NoVAS
Norovirus Attribution Study

Assessing the contribution made by the food chain to the burden of UK-acquired norovirus infection

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FS101040: Assessing the contribution made by the food chain to the burden of UK-acquired norovirus infection

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Contents

Investigators 2

Acknowledgments 2

1. Executive Summary 7

1.1. Summary 7

1.2. Recommendations 11

1.2.1. For research 11

1.2.2. For policy 12

2. Background to the project 13

3. Work Package 1: Foodborne and food-handler-associated norovirus outbreaks: Systematic review. 15

3.1. Summary 15

3.2. Introduction 15

3.3. Methods 17

3.3.1. Literature search 17

3.3.2. Study inclusion criteria 17

3.3.3. Data Collection 17

3.3.4. Analyses 18

3.4. Results 18

3.4.1. Literature Search 18

3.4.2. Norovirus Foodborne outbreaks 20

3.4.3. Norovirus Food handler associated outbreaks 20

3.5. Discussion 21

3.5.1. Main Findings 21

3.5.2. Limitations 22

3.6. Conclusion 23

4. Work Package 2: Determining the origins of human norovirus RT-qPCR signals 47

4.1. Summary 47

4.2. Application of the Capsid Integrity Assay (CIA) to CEN Methods 48

4.2.1. Introduction 48

4.2.2. Methods 50

4.2.3. Results 51

4.2.4. CIA Discussion 74

4.3. Application of VPg Magnetic Immuno0Capture RT-qPCR to CEN Methods 79

4.3.1. Introduction 79

4.3.2. Methods 81
4.3.3. Results 83
4.3.4. VPg Discussion 84
5. Work Package 3: Acquisition of data on norovirus contamination levels in oysters at retail 86
5.1. Summary 86
5.2. Introduction 86
5.3. Materials and Methods 87
5.3.1. Sampling Plan 87
5.3.2. Sample Collection 88
5.3.3. Sample Receipt 89
5.3.4. Detection and quantification of norovirus 89
5.3.5. Detection and quantification of *Escherichia coli* 91
5.3.6. Capsid Integrity Assay 92
5.3.7. Determination of ssRNA virus viability ratios using bacteriophage 92
5.3.8. Next Generation Sequencing (NGS) 93
5.4. Results/Discussion 94
5.4.1. Noroviruses 95
5.4.2. E.coli 107
5.4.3. Capsid Integrity Assay 109
5.4.4. Determination of ssRNA virus viability ratios using bacteriophage 110
5.4.5. More on Next Generation Sequencing (NGS) 111
5.5. Conclusion 114
6. Work Package 4: Prevalence of norovirus in fresh produce sold at retail 116
6.1. Summary 116
6.2. Introduction 116
6.3. How the survey was conducted 117
6.3.1. Survey Design 117
6.3.2. Sampling 118
6.3.3. Sample receipt 118
6.3.4. Sample treatment 118
6.3.5. Detection of Norovirus and mengovirus 119
6.3.6. Interpretation of RT-PCR results 119
6.3.7. Capsid Integrity Assay 119
6.3.8. Detection of Escherichia coli 119
6.4. Results 120
6.5. Discussion 130

7. Work Package 5: Prevalence of norovirus in the catering environment in outbreak and non-outbreak premises 133
7.1. Summary 133
7.2. Aims of the Study 133
7.3. Materials and Methods 134
7.3.1. Surveillance sampling 134
7.3.2. Sampling during outbreaks 135
7.3.3. Virology: Norovirus testing 136
7.3.4. Bacteriological testing 138
7.4. Results 139
7.4.1. Premises sampled 139
7.4.2. Virology results 141
7.4.3. Norovirus characterisation 143
7.4.4. Bacteriology results 144
7.5. Discussion 146
8. Work Package 6: Estimating the disease burden from foodborne norovirus in the UK, a microsimulation based quantitative microbial risk assessment 150
8.1. Summary 150
8.2. Introduction 152
8.3. Systematic Review 154
8.3.1. Methods 154
8.3.2. Results 154
8.3.3. Conclusions 155
8.4. Methods and Justification 157
8.4.1. Model Overview 157
8.4.2. Estimating dose per meal for oysters 167
8.4.3. Estimating dose per meal for lettuce and raspberries 169
8.4.4. Estimating dose per catered meals 170
8.4.5. Dose response model 173
8.4.6. Calculating risk per meal, per day and annually 174
8.4.7. Adjusting for acquired immunity from prior infection 176
8.4.8. Reporting uncertainty 178
8.5. Results 179
8.5.1. Virus exposure per meal 179
8.5.2. Risk results 180
8.5.3. Epidemic model – accounting for multiple prior infections 186
8.5.4. Sensitivity analyses 187
8.5.5. Synthesis 194

8.5.6. Validation 195

8.6. Discussion 196

8.7. Conclusions 199

9. Individual-based model to investigate the dynamics of norovirus infections in communities 200

9.1. Introduction 200

9.2. Methods 200

9.2.1. The individual-based model 200

9.2.2. Extending the individual-based model to include data on oysters, berry fruits and lettuce collected in the NoVAS project 204

9.2.3. Interpreting VIP scores 207

9.3. Results 207

9.3.1. Exposure risk and exposure levels to norovirus 207

9.3.2. Sensitivity analysis of exposure and transmission parameters on community burden of disease in norovirus naïve and norovirus non-naïve communities 208

9.4. Discussion 212

10. The models in context 215

10.1. Introduction 215

10.2. Re-cap of major outcomes from each model 215

10.3. Strengths 228

10.4. Limitations 229

10.5. Sources of Uncertainty 230

10.6. Conclusions 230

11. Recommendations 231

11.1. For research 231

11.2. For policy 232

12. List of abbreviations and acronyms used 233

13. Authors' Information 236

14. Acknowledgments 237

15. References for Chapter 3 (Work Package 1) 238

16. References for Chapter 4 (Work Package 2) 248

17. References for Chapter 5 (Work Package 3) 250

18. References for Chapter 6 (Work Package 4) 253

19. References for Chapter 7 (Work Package 5) 255

20. References for Chapter 8 (Work Package 6) 257

21. References for Chapter 9 262

22. References for Chapter 10 263

1 Executive Summary

1.1 Summary

Noroviruses (NoVs) are the most commonly identified cause of infectious diarrhoea and vomiting in the community, often causing outbreaks, especially in closed or semi closed communities. Like other organisms that affect the gut norovirus can be passed from person to person, or be picked up from a contaminated environment or through eating food contaminated at source or by infected food handlers. What is not reliably known is exactly what proportion of norovirus infection is food-related as opposed to transmitted by other means. Estimates from international research groups of the proportion of norovirus that is transmitted through contaminated food vary quite widely. Through a series of linked studies we sought to answer the following major research questions:-

a) How much norovirus is transmitted through contaminated food?
b) What is the role of infected food handlers in transmission?
c) Is it possible to differentiate between infectious and non-infectious virus in a variety of food matrices?

Specifically the objectives of this project were to:-

1. Review existing evidence for foodborne transmission of norovirus and undertake feasibility studies of new data collection.

A systematic review of outbreaks attributed to norovirus between January 2003 and July 2017 was conducted to assess the contribution of food handlers to the burden of norovirus, and to identify any foods commonly associated with norovirus outbreaks. Three thousand and eighty seven articles were retrieved, of which 27 met the definition of confirmed foodborne outbreaks and 47 met the criteria for definite food handler-associated norovirus outbreaks. Of all food types, shellfish were implicated in the greatest number of definite foodborne outbreaks. Food handlers contributed to definite food handler outbreaks involving a diverse range of foodstuffs and in a wide variety of settings, including weddings and military establishments. More genotypes of norovirus were found in people that were ill than in samples from food and food handlers. The potential for both food products and food handlers to contribute to the burden of norovirus infection was demonstrated conclusively.

2. Investigate the application of a capsid integrity assay to the CEN detection methods and so develop improved methods for the detection of infectious virus particles.

To meet this objective two molecular infectivity assays were developed and applied to determine the origins and significance of human norovirus (hNoV) RT-PCR signals, their application to the methods already developed in ISO/TS 15216, and the contribution of food and catering premises to hNoV infections in the UK. The first of these assays was based upon the measurement of the integrity of the virus coat or

capsid and is termed the “capsid integrity assay” or CIA. The second assay was based upon a novel approach termed “VPg immuno-capture RT-qPCR” that attempts to measure both the integrity of the norovirus capsid and its genome simultaneously.

Results obtained using the CIA showed excellent agreement in a collaborative blind trial between three different laboratories using six different hNoV positive stool samples belonging to different hNoV genogroups and genotypes. Although most samples showed extensive exposure of capsid-protected RNA following heat treatment, indicative of intact infectious particles, one of the hNoV samples (GII.1) was more resistant requiring increased heat treatment to achieve significant RNA exposure. The hNoV capsid was significantly more stable in water (pH 5.2) than seawater (pH 8.0) or phosphate buffered saline (pH 7.2).The GII.4 hNoV capsid was stable following three freeze thaw cycles at -80ºC, and at 37ºC for 48h showing that the freezing of food and environmental samples is not anticipated to influence capsid stability and that the GII.4 virus capsid is persistent in vitro.

The CIA was successfully applied to the ISO/TS 15216 method for berries and leafy greens. The application of the CIA to shellfish extract using the ISO/TS 15216 method was problematic and the CIA was apparently not compatible with the magnetic RNA extraction commonly used in the ISO/TS 15216 method as the heat treatment within the CIA produced changes to the sample that reduced extraction efficiency. Additionally RNA exposure was not observed for hNoV probably owing to the low pH (6.0) of the shellfish extract which appeared to stabilise the hNoV capsid. However, the use of sample buffer with an alkaline pH (8.8) and a solid phase extraction method resulted in the successful application of the CIA to hNoV spiked shellfish samples. Further studies are required to investigate the application of this method to naturally contaminated hNoV samples.

Results for VPg immuno-capture PCR demonstrated proof of principle of this approach. Rabbit polyclonal antibodies have been raised and purified against three conserved VPg peptide sequences present in human and murine noroviruses. Pooled antisera from all three peptide domains was bound to protein A magnetic beads. The resulting VPg immuno-magnetic affinity beads were directly used in RT-qPCR reactions and shown to capture RNA preferentially from heated norovirus particles (VPg exposed) compared with unheated samples (VPg not exposed). Furthermore since VPg is covalently linked to the genomic RNA the resulting RT-qPCR signal must have been derived from largely intact genomic RNA (>5000 nucleotides). This preliminary data showed for the first time that this novel method can be applied to simultaneously measure capsid and genomic integrity.

3. Acquire representative data on contamination with norovirus within high risk food chains at retail - oysters.

A one year survey of oysters collected from the point-of-sale to the consumer was carried out from March 2015 – March 2016. A total of 630 samples, originating in five different European Union Member States, were collected from 21 regions across the

UK using a randomised sampling plan, and tested for norovirus RNA using a qRT-PCR method compliant with ISO 15216-1, in addition to *Escherichia coli* as the statutory indicator of hygiene status.

As in a previous production area study, norovirus RNA was detected in a high proportion of samples (68.7%), with a strong winter seasonality noted. However, levels of norovirus RNA detected in positive samples were considerably lower than seen in a previous survey on oyster samples collected from production areas (Lowther *et al*., 2012). Levels >100 copies/g were found in 9.7% of samples. Some statistically significant differences in prevalence and levels in oysters from different countries was noted, with samples originating in the Netherlands showing lower prevalence and levels than those from either the UK or Ireland. Investigation of potential contributing factors to this pattern of results was carried out. Application of normalisation factors to the data from the two studies based on both the numbers of clinical laboratory reports of norovirus received by national surveillance systems, and the national average environmental temperatures during the two study periods resulted in a much closer agreement between the two data sets, with the notably different numbers of clinical laboratory reports explaining the major portion of the difference observed in norovirus levels in oysters.

The large majority of samples (76.5%) contained no detectable *E.coli*, however in a small number of samples (2.4%) levels above the statutory end product standard (230 MPN/100g) were detected. In these cases the results were reported on the same working day to the FSA to enable action to be taken if appropriate.

This study revealed the high prevalence of norovirus RNA in oysters directly available to the UK consumer, despite the high level of compliance with the existing *E.coli* based health standards, while also highlighting the difficulty in comparing the results of surveys carried out in different time periods, due to variability in risk factors.

4. Acquire representative data on contamination with norovirus within high risk food chains at retail - raspberries and lettuces.

One thousand one hundred and fifty two samples of fresh produce were collected and analysed for norovirus (1146 for *Escherichia coli*), to acquire UK prevalence data to be used within the NoVAS study. Of 568 samples of lettuce, norovirus RT-PCR signals were obtained from 79 (13.9%) samples; replicate RT-PCR signals (giving a greater indication of the presence of norovirus) were obtained from 30 (5.3%) lettuce samples. Eight lettuce samples (out of 568 analysed) tested positive for *E. coli*. Most (24/30) lettuce samples which tested positive for norovirus (replicate RT-PCR signals) were grown in the UK and contained norovirus GI (19/24). Twenty one of the UK-grown samples were collected from lettuce on sale between May and August 2015. Thirty seven / 310 (11.9%) samples of fresh raspberries gave norovirus RT-PCR signals; 7 samples (2.3%) gave replicate RT-PCR results. Most (6/7) of the positively-testing fresh raspberry samples in the NoVAS survey were

imported, but no predominance of a genogroup, or any seasonality, was observed. No (0/317) fresh raspberry samples tested positive for *E. coli*. Thirty four / 270 (~12.6%) samples of frozen raspberries gave norovirus positive results; 10 samples (3.6%) gave replicate RT-PCR results. The country of origin of the positively-testing frozen raspberry samples was not identified in most (7/10) instances. No (0/256) frozen raspberry samples tested positive for *E. coli*.

5. Determine the prevalence of environmental contamination with norovirus in outbreak and non-outbreak catering premises.

In total 256 catering premises were sampled - 247 were sampled for surveillance purposes, and 16 were sampled as part of an outbreak investigation. Overall 2,038 swabs were submitted for norovirus testing, with an average of 8 per premise (range 2 to 23) and a median of 7. The number of swabs submitted for outbreak investigation ranged from 10 to 23 with an average and a median of and 13 samples.

Overall, 11% (30/252) of the premises sampled yielded at least one norovirus positive sample (environmental, and/or hand swab), and 2.5% of the swabs were positive for norovirus in total. The proportion of premises in which norovirus was detected in the Greater London (GL) area was 21% compared with 4% in the North West (NW).

The proportion of norovirus positive premises was not significantly different during outbreak investigation or routine surveillance sampling: 25% (4/16) compared to 18% (44/247).

6. Compare the outputs from objectives 3 to 5 to generate an overall assessment of the contribution of norovirus to food-related illness in the UK, and the contaminated foods linked to most illnesses, including the role of infected food handlers.

A novel microsimulation-based method of performing a Quantitative Microbial Risk Assessment (QMRA) was developed, alongside an Individual-Based Model, to estimate the foodborne component of norovirus infection in the UK. Four food groups were chosen that historically are the most common food sources of norovirus infection, comprising oysters, lettuce, raspberries and catered food prepared in a commercial kitchen. New data acquired in Work Packages 3 to 5 were incorporated into the models where possible.

Overall, we estimated that the proportion of NoV transmission that is foodborne was 16% (range 2.1% to 22.9%) in a Quantitative Microbiological Risk Assessment and 35% (range 11% to 55%) in an Individual-Based Model so somewhere between a fifth and a third of all NoV illnesses could be attributed to the foodborne route. From the QMRA analyses, nearly 75% of foodborne infections occurred through contaminated catered food (a proxy for food handling), with contaminated lettuce accounting for around 20% of illness burden, followed by berries at 3% and oysters

at 3%. The major source of uncertainty surrounded assumptions about the duration of acquired human immunity. This had by far the biggest impact on the outputs of both models. Other important sources of uncertainty were virus viability, levels of contamination on food, hands and environmental surfaces, virus transfer from hands to food and the background incidence of NoV.

1.2 Recommendations

1.2.1 For research

To improve the QMRA and IBM we recommend that further research is needed to:-

1. Understand the proportion of gene copies in food commodities that represent virus with infectious potential. The VPg immuno-capture PCR and direct RT-qPCR using capture beads was not fully tested or optimised in this study and so further work is required to:-

    - Obtain more repeat data and extend studies to more GII and GI samples
    - Consider the inclusion of RNase inhibitors
    - Investigate optimal antibody selection for capture
    - Reduce or eliminate non-specific binding to increase sensitivity and specificity
    - Trial and compare the assay using shellfish or berry extract and the ISO methods.

2. Develop better dose response models for norovirus.

3. Understand the mechanisms of NoV transmission in commercial kitchens including:-

    - Studies of food handler behaviour during food preparation across the range of catered foods.[1]
    - Assessment of how frequently food is handled during preparation, especially when the food will be served directly to a diner with no further cooking.

4. Understand human immunity to norovirus.

5. Obtain data on seasonal consumption patterns to improve exposure assessment.

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[1] FSA has published research in this area carried out by Ipsos MORI, Food handlers and norovirus transmission: Social science insights (2017) which comprised a literature review followed by structured environmental and behavioural observations, surveys, and in-depth interviews with 32 food establishments: www.food.gov.uk/research/research-projects/food-handlers-and-norovirus-transmission-social-science-insights

6. Understand the likely impact of risk management interventions by industry in reducing consumer exposure to norovirus.

1.2.2 For policy

Given the importance foodborne NoV and that food eaten away from the home or take away food is the likely primary driver of foodborne NoV we recommend that:-

The Food Standards Agency maintains its emphasis on prevention of transmission of NoV in catering outlets (e.g. food handler instructions/procedures) to minimise/mitigate the risk of NoV infection from foods eaten away from the home.

2 Background to the project

Norovirus Attribution Study (NoVAS): Assessing the contribution made by the food chain to the burden of UK-acquired *norovirus* infection

Noroviruses (NoVs) are the most commonly identified cause of infectious diarrhoea and vomiting in the community. They often cause outbreaks, especially in closed or semi closed communities. Like other organisms that affect the gut norovirus can be passed from person to person, or be picked up from a contaminated environment or through eating food contaminated at source or by infected food handlers. What is not reliably known is exactly how much norovirus infection is food-related as opposed to transmitted by other means. Estimates from international research groups of the proportion of norovirus that is transmitted through contaminated food vary quite widely. Through a series of linked studies we sought to answer the following major research questions:

a) How much norovirus is transmitted through contaminated food?
b) What is the role of infected food handlers in transmission?
c) Is it possible to differentiate between infectious and non-infectious virus in a variety of food matrices?

Given critical data gaps identified in 2004, and the lack of progress in filling them, we conducted fieldwork in three crucial areas – first to determine the prevalence of norovirus contamination of three high risk food commodities on retail sale, namely oysters, salad leaves and soft berry fruits; secondly to assess whether or not the norovirus found is likely to be infectious or not and thirdly to determine the prevalence of norovirus contamination of the catering environment. These were essential data items for being able to conduct the quantitative microbiological risk assessment provided within this document.

Specifically the objectives of this project were to:-

1. Review existing evidence for foodborne transmission of norovirus and undertake feasibility studies of new data collection.

2. Investigate the application of a capsid integrity assay to the CEN detection methods and so develop improved methods for the detection of infectious virus particles.

