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A Systematic Review of the Survival of Norovirus in Foods and on Food Contact Surfaces

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EXECUTIVE SUMMARY

Human noroviruses are the single largest cause of gastrointestinal disease in the western world. However the efficacy of control measures to reduce the burden of foodborne norovirus infections remains largely unknown, partly owing to the inability to grow the virus and partly to the large number of surrogate studies of unknown relevance. A systematic review of the persistence and survival of human noroviruses (hNoVs) in foods and the environment was undertaken based upon PRISMA (Preferred Reporting Items for Systematic reviews and Meta Analyses) guidelines to answer the questions: Firstly, *"What are the natural human norovirus (hNoV) persistence characteristics in food and the environment?"*; and secondly, *"How can these properties be altered by applying physical and/or chemical treatments to foods or food contact surfaces?"* Over 10,000 citations relevant to viruses present in foods and the environment were screened using defined inclusion and exclusion criteria from which 126 citations were identified for further evaluation. Persistence data from each of these publications were extracted for the conditions of the treatment (dose, pH, and temperature, time, infectivity, and RT-qPCR results). The only marker for hNoV persistence and survival is RT-qPCR data and clinical studies. Therefore citations for inclusion were further restricted to only those that included hNoV RT-qPCR studies, in direct comparison with surrogate virus RT-qPCR and infectivity data; and also data from clinical trials. This resulted in 27 eligible laboratory-based studies and 3 clinical trials. Eligible studies were diverse and utilised different experimental approaches and methods. Few data were relevant to persistence and survival of hNoVs in real foods and on food contact surfaces. Data were further extracted for common treatments (heat and available chlorine) and for important additional experimental variables such as the matrix and RT-qPCR product size.

Comparison of extracted data showed that RT-qPCR data under-estimated reductions in infectivity for surrogate viruses and hNoV RT-qPCR signals are more persistent than those of comparative surrogates. The data suggest that hNoVs are more resistant to typical food and environmental control measures compared with cultivable surrogate viruses.

LAYPERSONS SUMMARY

Human noroviruses (hNoVs) are the single largest cause of gastrointestinal disease in the western world. However the efficacy of control measures to reduce the burden of foodborne norovirus infections remains largely unknown, partly owing to the inability to grow the virus and partly to the large number of studies that have used alternative cultivable viruses (surrogates). More recently molecular methods have been used to predict the persistence of hNoVs. However the relevance and significance of these studies for human noroviruses is unknown. The project was designed to answer two questions using a systematic review process. Firstly "*What are the natural human norovirus (hNoV) persistence characteristics in food and the environment?'* and secondly *"How can these properties be altered by applying physical and/or chemical treatments to foods or food contact surfaces?"* A systematic review is a defined structured approach to examining published data. The review process first designs and tests literature search methods to identify publications that contain relevant data. The data are analysed to detect common trends and determine if the trend resulting from a particular treatment is statistically significant – this is termed meta-analysis.

The initial search strategy identified over 10,000 citations. Of these, approximately 126 were relevant to norovirus in general (i.e. hNoV or surrogate studies) however only 27 of these and 3 clinical trials possessed sufficient data to allow comparison with hNoV. Comparison was also complicated by the large number of experimental differences between studies that prevented meta-analysis. However statistical analysis of two common treatment types (heat and disinfection using available chlorine (bleach) suggests that hNoV are more persistent than surrogate viruses and are more likely to resist food and environmental control strategies previously thought effective based on surrogate data alone.

LIST OF ABBREVIATIONS

CHCl3	Chloroform extracted virus
FBS	Foetal bovine serum
FCV F-9	Feline calicivirus vaccine strain F-9
MNV-1	Murine norovirus strain 1
hNoV	Human norovirus
NoV	Norovirus
PBS	Phosphate buffered saline
PRISMA	Preferred Reporting and Information Systematic review and Meta-Analysis
PV-1	Poliovirus 1
RT	Room temperature
RNPs	RNase resistant ribonucleoprotein complexes
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse Transcription quantitative polymerase chain reaction
TCM	Tissue culture medium
TV	Tulane virus

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INTRODUCTION

Aims and Objectives

The aim of this study was to conduct a systematic review and ideally meta-analysis of the survival of human norovirus (hNoV) on food and food contact surfaces in order to identify any knowledge gaps and determine the most effective control measures from the available published data. The overall project objectives were as follows:

- To conduct a systematic review of the available literature on the survival of norovirus in foods and on food contact surfaces, and where appropriate and feasible, undertake a meta-analysis of the data.
- To adopt a comprehensive search strategy considering studies relating to physical and chemical treatments and their impact on survival in/on different types of foods and on food contact surfaces.
- To use explicit and objective criteria for inclusion and rejection based on the quality of the studies being considered.
- To assess the robustness of existing studies on the survival and persistence of norovirus and highlight any research gaps which should be addressed to improve the evidence base in support of controlling norovirus in the food chain.

Systematic review is a structured approach to reviewing published scientific data in order to answer a specific question, and originally developed for the assessment of clinical interventions. Systematic review and meta-analysis can provide a statistical comparison of compiled published data that can help the user evaluate the success or failure of an intervention, and can provide a scientific justification for decision making. The application of systematic reviews within the agri-food sector has been previously described (Sargeant *et al.,* 2006). For example, systematic reviews have been used to identify, characterise, and measure the success of interventions designed to understand and control bacterial foodborne pathogens (Bucher *et al.,* 2012). This approach has recently been used to examine the success of infection control measures for hNoV; as well as the relative susceptibility of cultivable hNoV surrogate viruses to chemical disinfectants and the seasonality of hNoV outbreaks (Harris *et al.,* 2010, Hoelzer *et al.,* 2013, Ahmed *et al.,* 2013).

Human NoVs are recognised currently as the single most common cause of acute non-bacterial gastroenteritis in the industrialised world (Siebenga *et al.,* 2009). These viruses may be divided into different genogroups and genotypes, of which genogroup II, genotype 4 (GII.4) strains are detected most commonly in association with disease (Patel *et al.,* 2009), although there is a greater diversity of genotypes associated with foodborne outbreaks (Kroneman *et al.,* 2008, Stals *et al.,* 2012). Most hNoV foodborne disease outbreaks are associated with consumption of food outside of the home, where it appears that poor hygiene of infected food handlers is the major source of contamination (Mathijs *et al.,* 2012). Contamination of fresh produce (e.g. berries, leafy greens) may be associated with human handling or the use of raw sewage or contaminated water during production or processing (EFSA, 2014). Additionally hNoV disease is highly contagious and shows rapid person-to-person transmission directly or indirectly through the faecal-oral route, from contaminated fomites, and from aerosolized vomitus. Effective control measures for water treatment, food processing, cleaning and disinfection of surfaces, personal hygiene and hand washing, and/or sanitation are all required in order to control the spread of hNoV along the food chain, although the efficiency of current control measures for hNoVs is unknown.

The development of improved and suitable control measures for hNoVs is limited by the inability to culture these viruses *in vitro*. It is therefore not possible to determine the persistence and survival of hNoV without the use of carefully controlled human volunteer studies. These are expensive and are faced with substantial ethical and regulatory hurdles. Most data collected to date on the behaviour and control of hNoVs along the food chain have been obtained through the use of two cultivable surrogate virus models, most notably the feline calicivirus vaccine strain (FCV F-9) (Bidawid *et al.,* 2003, Duizer *et al.,* 2004, Cannon *et al.,* 2006) and murine norovirus (MNV-1) (Wobus *et al.,* 2006, Belliot *et al.,* 2008, Baert *et al.,* 2010, Li *et al.,* 2013). More recently, the primate-derived Tulane virus (TV) has been used (Hirneisen and Kniel, 2013; Li *et al.,* 2013).

The effect of food preservation methods on the inactivation of foodborne viruses including the norovirus surrogates FCV-F9 and MNV-1 has been extensively reviewed (Koopmans and Duizer, 2004; Rzezutka and Cook, 2004; Le Guyader and

Atmar, 2008; Baert *et al.,* 2009, Bertrand *et al.,* 2012; and in the grey literature (Sobsey and Meschke, 2003; Doyle, 2010).

