d
	Summer (control)	59.8	1 in 80 d
	Winter (irradiated)	89.3	<1 in 20 d
	Winter (control)	85.9	<1 in 20 d
NoV GII	Summer (irradiated)	20.5	4.5 in 80 d
	Summer (control)	41.0	2 in 80 d
	Winter (irradiated)	83.9	<1 in 20 d
	Winter (control)	101.5	<1 in 20 d

GE: genomic equivalents

3.20 Trisodium phosphate

Using RT-qPCR of NoV GI.1 particles which had bound to porcine gastric mucin Kingsley *et al.* (2014) observed GE reductions of ~1.6 log after 5 min exposure to 5% trisodium phosphate in suspension. A 1 min exposure had no effect.

3.21 Ultraviolet irradiation

Duizer *et al.* (2004) exposed NoV GII.4 suspensions to UV irradiances of 103 mJ·cm^{-2} and 206 mJ cm^{-2} and then performed RT-PCR. They expressed the results as change on Ct values obtained. Assuming that a 1 Ct value increase signifies a 50% reduction in starting GE number, their results indicate log GE reductions of <1 when the virus was exposed to either dose. Newland and Sobsey (2007) reported that a UV dose of 150 mW-sec·cm^{2} did not effect any GE reduction of NoV, MS2 or poliovirus type 3. The same treatment infectivity of the latter two viruses was reduced by 3.7 and 5.8 log, respectively. Wolf *et al.* (2009) used long-range reverse transcription to evaluate the genome damage caused by exposure of NoV to UV light. They exposed 5 ml suspensions of NoV GI and GII (~10^{8} GE ml^{-1}) in Petri dishes to

irradiance from 15W UV-C lamps (254 nm) set 70-90 cm above the dishes. After 3 min exposure, NoV GII GE titre was reduced by ~2 log and NoV GI GE titre by nearly 3 log.

Dancho *et al.* (2012), using RT-qPCR of NoV particles which bound to porcine gastric mucin, observed that treatment with 0.5 $J \cdot cm^{-2}$ reduced RT-PCR-detectable NoV GI by 1.8 log, while after a treatment with 2 $J \cdot cm^{-2}$ a 3.8 log reduction in binding was observed. Li *et al.* (2014) used a combination of binding to Caco-2 cells with subsequent long-range RT-qPCR. The latter technique employs priming of the nucleic acid amplification by oligonucleotides which bind to widely separated sequences on the virus genome, the hypothesis being that genome damage between the binding sites will prevent efficient amplification. Following exposure of NoV GII.4 to 20 $mJ \cdot cm^{-2}$ and 200 $mJ \cdot cm^{-2}$, ~3 log and >4 log reductions in detectable GE were observed, respectively. Wang and Tian (2014) used a method in which NoV was captured by histo-blood group antigens prior to RT-qPCR, to determine the effect of UV on NoV GII.4. Irradiances up to 0.5 $J \cdot cm^{-2}$ effected <1 log reductions in detectable GE, 0.75 $J \cdot cm^{-2}$ effected a ~1 log reduction, while 1 $J \cdot cm^{-2}$ effected a >3 log reduction. Ronnqvist *et al.* (2014) subjected NoV GII.4 (10^7 GE) on glass surfaces to UV irradiance, then employed an enzymatic treatment (proteinase + RNase) prior to RT-qPCR, to eliminate NoV RNA from damaged viral capsids. They observed a decrease in detectable GE of 2-3 log after a UV dose of 1.8 $J \cdot cm^{-2}$. They estimated that the probability of reducing NoV GE by 3 log was <1% with doses up to and including 0.9 $J \cdot cm^{-2}$, 11% after 1.35 $J \cdot cm^{-2}$ and 57% after 1.8 $J \cdot cm^{-2}$.

Jones *et al.* (2014) reported that NoV GII.4 replication in a novel cell culture system was inhibited by prior UV irradiance of the virus suspension used as inoculum, but provided no details of the UV dose.