3. Acquire representative data on contamination with norovirus within high risk food chains at retail - oysters.

4. Acquire representative data on contamination with norovirus within high risk food chains at retail - raspberries and lettuces.

5. Determine the prevalence of environmental contamination with norovirus in outbreak and non-outbreak catering premises.

6. Compare the outputs from objectives 3 to 5 above to generate an overall assessment of the contribution of norovirus to food-related illness in the UK, and the contaminated foods linked to most illnesses, including the role of infected food handlers.

3 Work Package 1[2]: Foodborne and food-handler-associated norovirus outbreaks: Systematic review.[3]

3.1 Summary

Noroviruses (NoV) are the commonest cause of gastrointestinal disease in the U.K. and many developed countries, causing millions of cases annually of diarrhoea and vomiting worldwide. Transmission is most often mediated from person to person. Norovirus infection has, however, additionally been associated with the consumption of food, either though the consumption of food contaminated at source such as seafood, berries and salad, or as a consequence of the foodstuff being contaminated in some way by a food handler during processing or serving. A systematic review of outbreaks attributed to norovirus between January 2003 and July 2017 was conducted to assess the contribution of food handlers to the burden of norovirus, and to identify any foods commonly associated with norovirus outbreaks. Three thousand and eighty seven articles were retrieved, of which 27 met the definition of confirmed foodborne outbreaks and 47 met the criteria for definite food handler-associated norovirus outbreaks. Of all food types, shellfish were implicated in the greatest number of definite foodborne outbreaks. Food handlers contributed to definite food handler outbreaks involving a diverse range of foodstuffs and in a wide variety of settings, including weddings and military establishments. More genotypes of norovirus were found in people that were ill than in samples from food and food handlers. The potential for both food products and food handlers to contribute to the burden of norovirus infection is demonstrated conclusively.

3.2 Introduction

Noroviruses (NoV) are the leading causes of gastrointestinal disease in people in many countries worldwide (Al-Thani *et al*. 2013; Scallan *et al*. 2011; Tam *et al*. 2012). Norovirus infection leads to a sudden onset of vomiting and diarrhoea. Symptoms usually last for between two and four days (Graham *et al*. 1994; Lopman *et al*. 2004; Rockx *et al*. 2002) in healthy adults (Murata *et al*. 2007). Symptom duration can be longer in hospitalised patients: one study showed that ten percent of hospitalised adults were still symptomatic after seven days (Lopman *et al*. 2004) and similarly in hospitalised children symptoms can last for six to seven days (Murata *et al*. 2007; O'Ryan *et al*. 2010). Both asymptomatic and symptomatic individuals have been shown to excrete the virus and are therefore able to transmit norovirus to other individuals (Akihara *et al*. 2005; Huynen *et al*. 2013; Krumkamp *et al*. 2015). Experimental evidence suggests that asymptomatic individuals shed the virus in

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[2] Note that the feasibility studies were presented as earlier deliverables in the contract and so are not described again here but are included as appendices to the final report (Appendix 1, Appendix 2 and Appendix 3).

[3] This work is published: Hardstaff JL, Clough HE, Lutje V, McIntyre KM, Harris JP, Garner P, O'Brien SJ. Foodborne and Food-Handler Norovirus Outbreaks: A Systematic Review. Foodborne Pathog Dis. 2018; 15(10):589-597.

smaller amounts than symptomatic individuals (Bernstein *et al*. 2015). Viral shedding can occur for 3-14 hours prior to symptom onset (Atmar *et al*. 2008). Cases who are immune-compromised, elderly and newborn infants have been shown to shed virus for longer periods than adults who are otherwise healthy (Atmar *et al*. 2008). Several studies have indicated peak shedding from two to five days post infection (Atmar *et al*. 2008; Graham *et al*. 1994; Kirby *et al*. 2014). The virus can be excreted in vomit and faecal matter. It can survive as a fomite as demonstrated in studies in care homes (Wu *et al*. 2005) and hospitals (Nenonen *et al*. 2014). Norovirus has experimentally survived as a fomite for long periods of time enabling a long period of potential exposure (Lamhoujeb *et al*. 2009; Liu *et al*. 2009).

Infection with norovirus occurs through the ingestion the virus, either as a result of unsterile contact with a contaminated environment, or more directly from contaminated food and water. Water samples contaminated with norovirus have been able to cause illness in subjects up to two months post contamination (Seitz *et al*. 2011), and contamination of water supplies by sewage containing norovirus has been implicated in large outbreaks, for example in Nokia, Finland (Rasanen *et al*. 2010). Food can be contaminated indirectly, for example through sewage being discharged in areas where sea food is farmed (Le Guyader *et al*. 2008) or from contaminated irrigation water (El-Senousy *et al*. 2013). It may also be contaminated directly through the hands of infected agricultural workers, for example Leon-Felix *et al.* 2010 (Leon-Felix *et al*. 2010) demonstrated contamination of peppers in the field and on the hands of pickers, classifiers and packers. Further, a food handler who returns to work after the acute symptoms of a norovirus infection have passed but before the infectious period has ended runs the risk of contaminating food products through unsterile contact with food in the preparation process (Mathijs *et al*. 2012). The presence of norovirus on the hands of food handlers means that it can easily be transferred to utensils; work surfaces and food; between utensils, work surfaces and foods once they themselves are contaminated as shown in a number of published studies (Ronnqvist *et al*. 2014; Sharps *et al*. 2012; Stals *et al*. 2013; Tuladhar *et al*. 2013; Verhaelen *et al*. 2013).

The aim of this review was to assess the contribution of particular foods to definitively foodborne outbreaks of norovirus, and to describe the contribution of food handlers to norovirus outbreaks.

3.3 Methods

3.3.1 Literature search

The search window for the review extended from January 2003 to July 2017. Databases and websites searched were: Web of Science, Medline, Embase, Biosis previews, CABI (CAB Abstracts® and Global Health®), Scopus, Biomed Central, Science Direct, OpenSigle, Proquest Dissertations and theses A&I, Foodbase website, Public Health England via www.gov.uk, Cefas via the DEFRA website and the World Health Organisation website.

The databases, dates of their use and number of articles retrieved can be found in supplementary Table S1 at the end of this chapter. The full list of search terms used for each database search can be found in the supplementary material S1. As an example, the search terms used for Web of Science were: TOPIC: (norovirus OR norwalk OR winter vomiting OR noroviral) AND TOPIC: (foodborne OR food-borne OR orofecal OR orofaecal OR sewage OR irrigation OR hand* OR hotel OR restaurant* OR catering or cook* OR waiter* OR cruise OR canteen OR contaminat* OR aerosol* OR spray* OR toilet* OR latrine* OR utensil* OR kitchen* OR shellfish OR fish* OR mussel* OR oyster* OR strawber* OR raspberr* OR lettuce OR salad* OR vegetable* OR green* OR fruit* OR ice or blueberr* OR onion* OR tomato*).

3.3.2 Study inclusion criteria

All titles and abstracts were screened by two reviewers. Articles were assessed for inclusion by one reviewer. A sub-sample of titles was then selected at random, and the decision to include or exclude each article was cross-validated by two reviewers.

An outbreak was defined as definitely foodborne if the article in which it was described provided formal evidence of laboratory confirmation of norovirus infection in both human cases and food stuffs.

Outbreaks were definitively attributed to food handlers if the outbreak report included laboratory confirmation of infection in both patrons and food handlers, with either the same strain being identified in either handlers and cases, or in handlers and in foods consumed by cases. Genotypes of strains found in food handlers and foods were reported where possible.

3.3.3 Data Collection

The data from each article were collated into a single data abstraction sheet, which can be found in supplementary section number two (S2). Briefly, for studies of foodborne infection, information was collected regarding the foods implicated in an outbreak and the number of samples taken. For food handler-associated studies, information was collected on the outbreak setting, foods handled and, where possible, data recorded on the number and types of samples tested from food handlers and norovirus genotypes.

3.3.4 Analyses

We calculated the proportion of foodstuffs and people in which norovirus was detected and described the genogroups and genotypes present, where this had been recorded. The heterogeneity between articles in terms of study design, timeframes and study populations prohibited the undertaking of a formal statistical meta-analysis: however descriptive statistics (proportions, medians and interquartile ranges) were calculated for data extracted from articles which met the inclusion criteria.

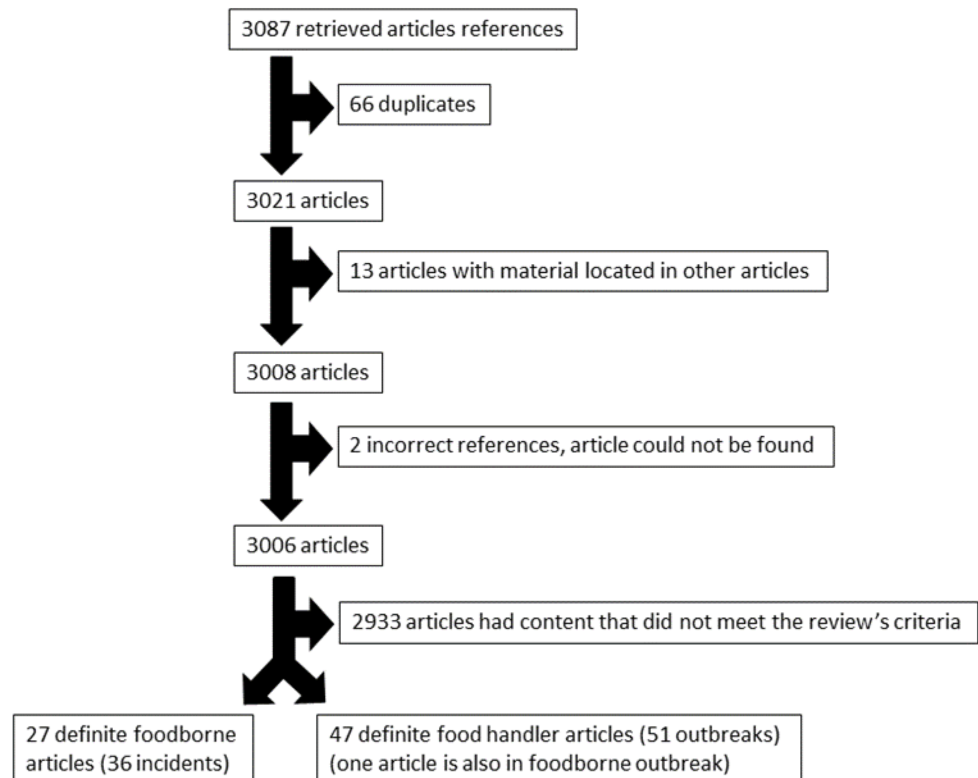
3.4 Results

3.4.1 Literature Search

Three thousand and eighty-seven articles were retrieved. Of these 2933 (95%) did not meet the inclusion criteria and were excluded because they were review articles or they concentrated on diagnostics, artificial contamination, efficacy of decontamination, aetiology and outbreak control; 66 (2.1%) were duplicates; 13 (0.4%) had information found in other articles; and two (0.06%) were incorrect and the paper could not be found (see Figure A1).

Twenty-seven articles met the criteria for inclusion as norovirus-definite foodborne outbreaks documenting 44 separate incidents (Baker *et al*. 2011; Cho *et al*. 2016; David *et al*. 2007; Doyle *et al*. 2004; Ethelberg *et al*. 2010; Fitzgerald *et al*. 2014; Furuta *et al*. 2003; Iizuka *et al*. 2010; Le Guyader *et al*. 2006; Le Guyader *et al*. 2010; Le Guyader *et al*. 2008; Le Guyader *et al*. 2004; Liko and Keene 2009; Maunula *et al*. 2009; Muller *et al*. 2016; Nenonen *et al*. 2009; Ng *et al*. 2005; Park *et al*. 2015a; Park *et al*. 2015b; Prato *et al*. 2004; Prevention 2012; Rasmussen *et al*. 2016; Research 2011b; Simmons *et al*. 2007; Viriot *et al*. 2011; Webby *et al*. 2007; Westrell *et al*. 2010).

Figure A1: Article selection process



Outbreaks that met the definition for definite food handler associated outbreaks of norovirus were identified in 47 articles documenting 51 different outbreaks (Baker *et al.* 2011; Barrabeig *et al*. 2010; Boxman *et al*. 2009; Cai *et al*. 2013; Centers for Disease and Prevention 2006; 2007; de Wit *et al*. 2007; Friedman *et al*. 2005; Furuya *et al*. 2005; Godoy *et al*. 2016; Godoy *et al*. 2005; Hirakata *et al*. 2005; Huang *et al*. 2013; Jung *et al*. 2015; Kim *et al*. 2005; Kimura *et al*. 2012; Lederer *et al*. 2005; Leshem *et al*. 2016; Lin *et al*. 2015; Liu *et al*. 2015; Made *et al*. 2016; Maritschnik *et al*. 2013; Mayet *et al*. 2011; Medici *et* al. 2009; Nicolay *et al*. 2011; Ohe 2013; Ohwaki *et al.* 2009; Oogane *et al*. 2008; Raj *et al*. 2017; Research 2011a; Ruan *et al*. 2013; Sakon *et al*. 2005; Sala *et al*. 2009; Sala *et al*. 2005; Sanchez *et al*. 2017; Schmid *et al*. 2011; Schmid *et al*. 2007; Showell *et al*. 2007; Smith *et al*. 2012; Smith *et al*. 2017; Tashima and Chijiwa 2003; Thornley *et al*. 2013; Wadl *et al*. 2010; Watier-Grillot *et al*. 2017; Yu *et al*. 2010; Zomer *et al*. 2010). A report from Rasmussen (2016) comprised an aggregated outbreak report from nine different venues but did not provide any further information that could individualise the outbreaks hence it remained as one aggregate outbreak.

One article had information that was relevant to both foodborne and food handler outbreaks (Baker *et al*. 2011)) (Figure A1).

3.4.2 Norovirus Foodborne outbreaks

Norovirus foodborne outbreaks were reported from around the globe. However, the largest proportion of foodborne outbreaks in this study (57%) were reported in Europe. Of all studies, the most commonly implicated food vehicle in documented outbreaks was seafood (61%), of which 89% were associated with oysters (Table A1).

The numbers of people reported as exposed to norovirus in each outbreak ranged from two (Muller *et al*. 2016) to 1580 people (Simmons *et al*., 2007), with a median of 59. The number of people reported as falling ill ranged between two (Muller *et al*. 2016) and 305 (Ng *et al*., 2005) (median = 23 cases). The number of ill people that provided samples for testing was between one (David *et al*. 2007; Fitzgerald *et al*. 2014) and 42 (Le Guyader *et al*., 2006) (median = 8 people). The median (inter-quartile range) for the proportion of samples in which norovirus was detected was 79% (52-100%), with the median and range of the number of positive samples being three (1-24) (2011b; 2012; David *et al*. 2007; Fitzgerald *et al*. 2014; Muller *et al*. 2016; Nenonen *et al*. 2009; Prato *et al*. 2004).

Most commonly, the norovirus genotypes found in food (Table A2) and patrons (Table A3) were mixed. Tables A3 and A4 indicate that a greater diversity of genotypes were recovered from people affected by the implicated foods than from the foods themselves. The most common genotypes include GII.4 recovered from food and GII.4, GI.4, GI.1 and GI.2 identified from people infected in foodborne outbreaks.

Many studies were outbreak reports and had used genotyping and attack rates to determine the mode of infection. However, 15 of 27 articles included the odds and risk ratios of foods implicated in associated outbreaks (Table A4). The risk factors were predominantly seafood-related.

3.4.3 Norovirus Food handler associated outbreaks

Food handler-associated outbreaks occurred in a wide-variety of settings and foods (Table A2). The most common setting was restaurants (12/51 outbreaks). In 33% of food handler-related outbreaks implicated food items were not categorised. When they were, the most commonly implicated foods, associated with 20% of outbreaks, were salad and vegetables, followed by dishes containing seafood (Table A5). The number of patrons with norovirus varied from three (Baker *et al*. 2011; Made *et al*. 2016; Sala *et al*. 2009) to 660 (Hirakata *et al*. 2005).

Food handling (mostly kitchen) staff were sampled for norovirus in 44 of 51 (86%) outbreaks. The median proportion of positive samples obtained from food handlers was 46% with an interquartile range of 25-76%.

The most common genotype recovered from food handlers (Table A6) and people whose illness was associated with food handlers (Table A7) was GII.4.

Many food handler-associated outbreaks had more than one risk factor (Table A8); some focussed on a time or place that an exposure occurred, whilst others implicated consumers' dishes that food handlers handled (Table A8).

3.5 Discussion

3.5.1 Main Findings

Oysters and other types of seafood dominated the list of foodstuffs tested for norovirus after clinical illness, and it is not clear whether this is a genuine food-related effect, or a consequence of one or more of a number of factors. First, investigator bias may arise because of a long-established association between seafood (Murphy *et al*. 1979) and norovirus (investigators looking for norovirus in preference to other microbial agents because they have an *a priori* suspicion that it is likely to be there). Secondly, seafood may additionally dominate food sources in our review because of the availability of oysters for testing from batches implicated in outbreaks: this is unlike salad and berries, which are likely to perish or be consumed in their entirety more quickly. Finally, the dominance of seafood may be due to virus attaching more easily to seafood compared with salad and berries (Tian *et al*. 2011). Lettuce and raspberries are also implicated in a number of outbreaks, either reflecting contamination of food through the roots as a result of infected irrigation water (Dicaprio *et al*. 2012; Hirneisen 2012), or contamination by food handlers, or a combination of both.

European law states that food handlers should notify their employers if they are ill and that no toxins should be shed into anywhere that food is present (European Union 2004). Current advice given to food handlers that have suffered from gastroenteritis is to stay away from work for 48 hours after the symptoms have disappeared (Food *et al*. 2009). Despite this, due to prodromal, prolonged and asymptomatic shedding, there is the potential for both symptomatic and asymptomatic individuals to contaminate the workplace. A study by Sabria *et al.* that tested food and healthcare workers whose workplace had had norovirus outbreaks, found that of those that tested positive (59.1% workers), 77.9% working with food reported being asymptomatic compared with 28.1% of workers in a healthcare setting (Sabria *et al*. 2016). Sabria *et al.* 2016 also demonstrated that both asymptomatic and symptomatic food workers shed virus for up to three weeks post-outbreak exposure (Sabria *et al*. 2016). Some articles in our review described workers becoming ill at work, resulting in workplace contamination (which incidentally could have made it easier to determine the cause of an outbreak) (Baker *et al*. 2011; Centers for Disease and Prevention 2007; Maritschnik *et al*. 2013; Thornley *et al*. 2013). Some food handlers, however, were not ill but were found to be shedding the virus (Ozawa *et al.* 2007) whilst others, who had been around ill people but had not exhibited symptoms themselves, may be asymptomatic shedders who run the same risk as symptomatic individuals of contaminating the workplace (Kuo *et al*. 2009; Lin *et al*. 2015).

A study by Verhoef *et al*. (Verhoef *et al*. 2013) found that few food handlers in catering companies (20%, k = 600, n = 1023) had heard of norovirus, compared with food handlers based in hospital (92%, k = 141, n = 154) and non-hospital (71%, k = 88, n = 101) institutions. Knowledge may impact on a worker's likelihood of staying away from work in the event that they experience an active infection. Fewer facilities necessary for kitchen staff to maintain high standards of hygiene, for example hand washing instructions for new staff and separate sinks for hand washing, were found in catering companies, compared with hospital restaurants and non-hospital institutional catering (Verhoef *et al*. 2013). The differences in kitchen standards, training and knowledge may help to explain why fewer food handler outbreaks were attributed to hospitals than to restaurants and caterers.