Examination of this published data has shown that the extent of virus inactivation varies dependent on the virus tested, the inactivation method, and the selected food matrix, (including oysters, fresh produce, milk, salad crops, and fruits). In general surrogate viruses appear to persist at reduced relative humidity or when tested in complex matrices that may confer protection e.g. fats. However in general the effect of food matrices has been poorly characterised although it and it is well known that the pH stability amongst surrogates can differ markedly (Cannon *et al.,* 2006). It is not known if current surrogate models are relevant to the survival of hNoVs. The environmental persistence and transfer of enteric viruses has recently been reviewed (Kotwal and Cannon, 2014) and concluded that enteric viruses are readily transferred and persist on hands, food, water and environmental surfaces. However the value and relevance of data obtained from surrogates compared with hNoVs is unclear and has been questioned (Richards, 2012). Alternatively researchers have sought to adapt molecular assays so that they can better measure infectivity of hNoV after inactivation treatments, many of which target measurement of the integrity of the virus capsid (Knight *et al.,* 2012).

Since hNoV cannot be grown in culture, the systematic literature review undertaken here also considered data from cultivable surrogate virus studies and alternative molecular measures of infectivity. Many different viruses (in addition to FCV F9, MNV-1, and TV) have occasionally been utilized as surrogates for hNoVs, including attenuated poliovirus and the bacteriophage MS2, although similarly their relevance is largely unknown. For this reason, the literature reviewed for this study focused exclusively on related enterically infecting single stranded (ss) RNA viruses. The rationale for this selection was that hNoVs possess a positive sense ssRNA genome and that ssRNA is less stable than double stranded (ds) RNA, or DNA (ss or ds) in general. Additionally some clear distinctions can be found between distantly related ssRNA viruses such as foot and mouth disease virus, rhinovirus and feline calicivirus. For instance, these latter viruses are not enterically infecting, acid labile, and share different cell entry pathways associated with the dissociation of the viral capsid (reviewed by Knight *et al.,* 2012). We therefore restricted comparison to other

enterically infecting viruses such as poliovirus, hepatitis A, cocksackie B, sapovirus and murine NoV (MNV). The only exception was the inclusion of data for the widely studied hNoV surrogate FCV as a reference point in order to ensure the most comprehensive review possible.

METHODS

The PRISMA approach

The approach used was to conduct the review according to the PRISMA guidelines http://www.prisma-statement.org/ (Moher *et al.,* 2009). This includes a structured approach to the review process and a check-list for reporting. It is intended that a formal PRISMA structured manuscript will be published as an academic publication in support of this report (in preparation). The reporting structure below is therefore based upon PRISMA guidelines to facilitate publication.

RESULTS

Study design

The PRISMA approach requires the definition of the central question(s) at the commencement of the study. These were agreed upon between the project partners and the Food Standards Agency and defined as firstly, “What are the natural hNoV persistence characteristics in food and the environment?” and secondly “How can these properties be altered by applying physical and/or chemical treatments to foods or food contact surfaces?” The term “environment” was included as part of the question since food and food contact surfaces are components of the natural environment of hNoV, which also includes the human host, as well as soil and water. These questions were refined according to the PRISMA PICOS criteria where “P” represents the participants; “I,” the intervention; “C,” the comparison; “O,” the outcomes; and “S,” the study design. In terms of this review, “P” represents the NoV population under review and “I” is the treatment type assigned as biological, physical, chemical or combined treatment. “C” represents the comparative data between hNoV and other viruses, while “O” is the quantitative survival data. “S” corresponds to the study design, which in this case is mainly represented by

observational studies related to survival of surrogate viruses and hNoV in food or food contact surfaces, and a smaller number of relevant clinical studies.

The PICOS questions were defined as:

1.1 What are the survival (O) characteristics of hNoV in the environment (P)?

1.2 What are the survival (O) characteristics of hNoV in food or on food contact surfaces (P)?

2.1 What are the comparative effects (C) of physical treatments (I) on the survival (O) of noroviruses, other enterically infecting single stranded RNA viruses, and FCV in foods or on food contact surfaces (P)?

2.2 What are the comparative effects (C) of biological treatments (I) on the survival (O) of noroviruses, other enterically infecting single stranded RNA viruses, and FCV in foods or on food contact surfaces (P)?

2.3 What are the comparative effects (C) of chemical treatments (I) on the survival (O) of noroviruses, other enterically infecting single stranded RNA viruses, and FCV in foods or on food contact surfaces (P)?

Eligibility criteria

Eligible citations were those identified from the search terms as satisfying the PICOS defined criteria. Citations irrelevant to the systematic review objectives and PICOS criteria were excluded. Excluded citations included those relevant to detection and detection methods, prevalence, detection or occurrence studies, descriptions of outbreaks, molecular epidemiology, diagnostics, and development of vaccines, drug development, clinical studies, immunology and fundamental studies of virus biology.

Information sources

Information sources included Medline, the USDA-NIFA Food Virology Collaborative (NoroCORE) database (a new comprehensive database for food virology), Agricola, Agris International, Foodline, CABI, , and the Cochrane library as shown in Table 1.

The information sources were limited to peer-reviewed publications with English abstracts published up to 31st December 2012. Additional relevant citations identified up to 1st September 2013 were also included, as collected over the course of the review process. They were identified using the same inclusion and exclusion criteria as part of the systematic review for publications up to 31st Dec 2012.

Search strategy

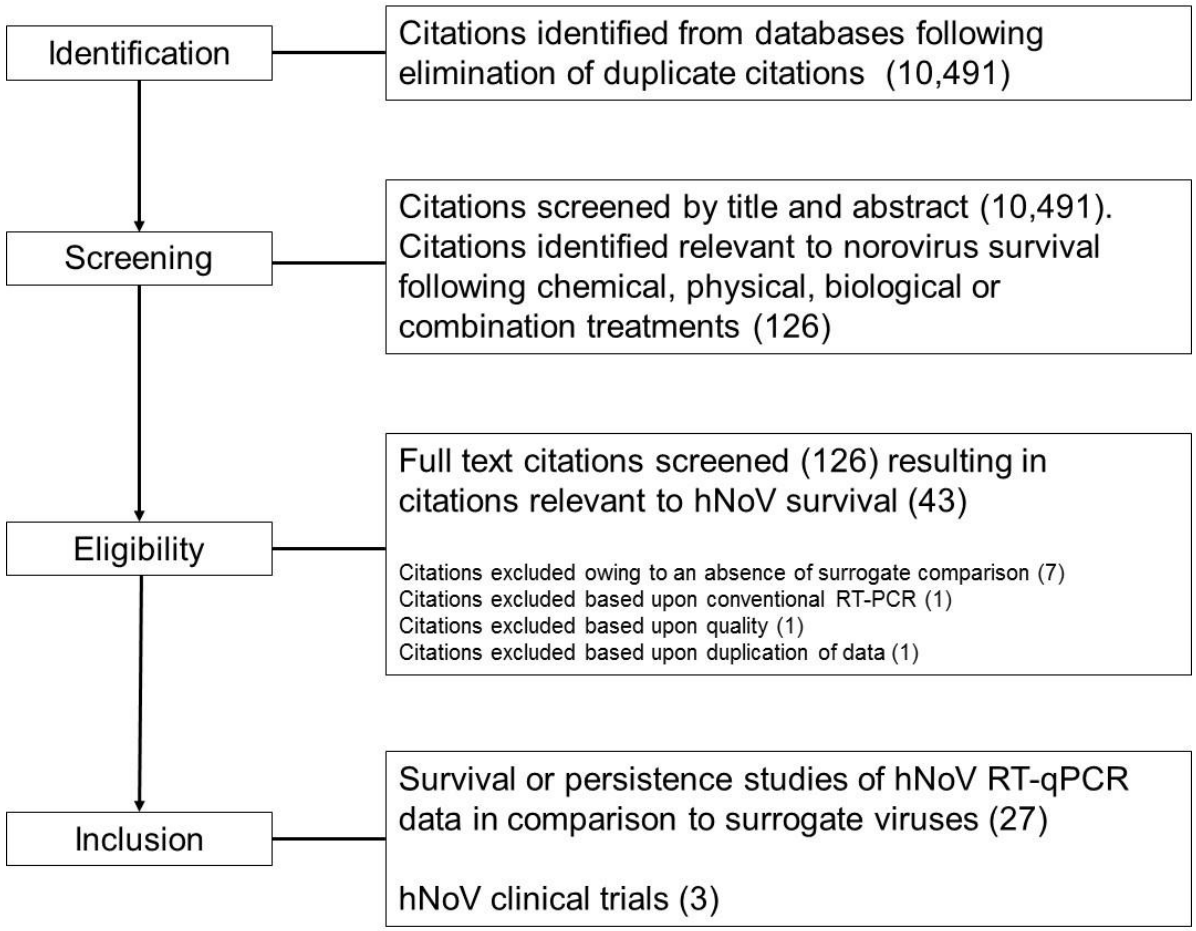
Database search strategies were based upon screening titles and abstracts. This strategy was first developed and tested on Medline using Boolean operators and wildcard * notations. Owing to the limitations of different search engines for different databases it was not possible to use a single universal search strategy. An overview of the information sources, search strings and results of searching is shown in Table 1, together with a summary figure (Figure 1). Citations resulting from the search strings were downloaded into Endnote (Version X5; Thomson Reuters; Carlsbad, CA, USA) with removal of duplicates, resulting in a database containing 10,047 citations.