3.22 Washing

Butot and colleagues (2008 & 2009) examined the effectiveness of washing for the elimination of NoV from berries and other produce types. To simulate post-harvest conditions, samples were artificially contaminated with 1.2×10^5 NoV GI GE and 2×10^6 NoV GII GE and then washed for 30 sec in 200 ml tap water at 18°C or 43°C. There was little difference in NoV removal between washing at each temperature (Table 3.14). There was higher removal of NoV GI GE from basil than the other produce types, which the authors speculated was possibly due to the virus being less able to bind to the surfaces of basil than the other types. Bae *et al.* (2011) found that several simple washing procedures – immersion,

rinsing, and a combination of both with and without the addition of detergent could remove ~1 log of NoV GII GE on artificially contaminated perilla (mint) leaf (Table 3.14). Immersion in tap water for 2 min and rinsing in tap water for 30 sec were not effective in removing NoV GII from iceberg lettuce. The reason for this was not discussed, but may have been due to the more convolute structure of this vegetable offering more niches where the virus could escape removal. Addition of detergent did not have a significantly different effect over immersion and washing in tap water alone. Tian *et al.* (2011) examined the effectiveness of washed in electrolysed water for removal of NoV from lettuce and raspberries. NoV GII.4 was inoculated onto the surface of produce samples which were then immersed in electrolysed water at acidic (pH ~2.3), neutral (pH 7.0-8.0) or basic (pH 10-11) pHs and rinsed for 15 min or washed by inverting the container 10 times. Only washing at neutral to basic pH produced GE reductions greater than 1 log, although the report is unclear which data is presented (from soaking or rinsing).

Table 3.14: Elimination of NoV GE on food products by washing

Product	**Treatment**	**Time**	**Temperature (^{o}C)**	**Approximate overall log decline in GE**	**Reference**
Basil	Water	30 sec	18	>3.4 (GI) 1.1 (GII)	Butot, *et al.* (2008)
Basil	Water	30 sec	43	>3.4 (GI) 1.1 (GII)	Butot *et al.* (2008)
Blueberries	Water	30 sec	18	< 1 (GI) 1.5 (GII)	Butot, *et al.* (2008)
Blueberries	Water	30 sec	43	< 1 (GI) 2.1 (GII)	Butot *et al.* (2008)
Iceberg lettuce	Water	2 min immersion + 30 sec rinsing	NG	<1	Bae *et al.* (2011)
Iceberg lettuce	Water	2 min immersion in 0.1% detergent + 30 sec rinsing	NG	1	Bae *et al.* (2011)
Parsley	Water	30 sec	18	<1(GI/GII)	Butot, *et al.* (2008)
Parsley	Water	30 sec	43	<1 (GI) 1.7 (GII)	Butot *et al.* (2008)
Perilla leaf	Water	2 min immersion	NG	<1 GII	Bae *et al.* (2011)

Perilla leaf	Water	30 sec rinsing	NG	1.2 GII	Bae *et al.* (2011)
Perilla leaf	Water	2 min immersion + 30 sec rinsing	NG	1.2 GII	Bae *et al.* (2011)
Perilla leaf	Water	2 min immersion in 0.1% detergent + 30 sec rinsing	NG	1 GII	Bae *et al.* (2011)
Raspberries	Water	30 sec	18	<1 (GI/GII)	Butot *et al.* (2008)
Raspberries	Water	30 sec	43	<1(GI/GII)	Butot *et al.* (2008)
Raspberries	Electrolysed water pH~2.3	Rinsing, or soaking 15 min	RT	<1	Tian *et al.* (2011)
Raspberries	Electrolysed water pH 7.0-8.0	Rinsing, or soaking 15 min	RT	~2	Tian *et al.* (2011)
Raspberries	Electrolysed water pH 10.0-11.0	Rinsing, or soaking 15 min	RT	1-2	Tian *et al.* (2011)
Romaine lettuce	Electrolysed water pH~2.3	Rinsing, or soaking 15 min	RT	<1	Tian *et al.* (2011)
Romaine lettuce	Electrolysed water pH 7.0-8.0	Rinsing, or soaking 15 min	RT	1-2	Tian *et al.* (2011)
Romaine lettuce	Electrolysed water pH 10.0-11.0	Rinsing, or soaking 15 min	RT	1-2	Tian *et al.* (2011)
Strawberries	Water	30 sec	18	< 1(GI/GII)	Butot *et al.* (2008)
Strawberries	Water	30 sec	43	< 1(GI) 1.1 (GII)	Butot *et al.* (2008)

NG: not given; possibly room temperature
RT: room temperature

4. Conclusions

NoV are the principal cause of gastroenteritis worldwide. Their detection and quantification have been principally achieved from molecular techniques. The advent of RT-qPCR has provided information on the presence and levels of NoV in clinical and environmental samples and in foods but is limited by the inability to differentiate infectious from non-infectious virus particles (Richards 1999). As stated in the introduction to this review, efforts to propagate NoV over the past four decades have been unsuccessful and propagatable surrogate viruses are not truly representative of NoV (Richards 2012); therefore, it has not been possible to monitor for the presence and survival of infectious viruses or to assess the ability of various processing techniques to inactivate NoV. Recent reports have suggested that NoV cultivation may now be possible (Jones *et al.*, 2014) and, if validated, would represent an important tool to further our understanding of the survival and elimination of NoV in foods and the environment.