Hedberg *et al*. (Hedberg *et al*. 2006) found that in restaurants with managers that had undertaken training on food safety outbreaks were less likely than in those without trained managers and staff. However, practices that reduced contamination such as using gloves and using designated utensils on different products did not always occur, even if the worker was aware that they should be doing this (Robertson *et al*. 2013). Hedberg *et al*. (Hedberg *et al*. 2006) additionally found that outbreaks were less likely in restaurants where sick pay was provided and a staff reporting policy in the event of illness was in place: this accords with the study by Carpenter *et al*. (Carpenter *et al*. 2013), which found that people continued to work through diarrhoea and vomiting for fear of losing their jobs and shifts if they were absent. It is difficult to make recommendations to stay at home in a culture within which many workers will not have fixed term contracts, with regulated hours, and will not necessarily receive sick pay if they are absent from work.

3.5.2 Limitations

There are various reasons that foodborne and food handler associated norovirus may be under-represented within the literature, and this has led to limitations in the results of our review. For example, varying time lags between falling ill and faecal sampling in different studies were observed and this will have affected the likelihood of finding virus. This is noted in two studies as a possible explanation for heterogeneity in shedding periods (Atmar *et al*. 2008; Murata *et al*. 2007). The length of time from acquisition of the virus to genotyping may determine the strains found and will not necessarily capture chance point mutations or gene transfer from other co-circulating strains. This might have resulted in identifying fewer food handler-associated outbreaks. Furthermore, food handlers will not necessarily admit to being ill (Verhoef *et al*. 2013; Carpenter *et al*. 2013), as they may lose work and may not want leave the work place under-staffed. This will also lead to an underestimate of the frequency of food handler-associated outbreaks.

The completeness of studies included in a systematic review was achieved through the use of a wide search strategy, which located studies for potential inclusion across a range of sources. However, the time-scale of the review was restricted in

an effort to ensure comparability of laboratory methods across studies, which resulted in studies outside this time window being omitted.

It was commonly the case that in study abstracts, more detailed information was provided for peer-reviewed papers than for government reports. This resulted in a comparatively large proportion of papers selected for inclusion in the review being from the peer-reviewed literature. Peer-reviewed publication usually requires reporting of novel findings (new virus type, new food vehicle etc.) and so outbreaks which provide high-quality evidence of long-established causes and exposure routes may not reach the peer-reviewed literature. This means that the burden of illness associated with particular food sources and risky environments may be under-represented in our systematic review.

The strict case definition resulted in comparatively few articles for which the quality of evidence confirming a food source or food handler involvement was judged to be high. Relatively few studies had tested both cases and foods, or cases and handlers.

Finally, studies from developed countries comprised the majority of those appearing in the review reflecting the fact that the technology, public health infrastructure and monetary resource required for the investigation of outbreaks and identification of causative microbiological agents are available in those countries. Further, the short duration of illness with norovirus may limit the number of outbreaks which are formally reported and investigated, for example small foodborne outbreaks may be expected and, therefore, not reported in countries in which a lot of seafood is eaten e.g. Japan (*Pers. Comm.* Dr Yamanaka).

3.6 Conclusion

Food and food handlers both contribute to outbreaks of norovirus. Some outbreaks were attributed to asymptomatic food handlers. Contaminated shellfish were implicated in the greatest number of definite foodborne outbreaks. Food handlers contributed to definite food handler outbreaks involving a diverse range of foodstuffs and in a wide variety of settings, including weddings and military establishments. More genotypes of norovirus were found in ill people than in samples from food and food handlers. The potential for both food products and food handlers to contribute to the burden of norovirus infection was demonstrated conclusively

Table A1. Food implicated in foodborne norovirus outbreaks

Country	Clams	Lettuce	Mussels	Oysters	Raspberries	Shellfish
Australia				1		
Canada				1		
Denmark		1				
Finland					1	
France				3		
Italy			1			
Italy and France				1		
Japan	1					1
New Zealand				2		
Singapore				1		
Sweden				1	1	
UK				1		
US				2		
France, Italy				1		
Denmark, France and UK				1		

Table A2. Genotypes from food

Qtr.	Year	GI-NLV/Steinbach/EG/2001/CA; GII-NLV/Tarrag/238/2001/Sp, Saitama U25 and Khs1-1997-JP.	GI.1	GI.1; GI.2; GI.4; GII.4	GI.1; GII.3	GI.1; GII.4; GII.8	GI.2	GI.2; GII.17	GI.3; GII.3; GII.6; GII.8; GII.13	GI.4	GI.4; GII.4	GII.2	GII.3	GII.4	GII.4; GII.14	NA
Q4	2001															2
Q1/2	2002	1														
Q4	2002					2										
Q4 2002 and Q1 2003											1					
Q4	2003													2		
Q4 2003 and Q1 2004																
Q1	2004						2							1		
Q1	2006			1												
Q2	2006								1							
Q4	2006															1
Q1	2007				1											
Q1	2008												1			
Q2	2008		1													
Q4	2009									1						
Q1	2010															6
Q3	2010															1
Q4	2011												1			
Q1	2012											2				
Q4	2012															1
Q2	2013										3				1	
Q1	2016							1								
Q2	2016															1

Table A3. Genotypes people from foodborne outbreaks

Qtr.	Year	G1-NLV/Steinbach/EG/2001/CA; G2 - NLV/ Tarrag/238/2001/ Sp, Saitama U25 and Khs1-1997-JP.	G1.4	GI.1	GI.1 GI.2 GI.9 GI.12 GI.14 GII.6	GI.1 GI.2 GII.2 GII.4 GII.7 GII.17 GIIb	GI.1; GII.17	GI.2	GI.2 GI.4 GII.3 GII.6 GII.7 GII.9	GI.4	GI.4 GI.6 GII.4 GII.8	GI.4 GII.4	GI.4 GII.4 GII.b	GI.P2 GI.2 GI.Pb-GI.6	GII.2 GII.4	GII.3 GII.6 GII.12	GII.4	GII.4 GII.11 GII.14	GII.5, GII.6, GII.7, GII.14 and GII.17	GII.P17 GII.17	GII GII.4 GII.5	NA
Q4	2001																					2
Q1/2	2002	1																				
Q4	2002										1		1									
Q4 2002 & Q1 2003											1											
Q4	2003								1													1
Q4 2003 & Q1 2004																					1	
Q1	2004							1									1				1	
Q1	2006					1																
Q2	2006															1						
Q4	2006																					1

Qtr.	Year	G1-NLV/Steinbach/EG/2001/CA; G2 - NLV/ Tarrag/238/2001/ Sp, Saitama U25 and Khs1-1997-JP.	G1 .4	GI. 1	GI.1 GI.2 GI.9 GI.12 GI.14 GII. 6	GI.1 GI.2 GII. 2 GII. 4 GII. 7 GII. 17 GIIb	GI.1 ; GII. 17	GI. 2	GI.2 GI.4 GII. 3 GII. 6 GII. 7 GII. 9	GI. 4	GI.4 GI.6 GII. 4 GII. 8	GI. 4 GII .4	GI.4 GII. 4 GII. b	GI.P 2 GI.2 GI.P b-GI.6	GII. 2 GII. 4	GII. 3 GII. 6 GII. 12	GII .4	GII. 4 GII. 11 GII. 14	GII. 5, GII. 6, GII. 7, GII. 14 and GII. 17	GII.P 17 GII.17	GII GII. 4 GII. 5	NA
Q1	2007			1																		
Q1	2008														1							
Q2	2008			1																		
Q4	2009		1																			
Q1	2010																					6
Q3	2010																					1
Q4	2011						1															
Q1	2012				1														1			
Q4	2012																1					
Q2	2013									3								1				
Q1	2016																			1		

Table A4. The odds and risk ratios calculated in studies describing foodborne norovirus outbreaks

Author	Year	Risk factor(s)	odds or risk ratio (95% confidence interval)
Doyle, A. *et al.*	2004	Oysters	55.3 (2.9-1058.7)
Prato, R. *et al.*	2004	Cooked mussels	1.5 (1.05-2.23)
Prato, R. *et al.*	2005	Cooked mussels	3.04 (1.26-7.30)
Prato, R. *et al.*	2006	Raw mussels	1.38 (1-1.91)
Prato, R. *et al.*	2007	Raw mussels	1.5 (1.18-1.89)
Ng, T. L. *et al.*	2005	Oysters	18.3 (9.9-33.2)
Simmons, G. *et al.*	2007	Oysters	11.9 (3.9-36.1)
Simmons, G. *et al.*	2007	Salmon contaminated by oysters	2.3 (1.2-4)
Simmons, G. *et al.*	2007	Tuatuas	3 (1.7-5.6)
Simmons, G. *et al.*	2007	Seafood chowder	2.5 (1-6.3)
Webby, R. J. *et al*	2007	Grilled oysters	17 (5 - 51)
Webby, R. J. *et al*	2007	Oyster cocktails	35 (5-243)
Liko, J. *et al.*	2009	Oysters	11.8 (2-50)
Maunula, L. *et al.*	2009	Frozen raspberries	3
Ethelberg *et al.*	2010	Lettuce	6.2 (1-38)
NZ public health surveillance	2011	Oysters at a wedding	8.5 (2.3-31.3)
Baker *et al.*	2011	Oysters	(11.7-inf)
Baker *et al.*	2011	Lamb	(3.8-inf)
Baker *et al.*	2011	Crème brûlée	16.8 (1.3-825.9)
Viriot, D. *et al.*	2011	Late cases oysters	32.22 (7.09-146.34)
Viriot, D. *et al.*	2011	Early cases - oysters	2.68 (1.36-5.27)
Viriot, D. *et al.*	2011	Early cases - knuckle of ham	3.75 (1.91-7.35)
Muller, L., *et al.*	2016	Salmon and leafy greens	7.7 (2.2-27)
Park, J. H., *et al.*	2015	Raw seaweed with vinegar and radish	7.9 (1.1-56.2)
Park, J. H., *et al.*	2015	Seasoned green seaweed with pears	5.1 (1.1-24.8)
Park, J.H., *et al.*	2015	School A: Cabbage kimchi (Lunch)	4.56 (2.96-7.02)
Park, J.H., *et al.*	2015	School A: Spicy potato stew (Lunch)	1.78 (1.05-3.02)
Park, J. H., *et al.*	2015	School A: Cabbage kimchi (Dinner)	1.9 (1.39-2.60)
Park, J. H., *et al.*	2015	School B: Cabbage kimchi	2.26 (1.24-4.15)
Park, J. H., *et al.*	2015	School C: Kimchi	2.10 (1.68-2.63)
Park, J. H., *et al.*	2015	School C: Water	1.56 (1.17-2.08)
Park, J. H., *et al.*	2015	School C: Jajangbap, rice and Chinese bean sauce	3 (1.48-6.09)

Author	Year	Risk factor(s)	odds or risk ratio (95% confidence interval)
Park, J. H., *et al*.	2015	School C: Bean paste soup with tofu	1.23 (1.01-1.50)
Park, J. H., *et al*.	2015	School C: Sweet and sour pork and salad	3.08 (1.59-5.98)
Park, J. H., *et al*.	2015	School C: Tangerine juice	2.55 (1.37-4.74)
Le Guyader, F.S. *et al*.	2008	Oysters	4.5 (1.6-13.3)

Table A5. Settings and foods handled in food handler associated norovirus outbreaks

Food handled	Bakery	Birthday Party	Care Home	Caterer	College	Healthcare Facility	Hotel	Military Base	Restaurant	School	Staff Canteen	Tourists	Wedding
Aemono sauce									1				
Antipasti and garlic mashed potatoes									1				
Bakery products	1	1								1			1
Cold sausage; meat dish with salad and a rolled spinach pancake.						1							
Cold shredded chicken set meal.									1				
Egg mayonnaise; sandwiches.							1						
Mushroom dish													1

Food handled	Bakery	Birthday Party	Care Home	Caterer	College	Healthcare Facility	Hotel	Military Base	Restaurant	School	Staff Canteen	Tourists	Wedding
Paella and beef												1	
Sandwiches							1						
Sandwiches and Vegetation						1							
Seafood									3				
Seafood, lamb and crème brûlée									1				
Seafood, passion fruit and lavender dish									1				
Seafood; spiced chicken and noodle salad				1									
Standard hospital diet						1							
Unknown			1	1	1	1	3		2	2	1		

Food handled	Bakery	Birthday Party	Care Home	Caterer	College	Healthcare Facility	Hotel	Military Base	Restaurant	School	Staff Canteen	Tourists	Wedding
Vegetation				1				1		1	3		
Vegetation, water fountain								1					
Vegetation, Sara udon noodles									1				

Table A6. Food handlers' genotypes

Quarter	Year	GI.12	GI.2	GI.3	GI.4	GI.6	GI.6; GII.4	GII.1	GII.12.g	GII.17	GII.2	GII.2; GII.4, GII.6	GII.3	GII.4	GII.7	GII.9; GII.13	GII.P21	GIIe; GII.4	NA
Q2	1999							1											
Q1	2001																		1
Q2	2002			1															2
Q4	2003												1						
Q2	2004			1			1												
Q3	2004																		1
Q4	2004	1																	4
Q2	2005																		1
Q3	2005										1								1
Q1	2006				1										1				
Q4	2006													1					
Q1	2007													1					
Q2	2007					1								1					
Q4	2007			1										1					
Q4 2008 & Q1 2009														1					
Q1	2009											1		2		1			
Q4	2009													1					1
Q1	2010								1										1
Q3	2010													1	1			1	
Q2	2011																		1

Quarter	Year	GI.12	GI.2	GI.3	GI.4	GI.6	GI.6; GII.4	GII.1	GII.12.g	GII.17	GII.2	GII.2; GII.4, GII.6	GII.3	GII.4	GII.7	GII.9; GII.13	GII.P21	GIIe; GII.4	NA
Q4	2011													3	1				
Q1 & Q2 2012				1															
Q2	2012													1					
Q4	2012													1					
Q4 2012 & Q1 2013														1					
Q1	2013													1					
Q2	2013																		1
Q1	2014																1		
Q3	2014		1																
Q3	2015					1													
Q1	2016									1									1

Table A7. Patrons ill via food handlers' genotypes

Quarter	Year	GI.12; GII.14	GI.2	GI.3	GI.3; GI.4; GI.5, GII.4; GII.6; GII.12; GII.14	GI.3; GI.4; GII.5; GII.8	GI.4	GI.6	GI; GII.3; GII.4; GII.6	GII.1	GII.12.g	GII.17	GII.2	GII.3	GII.4	GII.7	GII.9	GII.e; GII.pe/GII.2	GII.P21	GIIe; GII.4	NA
Q2	1999									1											
Q1	2001																				1
Q2	2002			1																	2
Q4	2003													1							
Q2	2004			1		1															
Q3	2004																				1
Q4	2004	1																			4
Q2	2005																				1
Q3	2005												1								1
Q1	2006						1									1					
Q4	2006														1						
Q1	2007														1						
Q2	2007							1							1						
Q4	2007			1											1						
Q4 2008 Q1 2009															1						
Q1	2009								1						2		1				
Q4	2009														1						1

Quarter	**Year**	**GI.12; GII.14**	**GI.2**	**GI.3**	**GI.3; GI.4; GI.5, GII.4; GII.6; GII.12; GII.14**	**GI.3; GI.4; GII.5; GII.8**	**GI.4**	**GI.6**	**GI; GII.3; GII.4; GII.6**	**GII.1**	**GII.12.g**	**GII.17**	**GII.2**	**GII.3**	**GII.4**	**GII.7**	**GII.9**	**GII.e; GII.pe/GII.2**	**GII.P21**	**GIIe; GII.4**	**NA**
Q1	2010										1										1
Q3	2010														1	1				1	
Q2	2011																				1
Q4	2011														3	1					
Q1 and Q2 2012					1																
Q2	2012														1						
Q4	2012														1						
Q4 2012 and Q1 2013															1						
Q1	2013														1						
Q2	2013																				1
Q1	2014																		1		
Q3	2014		1																		
Q3	2015							1													
Q1	2016											1						1			

Table A8. The odds and risk ratios calculated in studies about food handler norovirus outbreaks

Author	Year	Risk factor(s)	odds or risk ratio (95% confidence interval)
Wadl, M. *et al.*	2010	Salad	8.1 (1.5-45.4)
Zomer, T. P. *et al.*	2010	Eating tomatoes	5.6 (3.2-9.6)
Cai *et al.*	2013	Eating restaurant A	3.46 (1.07-11.16)
Cai *et al.*	2013	Cold shredded chicken set meal	17.82 (4.46-78.17)
Ruan, F. *et al.*	2013	Unknown	12 (5.4-28)
Friedman, D. S. *et al.*	2005	Wedding cake with strawberry filling	9.3 (6.2-13.8)
Baker *et al.*	2011	Oysters	(11.7-inf)
Baker *et al.*	2011	Lamb	(3.8-inf)
Baker *et al.*	2011	Crème brûlée	16.8 (1.3-825.9)
Godoy, P., *et al.*	2016	Eating in canteen	5.8 (1.8-19.3)
Lin, Y.C., *et al.*	2015	Eating a kebab	6.7 (3.4-28)
Sanchez, M.A., *et al.*	2017	Cake	10.1 (1.2-81.6)
Sanchez, M.A., *et al.*	2017	Pizza	3.6 (1.1-11.9)
Godoy, P. *et al.*	2005	Sandwiches	2.3 (1.1-5.1)
DeWit *et al.*	2007	Bread rolls	2 (1.6-2.4)
Hirakata, Y *et al.*	2005	Sara Udon	3.1 (1.1-8.7)
Hirakata, Y *et al.*	2005	Spring roll	2.3 (1.1-4.7)
Hirakata, Y *et al.*	2005	Broccoli	2.4 (1.2-4.6)
Centers for Disease Control	2006	Scalloped potatoes	2.8 (1.1-6.9)
Centers for Disease Control	2006	Chicken	2.2 (1.0-4.8)
Centers for Disease Control	2006	Self-reported direct contact ill people	2.3 (1.0 - 5.0)
Centers for Disease Control	2007	Antipasti platter	2.96 (1.08-8.14)
Centers for Disease Control	2007	Garlic mashed potatoes	4.05 (1.37-11.99)
Schmid, D. *et al.*	2007	Food exposure Wednesday	18.81 (11.82-29.96)
Schmid, D. *et al.*	2007	Food exposure Thursday	2.14 (1.65-2.79)
Schmid, D. *et al.*	2007	Salad	2.82 (1.0-7.94)
Showell, D. *et al.*	2007	Eating salad on day 1.	74 (8-1685)