Search strings were: S1: norovirus* AND (infectivity OR infectious OR inactivation OR survival OR disinfect* OR decontaminat* OR persist* OR virucid* OR gastroenteritis OR detection OR prevalen* OR occurrence). S2: (norovirus* OR poliovirus* OR coxsackie b OR calicivirus* OR norwalk OR sapovirus* OR small round structured virus* OR SRSV) AND (infectivity OR infectious OR inactivation OR survival OR disinfect* OR decontaminat* OR persist* OR virucid* OR gastroenteritis). S3: hepatitis A[Title] OR hepatitis E[Title] OR enteric virus*[Title] OR sapporo virus*[Title]) AND (infectivity OR infectious OR inactivation OR survival OR disinfect* OR decontaminat* OR persist* OR virucid* OR gastroenteritis). S4: norovirus OR poliovirus OR "coxsackie b" OR calicivirus OR norwalk OR sapovirus OR "small round structured viruses" OR srsv. S5: ("hepatitis A" OR "hepatitis E" OR "enteric virus" OR "sapporo virus") NOT ("vaccin*"). S6: Norovirus, Norwalk, small round structured virus, srsv. S7: S1 (without gastroenteritis term).

Table 1. Summary of results obtained from the search strategy of the systematic review process

Database	Search String	No Citations	Duplicates removed	Source	Date
Medline	S1, S2, S3	11143	2137	http://www.ncbi.nlm.nih.gov/pubmed/	13/12/2012
Norocore	N/A	2436	2435	http://norocorelit.com/	31/12/2012
Cochrane	S4,S5	651	4	http://www.thecochranelibrary.com/	13/12/2012
Agricola	S6	318	0	http://agricola.nal.usda.gov/	13/12/2012
Agris	S6	131	3	http://agris.fao.org/	13/12/2012
CABI	S7	578	384	http://www.cabdirect.org/	20/05/2013
Foodline	S1, S2	520	323	http://www.foodlineweb.com/foodline/scier	14/06/2013
Total		15777	5286		
Unique citations		**10,491**			
Selection 1					
hNoV & relevant surrogate studies	**Categories**		**Outcome**		
	Biological	12	INCLUDED		
	Physical	27	INCLUDED		
	Chemical	45	INCLUDED		
	Combination	42	INCLUDED		
	Total	**126**	INCLUDED		
	Irrelevant	**10,365**	EXCLUDED		
Selection 2					
hNoV studies (from 126 included)	**Categories**	43/126			
	hNoV RTqPCR + Surrogate infectivity & RT-qPCR	**27**	**FINAL INCLUSION**		
	hNoV clinical trials	**3**	**FINAL INCLUSION**		
	hNoV RT-qPCR data only	7	EXCLUDED		
	Conventional RT-PCR	4	EXCLUDED		
	Duplication	1	EXCLUDED		
	Quality	1	EXCLUDED		
	Total included	**30**			
	Total excluded	**10,461**			
	Total citations screened	**10,491**			

Figure 1. Process flow diagram for the systematic review



Study selection

Table 1 details the search strategy. Screening of the citation database by title and abstract showed many irrelevant citations and citations limited only to the survival or persistence of a wide range of surrogate viruses in foods and the environment (without inclusion of hNoV data). These were largely unique observational studies that have been reported and reviewed elsewhere (see above). The relevance of these studies to the survival of hNoV is unknown. The citation database was further refined by restricting to only those studies reporting data for noroviruses (either murine or human) and meeting the PICOS eligibility criteria (Selection 1, Table 1). Citations were screened for eligibility and categorised according to the treatment type (biological, physical, chemical, combination or irrelevant). This screening resulted in 126 selected citations (Selection 1) that were subjected to full text analysis and used for data collection.

Data collection process

Relevant data were extracted from the 126 selected citations, marked, dated and highlighted within citations. Data were extracted and summarised within an Excel spreadsheet and pivot table under the following headings relevant to the PICOS criteria: citation, assay type, treatment type, dose, pH, temperature, time, log reduction by infectivity assay, log reduction by RT-qPCR assay, and comments. Full text screening and data collection showed that this represented a large group of heterogeneous studies with limited relevance to real food systems that was not suitable for meta-analysis. Additionally many of these studies were based solely upon surrogate viruses of unknown relevance to the persistence of hNoVs. In order to address this problem, the database was further restricted to those citations meeting refined inclusion criteria to include only those studies related to hNoVs. This resulted in 43 citations that were subjected to further eligibility screening (Selection 2, Table 1).

The only markers for the natural resistance and persistence of hNoVs are data from the RT-qPCR or clinical studies. However the significance of RT-qPCR signals and any exact relationship with hNoV infectivity is unknown. In order to provide a comparison with infectivity data, final inclusion was based upon those citations that measured both reductions by RT-qPCR and infectivity for a surrogate virus *in direct comparison* to the reduction or persistence of hNoV RT-qPCR signals and clinical trial data. Final inclusion from the 43 selected citations resulted in a total of 30 citations with 27 providing a direct comparison between surrogate persistence data and hNoV RT-qPCR data and 3 clinical trials involving human volunteers (Table 2). The 3 clinical trials included the survival and persistence of hNoV after chlorine treatment of drinking water (Keswick *et al.,* 1985); high pressure processing of artificially contaminated oysters (Leon *et al.,* 2011); and virus persistence in purified groundwater (Seitz *et al.,* 2011).

Final exclusion from the 43 selected citations (Table 3) resulted in 7 excluded citations owing to a lack of comparative surrogate infectivity data; 4 citations were excluded since the studies utilised semi-quantitative conventional RT-PCR; and 2 additional studies were rejected, one owing to limited hNoV data, (Kitajima *et al.,*

2010) and another a review article where some of the data sources were duplicated (Bertrand *et al.,* 2012). The citations resulting from the final screening of the 43 selected hNoV studies (Selection 2 Table 1) are shown in Table 2 (eligible studies) and Table 3 (excluded studies).

Table 2. Results of eligibility screening showing eligible observational studies and clinical trials.

Eligible Observational Studies		
Citation	**Description**	**Matrix/Surface**
Shin & Sobsey, (2003)	Ozone disinfection of water	Water
Duizer et al., (2004)	Temperature, UV, and chlorine treatments	Tissue culture medium
Hewitt & Greening (2004)	Marinated mussel processing - acid marinade	Mussels
Hewitt & Greening (2006)	Marinated mussel processing -heat treatment. Comparison with HAV	Mussels
Park et al., (2007)	Survival on stainless steel or ceramic following fog and solution based hypochlorous acid treatment	Stainless steel
Poschetto et al., (2007)	Survival of hNoV and FCV-F9 following chemical disinfection with high organic load	Water
Butot et al., (2008)	Effect of freezing and frozen storage on survival of hNoVs and FCV F-9 on berries	Berries
Bae & Schwab (2008)	Persistence in surface and groundwater	Water
Shin & Sobsey 2008	Survival following chlorine disinfection	Buffer (PBS)
Butot et al., (2009)	Effect of freeze drying and dry heat on survival of hNoVs and FCV F-9 on berries	Berries
Topping et al., (2009)	Temperature stability	Buffer (PBS)
Wolf et al., (2009)	Long range RT-qPCR and UV	Buffer (PBS)
Nowak et al., (2011a)	Chlorine disinfection	Water
Li et al., (2011)	Temperature and cell binding	Buffer (PBS)
Park & Sobsey 2011	Survival following chlorine disinfection of faecally soiled stainless steel surfaces	Stainless steel
Sanchez et al, 2011	High pressure processing	Buffer (PBS) & protein
Dancho et al., (2012)	Temperature and high pressure differentiation of infectious and non-infectious hNoV by gastric mucin binding	Buffer (PBS)
Escudero et al., (2012)	Persistence and transfer between surfaces and foods (lettuce and turkey slices)	Stainless steel, ceramic, formica, lettuce and turkey
Girard et al., (2012)	Attachment to stainless steel and disinfectant inactivation.	Stainless steel
Li et al., (2012)	Temperature stability	Buffer (PBS)
Liu et al., (2012)	Comparison of persistence on stainless steel surfaces with MS2 and E.coli	Stainless steel and PBS
Sharps et al., 2012	Transfer of hNoV to stainless steel and small fruits	Stainless steel & fruit
Tuladhar et al., (2012a)	Temperature stability	Buffer (PBS)
Tuladhar et al., (2012b)	Cleaning study of contaminated stainless steel surfaces.	Stainless steel
Tuladhar et al., (2012c)	Hydrogen peroxide vapour	Room surfaces
Verhaelen et al., (2012)	Persistence on soft berries	Berries
Tung et al., (2013)	Disinfectants	Disinfectant
Eligible clinical trials		
Keswick et al., (1985)	Chlorine disinfection	Water, veal heart infusion broth + 2% Sodium bicarbonate
Leon et al., (2011)	High pressure processing of hNoV inoculated oysters	Oysters
Seitz et al., (2011)	Human trial of hNoV persistence in water with RT-qPCR data	Water