The majority of the data in this review is based upon the results of measurements using RT-qPCR. Unfortunately the relationship between RT-qPCR signals and infectivity is complex and this relationship is dependent both on the mechanism of inactivation and the experimental approach. Reductions in RT-qPCR signals generally underestimate infectivity for surrogate viruses and this is most likely true for NoVs; so small (2 log) reductions in RT-qPCR signals may be of greater significance than is immediately apparent. Results of a systematic review have provided qualitative evidence that NoVs RT-qPCR signals are more persistent than surrogate RT-qPCR signals following either heat or available chlorine treatment suggesting that NoVs are more persistent than surrogates (Knight *et al.*, submitted). This persistence is supported by a number of unique observational studies identified in this review including resistance to freezing (Richards *et al.*, 2012), persistence in water (Seitz *et al.* (2011), on berries (Verhaelen 2012) and in organic waste (Meschke *et al.*, 2000). Interpretation of RT-qPCR data from unique observational studies is complicated by both differences in inactivation mechanism and experimental approach.

Although molecular methods for predicting infectivity have progressed for measuring virus inactivation there remain gaps in corresponding data for determining if a detected virus RT-qPCR signal is derived from a viable particle. Newer methods have been developed to facilitate the identification of infectious NoV by means of binding infectious viruses to histo-

blood group antigens found on porcine mucin and then performing RT-qPCR on eluted viruses (Dancho *et al.*, 2011). This work is undergoing validation to ascertain its effectiveness and could provide a better indication for the presence of infectious NoV particles using RT-qPCR. Capsid integrity has also been evaluated using propidium monoazide intercalation and RNase treatment (Escudero-Abarca *et al.*, 2014), virus binding to Caco-2 cells followed by long-range RT-qPCR (Li *et al.*, 2012) and other techniques. In the meantime, comparisons of past molecular studies involving NoV are complicated by the lack of harmonization of virus extraction and assay methods and the different types of matrices examined. This review used what are referred to as genomic equivalents to compare relevant data; however, it is impossible to directly compare the data, since laboratory methods are highly variable from one study to another. It is possible in some cases, however, to identify common features in regard to NoV persistence and inactivation, particularly when several studies show similar trends.

Survival of Norovirus

NoV is highly resilient to most of the environmental pressures and processing techniques evaluated to date. This persistence is supported by a number of unique observational studies identified in this review including resistance to freezing (Richards *et al.*, 2012), persistence in water (Seitz *et al.* (2011) and shellfish (Grohmann *et al.*, 1981), on berries (Verhaelen 2012) and in organic waste (Meschke *et al.* 2000). Depending on the type of water, NoV is able to persist for 60 – 728 d (Seitz *et al.*, 2011; Ngazoa *et al.*, 2007; Bae and Schwab, 2008; Charles *et al.*, 2009). NoV also persisted on berries and vegetables (Verhaelen *et al.*, 2012; Escudero *et al.*, 2012; Lamhoujeb *et al.*, 2008), often showing <1 log reduction in counts within 1-2 weeks. The persistence of NoV in fruits and vegetables may easily exceed the shelf-life of the products. Studies on bivalve shellfish showed a persistence of NoV within shellfish subjected to depuration for 7 d (Grohmann *et al.*, 1981), a sign that NoV like other enteric viruses may persist for long periods within shellfish tissues (Provost *et al.*, 2011). NoV can persist for long periods of time in other food products, like cooked turkey (at least 10 d at 7^{o}C), processed foods (at least 2 days at 6^{o}C), and apples (at least 7 d at 11^{o}C) (Lamhoujeb *et al.*, 2008; Mormann *et al.*, 2010). On environmental and food processing surfaces NoV was resilient on carpets, Formica, stainless steel, PVC and ceramic surfaces (Cheesebrough *et al.*, 1997; Liu *et al.*, 2009, 2012; Park *et al.*, 2007; Lamhoujeb *et al.*, 2008). On hands, NoV could persist for at least 2 h with a < 1 log reduction(Liu *et al.*, 2009); washing with water for 10 sec could effect a reductions of up to 1.5 log NoV (Liu *et al.*, 2010).