Author	Year	Risk factor(s)	odds or risk ratio (95% confidence interval)
Showell, D. *et al*.	2007	Eating salad on day 2.	27 (6-138)
Ohwaki *et al*.	2009	Eating standard diet (workers)	18.13 (5.76-57.03)
Ohwaki *et al*.	2009	Eating standard diet (patients)	2.12 (1.05-4.31)
Nicolay *et al*.	2011	Egg mayonnaise sandwich	2.3 (1.4-3.9)
Nicolay *et al*.	2011	Turkey and stuffing sandwich	1.9 (1.2-3.2)
Nicolay *et al*.	2011	Chicken sandwich	1.9 (1.1-3.1)
Schmid, D. *et al*.	2011	Sliced pork with salad	1.8 (1.1-2.99)
Schmid, D. *et al*.	2011	Rolled pancake filled with spinach	1.86 (1.19-2.93)
Smith, A. J. *et al*.	2012	Oyster, passion fruit and lavender dish	7 (1.1-45.2)
Maritschnik, S. *et al*.	2013	Females eating a mushroom dish	2.3 (1.21-4.34)
Ruan, F. *et al*.	2013	Eating delicatessen food from a shop on 14th November	9.7 (2.6-36)
Ruan, F. *et al*.	2013	Eating delicatessen food from a shop on 15th November	8.8 (3.2-24)
Thornley, C. N. *et al*	2013	Italian sushi	3.4 (1.2-9.5)
Thornley, C. N. *et al*	2013	Consuming food prepared manually	6.6 (2.2-39.2)
Thornley, C. N. *et al*	2013	Attending an event before 11.45am	7.2 (24.-43.2)
Kimura, H. *et al*.	2012	Eating on 23rd March	18.1 (9.2-35.4)
Liu, Y., *et al*.	2015	Roasted duck	4.94 (2.01-12.35)
Raj, P., *et al*.	2017	Event two: prawn salad	3.92 (1.39-11.08)
Raj, P., *et al*.	2017	Event two: Chicken simmered in wine	3.92 (1.39-11.08)
Raj, P., *et al*.	2017	Event three: spring rolls	11.52 (4.31-30.79)
Raj, P., *et al*.	2017	Event six: Prawn salad	11.07 (1.33-92.46)
Raj, P., *et al*.	2017	Event six: spicy jelly fish	15.58 (4.41-55.13
Raj, P., *et al*.	2017	Event six: deep fried prawn	5.45 (1.43-20.72)
Smith, K. C., *et al*.	2017	Ham hock	6.62 (2.19-20.03)
Watier-Grillot, S., *et al*.	2017	Shrimp salad	2.6 (1.2-6.0)
Watier-Grillot, S., *et al*.	2017	Pasta salad	2.9 (1.3-6.4)
Centers for Disease Control	2017	Mashed potatoes	2.4 (1.0-5.4)

Supplementary material for work package

Table S1. The databases, dates of their use and number of articles retrieved for the systematic review

Search No.	Date	Database searched	Hits (before duplicate removal)
1	28/07/2017	Medline (OVID)	1920
2	28/07/2017	Embase (OVID)	2265
3	28/07/2017	Web of Science (Science Citation Index Expanded (SCI-EXPANDED)	2229
4	28/07/2017	Biosis previews	1368
5	28/07/2017	CABI (CAB Abstracts® and Global Health®)	862
6	28/07/2017	Scopus	835
7	28/07/2017	Biomed Central	74
8	28/07/2017	Science Direct	121
9	28/07/2017	OpenSigle	35
10	28/07/2017	Proquest Dissertations and theses A&I	65
11	28/07/2017	Foodbase website	68 (separate Word document)-same as previous search
12	28/07/2017	PHE (www.gov.uk)	1 (see separate Word document)-same as previous search
13	28/07/2017	WHO website	18(separate document)
14	28/07/2017	Cefas (Defra.gov.uk)	19 (separate document)-same as previous search
			FINAL NUMBER OF REFERENCES IN ENDNOTE AFTER DELETING DUPLICATES = **3326**

S1. Search strategies

OVID MEDLINE(R) In-Process & Other Non-Indexed Citations and Ovid MEDLINE(R) <1946 to Present>

Search terms:

1 norwalk virus.mp. or Norwalk virus/ (881)

2 norovirus.mp. or exp norovirus/ (3884)

3 small round structured virus.mp. (73)

4 (norovirus or noroviral).ab. or (norovirus or noroviral).ti. (2868)

5 (winter and vomiting).ab. or (winter and vomiting).ti. (148)

6 1 or 2 or 3 or 4 or 5 (4129)

7 limit 6 to yr="2003 -Current" (3354)

8 Foodborne Diseases/ (7844)

9 Food Contamination/ (31122)

10 (foodborne or food-borne).mp. [mp=title, abstract, original title, name of substance word, subject heading word, keyword heading word, protocol supplementary concept word, rare disease supplementary concept word, unique identifier] (15629)

11 (faecal-oral or oro-fecal or oro-faecal).ab. or (faecal-oral or oro-fecal or oro-faecal).ti. (243)

12 (sewage or irrigation or hand* or hotel or restaurant* or catering or cook* or waiter*).ab. or (sewage or irrigation or hand* or hotel or restaurant* or catering or cook* or waiter*).ti. (504425)

13 fomites.mp. or Fomites/ (582)

14 (contaminat* or aerosol* or spray* or toilet* or latrine* or utensil* or kitchen*).ab. or (contaminat* or aerosol* or spray* or toilet* or latrine* or utensil* or kitchen*).ti. (193960)

15 Shellfish Poisoning/ or Shellfish/ or shellfish.mp. (6118)

16 (fish* or mussel* or oyster* or strawber* or raspberr* or lettuce or salad* or vegetable* or green* or fruit* or ice or blueberr* or onion* or tomato*).ab. or (fish* or mussel* or oyster* or strawber* or raspberr* or lettuce or salad* or vegetable* or green* or fruit* or ice or blueberr* or onion* or tomato*).ti. (402656)

17 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 (1086569)

18 6 and 17 (1373)

EMBASE 1947-Present, updated daily

Search terms:

1 norwalk virus.mp. or Norwalk virus/ (3840)

2 norovirus.mp. or exp norovirus/ (3989)

3 small round structured virus.mp. (115)

4 (norovirus or noroviral).ab. or (norovirus or noroviral).ti. (3133)

5 (winter and vomiting).ab. or (winter and vomiting).ti. (242)

6 1 or 2 or 3 or 4 or 5 (4685)

7 limit 6 to yr="2003 -Current" (4121)

8 Foodborne Diseases/ (13308)

9 Food Contamination/ (35621)

10 (foodborne or food-borne).mp. [mp=title, abstract, subject headings, heading word, drug trade name, original title, device manufacturer, drug manufacturer, device trade name, keyword] (10775)

11 (faecal-oral or oro-fecal or oro-faecal).ab. or (faecal-oral or oro-fecal or oro-faecal).ti. (328)

12 (sewage or irrigation or hand* or hotel or restaurant* or catering or cook* or waiter*).ab. or (sewage or irrigation or hand* or hotel or restaurant* or catering or cook* or waiter*).ti. (684705)

13 fomites.mp. or Fomites/ (618)

14 (contaminat* or aerosol* or spray* or toilet* or latrine* or utensil* or kitchen*).ab. or (contaminat* or aerosol* or spray* or toilet* or latrine* or utensil* or kitchen*).ti. (262096)

15 Shellfish Poisoning/ or Shellfish/ or shellfish.mp. (7176)

16 (fish* or mussel* or oyster* or strawber* or raspberr* or lettuce or salad* or vegetable* or green* or fruit* or ice or blueberr* or onion* or tomato*).ab. or (fish* or mussel* or oyster* or strawber* or raspberr* or lettuce or salad* or vegetable* or green* or fruit* or ice or blueberr* or onion* or tomato*).ti. (493471)

17 8 or 9 or 10 or 11 or 12 or 13 or 14 or 15 or 16 (1415428)

18 6 and 17 (1548)

Biosis previews

TOPIC: (norovirus OR norwalk OR winter vomiting OR noroviral) AND TOPIC: (foodborne OR food-borne OR orofecal OR orofaecal OR sewage or irrigation or hand* or hotel or restaurant* or catering or cook* or waiter* OR cruise OR canteen)

CABI (Abstracts® and Global Health®)

TOPIC: (norovirus OR norwalk OR winter vomiting OR noroviral) AND TOPIC: (foodborne OR food-borne OR orofecal OR orofaecal OR sewage or irrigation or hand* or hotel or restaurant* or catering or cook* or waiter* OR cruise OR canteen)

SCOPUS

History Search Terms:

((TITLE-ABS-KEY (foodborne OR food-borne OR orofecal OR orofaecal OR sewage OR irrigation OR hand* OR hotel OR restaurant* OR catering OR cook* OR waiter* OR cruise OR canteen OR contaminat* OR aerosol* OR spray* OR toilet* OR latrine* OR utensil* OR kitchen* OR shellfish) AND SUBJAREA (mult OR agri OR bioc OR immu OR neur OR phar OR mult OR medi OR nurs OR vete OR dent OR heal)) OR (TITLE-ABS-KEY (fish* OR mussel* OR oyster* OR strawber* OR raspberr* OR lettuce OR salad* OR vegetable* OR green* OR fruit* OR ice OR blueberr* OR onion* OR tomato*) AND SUBJAREA (mult OR agri OR bioc OR immu OR neur OR phar OR mult OR medi OR nurs OR vete OR dent OR heal))) AND (TITLE-ABS-KEY (norovirus OR norwalk OR winter vomiting) AND SUBJAREA (mult OR agri OR bioc OR immu OR neur OR phar OR mult OR medi OR nurs OR vete OR dent OR heal))

163 document results

WHO – 9th November 2014, 27 results

Foodborne disease outbreaks: Guidelines for investigation **...**pdf

... Many **foodborne** pathogens (such as **norovirus**, hepatitis A, *Shigella* and *E. coli*) commonly exhibit this mode of spread. **...**
www.who.int/foodsafety/publications/foodborne_disease/outbreak_guidelines.pdf - 1013k

WHO Consultation to Develop a Strategy to Estimate the **...**pdf

... Table 2 - A fully integrated approach to **foodborne** diseases combining syndromic and agent-specific **... Norovirus** • Bacterial toxins • Yersinia sp. **...**
www.who.int/foodsafety/publications/foodborne_disease/fbd_2006.pdf - 298k
[More results from www.who.int/foodsafety/publications/foodborne_disease]

Progress Reportpdf

... include case studies, learning modules, manuals and articles focused on **foodborne** disease topics such as Salmonella, botulism and **norovirus**. **...**
www.who.int/entity/salmsurv/links/GSSProgressReport2005.pdf - 263k

FIVE KEYS TO SAFER FOOD MANUALpdf

... Examples of common dangerous **foodborne** microorganisms include: **...** Parasites -
Giardia, Trichinella; and Viruses – Hepatitis A, **Norovirus**. **...**
www.who.int/foodsafety/publications/consumer/manual_keys.pdf - 109k

WHO GLOBAL SALM-SURV PROGRESS REPORT (2000 **...**pdf

... Articles focused on **foodborne** and other infectious enteric diseases topics such as Salmonella, botulism, and **Norovirus**. **...**
www.who.int/salmsurv/GSSProgressReport2005.pdf - 235k

CAMPYLOBACTERIOSISpdf

... occur more commonly in the general population (**norovirus** incidence is four **...** illness have been published to support WHO's **Foodborne** Disease (8 **...**
www.who.int/iris/bitstream/10665/80751/1/9789241564601_eng.pdf - 569k

Viajes internacionales y Salud (situación a 1 de enero de **...**pdf

... En los úl timos años, los brotes de gripe y **norovirus** han supuesto un desafío de salud pública para el sector de los cruceros. **...**
www.who.int/iris/bitstream/10665/77945/1/680120496_spa.pdf?ua=1 - 2033k
[More results from www.who.int/iris/bitstream/10665/77945/1/680120496_spa.pdf]

WHO | UN strengthens regulations on melamine, seafood **...**

... Common **food**-**borne** viral diseases are caused by hepatitis A virus and **norovirus**. The Commission noted that the main **...**
www.who.int/entity/mediacentre/news/releases/2012/codex_20120704/en - 31k

WHO | New UN food safety and nutrition standards will benefit **...**

... contamination and have been associated with several **foodborne** illness outbreaks caused by viruses (Hepatitis A, **Norovirus**), bacteria (*E.coli*) and **...**
www.who.int/entity/mediacentre/news/notes/2013/codex_alimentarius_20130708/en - 31k
[More results from www.who.int/entity/mediacentre/news]

Communicable disease alert and response for mass **...**pdf

... of special prevention programmes to reduce the risk of **food**-**borne**, water-borne **...**
diseases at the Hajj pilgrimage and the **norovirus** outbreak during **...**
www.who.int/csr/Mass_gatherings2.pdf - 841k

Guidelines for Drinking-water Quality.pdf

Page 1. Guidelines for THIRD EDITION Volume 1 Recommendations WORLD HEALTH ORGANIZATION 2004 Drinking-water Quality Geneva **...**
www.who.int/water_sanitation_health/dwq/GDWQ2004web.pdf - 2560k

Guidelines for Drinking-water Quality.pdf

Page 1. Guidelines for Drinking-water Quality FIRST ADDENDUM TO THIRD EDITION Volume 1 Recommendations Page 2. **...**
www.who.int/water_sanitation_health/dwq/gdwq0506.pdf - 2560k
[More results from www.who.int/water_sanitation_health/dwq]

Emerging Issues in Water and Infectious Disease.pdf

... **norovirus** (formerly Norwalk virus). **...** developed countries Cyclospora cayetanensis has been primarily associated with **foodborne** disease outbreaks **...**
www.who.int/water_sanitation_health/emerging/en/emerging.pdf - 187k

Guide to Hygiene and Sanitation in Aviation.pdf

... Incidents of **foodborne** illness associated with international air travel that are **...**
airport may be advised if diseases of concern (eg **norovirus** or cholera **...**
www.who.int/ihr/ports_airports/guide_hygiene_sanitation_aviation_3_edition_wcov.pdf - 433k

Voyages internationaux et santé.pdf

... Ces dernières années, des flambées de grippe et d'infections à **norovirus** ont posé des problèmes de santé publique importants aux croisiéristes. **...**
www.who.int/ith/ITH2009fr.pdf - 1460k

Waterborne Zoonoses.pdf

... by the WHO units dealing with Water, Sanitation and Health and with Strategy Development and Monitoring of Zoonoses, **Foodborne** Disease and **...**
www.who.int/entity/water_sanitation_health/diseases/zoonoses.pdf - 2560k

Water Recreation and Disease.pdf

... Immunopathogenic aspects of **foodborne** microbial disease. **...** Chronic sequelae of **food-borne** disease. **...** enteric viruses, neither **Norovirus** nor HAV **...**
www.who.int/water_sanitation_health/bathing/recreadis.pdf - 1660k

Opensingle results

Title: Contamination de l'environnement par les norovirus (impacts sanitaires pour l'homme suite à la consommation de coquillages contaminés)

Author: THOMAS, Adeline ;

Thesis advisor: LE GUYADER, Soizick ; DIMIER-POISSON, Isabelle ;

University: Université François Rabelais, Tours ;

Publication year: 2011

Language: French ;

Title: Diarrhoeal disease in children under the age of five in Ho Chi Minh City, Vietnam

Author: My, Phan Vu Tra ;

University: University of Oxford ;

Publication year: 2013

Language: English ;

Title: Infection à Norovirus (évaluation au CHU de Rennes, au cours de l'épidémie de l'hiver 2008-2009)

Author: LE GUILLOU, François-Xavier ;

Thesis advisor: CHA-MINJOLLE, Sophie ;

University: Université François Rabelais, Tours ;

Publication year: 2009

Language: French ;

Pagination/Size: 1 vol., 105 p., Bibliogr. f. 94-105. Référence de site internet p. 103., Illustration ;

SIGLE classification: 06O - Pharmacology, pharmacy, pharmaceutical chemistry ; 06E - Medicine ;

Keyword(s): Pharmacology ; Pharmacologie ; Virus d'entérite ; Entérite virale ; Norovirus ; Épidémiologie ; Diagnostic moléculaire ;

S2. Data collected

Data were collected for foodborne outbreaks using the following categories: name (author), year, journal, title, country, region, date of outbreak, duration of the outbreak, problems accessing the article, who carried out the investigation, how the virus was detected, case definition for both primary and secondary cases, suspected cause, type of study, period of recall, did the study include food handlers, demographics, setting, age, gender ratio, number of people at the event (exposed), number of people at the event contacted, number that responded, Number of cases, number of primary cases, number of secondary cases, number of samples, number of samples positive, single or multiple genotypes, genotypes from cases, genotypes from food, other pathogens in cases, number of cases, number of controls, were there matching criteria, number of cases exposed, number of cases unexposed, controls exposed, number of controls unexposed, confounding factors explored, odds ratios and their confidence intervals, population attributable risks and their confidence intervals and risk ratios and their confidence intervals.

Additional data were collected for food handler associated outbreaks these included: number of kitchen handlers, number of ill kitchen handlers, number of kitchen handlers sampled, number of kitchen handlers sampled that were ill, single of multiple strains from kitchen handlers, genotypes from kitchen handlers, other pathogens found in kitchen handlers and foods handled by kitchen handlers.

4 Work Package 2: Determining the origins of human norovirus RT-qPCR signals

4.1 Summary

The European Committee for Standardisation (CEN) have developed and published an ISO standard method for the detection of human noroviruses (hNoVs) in foods (ISO/TS 15216). Unfortunately, these methods only specifically detect part of the hNoV genome using signals obtained using the reverse transcription quantitative polymerase chain reaction (RT-qPCR) and cannot determine hNoV infectivity; in addition hNoVs cannot at present be grown in culture. This report provides results and a description of progress in the development and application of molecular infectivity assays for determining the origins and significance of human NoV RT-PCR signals, their application to the methods already developed in ISO/TS 15216, and the contribution of food and catering premises to hNoV infections in the UK. The first of these assays is based upon the measurement of the integrity of the virus coat or capsid and is termed the “capsid integrity assay” or CIA. The second assay is based upon a novel approach termed “VPg immuno-capture RT-qPCR” that attempts to measure both the integrity of the norovirus capsid and its genome simultaneously.

Results obtained using the CIA showed excellent agreement in a collaborative blind trial between three different laboratories using six different hNoV positive stool samples belonging to different hNoV genogroups and genotypes. Although most samples showed extensive exposure of capsid-protected RNA following heat treatment, indicative of intact infectious particles, one of the hNoV samples (GII.1) was more resistant requiring increased heat treatment to achieve significant RNA exposure. The hNoV capsid was significantly more stable in water (pH 5.2) than seawater (pH 8.0) or phosphate buffered saline (pH 7.2). The GII.4 hNoV capsid was stable following three freeze thaw cycles at -80ºC, and at 37ºC for 48h showing that the freezing of food and environmental samples is not anticipated to influence capsid stability and that the GII.4 virus capsid is persistent in vitro.

The CIA was successfully applied to the ISO/TS 15216 method for berries. The application of the CIA to shellfish extract using the ISO/TS 15216 method was problematic. The CIA was apparently not compatible with the magnetic RNA extraction commonly used in the ISO/TS 15216 method as the heat treatment within the CIA produced changes to the sample that reduced extraction efficiency. Additionally, RNA exposure was not observed for hNoV probably owing to the low pH (6.0) of the shellfish extract which appeared to stabilise the hNoV capsid. However, the use of sample buffer with an alkaline pH (8.8) and a solid phase extraction method resulted in the successful application of the CIA to hNoV spiked shellfish samples. Further studies are required to investigate the application of this method to naturally contaminated hNoV samples.