Table 3. Results of eligibility screening showing excluded studies with reasons.

Excluded Studies		
Citation	**Description**	**Reason**
Lamboujheb et al (2008)	Survival on turkey and lettuce with RNase and Proteinase K pretreatment	Single study based on NASBA
Nowak et al., (2011b)	Disinfectants and sanitisers in solution	hNoV RT-qPCR data only
Liu et al., (2010)	Liquid soap and hand sanitisers on contaminated hands	hNoV RT-qPCR data only
Liu et al., (2011)	Alcohol based hand rubs	hNoV RT-qPCR data only
Mormann et al., (2010)	Reduction in RT-qPCR signals following food processing conditions	hNoV RT-qPCR data only
Ngazoa et al ., (2008)	Attachment to stainless steel and household disinfection	hNoV RT-qPCR data only
Richards et al., (2012)	Freezing and thawing & capsid integrity	hNoV RT-qPCR data only
D'Souza et al., (2006)	Persistence on stainless steel, ceramic and formica and transfer to lettuce	Qualitative RT-PCR
Hudson et al., (2007)	Ozone fog disinfection of surfaces	Qualitative RT-PCR
Park et al., (2007)	Survival on stainless steel or ceramic following fogand solution based hypochlorous acid treatment	Qualitative RT-PCR
Barker et al., (2004)	Cleaning and decontamination study	Qualitative RT-PCR
Bertrand et al., (2012)	Review paper	Avoidance of duplication
Kitajima et al, (2010)	Chlorine inactivation in water. hNoV data was limited by the scale of the study.	Quality

Comparison of RT-qPCR signals obtained for hNoVs with surrogate infectivity and RT-qPCR assays for different treatments

Full text screening of the 30 eligible citations (27 observational studies and 3 clinical trials) showed a heterogeneous range of studies and insufficient common data to compare persistence in foods and on food contact surfaces.

However, data extracted from a number of studies and different treatments, e.g. temperature, sodium hypochlorite, and UV (Duizer *et al.,* 2004; Girard *et al.,* 2010); Wolf *et al.,* 2009) suggested that RT-qPCR signals for hNoVs were more persistent following treatments compared with surrogate viruses. Although hNoV persistence in foods and on food contact surfaces could not be examined directly, sufficient data were available to compare the effect of certain treatments (i.e., heat and chlorine) between different studies, and examine the comparative persistence and survival of surrogates and hNoVs. For these studies data were extracted specifically for the reductions in RT-qPCR signals for hNoVs and surrogates that corresponded to the minimal treatment required to produce the maximum reduction in surrogate infectivity. Where necessary, data were extracted from graphs. To reduce potential bias in any meta-analysis across the selected studies, data for key variables were also extracted. In this case, those variables included treatment conditions, RT-qPCR target amplicon size, any enzymatic pre-treatments, and matrix composition, all of which may influence the relative stability of the RT-qPCR target, the final RT-qPCR results, and ultimately the evaluation of the effectiveness of the treatment. Additionally, for each data set a qualitative comparison was made of the quantitative RT-qPCR data to determine if the hNoV RT-qPCR signal was more or less persistent than that of the surrogate.

The effect of similar heat treatments on hNoV RT-qPCR signals compared with surrogate viruses and surrogate infectivity data.

Table 4 shows extracted data for five studies for similar heat treatments ranging from 70 - 76°C for 2-3 min. Data are shown for different hNoV genotypes and in comparison to surrogate virus data for MNV-1 and FCV F-9. The RT-qPCR assay

data also includes the use of RNase pre-treatment in some studies. RNase treatment following heat inactivation conditions is intended to abolish RNA signals resulting from the release of RNA from heat inactivated viruses. However, following heat treatments that inactivate viruses, RT-qPCR signals can persist as RNase resistant ribonucleoprotein complexes (RNPs), and hence reductions in RT-qPCR signals are not directly equivalent to reductions in infectivity (Nuanualsuwan and Cliver, 2002; Nuanualsuwan and Cliver, 2003; reviewed by Knight *et al.,* 2012).

On average for MNV-1, small reductions in RT-qPCR signals ranging between 0.4 $\pm$ 0.1 $\log_{10}$ and 0.8 $\pm$ 0.29 $\log_{10}$ were associated with significant reductions in infectivity (ranging from >3.5 to >6 $\log_{10}$, depending on the study). These reductions in RT-qPCR signal although small were significant at the 95% confidence interval. In contrast, mean reductions in RT-qPCR signals for GI and GII hNoVs were not significant at the 95% confidence interval compared with control signals (0.13 $\log_{10}$ $\pm$ 0.10) for two out of three studies that also included MNV data. The only exception was for a GII.4 hNoV strain for which there was a very small but significant reduction in RT-qPCR signal as a function of heat treatment (0.16 $\pm$ 0.04) which appeared greater following RNase pre-treatment (0.44 $\pm$ 0.04 $\log_{10}$ reduction).

Reductions in RT-qPCR signals for heat-treated MNV-1 were not as great as those observed for FCV F-9 or hNoV that had been pre-treated with RNase prior to RT-qPCR. For example, a maximum 98.5% reduction (equivalent to a 1.99 $\log_{10}$ reduction) in RT-qPCR signal (with RNase pre-treatment) for FCV F-9 corresponded to complete loss of detectable infectivity (> 4 $\log_{10}$). Similar reductions for hNoV GII.4 required increased heat treatment. No reductions in RT-qPCR signals for either FCV F-9 or GII.4 hNoV were observed in the absence of RNase pre-treatment (Topping *et al.,* 2009).

The only comparable published data for foods was the effect of steam blanching of herbs at 75°C for 150s using hNoVs and the surrogate FCV F-9 (Butot *et al.,* 2009). These data showed that for survival on herbs, small (1-2 $\log_{10}$) reductions in RT-qPCR signals for FCV F-9 corresponded to large (>3.5 $\log_{10}$) reductions in infectivity. Under equivalent conditions, a 1-2 $\log_{10}$ reduction occurred for GII.4 hNoV and a 0.5 to >3 $\log_{10}$ reduction for GI hNoV by RT-qPCR (without added RNase pre-treatment).

Qualitative data comparison showed that for nineteen out of twenty three comparisons, greater persistence of RT-qPCR signals was observed for hNoV compared with surrogate MNV-1 or FCV F-9 RT-qPCR signals (Table 3).

The effect of available chlorine treatments on hNoV RT-qPCR signals compared with surrogate viruses and surrogate infectivity data.

Table 5 summarizes extracted data for 10 studies based upon treatment with available chlorine. Quantitative analysis of the data shows a complex range of results owing to the large number of experimental variables and different study designs. Variables included the amplicon size; the oxidative demand of the virus and matrix resulting from both the virus preparation and experimental conditions (particularly inclusion of faeces); the concentration, duration and temperature of treatment; and the particular surface or solution. In general, surrogate RT-qPCR signals resulting in complete loss of infectivity were 1-2 $\log_{10}$ less than corresponding infectivity data. Qualitative data analysis obtained from 29 out of 35 comparisons showed a greater persistence of hNoV RT-qPCR signals compared with surrogate MNV-1 or FCV F-9 signals (Table 5).