The effect of elimination procedures on Norovirus

Numerous studies were reviewed on the inactivation of NoV by various interventions. Studies involving heating as a means to degrade NoV RNA on surfaces or suspended in various solutions show widely varying results, depending on the technique applied and the composition of the solutions. Autoclaving was effective in reducing NoV by at least 5 log (Choi *et al.*, 2014). Heating of NoV in various suspensions reduced NoV RNA levels, although temperatures required to destroy the RNA depended greatly on the suspension solutions and the duration of application (Duzier *et al.*, 2004; Dancho *et al.*, 2012; Croci *et al.*, 2012; Escudero-Abarca *et al.*, 2014; Li *et al.*, 2014; Wang and Tian, 2014).

Chemical disinfection of NoV has been mostly restricted to an evaluation of RNA loss after various treatments. Chlorine appears to be a suitable disinfectant. In a volunteer study, treatment of water with 10 mg^{-l} of chlorine for 30 min (reportedly 5-6 mg ml^{-1} free chlorine) prevented infection of volunteers (Keswick *et al.*, 1985). Other studies evaluated the effectiveness of chlorine/sodium hypochlorite to reduce NoV RNA with generally good results (Duizer *et al.*, 2004; Shin and Sobsey, 2008; Kitajama *et al.*, 2010; Liu *et al.*, 2010; Tung *et al.*, 2013; Kingsley *et al.*, 2014). Chlorine solutions were also effective as a surface disinfectant for steel, melamine, berries and herbs (Barker *et al.*, 2004; Girard *et al.*, 2010; Park and Sobsey, 2011; Tuladhar *et al.*, 2012b; Butot *et al.*, 2008). Hypochlorous acid was also useful for reduction of NoV RNA (Park and Sobsey, 2007). Ineffective as disinfectants were hydrogen peroxide, quaternary ammonium compounds, ethoxylated alcohol-based disinfectants and antiseptics at normally used concentrations (Park *et al.*, 2007; Girard *et al.*, 2010; Tuladhar *et al.*, 2012b; Kingsley *et al.*, 2014, Tung *et al.*, 2013). Others found ethanol to be ineffective against NoV even at concentrations ≥90% alcohol (Liu *et al.*, 2010; Tung *et al.*, 2013). However hand sanitizers containing ethanol in combination with other compounds could be effective in reducing NoV (Liu *et al.*, 2011). Liquid soap containing 0.5% triclosan and a water rinse alone reduced the levels of NoV on finger pads of volunteers by 1-1.5 log (Liu *et al.*, 2010).

Finally, various processing techniques have shown some promise in reducing NoV loads from foods, namely the use of heat, as mentioned above, high hydrostatic pressure to inactivate NoV in oysters, as determined by a volunteer study (Leon *et al.*, 2011) and on berries (Li *et al.*, 2013); ozone on solutions containing NoV (Shin and Sobsey, 2003) and for

moderate reductions of NoV on surfaces (Hudson *et al.*, 2007). High pH (but not neutral or low pH) appears to destabilize NoV capsids and could be useful as a rinse to disinfect the surface of some food products. Heating and marinating mussels in a pH 3.75 solution did little to reduce NoV levels (Hewitt and Greening, 2004). Depuration has also been unsuccessful in totally eliminating NoV from shellfish (Grohmann *et al.*, 1985; Ueki *et al.*, 2007; Savini *et al.*, 2009; McLeod *et al.*, 2009; Nappier *et al.*, 2008, 2010; Polo *et al.*, 2014). Freezing of NoV in solution was also shown to have little to no effect on NoV RNA levels based on RT-PCR and infectivity-based assays involving binding of the virus to porcine mucin (Richards *et al.*, 2012). Freeze drying of some products, like blueberries and parsley, reduced NoV levels by up to 3.5 log; however, reductions were generally better for GII NoV (Butot *et al.*, 2008, 2009). Gamma irradiation appeared to alter the capsid structure, but no conclusions could be drawn in regard to viral RNA levels or virus infectivity (Feng *et al.*, 2011). UV irradiation reduced RT-PCR products slightly in some studies (Duizer *et al.*, 2004; Newland and Sobsey, 2007), and more extensively in others (Li *et al.*, 2014; Wang and Tian, 2014; Dancho *et al.*, 2012; Ronnqvist *et al.*, 2014). Overall, it appears that NoV is inactivated by UV. From an industrial standpoint, thorough washing of basil with water was effective in reducing NoV GI by up to 3.4 log and GII by 1.1 log; while, similar washing of blueberries reduced G1 NoV by <1 log and GII by ≤2.1 log (Butot *et al.*, 2008). Additional research is needed.

5. Recommendations for further research

To determine the presence, persistence, and methods for the inactivation of NoV require efforts on several fronts as follows:

Reports and data should be reviewed on the culture and quantification of NoV and additional funding to support propagation studies should be considered. Questions that need to be answered include: Can the data be reproduced? Is the culture system limited to specific genogroups or genotypes? What is the apparent multiplicity of infection and could it be applied to environmental samples? In the future, culturing may well be the principle method to monitor NoV infectivity.