Results for VPg immuno-capture PCR have demonstrated proof of principle of this approach. Rabbit polyclonal antibodies have been raised and purified against three conserved VPg peptide sequences present in human and murine noroviruses. Pooled antisera from all three peptide domains has been bound to protein A magnetic beads. The resulting VPg immuno-magnetic affinity beads have been directly used in RT-qPCR reactions and shown to capture RNA preferentially from heated norovirus particles (VPg exposed) compared with unheated samples (VPg not exposed). Furthermore, since VPg is covalently linked to the genomic RNA the resulting RT-qPCR signal must be derived from largely intact genomic RNA (>5000 nucleotides). This preliminary data shows that this novel method can be applied to simultaneously measure capsid and genomic integrity for the first time.

4.2 Application of the Capsid Integrity Assay (CIA) to CEN Methods

4.2.1 Introduction

To comply with developing food safety legislation the European Committee for Standardisation (CEN) have developed standardised methods for the detection of hNoVs (and also Hepatitis A virus) in food and environmental samples; these methods were published as an ISO in 2013 (ISO/TS 15216). These detection methods are based upon quantitative reverse transcription polymerase chain reaction (RT-qPCR) or qualitative RT-PCR. This study reports data from RT-qPCR studies. Unfortunately, these methods cannot determine if the signals obtained originate from infectious or non-infectious virus particles. There is a potential problem in assessing the risk to human health from norovirus contaminated food samples detected as positive in both RT-qPCR and RT-PCR assays and it would be beneficial to determine if these RT-qPCR signals originate from infectious particles (Knight *et al*., 2012).

The objective of Work Package 2 (WP2) of this project is to investigate if it is possible to differentiate between infectious and non-infectious norovirus in food matrices using molecular approaches and to develop a standard operating procedure for with minimal modifications to ISO/TS 15216methods. The first of these is based upon measuring the integrity of the virus capsid using a capsid integrity assay (CIA) (reported in Part 1) and the second (reported in Part 2) is based upon a combined measurement of capsid and genomic integrity (VPg immuno-capture). It is anticipated that this research will allow the development of a standard operating procedure(s) to be used in surveys of fresh produce and shellfish in WP3 and WP4 of this project in parallel to testing using the unmodified ISO methods.

The loss of capsid integrity and infectivity following high heat treatment (typically 70-80ºC for 2 minutes) in RNA viruses is accompanied by exposure of virus RNA from intact virus particles resulting in particles with a decreased sedimentation coefficient

as determined by ultra-centrifugation (termed 80S empty capsids). This exposure of RNA from intact and infectious virus particles can be measured using a combination of RNase treatment and RT-qPCR in a capsid integrity assay (CIA). This observation can be applied to investigate the origins of RT-qPCR signals from intact presumed infectious particles.

RNA within intact particles is protected from RNase but is exposed and digestible by RNase following heat treatment. RNase digestion is never complete owing to residual protein RNA complexes, termed ribonucleoprotein or RNP. Free "naked" RNA is digested by RNase, and RNPs are resistant to both RNase and heat treatment, reviewed by (Knight, *et al*., 2012).

A typical CIA experiment e.g. to determine capsid integrity after heating to 80ºC for 2 minutes, measures RT-qPCR signals before and after heat treatment, with or without added RNase. The RNase is added to ensure that the maximum digestion of any RNA exposed from intact virus following heat treatment is achieved. The CIA requires four different measurements resulting in copy number/Cq determinations and results A, B, C, and D for each sample as shown in Table 1. Absolute copy number determination is not actually required since results are compared within the same experiment resulting in ΔCq values, this minimises errors in RT-qPCR associated with comparisons using separate determinations.

Table 1: Cq or copy number measurements A, B, C and D required for measuring capsid integrity

	Unheated control	**Heat treatment (e.g. 80ºC 2min)**
- RNase digestion	A	C
+ RNase digestion	B	D

Results from A measure the starting copy number. Results from B show that free "naked RNA" is or is not present in the sample when compared with results from A. Results from C show that the heat treatment does or does not affect the starting copy number when compared with results from A. Finally, results from D when compared with results from A show that the heat treatment does or does not result in the exposure of RNase sensitive RNA.

In practice control B is not required and results are identical to A since "naked" RNase sensitive RNA is not present in clinical sample or tissue culture virus lysates owing to endogenous RNase activity, and is likely to be similarly not present in food samples. Additionally, all published literature (based upon RNA transfection studies and RT-qPCR) shows that heat treatment < 90 ºC affects the virus capsid and does not degrade RNA sufficiently to influence small fragment RT-qPCR assays; consequently, control C is also not required absolutely. This means in practice that

the three results from A, B and C are the same. Results from D in comparison to any of the controls A, B or C shows if exposure of RNase sensitive RNA from the intact virus capsid has occurred following heat treatment (indicative of intact virus particles). The purpose of adding RNase is to ensure as far as possible that any endogenous RNase activity goes to completion. Typical results for D obtained from infectious virus particles using surrogate feline calicivirus (FCV) show a Δ 5-6 Cq increase when compared with A, equivalent to a 98% reduction in the starting copy number and corresponding to a predicted > 4 log reduction in infectivity following heat treatment at 62ºC for 2 minutes. Similar reductions were observed for three epidemiologically unlinked GII.4 noroviruses, (Topping *et al*., 2009) resulting in predicted hNoV inactivation following heat treatment at 76ºC for 2 minutes. Based upon published studies, the residual 2 % RT-qPCR signal that remains following heat treatment is resistant to RNase digestion and protected by protein and corresponds to ribonucleoprotein complex (RNP). The persistence and occurrence of RT-qPCR signals derived from RNPs in food and environmental samples is unknown. This study attempts to apply the CIA assay to the methods in ISO/TS 15216 with minimal method modification.

4.2.2 Methods

Following initial assessment by participants, a standard operating procedure (SOP) for the CIA assay was provided by LFR (Appendix 4). hNoV positive stool samples were kindly supplied by Public Health England (PHE) and are listed in Table 2. Unless otherwise stated the CIA used a heat treatment at 80ºC for two minutes. This temperature and time combination was selected based upon LFR published data showing that maximal exposure of capsid RNA required a minimum heat treatment of 76 ºC for 2 minutes for GII.4 hNoVs (Topping *et al*., 2009). RT-qPCR and RNA extraction from shellfish and berries was according to ISO/TS 15216. Unless otherwise stated, for all experimental samples, duplicate subsamples were subjected to treatments/extraction, with each subsample RNA extract then subjected to triplicate RT-qPCR. Results are presented as average Cqs for the six RT-qPCR reactions per sample. Error bars represent the standard deviation from the mean. RNase activity was measured using a commercially available kit (RnaseAlert Life Technologies) as relative fluorescence units (RFU) at 525nm. Lab on a Chip (LOC) capillary electrophoresis (Agilent) was in accordance with manufacturer's instructions and used 1 µl RT-qPCR product. Viable FRNA bacteriophage was enumerated using a double overlay plaque assay method as described in ISO 10705-1.

Table 2: hNoV stool samples provided by PHE

Stool sample	Norovirus genotype
NVREFFS0001	GI.3
NVREFFS0002	GI.6
NVREFFS0003	GI.7
NVREFFS0004	GII.1
NVREFFS0005	GII.4
NVREFFS0006	GII.5

4.2.3 Results

4.2.3.1 Inter-laboratory comparison of the ISO/TS 15216 RT-qPCR method

Independent blind RT-qPCR of hNoV samples using the ISO real-time PCR protocol showed that Cq determinations obtained from the three test laboratories were very similar despite differences in sampling, reagents, equipment, personnel, and extraction methods (Figure 1).

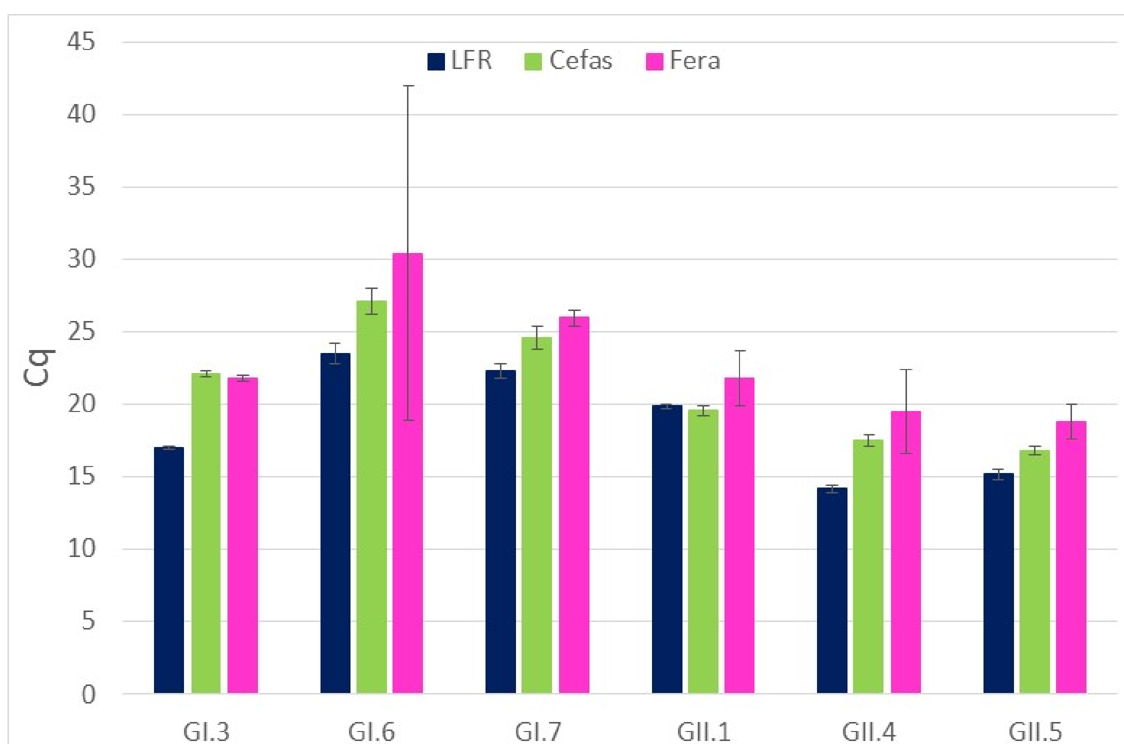


Figure 1: Cq values obtained for the different hNoV samples obtained in the different laboratories at the commencement of this study

4.2.3.2 Inter-laboratory comparison of the CIA method

Each participant evaluated the CIA assay in a blind study according to the supplied SOP using independent stool samples and dilution in PBS to 0.1% (v/v), the ISO RT-qPCR protocol (with DNA standards supplied by Cefas), independent sources of all reagents and different commercially available RNA extraction protocols. A magnetic bead extraction method (Biomerieux) was used by Cefas, and LFR and Fera used a solid phase extraction method Qiagen. Following confirmation by partners during the course of this study control B was not performed in all experiments, since no evidence could be found for the occurrence of “naked” RNase sensitive RNA in any of the samples tested (in agreement with published data).

Results for heat treatment at 80ºC for 2 minutes are shown in Figures 2 & 3. Figure 2 shows the ΔCq values obtained in the different laboratories comparing results obtained following heat treatment at 80ºC for 2 minutes, followed by either addition of RNase and incubation at 37ºC for 15 minutes or RNase buffer alone i.e. comparing results C and D above. The results were very similar between laboratories with 4/6 isolates showing significant RNA exposure (ΔCq values of 5-6 or greater), however two hNoV samples (GI.3 and GII.1) showed minimal exposure.

Overall the comparison of ΔCq values obtained between the different laboratories shown in Figure 2 and 3 was very similar showing that the heating of samples without adding RNase did not affect the ΔCq value obtained. However, the GI.3 sample which showed minimal differences in ΔCq in comparison with the heated control without added RNase (as shown in Figure 2.) shows a significant (>6) ΔCq increase when compared with the unheated control. However, and in contrast, the GII.1 sample showed minimal ΔCq differences upon heat treatment independent of the choice of control.

Figure 2: ΔCq values obtained from a blind inter-laboratory comparison of the CIA using the six different 0.1% (v/v) hNoV samples comparing values obtained following heat treatment at 80ºC for 2 minutes with control values obtained after heating to 80ºC for 2 minutes without added RNase. * denotes a ΔCq > 8 *(simply denotes that following heat and RNase the RT-qPCR signal was abolished and therefore ΔCq could not be measured. The figure of 8 was based on a Cq cut-off value of 40)*

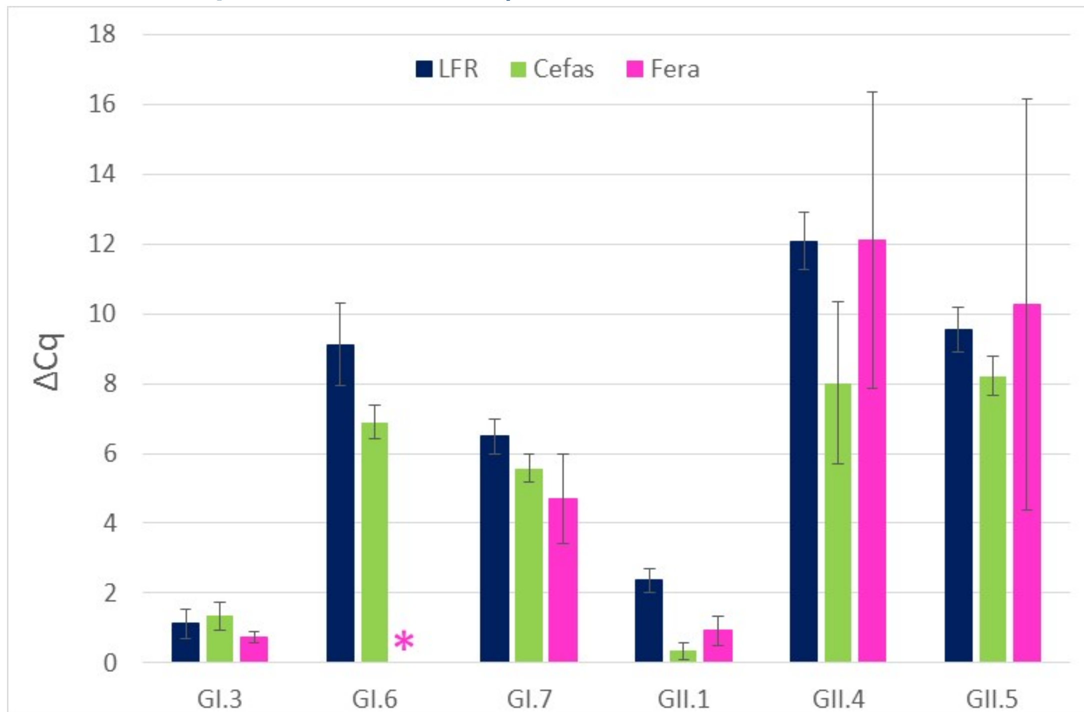
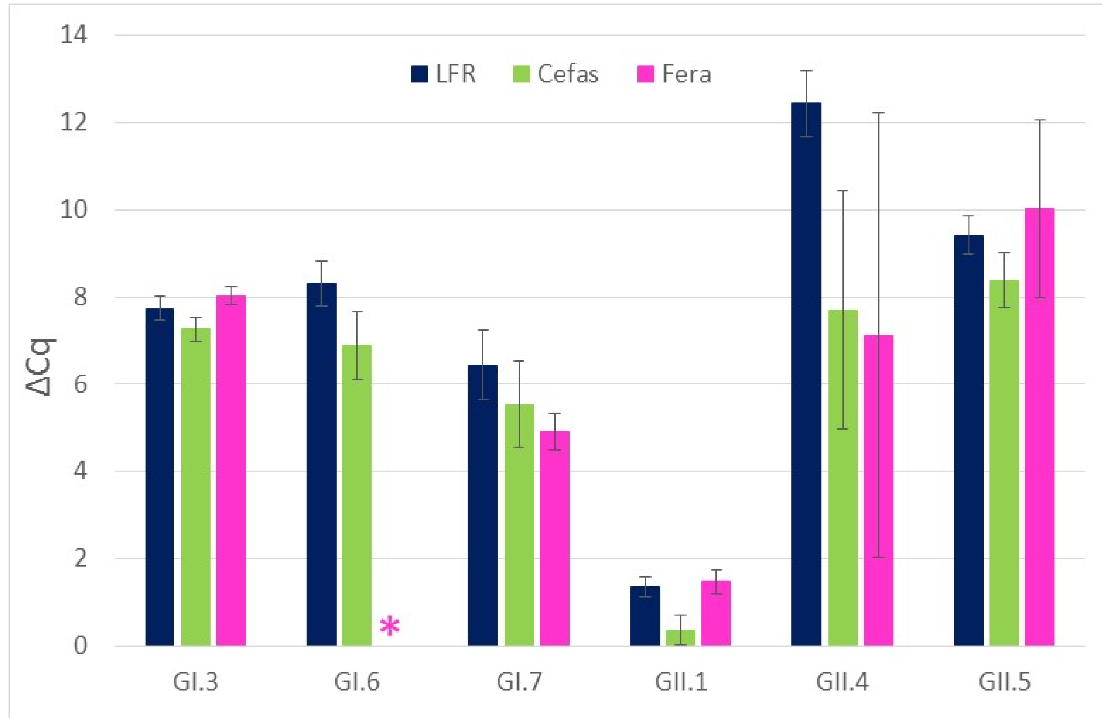


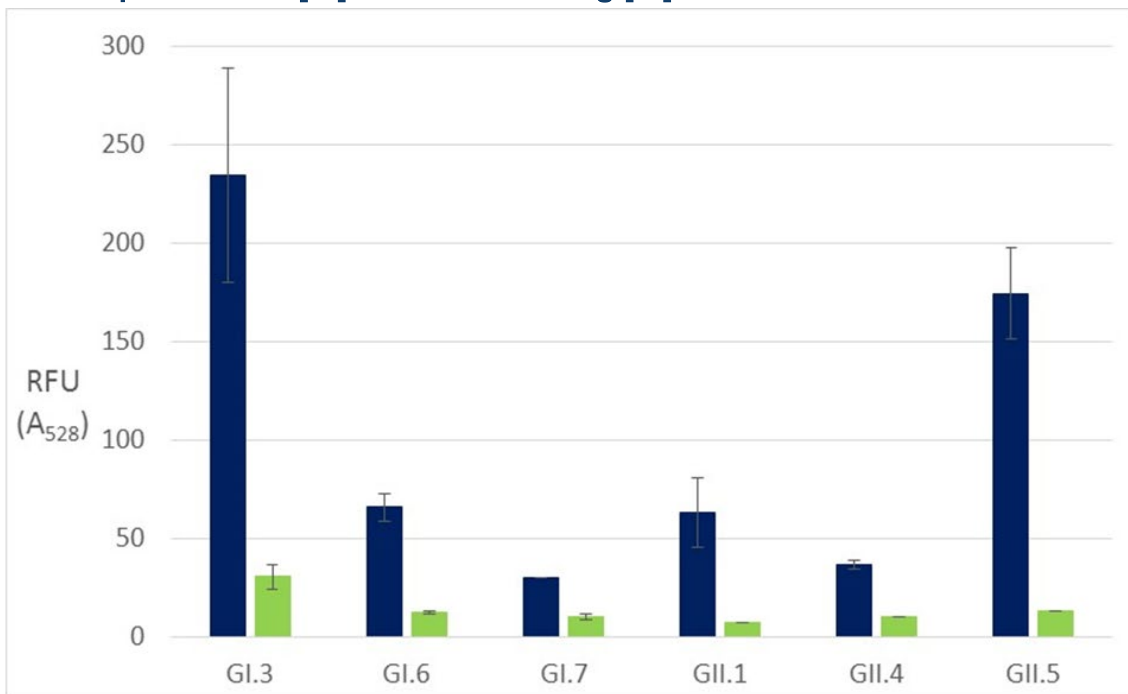
Figure 3: ΔCq values obtained from a blind inter-laboratory comparison of the CIA using the six different hNoV 0.1% (v/v) stool samples and comparing results obtained following heat treatment at 80ºC for 2 minutes with added RNase compared with unheated (4ºC) controls without added Rnase. * denotes a ΔCq > 8.