Statistical analysis

Owing to the high level of heterogeneity within the methods used for analysis for heat and chlorine treatments, a meta-analysis of data from each study was not possible. However qualitative comparisons of data within and between studies allowed a statistical assessment of the relative persistence of hNoV RT-qPCR signals in comparison to surrogates RT-qPCR signals. This is shown in Tables 4 and 5 and summarized in Table 6 as a two-tailed binomial p value, based upon the likelihood that hNoVs are equally persistent when compared with surrogates.

Table 4. Characteristics of the studies comparing RT-qPCR reductions and infectivity data for the surrogates murine norovirus and feline calicivirus in comparison to RT-qPCR data for human noroviruses for similar heat treatments.

Citation	Heat (°C/s)	Matrix	RT-qPCR Pre-treatment	Murine Norovirus MNV-1					Human Norovirus					Comparative persistence by RT-qPCR
				RT-PCR (bp)	-Δ Log RT-PCR U	SD	2 X SD	-Δ Log Infectivity	RT-PCR (bp)	-Δ Log RT-PCR U	SD	2 X SD	Genotype	
Hewitt et al., (2009)	72°C 120s	Water	None	125	0.44	0.2	0.4	>3.5	84	0	0.1	0.2	GI	GI > MNV-1
	72°C 120s	Milk	None	125	0.67	0.04	0.08	>3.5	84	0.05	0.1	0.2	GI	GI > MNV-1
	72°C 120s	Water	None	125					97	0	0.1	0.2	GII	GII > MNV-1
	72°C 120s	Milk	None	125					97	0.05	0.1	0.2	GII	GII > MNV-1
Li et al., (2012)	70°C 120s	TCM (MNV-1) PBS (hNoV)	None	108	0.8	0.29	0.58	>6	88	0.16	0.04	0.08	GII.4	GII.4 >MNV-1
	70°C 120s		RNase	108	0.45	0.1	0.2	>6	88	0.44	0.04	0.08	GII.4	GII.4 >MNV-1
Tuladhar et al., (2012)a	73°C 180s	TCM	None	159	0.8	0.2	0.4	>5	206	0.1	0.1	0.2	GII.4	GII.4 >MNV-1
	73°C 180s	1% stool in PBS	None	159	0.4	0.1	0.2	>5	206	0	0	0	GII.4	GII.4 >MNV-1
TOTAL					3.56	0.93	1.86			0.8	0.58	1.16		
MEAN					0.59	0.16	0.31			0.13	0.10	0.19		GII.4 >MNV-1
				Feline Calicivirus FCV F-9										
Butot et al., (2009)	75°C 150s	Basil	None	111	1.63	0.21	0.42	3.98	85	0.48	0.08	0.16	GI	GI > FCV F-9
	75°C 150s	Chives	None	111	2.01	0.13	0.26	>4	85	>3	0	0	GI	GI > FCV F-9
	75°C 150s	Mint	None	111	1.26	0.15	0.3	>4	85	0.97	0.07	0.14	GI	GI > FCV F-9
	75°C 150s	Parsley	None	111	3.09	0.15	0.3	3.65	85	1.6	0.19	0.38	GI	GI > FCV F-9
	75°C 150s	Basil	None						88	1.46	0.08	0.16	GII	GII > FCV F-9
	75°C 150s	Chives	None						88	1.35	0.12	0.24	GII	GII > FCV F-9
	75°C 150s	Mint	None						88	1.58	0.12	0.24	GII	FCV F-9 > GII
	75°C 150s	Parsley	None						88	1.52	0.08	0.16	GII	GII > FCV F-9
Topping et al., (2009)	63 °C 120s	PBS	Rnase	97	1.99 (max)	>4								
	76 °C 120s	PBS	Rnase						89	1.99 (max)			GII.4 (249)	GII.4 > FCV F-9
	76 °C 120s	PBS	Rnase						89	1.99 (max)			GII.4 (432)	GII.4 > FCV F-9
	76 °C 120s	PBS	Rnase						89	1.99 (max)			GII.4 (533)	GII.4 > FCV F-9
	Control		No Rnase										GII.4 (249)	GII.4 = FCV F-9
	Control		No Rnase										GII.4 (432)	GII.4 = FCV F-9
	Control		No Rnase										GII.4 (533)	GII.4 = FCV F-9

Table 5. Characteristics of studies comparing RT-qPCR reductions and infectivity data for the surrogates murine norovirus, feline calicivirus and poliovirus in comparison to RT-qPCR data for human noroviruses for chlorine treatment.

Citation	Chlorine treatment	Matrix	Food or Food Surface	Surrogate RT-qPCR & Infectivity data				hNoV RT-qPCR data			Comparative persistence of RT-qPCR signals
				Surrogate	PCR (bp)	- Δ Log RT-PCR	- Δ Log infectivity	hNoV	PCR (bp)	- Δ Log RT-PCR	
Butot et al., 2008	200ppm 18°C 30s	Tap water + berry or herb	Blueberries	FCV F-9	111	4	>3.5				
	200ppm 18°C 30s	Tap water + berry or herb	Raspberries	FCV F-9	111	>3.0	>3.5				
	200ppm 18°C 30s	Tap water + berry or herb	Strawberries	FCV F-9	111	3.4	>3.5				
	200ppm 18°C 30s	Tap water + berry or herb	Basil	FCV F-9	111	2.5	>3.5				
	200ppm 18°C 30s	Tap water + berry or herb	Parsley	FCV F-9	111	2	>2.7				
	200ppm 18°C 30s	Tap water + berry or herb	Blueberries					GII	88	3	GII > FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Raspberries					GII	88	0.9	GII > FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Strawberries					GII	88	1.4	GII > FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Basil					GII	88	1.6	GII > FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Parsley					GII	88	1.8	GII > FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Blueberries					GI	85	3.4	GI > FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Raspberries					GI	85	0	GI > FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Strawberries					GI	85	>3.1	GI ≤ FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Basil					GI	85	>3.4	GI < FCV F-9
	200ppm 18°C 30s	Tap water + berry or herb	Parsley					GI	85	0.9	GI > FCV F-9
Duizer et al., (2004)	3000 ppm 10min RT	TCM + 0-2% FBS	Solution	FCV F-9	83	2.47	>5				
	6000 ppm 2 min RT	TCM + 0-2% FBS	Solution	FCV F-9	83	3.91	NS				
	6000 ppm 10 min RT	TCM + 0-2% FBS	Solution	FCV F-9	83	4.21	>5				
	3000 ppm 10min RT	TCM + 0-2% FBS + 5% stool	Solution					GII.4 (CHCl3)	209	0.84	GII.4 (CHCl3) > FCV F-9
	6000 ppm 2 min RT	TCM + 0-2% FBS + 5% stool	Solution					GII.4 (CHCl3)	209	2.2	GII.4 (CHCl3) > FCV F-9
	6000 ppm 10 min RT	TCM + 0-2% FBS + 5% stool	Solution					GII.4 (CHCl3)	209	>2.71	GII.4 (CHCl3) ≥ FCV F-9
Poschetto et al., (2007)	4500ppm 15min	HW No protein	Solution	FCV F-9	126	>3	>3				
	4500ppm 15min	HW + 40% FBS	Solution	FCV F-9	126	0	>3				
	5500ppm 15 min	HW + 25% stool	Solution	FCV F-9	126	0	>3				
	6000ppm 15 min	HW + 25% stool	Solution	FCV F-9	126	3	>3				
	7000ppm 15 min	HW + 25% stool	Solution	FCV F-9	126	3	>3				
	6000ppm 15 min	HW + 25% stool	Solution					GII	338	>4	FCV F-9 > GII
	7000ppm 15 min	HW + 25% stool	Solution					GII	338	>4	FCV F-9 > GII
Shin & Sobsey (2008)	1 ppm 5°C pH6 10min	Water	Solution	PV-1	197	1.5	>3.8				
(data from graph)	1 ppm 5°C pH6 10min	Water	Solution	PV-1	866	2	>3.8				
	1 ppm 5°C pH6 10min	Water + 0.1-0.2% stool	Solution					GI 8FIIa (CHCl3)	260	>3	PV1 ≥ GI 8FIIa (CHCl3)
	1 ppm 5°C pH6 10min	Water + 0.1-0.2% stool	Solution					GI 8FIIa (CHCl3)	1033	>3	PV1 ≥ GI 8FIIa (CHCl3)
	5 ppm 5°C pH6 1 min	Water	Solution	PV-1	197	1.6?	1.5				
	5 ppm 5°C pH6 1 min	Water	Solution	PV-1	866	0	1.5				
	5 ppm 5°C pH6 1 min	Water + 0.1-0.2% stool	Solution					GI 8FIIa (CHCl3)	260	>2	PV1 > GI 8FIIa (CHCl3)
	5 ppm 5°C pH6 1 min	Water + 0.1-0.2% stool	Solution					GI 8FIIa (CHCl3)	1033	>3	PV1 > GI 8FIIa (CHCl3)

Table 5 (cont'd). Characteristics of studies comparing RT-qPCR reductions and infectivity data for the surrogates murine norovirus and feline calicivirus in comparison to RT-qPCR data for human noroviruses for chlorine treatment.