Potential methods to determine infectious versus inactivated NoV, such as the porcine mucin binding assay as a possible indicator of virus infectivity, have been reported in the literature and some of them are under further evaluation. Progress in this area should be closely monitored and support for validation studies of those methods deemed most promising should be explored.

Volunteer studies are the only conclusive means currently available to identify NoV infectivity. Such studies offer the opportunity to determine the ability to inactivate NoV under various environmental conditions or processing scenarios. In the absence of other infectivity assays, volunteer studies can provide definitive information and should be considered for funding priority.

Research with NoV surrogates, like feline calicivirus and murine norovirus, have provided little to no insight into the inactivation of human NoV. Work with NoV surrogates should be discontinued unless the inactivation kinetics of the surrogate are directly compared with the inactivation kinetics of NoV under the same, well defined conditions. Publication of papers on surrogate research should be discontinued unless it can be shown that the surrogate mimics NoV. The literature on surrogate viruses was eliminated from this review, unless it was compared directly with NoV. Unfortunately, most surrogate studies do not involve any comparisons with NoV and are of little value.

The development of better assays for NoV infectivity are important; however, equally important is the development of methods to extract and concentrate viruses from environmental samples and foods. Without sensitive and reproducible extraction protocols, comparisons of data from one laboratory to another or even within a laboratory may not be possible. Much progress has been made in recent years in developing harmonised methods for NoV extraction from various commodities, but further research is needed. Current methods for NoV detection generally require the use of RT-qPCR, which requires that viral RNA be extracted for analysis; therefore, current extraction procedures for NoV are designed to extract viral RNA. With the advent of cell culture propagation methods potentially on the horizon, methods should be developed and evaluated to extract and concentrate intact NoV virions for use in future cell culture analyses.

The limitations in the use of RT-qPCR must be recognised and assay methods should be harmonised to the extent possible. There are many different NoV within several genogroups and all represent unique challenges for their detection and quantification. The use of RT-qPCR may well be discontinued once cell culture propagation methods are developed for NoV. Until that time, RT-qPCR should be standardised in regard to primers and reagents used, RT and PCR cycling conditions, and detection methods (probes, SYBR-green, etc. and detection wavelengths used).

Data on the occurrence and survival of NoV in the environment are limited by the very low levels of contamination present. This could be improved by the application of rapid and efficient separation and concentration methods.

Collaborative research on NoV should be supported as it is useful to ensuring repeatability of methods and adds confidence to experimental results.

Annex 1: How the review was performed

The project commenced on 8th December with an inception teleconference involving all partners. A refinement of the key words to be used in the literature search which were listed in the project proposal was discussed. Project partners completed the refinement of the terms later that day.

The following databases were then searched:

BIOSIS Citation IndexSM
CABI
Zoological Record®
Medline
Web of ScienceTM Core Collection
FSTA® - the food science resource
SciELO Citation Index
Current Contents Connect®
KCI-Korean Journal Database

A search of the Web of Knowledge host was completed by 23rd December, and had 1846 hits. Overall the logic was: (all suggested terms for survival, persistence, inactivation) and (terms for Norovirus, including previous names such as Norwalk virus and SRSV, and strain names such as Desert Shield and Snow Mountain). The database was placed on an EndNote file.

An initial screening of the abstracts in the database led to the rejection of 1618 papers which described work that was not relevant to the survival and elimination of NoV (e.g. the effect of heat on replication of the virus, effect of antivirals in human infection, studies exclusively on surrogates, etc.). The remaining references were grouped into two categories, "Survival" (70 references) and "Elimination" (157 references), to reflect the final structure of the review. A second screening of the database removed a further 38 references from the Survival group and a further 91 references from the Elimination group. These references were removed on various grounds (e.g. repetition, focus on surrogates, focus on transfer from one surface to

another, general details on controlling infection in nursing homes). Two "unfiled" references were found to be duplicates of references in the Survival group. Finally, the Survival group contained 32 references, and the Elimination group contained 66 references. When the draft review was written, one reference from the Survival group had yet to be obtained. Nineteen additional papers (mostly background information) were included in the draft review.

The draft review was sent to the Food Standards Agency for comment. When the comments were received, the project partners held a teleconference to discuss response to the comments and to plan the completion of the review.

During the final revision, the last reference from the literature search was obtained and reviewed, along with 1 further additional publication.

Finally, 118 publications were reviewed for this report.

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