4.2.3.3 Rnase activity in hNoV samples

The differences in ΔCq values obtained for the GI.3 strain following heat treatment in comparison to an unheated control was considered attributable to endogenous Rnase activity present in the sample. Therefore Rnase activity was measured in the diluted extracts using a commercially available kit (Figure 4).

Figure 4: Endogenous Rnase activity present in the six diluted (0.1% v/v) hNoV stool samples before [■] and after heating [■] to 80ºC for 2 minutes.

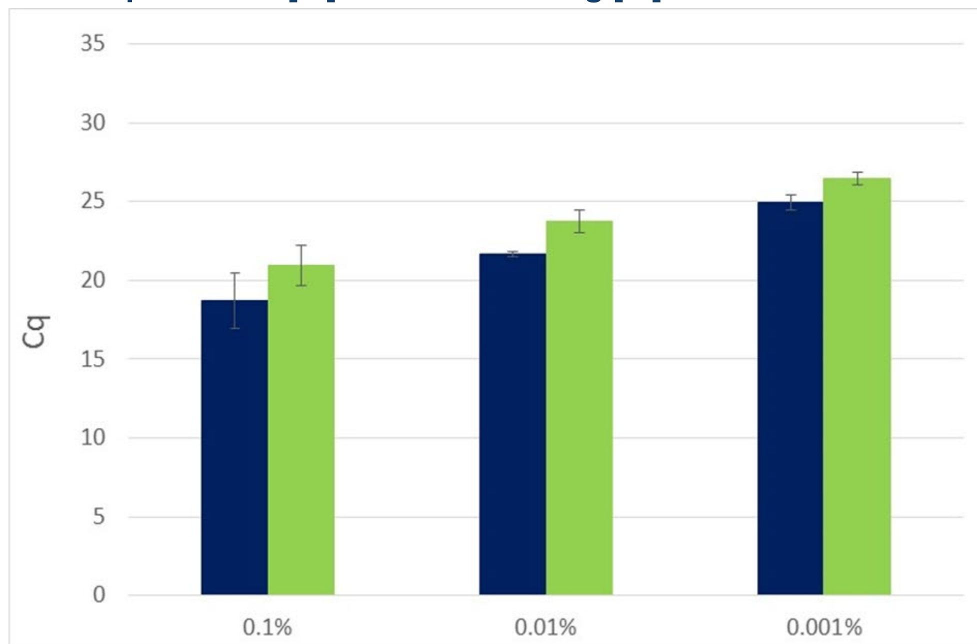


These data also show that data comparison using the CIA only requires a comparison between a heated sample + RNase and an unheated control sample i.e. results from D (typically heating to 80ºC for 2 minutes followed by RNase digestion) vs A (unheated, +4ºC control) this eliminates the need for controls B and C and consequently minimises the impact of sample splitting when testing very low Cq value samples typically found in foods and the environment.

4.2.3.4 Thermal Stability of hNoV GII.1

The apparent failure of the GII.1 sample to expose RNA following heat treatment in comparison to the other hNoV samples suggested that this was owing to either a matrix effect, or an intrinsic property of the virus capsid within this sample resulting in an increased thermal stability of the virus capsid or a potentially false positive result. To investigate any dilutable matrix effect the sample was further diluted and the ΔCq values measured. Figure 5 shows the effect of further dilution of the sample.

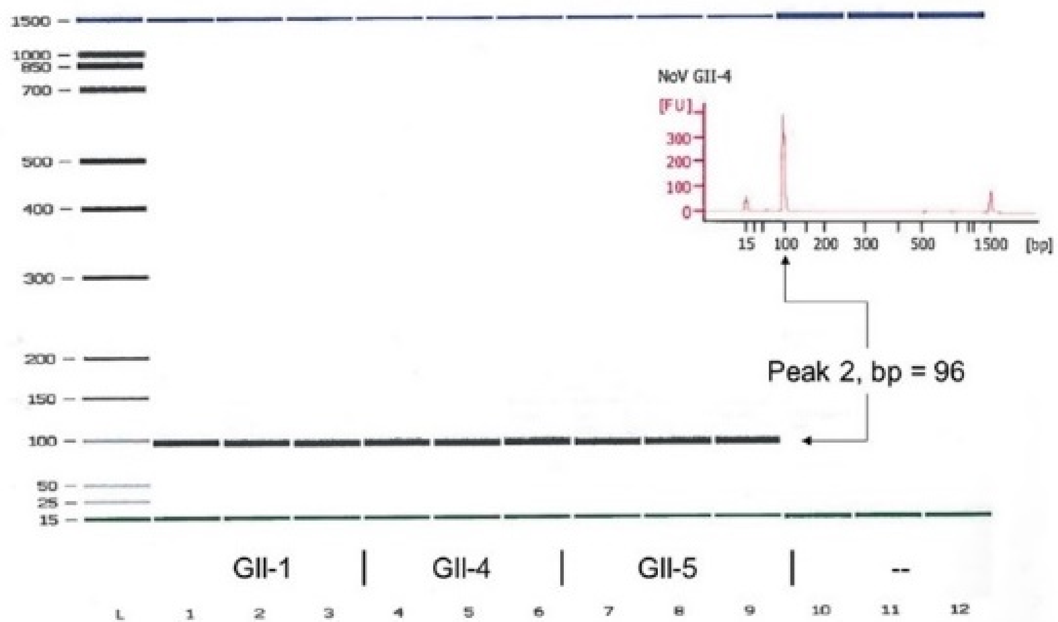
Figure 5: Cq values obtained using the CIA and 0.1 – 0.001 % (v/v) dilutions of GII.1 stool sample before [■] and after heating [■] to 80ºC for 2 minutes.



The data (Figure 5) shows that the ΔCq values obtained using different dilutions of stool samples for the heat resistant GII.1 sample did not change significantly showing that there was not a dilutable matrix effect resulting in increased capsid stability. Additionally spiking the GII.1 with GI.6 hNoV in GI RT-qPCR did not confer a protective effect on the GI.6 sample (data not shown) again showing that no diffusible protective effect was present.

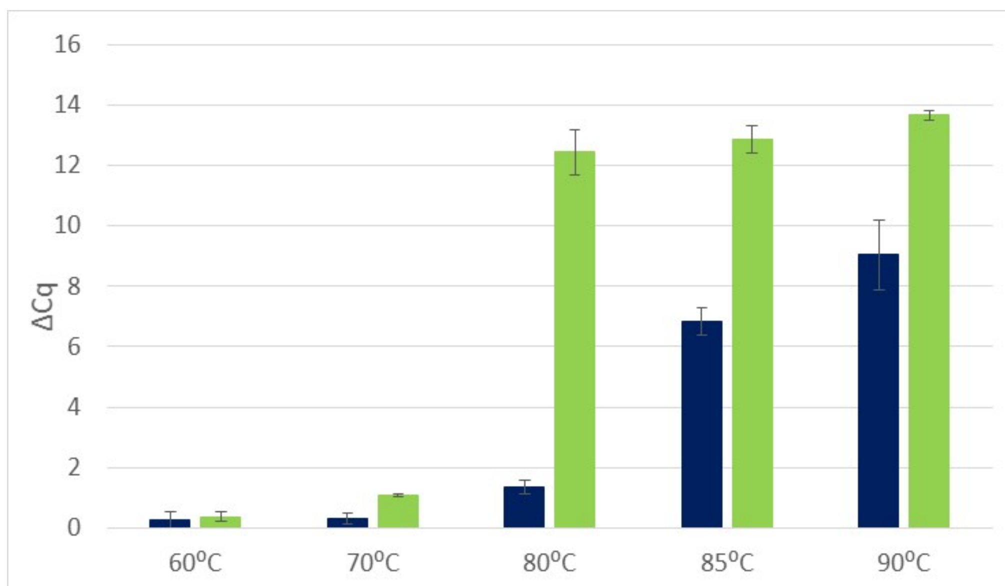
Evidence that the GII.1 RT-qPCR product was of the expected size and not owing to a false positive was provided by Lab-on-a- Chip (LOC) capillary electrophoresis (Figure 6).

Figure 6: LOC capillary electrophoresis of RT-qPCR products derived from GII.1, GII.4 and GII.5 samples.



Finally the thermal stability of the GII.1 sample was compared with that of the GII.4 sample.

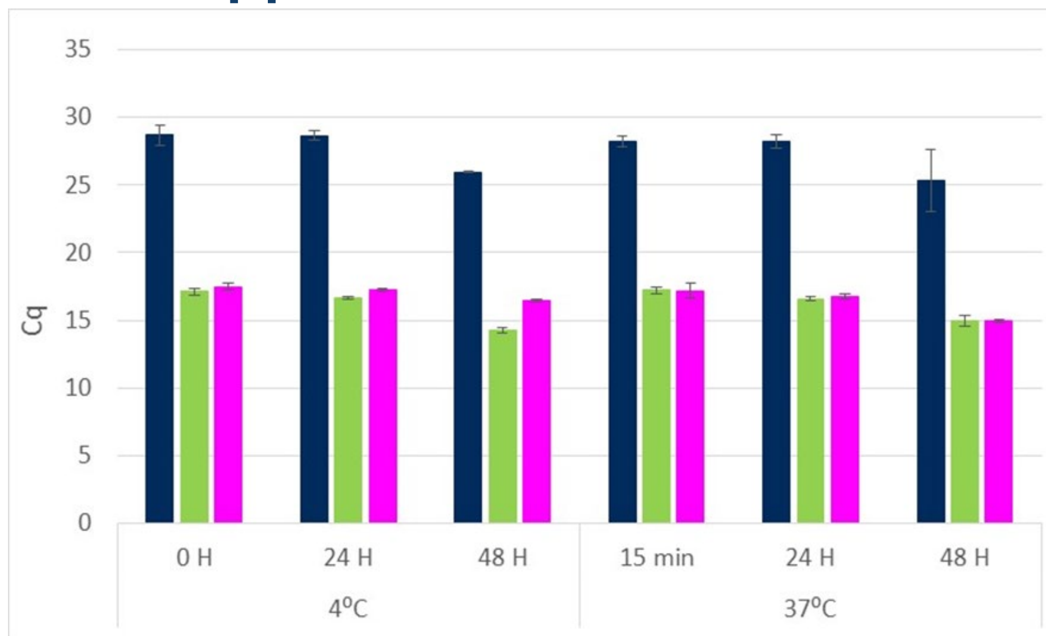
Figure 7: ΔCq values obtained using the CIA following heating at different temperature for 2 minutes for the GII.1 sample [■] in comparison to the GII.4 sample [■].



4.2.3.5 Thermal stability of GII.4 hNoV at 37ºC

Although heat treatment results in the formation of RNase resistant RT-qPCR signals due to RNP there are currently no data to show that RNP occurs naturally. We therefore conducted an accelerated test by incubating 0.1% hNoV in PBS at 37ºC for 48hr in comparison to normal storage at 4ºC. The results are shown in Figure 8.

Figure 8: Accelerated stability testing of GII.4 hNoV at 37ºC, with RNase treatment [■] and without RNase treatment [■] after heating at 80ºC for 2 minutes and at 4ºC [■].



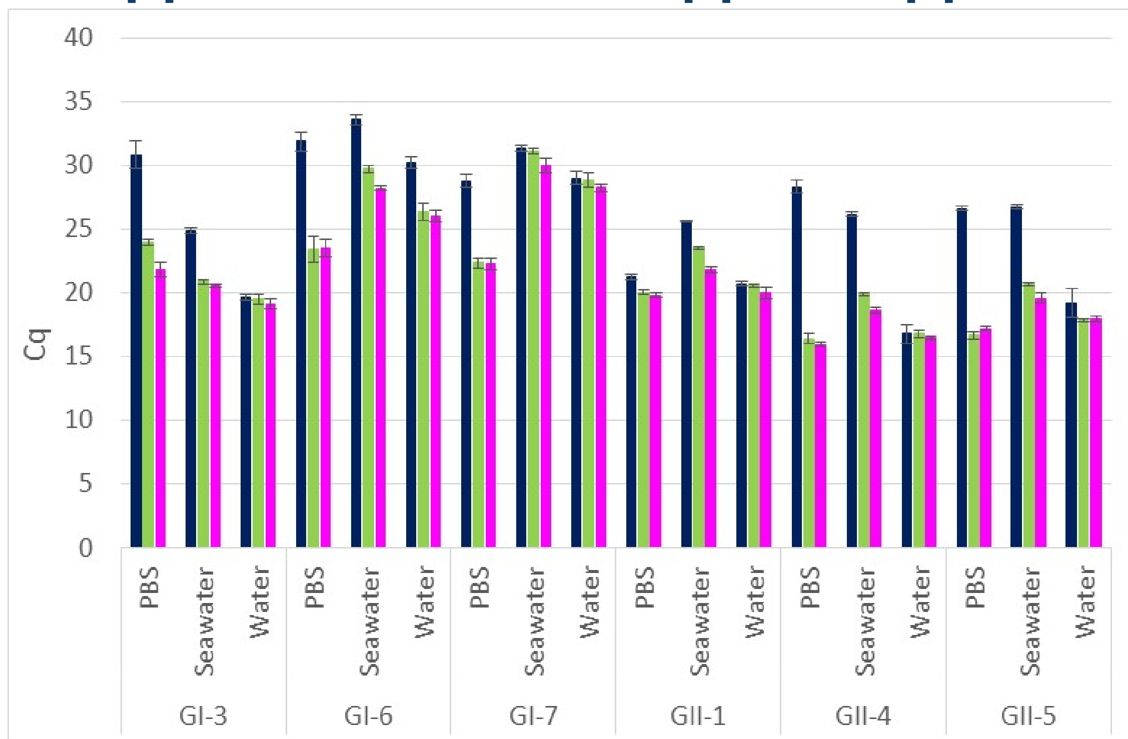
The results (Figure 8) show that the GII.4 hNoV sample was stable at 37ºC for 48h in comparison to the controls kept at 4ºC.

4.2.3.6 Capsid stability in PBS, water, and seawater

Capsid stability was determined in PBS, water, and finally in seawater using the CIA and heat treatment at 80ºC for 2 minutes to assess the impact of simple environmental conditions on capsid stability (Figure 9). As anticipated RNase I activity was unaffected by PBS, water, or seawater (data not shown). For GI.3, GI.4 and GII.5 hNoV samples the capsid protected RNA was exposed when heated in PBS or seawater but was stabilised in water. The GI.6 sample showed exposure of capsid protected RNA in PBS, seawater and to a lesser extent in water. Results for GII.1 did not expose capsid protected RNA at 80ºC in PBS (as already shown at this temperature above) in water or seawater. The GI.7 sample showed exposure of capsid protected RNA in PBS but not in water or seawater; however, this difference was considered attributable to a sample storage effect since repeat analysis in PBS showed an increased starting Cq value with concomitant loss of the ability of the capsid to expose capsid protected RNA. A similar overall effect is seen for the seawater test samples where the capsid exposure was often decreased compared

with PBS but was accompanied by an increased starting Cq suggesting that intact particles had become degraded with concomitant loss of capsid integrity resulting in residual RNP.

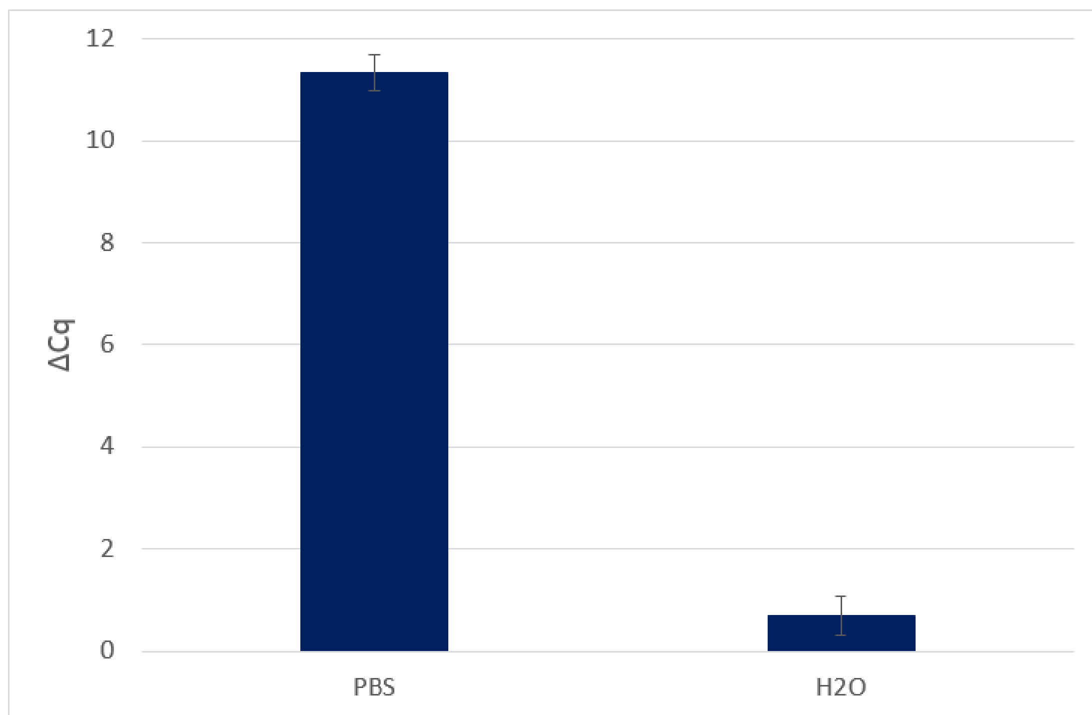
Figure 9: Comparison of hNoV capsid stability in the CIA assay following heat treatment at 80ºC for 2 minutes for 0.1% (v/v) hNoV stool samples diluted in PBS (pH 7.2), artificial seawater (pH 8.0) and water (pH 5.2), heated with RNase treatment [■] heated without RNase treatment [■] and at 4ºC [■]



4.2.3.7 Freeze-thaw stability of GII.4 hNoV

Freeze-thaw stability of the GII.4 hNoV capsid was investigated in water and PBS by repeatedly (3X) freezing 0.01% hNoV samples (100 µl) at -80ºC for 1 hour followed by defrosting at 4ºC for 10 minutes and then testing for capsid integrity using the CIA. The results are shown in Figure 10. The results for PBS show that GII.4 hNoV continues to expose capsid protected RNA following three freeze thaw cycles indicating that the capsid remained intact. However freezing in water appeared to result in a loss of the ability of the capsid to expose RNA suggesting that the capsid had either been damaged resulting in RNP or stabilised by the water. This was further investigated by freeze thawing 10% hNoV stool in water followed by dilution in PBS. The results showed that the GII.4 capsid regained the ability to expose RNase sensitive RNA and therefore that water stabilised the capsid (data not shown) consistent with the observed increased capsid stability when hNoVs were analysed using the CIA following dilution in water (above).