Citation	Chlorine treatment	Matrix	Food or Food Surface	Surrogate RT-qPCR & Infectivity data				hNoV RT-qPCR data			Comparative persistence of RT-qPCR signals
				Surrogate	PCR (bp)	- Δ Log RT-PCR	- Δ Log infectivity	hNoV	PCR (bp)	- Δ Log RT-PCR	
Girard et al., (2010)	(+ PK and Rnase)										
(data from graph)	3% Sodium hypochlorite pH 12	PBS	Stainless steel	MNV-1	83	4.2	4.75				
	3% Sodium hypochlorite pH 12	10% stool	Stainless steel					GII	97	1.9	GII > MNV-1
Nowak et al., (2011)	(+ Rnase One)										
(data from graph)	48 ppm 30min RT (predicted)	0.03% w/v BSA in hard water	Solution	FCV F-9	89	>4	>4				
	66 ppm 30min RT (predicted)	0.03% w/v BSA in hard water	Solution	FCV F-9	89	>4	>4				
	250 ppm 30min RT	0.03% w/v BSA in HW + 1% stool (249)	Solution	FCV F-9	89	>4	ND				
	250 ppm 30min RT	0.03% w/v BSA in HW + 1% stool (432)	Solution	FCV F-9	89	2.2	ND				
	250 ppm 30min RT	0.03% w/v BSA in HW + 1% stool (533)	Solution	FCV F-9	89	3.5	ND				
	250 ppm 30min RT	0.03% w/v BSA in HW + 1% stool (249)	Solution					GII.4 (249)	97	1.6	GII.4 > FCV F-9
	250 ppm 30min RT	0.03% w/v BSA in HW + 1% stool (432)	Solution					GII.4 (432)	97	2.1	GII.4 > FCV F-9
	250 ppm 30min RT	0.03% w/v BSA in HW + 1% stool (533)	Solution					GII.4 (533)	97	1.4	GII.4 > FCV F-9
Park & Sobsey (2011)	500ppm 5min	10% stool	Stainless steel	MNV-1	230	0.05	0.4				
(data from graph)	500ppm 5min	10% stool	Stainless steel	FCV F-9	229	0.3	1.25				
	500ppm 5min	10% stool	Stainless steel					GII.4	211	0	GII.4 > MNV-1 > FCV F-9
	5000ppm 4min	10% stool	Stainless steel	MNV-1	230	1.25	3.75				
	5000ppm 4min	10% stool	Stainless steel	FCV F-9	229	1.25	>4.5				
	5000ppm 4min	10% stool	Stainless steel					GII.4	211	1.25	GII.4 = MNV-1 = FCV F-9
Tung et al., (2013)	250 ppm 30s	TCM + 1% FBS	Solution	MNV-1	121	3	3.9				
	250 ppm 30s	TCM + 1% FBS	Solution	FCV F-9	92	2.2	2.5				GII.2 > FCV F-9 > MNV-1
	250 ppm 30s	1% stool	Solution	MNV-1	121	0	0				GII.4 > FCV F-9 > MNV-1
	250 ppm 30s	1% stool	Solution	FCV F-9	92	1	1				
	250 ppm 30s	1% stool	Solution					GII.2	97	0	GII.2 = MNV-1 > FCV F-9
	250 ppm 30s	1% stool	Solution					GII.4	97	0	GII.4 = MNV-1 > FCV F-9

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Table 6. Statistical analysis and p values for the qualitative persistence of hNoV RT-qPCR signals in comparison to those for surrogate MNV-1 and FCV F-9 viruses following heat or available chlorine treatment.

Treatment	Studies	Comparisons	hNoV > MNV -1	p value
Heat	3	9		0.01
Chlorine	3	7		0.02
			hNoV > FCV F-9	
Heat	2	14		0.18
Chlorine	6	24		0.00
			1 5 10 15 20	
			No. of RT-qPCR comparisons showing greater hNoV persistence	

DISCUSSION

The project was designed to answer two questions Firstly "*What are the natural human norovirus (hNoV) persistence characteristics in food and the environment?'* and secondly *"How can these properties be altered by applying physical and/or chemical treatments to foods or food contact surfaces?"* The approach of this study was to conduct a systematic review designed to answer the defined PICOS questions. Of the 30 eligible citations identified, all were unique observational studies employing different methods and approaches. The survival and persistence of hNoVs on foods and food contact surfaces is difficult to predict owing largely to the lack of a suitable culture system and the uncertain relevance of surrogate infectivity and their corresponding RT-qPCR data. In order to better understand hNoV survival and persistence in the food chain, we directly compared RT-qPCR and infectivity data from surrogates to hNoV RT-qPCR data for key treatments (excluding studies that reported on surrogates alone or hNoV RT-qPCR data alone). Data were extracted from a wide range of independent studies but there were limited data for foods and food contact surfaces. Only heat treatment and exposure to available chlorine provided a sufficient number of studies to warrant comparative analysis. For surrogate viruses, reductions in infectivity, following heat or chlorine treatment, were nearly always accompanied to some extent by reductions in RT-qPCR signal. The corresponding reductions in RT-qPCR signals for hNoVs under comparable conditions were nearly always less than that observed for the surrogates (Tables 3 and 4).

For heat treatment, small and sometimes barely significant reductions in RT-qPCR signals were indicative of complete loss of infectivity for surrogates and by implication for hNoVs (Table 3). A wide range of reductions in RT-qPCR signals was observed following similar heat treatments, probably resulting from differences in procedures and in particular the presence or absence of endogenous, or added, RNase activity. Heat treatment is generally considered to act by degrading the virus capsid and not the viral RNA. The maximum observed reductions in RT-qPCR signals following heat treatment can reach a plateau, and varies in different studies, complicating direct comparison between studies. For surrogate viruses this has been reported as < 0.5 $\log_{10}$ for MNV-1 (Li *et al.,* 2012, Diez-Valcarce *et al.,* 2011) to 2 $\log_{10}$ for FCV F-9 (with RNase pre-treatment, Topping *et al.,* 2009) and > 4 $\log_{10}$ for the bacteriophage MS2 (with proteinase K and RNase pre-treatment) (Pecson *et al.,* 2009) for temperatures up to 85°C. These differences are probably due to inherent differences in the heat resistance of these viruses. However, they likely also reflect experimental differences as discussed above, including the proportion of RNase resistant RNP compared with infectious virus present in the original samples. Similar issues may also result in variability in survival and persistence of hNoV exposed to heat. Overall, statistical analysis of the qualitative data showed that RT-qPCR signals are more persistent for hNoV than are surrogate RT-qPCR signals following heat treatment (Table 5). Unfortunately the heterogeneity of these studies does not allow consideration of the impact of endogenous or added RNase activity.

A closer relationship between RT-qPCR signals and infectivity was observed for chlorine inactivation (Table 5), presumably owing to the ability of available chlorine to oxidize and degrade RNA (O'Brien and Newman, 1979) rendering it insensitive to amplification by RT-qPCR. Reductions in RT-qPCR signals were approximately one $\log_{10}$ less than that observed for infectivity for surrogates, and comparable RT-qPCR reductions for hNoVs were again generally less than those for surrogates. Treatments with alternative oxidizing agents such as commercially available hypochlorous acid and ozone are also effective (>3 $\log_{10}$ reduction) at reducing hNoV RNA RT-qPCR signals and reducing both infectivity and RT-qPCR signals for surrogate viruses (Park *et al.,* 2007, Hudson *et al.,* 2007, Shin and Sobsey, 2003), although attention to cleaning to remove organic load is essential for an effective disinfection treatment (Barker *et al.,* 2004)

Statistical analysis of the available qualitative comparisons (Table 6) for heat and chlorine strongly suggest that hNoV RT-qPCR signals are more persistent than those from the surrogates MNV-1 or FCV F-9. This apparent persistence might be a function of RT-amplicon size and PCR efficiency, since larger RNA fragments are thought to be more susceptible to degradation than smaller fragments, and (as a generalization) reverse transcription and amplification efficiency is inversely proportional to product size. Therefore, the apparent persistence of hNoV RT-qPCR signals might be associated with reduced amplicon size or loss of a particular part of the genome. However, in most of these studies the size of the hNoV amplicons was similar to, or smaller than the size of the surrogate virus amplicons. Also, a number of different RT-qPCR amplification regions were targeted. Surrogate RT-qPCR signals were more resistant to heat treatment and available chlorine when suspended in stool samples, suggesting that the faecal matrix contributes towards the persistence of hNoV RT-qPCR signals (Tung *et al.,* 2013, Nowak *et al.,* 2011a, Topping *et al.,* 2009). Interestingly, the only two studies that did not consistently show greater persistence of hNoV RT-qPCR signals either had a large difference in amplicon size (Poschetto *et al.,* 2007) when comparing hNoV and the surrogate; or used a different surrogate (poliovirus) compared to chloroform extracted hNoV (Shin and Sobsey, 2008).

The reason for the comparatively higher persistence and resistance of hNoVs is unclear but may be related to the fact that, unlike in surrogate studies, hNoVs are commonly analysed within their natural faecal matrix. It is well known that the survival of surrogate viruses following available chlorine treatment is dependent on a number of factors, e.g. dispersion and organic load (including faecal matter). This may explain why RT-qPCR signals from chloroform extracted hNoV appeared less persistent when challenged with available chlorine as compared to the surrogate poliovirus (Shin and Sobsey, 2008). Additionally Vertrel (a proprietary organic hydrofluorocarbon solvent) extracted and filter purified hNoV GI.I 8FIIb possessed comparable stability at 25°C to MNV as measured by RT-qPCR (Bae and Schwab, 2008).

Data for clinical trials investigating the persistence of hNoV was obtained for chlorination, high pressure processing (of oysters), and virus persistence in ground water (Keswick *et al.,* 1985, Seitz *et al.,* 2011, Leon *et al.,* 2011). These were based upon results from 32, 13 and 44 subjects, respectively. Both infectivity and clinical trial data has suggested that the hNoV GI.I 8FIIa strain was more resistant to available chlorine disinfection conditions than surrogate viruses including rotavirus and poliovirus (Keswick *et al.,*1985). However as noted by Shin and Sobsey (2008), these data were limited by a number of factors including oxidative demand, aggregation, and dose, and perhaps in retrospect by more recent findings regarding secretor status and genetic susceptibility to hNoV infection (Lindesmith *et al.,* 2003).

Comparative data between MNV-1 and hNoV from High Pressure Processing (HPP) studies (Sanchez *et al.,* 2011) has shown that the RT-qPCR signals of MNV are less persistent than hNoV following HPP treatment. Human challenge data of high pressure-treated contaminated oysters has shown that treatment at 600MPa at 25°C (the highest condition of pressure treatment studied) was the only one that completely prevented infection of volunteers using a low dose (1×10^4 genome copies/ml) of GI.I 8FIIb hNoV (a human passage of GI.I 8FIIa (Teunis *et al.,* 2008). This high degree of pressure resistance of hNoVs is consistent with laboratory-based work using hNoV virus-like particles (Lou *et al.,* 2012).

A high degree of persistence for hNoVs is further supported by clinical data showing that the GI.I 8FIIb strain retained infectivity in human subjects for at least 61 days when stored in purified groundwater, with corresponding RT-qPCR signals persisting for over three years (Seitz *et al.,* 2011).

CONCLUSIONS

This review systematically set out to answer the questions “What are the natural hNoV persistence and resistance characteristics in food and the environment?, and how can these properties be altered by applying physical and/or chemical treatments to foods or food contact surfaces?” Since the only commonly available marker of hNoV survival is measurement of RT-qPCR signals, these questions could not be answered in isolation without a corresponding assessment of the significance of RT-qPCR signals and infectivity of surrogate viruses in comparable studies.

Systematic review of the published data showed that when inactivation treatments resulted in reduction in surrogate virus infectivity, this was nearly always accompanied to some degree by corresponding reduction in surrogate RT-qPCR signals, and to a lesser extent by reductions of the magnitude of hNoV RT-qPCR signals. This together with the results of clinical trials, and other supporting data, provides a large body of evidence showing that hNoVs are both more persistent and more resistant than surrogate viruses. Therefore the evidence suggests that infectious hNoVs are more likely to persist in foods and on food contact surfaces than currently predicted by most surrogate studies. The relative contribution of the faecal matrix and the structural characteristics of the capsid to the high degree of hNoV persistence remain unknown. Virus particles within the faecal matrix may be aggregated and components of the faecal matrix may all contribute to protection from inactivation by heat or chlorine (Sobsey and Meschke, 2003).

Although reductions in infectivity following different treatments were frequently accompanied by reductions in RT-qPCR signal, the use of RT-qPCR signals to predict the effectiveness of control measures beyond that already observed in comparison to surrogates is unclear and limitations remain. A number of variables including the test virus, treatment type and treatment conditions, PCR amplicon size, amplification efficiency, the presence of RNPs, endogenous RNase activity, and enzymatic pre-treatments all potentially affect the reliability of the survival endpoint determined by RT-qPCR.

RT-qPCR results can be subject to significant variation between and within experiments. Relative comparisons within experiments can be more accurate provided adequate controls for PCR inhibition and/or RNA degradation are present

(Pfaffl, 2004; Bustin *et al.,*2009). In order to ensure reproducibility, great care should be taken to avoid the action of endogenous RNase in control samples, and maximize the degradation of any exposed RNA in test samples when pre-treatments are applied. It is also clear that RT-qPCR signals may persist in the absence of infectivity due to RNase resistant RNP (Nuanualsuwan and Cliver, 2002*;* Nuanualsuwan and Cliver, 2003*;* Knight *et al.,* 2012) and this provides both a challenge and opportunity for evaluation of the relevance of RT-qPCR signals.

Most studies reviewed here and reported thus far are observational in nature, with no coordination, harmonization, or replication between laboratories, preventing analytical determination of the robustness of the methods by inter-laboratory comparison.

Recently (Hoelzer *et al.,* 2013) completed a systematic review and meta-analysis of the inactivation of hNoV surrogates on hard surfaces and in suspension by chemical disinfectants. The studies concluded that the hNoV surrogates hepatitis A virus, MNV-1 and the bacteriophage MS2 are more resistant to disinfection than FCV F-9. However, the strict quantitative inclusion criteria excluded the analysis or assessment of the value of surrogate or hNoV RT-qPCR data, and actually concluded that meta-analysis of these data was not possible resulting in the inclusion of data from only 6 studies. The approach described here although not quantitative can provide a qualitative approach to meta-analysis without discarding valuable data.

Despite the difficulties of applying the PRISMA approach to heterogeneous observational studies, this work provides evidence that, although reductions in RT-qPCR data may underestimate infectivity, hNoV RT-qPCR signals are nearly always more persistent than are RT-qPCR signals for the surrogates MNV-1 or FCV-F9 under similar treatment conditions. This suggests that control measures based on surrogate virus infectivity data alone are likely to be less effective in controlling hNoV contamination. Epidemiological data for hNoVs repeatedly show a high degree of virus persistence in foods and on environmental surfaces, with hNoV outbreaks attributable to a number of sources most notably shellfish and berries, and in hospitals and on cruise ships. It is already known from surrogate studies that viruses can survive quite well in chilled, acidified, and frozen foods; in foods packed under

modified atmosphere; and in dried foods (Baert *et al.,* 2009). Surrogate studies have also shown that the food matrix and environmental factors impact significantly on virus survival (Le Guyader and Atmar, 2008; Sobsey and Meschke, 2003). Additionally, hNoV RT-qPCR signals appear more persistent compared with surrogate MNV RT-qPCR signals when present on the surface of raspberries and strawberries kept at various storage temperatures (Verhaelen *et al.,* 2012). There is also little apparent impact of freezing and thawing on the persistence of hNoV RT-qPCR signals (Richards *et al.,* 2012) when spiked on the surface of berries and herbs (Butot *et al.,* 2008).