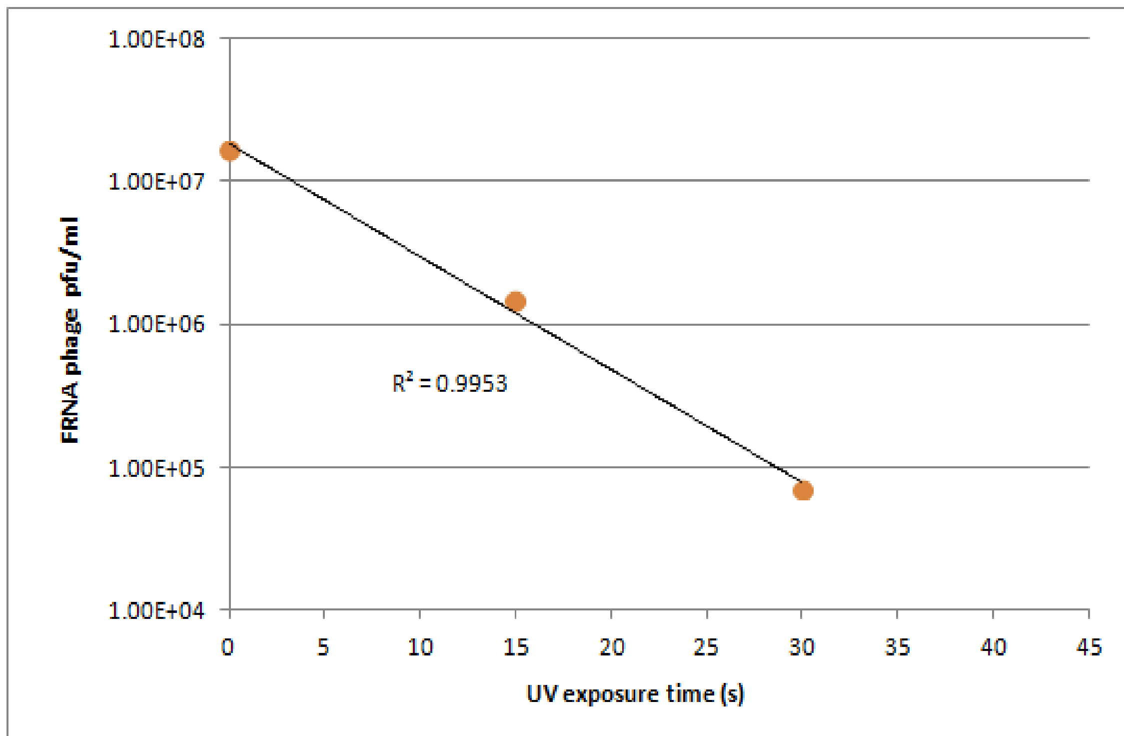
Figure 10: ΔCq values obtained using the CIA following three freeze thaw cycles at -80°C in PBS or water.



4.2.3.8 A comparison of the effect of UV light treatment on MS2 infectivity, MS2 and hNoV capsid integrity

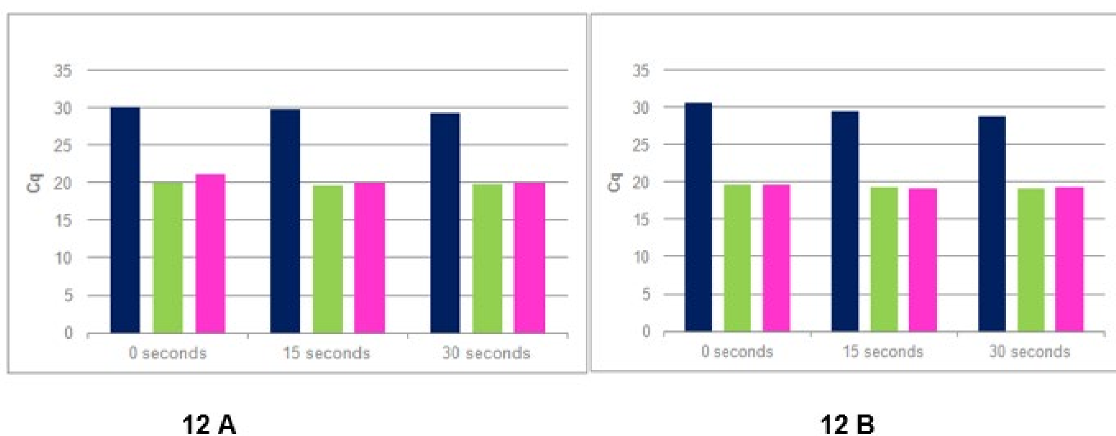
UV treatment is commonly used as a final step in the treatment of sewage effluent before discharge, and UV disinfected virus may therefore contribute to RT-qPCR positive results in bivalve shellfish samples. UV treatment resulting in a 2 log reduction in infectivity for FRNA bacteriophage is considered effective. Experiments were therefore performed to investigate if such UV treatments had any effect on RT-qPCR measurements or CIA results for both FRNA bacteriophage and hNoV. Following calibration experiments to determine appropriate UV exposure times a virus mix was prepared using MS2 strain FRNA bacteriophage and GII.5 hNoV. Portions of this mix were then exposed to germicidal UV light at 254 nm for different time-periods; 0 seconds, 15 seconds (calibrated to achieve a ~1 log reduction in viable FRNA bacteriophage) and 30 seconds (calibrated to achieve a ~2 log reduction). Measurements of viable FRNA bacteriophage in the different portions (using the plaque assay) are shown in Figure 11.

Figure 11: Reductions in infectivity of MS2 FRNA bacteriophage in a norovirus/MS2 mix following a time-course of germicidal UV exposure.



The CIA was then applied to each UV-treated portion of the virus mix. After the CIA separate RT-qPCR analyses for hNoV GII (Figure 12A) and MS2 strain FRNA bacteriophage (Figure 12B) were performed on each RNA extract.

Figure 12: CIA data obtained using 12A) hNoV GII and 12B) MS2 RT-qPCR assays from a norovirus/MS2 mix following a time-course of germicidal UV exposure. For each data set results are shown following incubation; heated with RNase treatment [■] heated without RNase treatment [■] and at 4ºC [■].



12 A **12 B**

4.2.3.9 Effect of the Proteinase K step on extraction efficiency of hNoV from shellfish using the ISO/TS 15216 method

The ISO/TS 15216 extraction method for the purification of RNA from bivalve mollusc and shellfish (section 8.2.6) includes a proteinase K digestion step whereby a proteinase K solution is added to the digestive tissues of the shellfish sample then incubated at 37ºC for 60 minutes followed by a secondary incubation at 60 ºC for 15 minutes. Potentially the use of Proteinase K in this step might expose RNA from initially viable virus particles and therefore distort the results of the CIA if this is subsequently applied to the extract. Experiments were therefore performed at Cefas to determine if this step of the extraction protocol could be modified to accommodate the CIA. Results showed that replacement of proteinase K solution with water only had a minor effect on virus recoveries; no significant increases in norovirus GI and GII Cq values were noted in either spiked or naturally contaminated samples when extracted using water instead of proteinase K. In the experiments below, virus extraction protocols where proteinase K solution was replaced with either water, 1X PBS or 10X PBS were therefore used.

4.2.3.10 Application of the CIA to hNoV in shellfish extracts using magnetic bead RNA extraction

To investigate the application of the CIA to shellfish extracts, Cefas conducted a series of experiments designed to assess capsid exposure in virus extracts from shellfish naturally contaminated with hNoV GI and GII as well as extracts spiked with hNoV and/or MS2 bacteriophage (all results shown in Table 3). RNA extraction used the BioMerieux NucliSens magnetic bead RNA extraction method (included as an informative annex in ISO/TS 15216). Through the course of these experiments minor modifications to the virus extraction and CIA protocols were trialled to neutralise the extract including the use of 1x or 10x PBS as sample diluent in preference to water. In later experiments a centrifugation step (10,000g for 1 minute) was added to the CIA protocol after heating, to clear extracts of the precipitate that had been observed in earlier experiments following the heat treatment of shellfish extract, and which had negatively impacted extraction efficiencies. To compensate for the difficulties with RNA extraction following heating possibly distorting results (artificially increasing ΔCqs), mengovirus was added to all samples as a specific RNA extraction control after virus extraction and CIA. Raw Cq values obtained for the target viruses were then normalised using the mengovirus Cq values prior to determination of ΔCqs (NOTE: in ISO/TS 15216 mengovirus is not used as a control for the efficiency of RNA extraction, but for the entire extraction process, and mengovirus Cq values are not used to normalise results for target viruses). For each set of experiments using spiked MS2 or hNoV a reference ΔCq (average normalised Cq following 80ºC for 2 minutes + RNase I, minus average normalised Cq following 4ºC w/o RNase I) was determined using the spike virus in buffer only (no shellfish extract).

Where hNoV was spiked into shellfish (SF) extracts (experiments 1 and 5) ΔCqs were significantly reduced (range -1.5 to 1.8) compared with the reference ΔCqs (determined with the same virus stock in buffer only, range 7.0 to 9.3). These results indicate that the shellfish matrix for the samples tested (SF1, SF2 and SF8) has a protective effect on the hNoV capsid. For the other five naturally contaminated shellfish samples tested in experiments 2-4, ΔCqs for GI and GII hNoV were also small (maximum 0.5) indicating that the same protective effect may have occurred, although reference ΔCqs cannot be determined for naturally contaminated samples and other virus-related mechanisms leading to low ΔCqs in these cases cannot therefore be ruled out. The low levels of virus present in these samples, coupled with reductions in sensitivity due to the modifications applied to the ISO/TS 15216 method, means that accurate determination of ΔCqs were not always possible.

Interestingly for MS2 bacteriophage, although the protective effect of the shellfish extract on the capsid was observed to some extent in samples SF3 and SF4 in experiment 3 (ΔCqs 1.6 to 3.3 cf. reference ΔCq of 8.4), for samples SF7 and SF8 tested in experiments 4 and 5 no protective effect on MS2 was observed, even though these SF extracts exerted a protective effect on hNoV.

The theoretical possibility that the observed protective effect of the shellfish extracts resulted from inhibition of RNase by substances in the matrix was excluded, both by the observed ΔCqs for MS2 in experiment 5, and by direct measurement of RNase activity on the extracts used in the same experiment (Figure 13).

Taking into account the results of previous experiments on the stabilising effects of low pH on virus capsids, it is possible that the protective effect observed was a function of the acidic pH of the shellfish digestive tissues. In experiments 4 and 5 treatment of the tissues with buffers (1x PBS, 10x PBS) resulted in no observable effect compared with the use of water only. However direct pH measurements on the extracts in experiment 5 (pH 6.2) indicated that even the use of 10 x PBS as diluent was not sufficient to fully neutralise the extract. Further neutralising modifications of the method for preparing shellfish samples for the CIA may therefore eliminate the protective effect of the matrix, and allow further investigation of the application of the CIA to shellfish.

An additional complication with the application of the CIA protocol to SF extracts was observed with the formation of precipitates following heating at 80°C, leading to difficulties with RNA extraction. In most cases heated extracts showed RNA extraction efficiencies that were markedly reduced compared to non-heated extracts, leading to a significant reduction in sensitivity and corresponding problems with determinations of reliable ΔCqs. In experiment 5 a centrifugation step was trialled to remove the precipitate. Although this step was successful in reducing the problems with RNA extraction as measured by mengovirus Cq values, centrifugation also resulted in a reduction in recovery of hNoVs from the samples cf. heat-treatment only

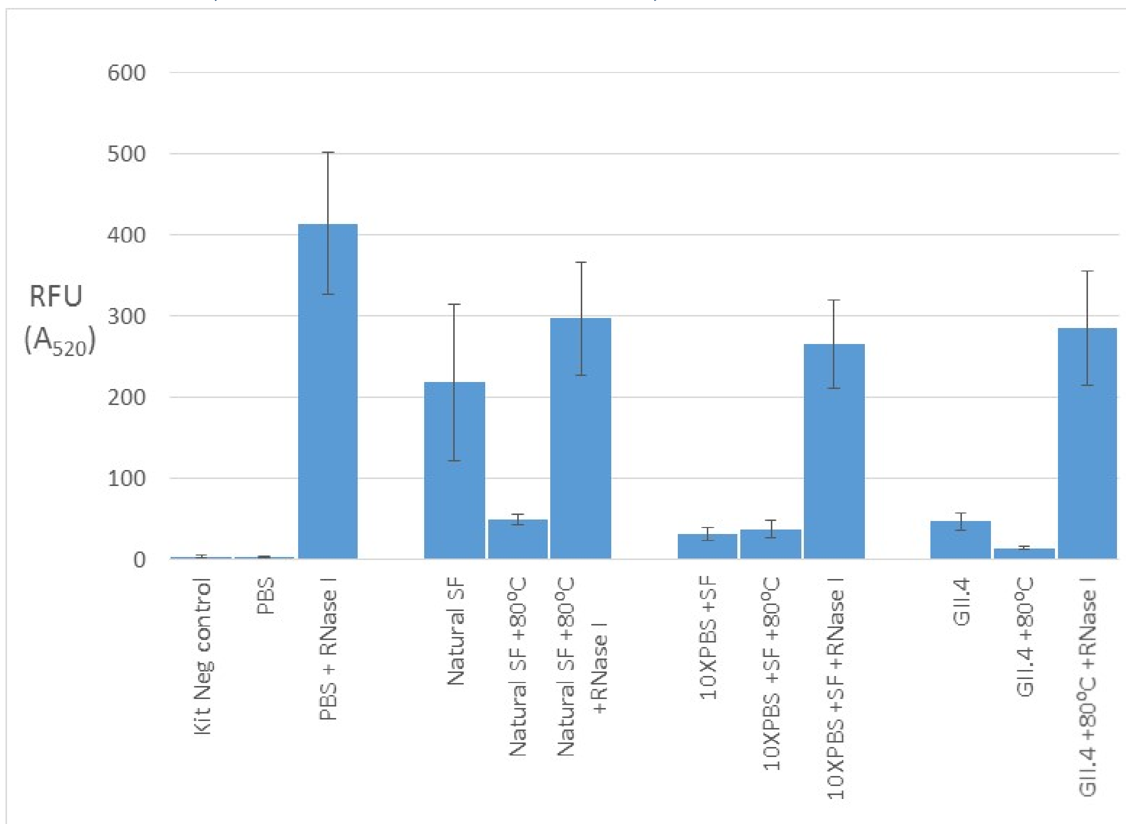
(non-normalised Cq for GII hNoV for 80ºC + centrifugation + RNase = 22.1, for 80ºC + RNase =19.3), presumably due to co-sedimentation of the spiked hNoV particles with the precipitate. Due to this additional reduction in recovery of hNoV leading to lower overall sensitivity, removal of precipitates by the particular centrifugation step as trialled here is not a suitable modification to the extraction/CIA protocol.

If the temperature applied for the heat treatment step is increased as may be necessary to expose RNA from certain hNoV strains it is to be anticipated that the problem with reduced sensitivity due to the formation of precipitates upon heating will be further exacerbated, complicating application of the CIA to shellfish samples.

Table 3: Application of the CIA to shellfish extracts. NC = ΔCq not calculated (one or other result negative), NQ = ΔCq not quantifiable (one or other result less than the limit of quantification), x = no reference ΔCq available (naturally contaminated samples).

Experiment number	Sample number	diluent	reduction in RNA extraction efficiency due to heating	centrifugation after heat treatment?	ΔCq (normalised for extraction efficiency)						source of norovirus
					MS2		GI		GII		
					sample	**reference (no SF extract)**	sample	**reference (no SF extract)**	sample	**reference (no SF extract)**	
Exp 1	SF1	h2o	7.7x	x	-	**-**	-	**-**	0.4	**9.3**	NVREFFS0006 (GII.5)
"	SF2	h2o	3.6x	x	-	**-**	-	**-**	-0.1	**9.3**	
Exp 2	SF3	h2o	4.6x	x	-	**-**	NC	**x**	-3.2 (NQ)	**x**	naturally contaminated
"	SF4	h2o	8.8x	x	-	**-**	0.3	**x**	0.5	**x**	
"	SF5	h2o	14.5x	x	-	**-**	0.2 (NQ)	**x**	NC	**x**	
"	SF6	h2o	1.3x	x	-	**-**	-1.1 (NQ)	**x**	-0.2 (NQ)	**x**	
Exp 3	SF3	h2o	76.4x	x	3.3	**8.4**	-0.7 (NQ)	**x**	NC	**x**	naturally contaminated
"	SF4	h2o	2.6x	x	1.6	**8.4**	-0.2	**x**	-0.3	**x**	
Exp 4	SF7	1 x PBS	1.3x	x	8.1	**7.3**	-0.3	**x**	0.2	**x**	naturally contaminated
"	"	h2o	23.0x	x	6.3	**7.3**	0.4	**x**	0.5 (NQ)	**x**	
Exp 5	SF8	10 x PBS	6.2x	x	7.0	**7.0**	-	**-**	-1.2	**7.0**	NVREFFS0005 (GII.4)
"	"	10 x PBS	none	YES	9.5	**7.0**	-	**-**	1.8	**7.0**	
"	"	h2o	9.0x	x	7.8	**7.0**	-	**-**	-1.5	**7.0**	

Figure 13: Endogenous RNase activity and added RNase I activity in PBS, shellfish extract, shellfish extract in 10X PBS, and in GII.4 hNoV.



4.2.3.11 Optimisation of the CIA for shellfish digestive tissues using solid phase RNA extraction

The results of the direct application of the CIA to the ISO/TS 15216 method in conjunction with the BioMerieux NucliSens magnetic bead RNA extraction method (included as an informative annex in ISO/TS 15216) to hNoV contaminated shellfish and spiked shellfish samples were unsuccessful (above) and showed that further studies were required to:

- Neutralise the pH of the shellfish extract and investigate whether this reduced the protective effect of the matrix
- Minimise the effect of precipitates on hNoV and mengovirus spike recovery from heated shellfish extract

In order to investigate this further the overall approach was to use a different RNA extraction method compatible with the ISO/TS 15216 method (Qiagen, solid phase, extraction) and a high pH sample buffer (7.2 or greater) to allow both improved extraction efficiency and promote capsid instability respectively.

4.2.3.12 pH of Shellfish extract

Cefas prepared and shipped four samples of chopped shellfish digestive tissues (SF1-4) to LFR on dry ice and LFR purchased retail rock oysters and prepared two further samples (SF5-6) according to the same ISO/TS 15216 protocol. pH adjustment of the Cefas shellfish digestive tissue samples was not required since the pH upon delivery was alkaline pH 8.8 (+/- 0.2). In contrast, the pH of the LFR oyster samples was acidic pH 5.9 (+/- 0.2) as normally observed for extracts prepared at Cefas and LFR. This was both fortuitous and convenient since no pH adjustment of the Cefas samples was required. To compare data between Cefas and LFR samples and provide a simple method of adjusting extract to alkaline pH, the LFR samples were adjusted from pH 5.9 to pH 8.0 or 8.8 by the addition of an equal weight for volume of 1M Tris HCl pH 8.0 or 8.8 respectively.