There are very little data on hNoV persistence or survival in real-food systems under many commonly used food processing conditions, including heat. Further, currently available data for hNoV survival and persistence in foods lacks direct and comparable surrogate data (Mormann *et al.,* 2010, Lamhoujeb *et al.,* 2008). In the absence of data and relevant studies, it is difficult to make conclusions or recommendations about the survival of hNoV in foods and on food contact surfaces. However where comparisons could be made in answering the PRISMA questions a qualitative meta-analysis shows that hNoVs appear more persistent than surrogate feline calicivirus or murine norovirus in response to heat and available chlorine than recognised previously. Unfortunately a lack of data meant that quantitative meta-analysis could not be used to define conditions that might more accurately predict the inactivation of hNoVs – a problem also recognised by Hoelzer *et al.,* 2013

There remain many unanswered questions, including the best means by which to use RT-qPCR to measure virus infectivity (e.g. enzymatic pre-treatments, ligand-based capsid capture); the impact of the sample matrix on virus inactivation; strain differences; and others. Further studies are necessary to determine the nature of hNoV persistence and to define optimal methods to evaluate hNoV inactivation in the absence of cultivable strains. Finally the database of extracted data and pivot table created during the course of this project should provide a valuable searchable data source for Norovirus research.

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Appendix 1 : Reviewers and Qualifications

Leatherhead Food Research

Dr Angus Knight PhD

Research & Development Manager - Principal Investigator (PI)

Angus obtained his PhD in microbial molecular genetics from Cranfield University in 1986. Following post-doctoral research in at Surrey University on bacterial meningitis and at the National Institute for Medical Research on HIV he joined LFR in February 1994. By background Angus is a molecular biologist with extensive experience in the application of molecular techniques to industrial and academic R&D. Angus has held different positions within LFR including responsibility for different departments including food safety and food ingredients and is currently responsible for the development and management of major external contract research projects. This includes the development of confidential contract R&D, collaborative R&D, project management and consultancy across a broad spectrum of activities and for a variety of clients including, industry, venture capital, academia, DEFRA, DTI (now TSB), EU and the FSA.

R&D at LFR has included the development of the first molecular tests for genetically modified food ingredients in the UK, the application of ICT in food manufacturing, and research on quantitative molecular approaches for fruit juice authentication. Recent work has focused on interim scientific management for a biotechnology company and the development of collaborative DEFRA LINK and NHS funded R&D for controlling Noroviruses within food manufacturing and the environment. This has resulted in a number of peer-reviewed publications. Angus is a recognized EFSA expert and has acted as a scientific referee for journals and grant applications, participated in working groups for food authentication and has also acted as an expert witness in the high court. Angus is a member of British Standards Institute CH216 for the developments of standards for disinfectant testing.

Data extracted from included citations were received or checked by AK and Mr John Haines.

John Haines Bsc

Senior scientist. Over 15 years experience in the development and validation of molecular assays. Participated in confidential virology projects. and a key researcher in a past DEFRA LINK norovirus project.

Dr Agnieszka Olejnik-Schmidt PhD

Assistant Professor. Department of Biotechnology and Food Microbiology, Faculty of Food and Nutrition Sciences, Poznań University of Life Sciences. (Visiting fellowship at Leatherhead Food Research)

University of Ghent

Prof. dr. ir. Mieke Uyttendaele PhD

Project partner & PI

Prof. dr. ir. Uyttendaele is research professor in the Laboratory of Food Microbiology and Food Preservation (LFMFP) Dept Food Safety & Food Quality, Ghent University (UGent) and has 20 years of experience in the area of Microbial Analysis of Foods, Food Safety and Risk Assessment. Being graduated and presently still situated at the Faculty of Bio-Science Engineering (Ghent University) Mieke Uyttendaele has a background and widespread experience in food microbiology in general as well as product technology and food processing, mild preservation methods, decontamination of foods, packaging of food products etc. She has a track record of over 100 scientific publications and was/is promoter of more than 25 Ph.D students with research related to *Listeria monocytogenes, Bacillus cereus, Campylobacter*, VTEC, *Salmonella*, Norovirus etc and present projects related to ready-to-eat foods with prolonged shelf life, poultry and red meat and meat products, dairy products,

cooked chilled foods, fresh-cut produce, etc. She was a member of multiple EFSA Working groups, is active in the scientific committee of the Belgian Food Safety Agency and provides expertise to national government in the frame of Codex Alimentarius meetings. In particular related to food borne viruses she is/was the promoter of 4 Ph.D students on the topic dealing with „'Risk assessment of foodborne viruses in the fresh produce chain"; „Development and implementation of an integrated cell culture RT-PCR methodology as indicator for infectivity of foodborne viruses"; "Molecular detection of noroviruses in ready-to-eat foods and fruits"; "Molecular detection of, and strategies to reduce Norovirus load or infectivity in foods" and current promoter or of 2 projects on the topic funded by Belgian Ministry of Public Health (TRAVIFOOD) & the Belgian Ministry of Scientific Affairs (Belspo). She currently also coordinates the FP7 EU project Veg-i-Trade in which research on Norovirus methods, prevalence quality-assurance and elaboration of control measures & risk assessment in particular for (processed) berries and leafy vegetables are also part of WP 3 and WP 6 of the project(www.veg-i-trade.org)

Data extracted from included citations were received and checked from:

Dr. Ambroos Stals

Senior scientist

Dr. Ambroos Stals is a post-doctoral researcher in the Laboratory of Food Microbiology and Food Preservation (LFMFP), Department Food Safety & Food Quality, Ghent University (UGent). He obtained his PhD in norovirus research and has six years experience studying food borne viruses.

Dr Dan Li

Senior scientist

Dr Li Recently is a post-doctoral researcher in the Laboratory of Food Microbiology and Food Preservation (LFMFP), Department Food Safety & Food Quality, Ghent University (UGent). Dr Li obtained her PhD in Norovirus research in 2012

North Carolina State University

Professor Lee Ann Jaykus

Project role: Project partner & PI

Professor Jaykus is a William Neal Reynolds Distinguished Professor in the Food, Bioprocessing, and Nutrition Sciences Department at North Carolina State University (NCSU) in Raleigh, NC, USA. Prof. Jaykus has an internationally recognized track record of peer reviewed research in food borne viruses Prof. Jaykus' teams efforts have focused on investigating viral foodborne disease outbreaks; developing practical methods to detect viral contamination in complex sample matrices; evaluating virus persistence, resistance and transfer; and characterizing viral foodborne disease risks. She has served on a variety of national and international committees, including the National Advisory Committee on Microbiological Criteria for Foods (NACMCF); the Food and Agriculture Organization-World Health Organization (FAO-WHO) food borne virus expert consultation; several National Academy of Sciences (Institute of Medicine/National Research Council) food safety panels; and as past-president of the International Association for Food Protection (IAFP). Prof. Jaykus currently serves as the scientific director of the USDA-NIFA Food Virology Collaborative. Also called NoroCORE, the Collaborative is a 5 year, $25 million project intended to reduce the burden of foodborne disease associated with enteric viruses.

Data extracted from included citations were received and checked from:

Dr. Rebecca Goulter, Laboratory Manager

Dr. Blanca Escudero-Abarca, Senior Researcher

Dr. Jennifer Shields, post-doctoral research associate

Dr. Soo-Hwan Suh, post-doctoral research associate

Grace Tung, Jon Baugher, Matthew Moore, and Clyde Manuel - senior Ph.D. candidates

Dr Alastair D. Knight

Project partner & PI

Dr Alastair Knight is founder and Director of Evicom Ltd www.evicom.co.uk an outcome research company with specialist skills in evidence-based reviews, statistical analyses, epidemiology and pharmacoeconomics. Evicom provided technical expertise in search strategy development, systematic review and meta-analysis.

Appendix 2 : Background information regarding searches and data extraction

Literature searches

The original search strategies S1,S2 and S3 were devised, developed, tested, and performed by Dr Angus Knight and Dr Ambroos Stals in conjunction with Dr Alastair Knight. These search strategies were subsequently modified for different search engines as described in results. (Table 1).

Database extraction

Selection of citations from the database resulting from the search strategies was performed by Dr Angus Knight and Mr John Haines. Where uncertainty over inclusion was present this was resolved by reference to the original text. Selected citations were mailed to project partners for data extraction.

Data from the selected citations was extracted and checked by the researchers (above) and identified by initials and date within the data extraction database. Data from the final 30 citations selected for inclusion were additionally examined to extract specifically the reductions in RT-qPCR signals for hNoVs and surrogates that corresponded to the minimal treatment required to produce the maximum reduction in surrogate infectivity. Extracted data was highlighted within these papers by Dr Angus Knight and further checked by Dr Agnieszka Olejnik-Schmidt and Mr John Haines