4.2.3.13 Recovery of spiked mengovirus and naturally contaminated GII hNoV and from heat treated alkaline shellfish extracts

Previous experiments (above) had shown that the recovery of control mengovirus was reduced when spiked into already heated shellfish extract. Experiments were therefore performed to investigate the recovery of mengovirus spiked into pre-heated (80ºC, 2 min) alkaline (pH 8.8) shellfish extracts using a solid phase (Qiagen) extraction method. Coincidentally, RT-qPCR data also showed evidence of natural (indigenous) GII hNoV contamination in the shellfish extracts and therefore this approach also allowed the opportunity to investigate the effect of the heat treatment step on the recovery of RNA from indigenous GII hNoV. The results are shown in Table 4.

Table 4: Cq and ΔCq RT-qPCR values obtained for the recovery of mengovirus RNA (spiked with virus after heat treatment) and the recovery of RNA from naturally contaminated (indigenous) GII hNoV positive shellfish samples (SF1-6) following heat-treatment (80ºC for 2 minutes) in comparison to control samples kept on ice (4ºC). Results are the mean of duplicate analysis with triplicate RT-qPCR reactions with the exception of SF1 data which was obtained in singlicate. ND = Not done.

Indigenous GII Norovirus					Spiked Mengo virus				
	Cq 80°C	Cq 4°C	ΔCq	Run No.		Cq 80°C	Cq 4°C	ΔCq	Run No.
SF1	34.26	35.01	-0.75	77	**SF1**	ND	ND	ND	ND
SF2	37.39	38.79	-1.40	80	**SF2**	33.20	37.09	-3.89	80
SF3	38.46	37.96	0.50	80	**SF3**	34.47	36.11	-1.64	80
SF4	38.36	38.91	-0.55	80	**SF4**	29.09	31.50	-2.41	80
SF5	33.62	32.55	1.07	81	**SF5**	25.08	26.22	-1.14	83
SF6	37.34	38.68	-1.34	89	**SF6**	26.73	26.38	0.35	89
Mean			-0.41		Mean			-1.75	
SD			1.00		SD			1.57	

For mengovirus the results from 4/5 independent pooled shellfish samples, showed increased recovery of mengovirus following spiking into pre-heated shellfish extract. The probability that the mean values obtained at 80°C and at 4°C were equal was 0.07 (Student's paired t-test), showing that spiking MS2 into pre-heated extract increased RNA recovery. Although protein precipitate was visible following the heat treatment the results show that, unlike the results obtained using the magnetic extraction method, no significant loss of recovery was evident using the Qiagen method.

For GII hNoV the results from 6 independent pooled shellfish samples showed no significant difference in the recovery of naturally contaminated GII RNA following heat treatment in comparison to controls. The probability that the mean values obtained at 80°C and at 4°C were equal was 0.36 (Student's paired t-test). Results showed that heat treatment did not significantly affect the recovery of indigenous GII RNA signals. Although not statistically significant, results from 4/6 samples showed slightly more efficient RNA recovery following heat treatment which has the potential to lead to an underestimation of capsid RNA exposure.

4.2.3.14 Capsid Integrity Assay Calculator

During the course of this work, Dr James Lowther (Cefas) developed an analytical spreadsheet for calculating ΔCqs and the % exposure of the RT-qPCR target following heat treatment. This was applied to measure the upper and lower limits of exposure of naturally contaminated and hNoV spiked shellfish samples. The calculator incorporated a one-sided unpaired Student's t-test to measure the probability that there was no difference between the mean Cq values for unheated samples and those heated to 80oC for two minutes with subsequent RNase treatment. Analysis of representative hNoV data is shown in Table 5.

4.2.3.15 Application of the CIA to spiked alkaline shellfish extracts

The results for the recovery of indigenous GII hNoV following heat treatment suggested that the CIA could be applied to alkaline shellfish extracts. This was tested in spiking experiments using dilute hNoV stool samples. Dilute GII.4 and GII.1 hNoV stool samples were spiked into alkaline shellfish extracts SF1, SF5 and SF6 to a final concentration of 0.1% v/v and tested using the CIA method following heat treatment at 80ºC for two minutes. The results are shown in Table 5. Recovery of spiked hNoV was comparable to that observed in PBS (data not shown). RNA exposure was obtained for GII.1 and GII.4 in all three extracts.

Table 5: Cq values (uncensored data above) obtained from spiking alkaline shellfish extracts (SF 1, 5 and 6) with either GII.4 or GII.1 dilute (0.1%) hNoV stool. SF 1 and 5 extract was pH 8.0, SF extract 6 was pH 8.8. Results are the mean of duplicate analysis with triplicate RT-qPCR reactions (except for SF1 which was carried out in singlicate). Data was analysed using the CIA calculator kindly supplied by Dr James Lowther (Cefas) to calculate the ΔCq value and the upper and lower limits of RT-qPCR target exposure.

Run	Sample details	slope	intercept	UNCENSORED DATA Set 1 (untreated)						UNCENSORED DATA Set 2 (heat/ RNase treated)						ΔCq censored (a)	t-test	lower limit ΔCq	upper limit ΔCq	% exposed RT-qPCR target (x)	lower limit (y)	upper limit (z)
	SF 1, 5 and 6 spiked with GII.1																					
Run 77	LFR - Shellfish 1 - GII.1 spike - Tris pH 8.0*	-3.205	33.528	20.12	20.82	20.67	20.12	20.82	20.67	25.86	26.11	25.78	25.86	26.11	25.78	**5.38**	0.0000	5.111	5.649	**97.90%**	**97.46%**	**98.27%**
Run 84	LFR - Shellfish 5 - GII.1 spike - Tris pH 8.0	-2.916	35.717	25.66	25.72	25.85	25.8	25.35	25.69	30.11	30.19	30.69	29.83	29.74	29.28	**4.30**	0.0000	3.919	4.671	**96.63%**	**95.47%**	**97.50%**
Run 90	LFR - Shellfish 6 - GII.1 spike - Tris pH 8.8	-3.195	32.832	23.43	23.41	23.71	23.19	23.6	23.49	26.15	25.9	26.27	27.15	26.26	26.84	**2.96**	0.0000	2.585	3.328	**88.13%**	**84.48%**	**90.91%**
	SF 1, 5 and 6 spiked with GII.4																					
Run 77	LFR - Shellfish 1 - GII.4 spike - Tris pH 8.0*	-3.205	33.528	18.04	17.99	17.78	18.04	17.99	17.78	20.99	21.21	21.39	20.99	21.21	21.39	**3.26**	0.0000	3.099	3.421	**90.39%**	**89.21%**	**91.44%**
Run 81	LFR - Shellfish 5 - GII.4 spike - Tris pH 8.0	-3.438	33.123	18.64	18.8	18.98	19.38	19.13	19.53	24.54	24.63	24.74	25.15	25.05	24.88	**5.76**	0.0000	5.448	6.062	**97.88%**	**97.40%**	**98.28%**
Run 90	LFR - Shellfish 6 - GII.4 spike - Tris pH 8.8	-3.195	32.832	15.74	15.72	15.4	18.02	17.31	17.87	25.9	26.47	25.84	26.76	26.82	26.81	**9.76**	0.0000	8.816	10.698	**99.91%**	**99.83%**	**99.96%**

4.2.3.16 Effect of pH on RNA exposure in naturally contaminated and spiked shellfish extracts SF5 and SF6

To further assess the importance of pH, the effect of pH on capsid stability within shellfish extracts was compared. Extracts SF5 and SF6 were tested, since the starting pH of these samples was 5.8 and 6.0 respectively. The sample pH was adjusted by suspending samples either in PBS pH 7.2, 1M Tris HCl pH 8.0 or 1M Tris HCl pH 8.8. Samples were spiked with either GII.1, or GII.4 hNoV (0.1%) dilute stool samples and tested in the CIA in comparison to unspiked controls. Since the unspiked controls were found to contain indigenous bio accumulated GII hNoV this also allowed an assessment of the capsid integrity of hNoV within naturally contaminated GII hNoV samples.

Results in all experiments showed some improved RNA recovery and RNA exposure of spiked GII.4 hNoV at pH 8.0 and 8.8 compared with that obtained in PBS (Figures 14 and 15). Data for spiked GII.1 showed similar RNA recovery in SF5 and SF6 and significant RNA exposure following heat treatment.

There was no obvious exposure of indigenous capsid protected RNA in SF5 when significant (>75%) capsid RNA exposure for both the GII.1 and GII.4 spiked samples were simultaneously observed in spiked samples. The lower ΔCq recorded with the SF5 indigenous sample (1.15) is possibly indicative of the presence of levels of heat/RNase resistant particles (RNPs) in the shellfish that are considerably higher than those seen in clinical samples, but the presence of a significant proportion (>50%) of intact particles cannot be ruled out (however due to the low levels present the confidence in these results is not high). Some evidence of exposure of indigenous capsid protected RNA was apparent in SF6 although this was not significant in comparison to the high Cq values obtained from this sample.

Figure 14: Cq values obtained from CIA data obtained for SF5 comparing RNA exposure of indigenous GII hNoV, spiked GII.1 and spiked GII.4 hNoV (0.1% stool) in PBS and 1M Tris pH 8.0. Results are the mean of duplicate analysis with triplicate RT-qPCR reactions.

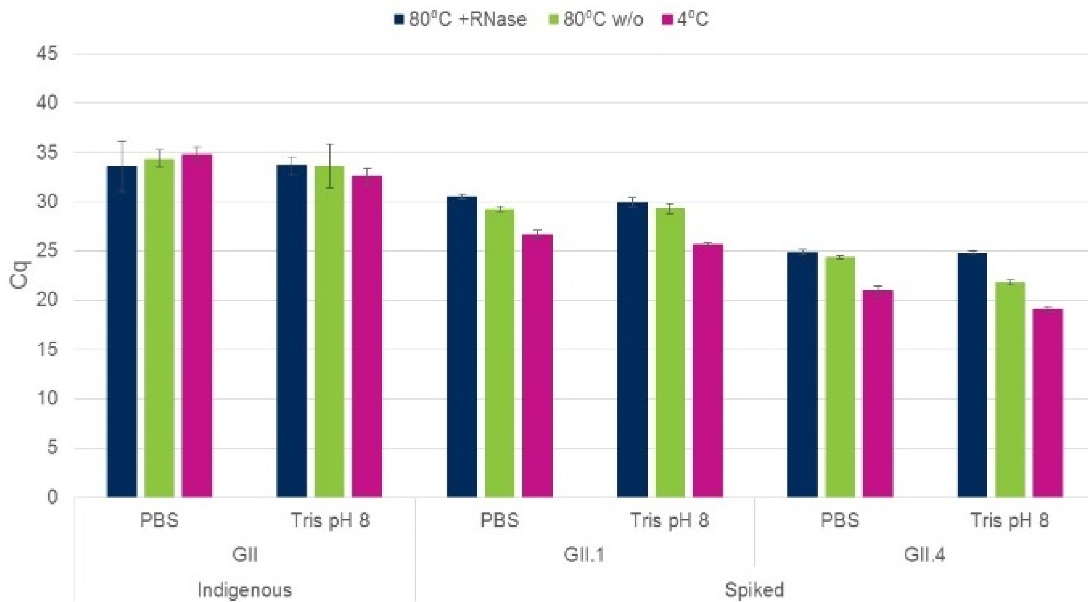
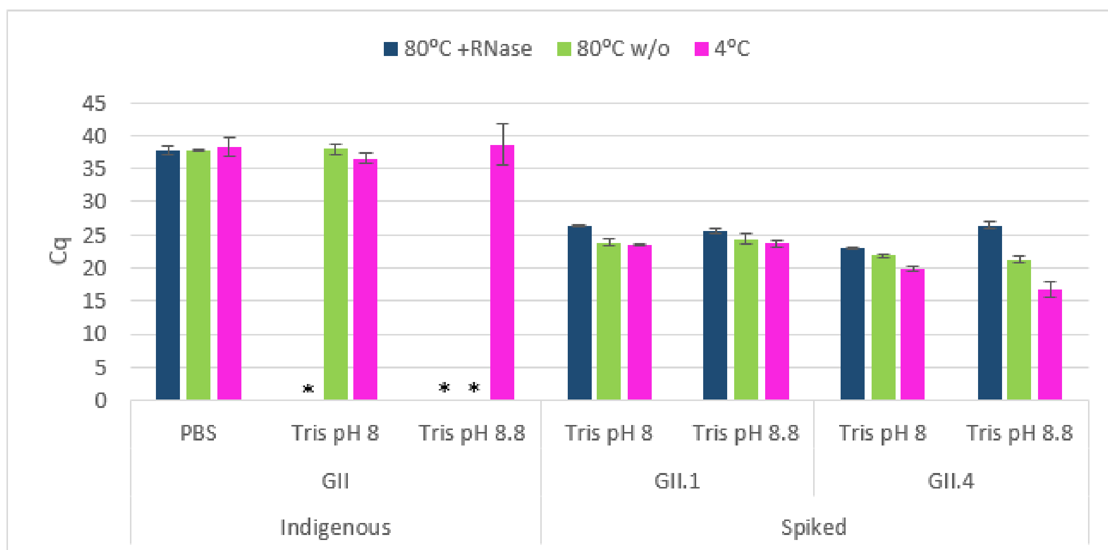


Figure 15: Cq values obtained for CIA data obtained for SF6 comparing RNA exposure of indigenous GII hNoV, spiked GII.1 and spiked GII.4 hNoV (0.1% stool) in PBS, 1M Tris pH 8.0 and 1M Tris pH 8.8, Results are the mean of duplicate analysis with triplicate RT-qPCR reactions. * Cq > 40



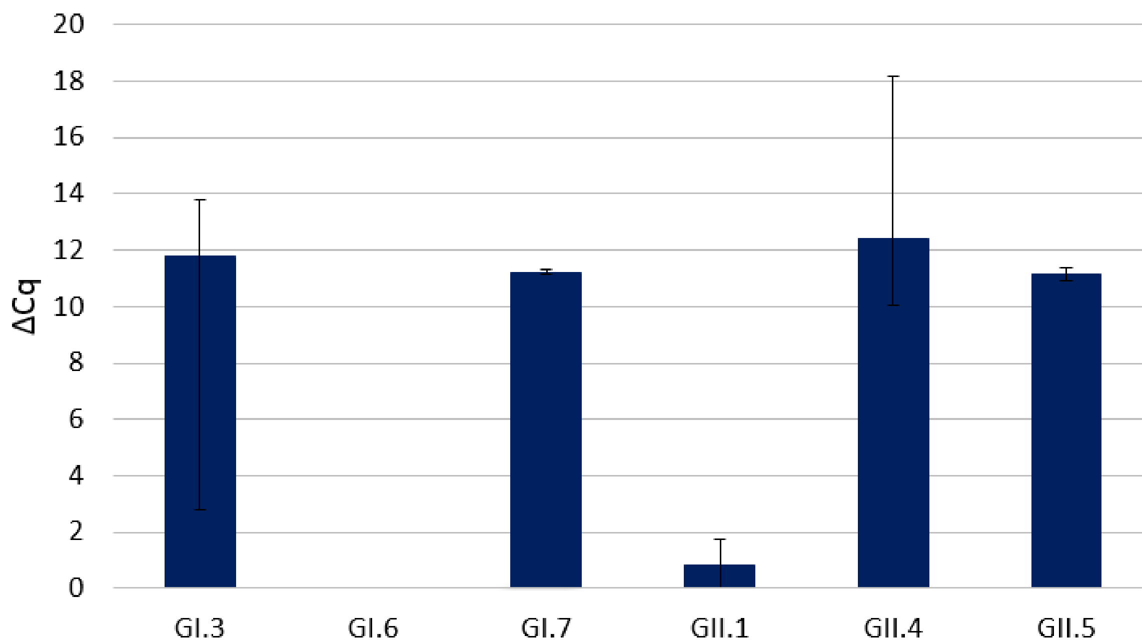
4.2.3.17 Application of the CIA to berries and leafy greens

Re-analysis of faecal extracts containing hNoV strains

This test was performed to ascertain whether any loss of capsid integrity occurred in the hNoV strains through storage in faecal extracts at 4ºC prior to the analysis of raspberries.

The faecal extracts used in the inter-laboratory comparison of the CIA method were re-analysed 3 months after the analysis reported above. This was required to assess any potential storage effects that might influence the analysis of raspberries. The faecal samples had been stored at 4ºC and diluted in PBS to 0.1% (v/v). Figure 16 shows the results obtained. The main difference from the first set of results is that the mean ΔCq obtained from the GI.3 is greater than the first time the CIA was performed on this strain. This may signify that the virus capsids have become more fragile over the period of storage and therefore more susceptible to disruption by the 80ºC treatment. The GI.6 sample appeared to have completely degraded.

Figure 16: ΔCqs obtained from the CIA on 6 hNoV strains 3 months after the inter-laboratory comparison



4.2.3.18 Application of the CIA to raspberries

This test was performed to determine whether the CIA could be applied to NoV extracted from raspberries by the CEN method.

Two samples of 25-30 g fresh raspberries were each spiked with 10 µl of a faecal extract (10 % neat extract in PBS) containing norovirus GII.4. Each sample was extracted following the CEN method. After chloroform: isobutanol extraction, the

extracts were divided into replicate 4 aliquots of 100 µl. Two aliquots of each extract were kept at 4ºC prior to nucleic acid extraction. Two aliquots of each extract were heated to 90ºC for 2 minutes treated, then RNase was added and the extracts incubated at 37ºC for 15 minutes; nucleic acid extraction was then performed. RT-qPCR was then performed in duplicate on all nucleic acid extracts. The results are shown in Table 6.

Table 6: Cq values obtained from RT-qPCR amplification of hNoV GII.4 sequences extracted from spiked-raspberry extracts subjected to the CIA. *No Cq values above 40 were recorded by the thermocycler.

Sample	Treatment	
	4°C +RNase buffer	90°C+ RNase
1	25.8	35.9
	25.7	>40*
	25.7	>40
	25.9	>40
2	26.5	>40
	26.9	37.6
	26.7	>40
	26.8	>40

Cq values obtained from RT-qPCR analysis of each unheated sample were similar. After 90ºC / RNase treatment, ΔCqs from ~10 to >14 were obtained. Following the capsid integrity hypothesis this indicates that the majority of the hNoV particles extracted from the raspberries were intact, and that few RNP complexes were present. The results also demonstrate that the CIA can be applied successfully to raspberry samples, and consequently it will be applied to extracts from retained berries from hNoV-positive samples during the survey in WP4.

4.2.3.19 Application of the CIA after freezing of fresh raspberries spiked with hNoV strains

This test was performed to test whether freezing of raspberries at -20ºC resulted in any loss of capsid integrity of contaminating hNoV strains.

Six samples of 25-30 g fresh raspberries were spiked with 10 µl of a faecal suspension containing hNoV GI.3, and 6 similar samples were spiked with 10 µl of a faecal suspension containing GII.4. Three samples of each spiking type were immediately treated following the CEN method, and 3 samples were placed in a freezer at 20ºC and stored overnight prior to treatment. For each sample, after chloroform; isobutanol extraction the extracts were divided into 2 replicate aliquots of 100 µl. One aliquot was kept at 4ºC prior to nucleic acid extraction. One aliquot of each extract was heat treated to 80ºC for 2 minutes, then RNase was added and the extracts incubated at 37ºC for 15 minutes; nucleic acid extraction was then

performed. RT-qPCR was then performed in duplication all nucleic acid extracts. The results are shown in Table 7.

Table 7: Cq values obtained from RT-qPCR amplification of hNoV sequences following the CIA using fresh or frozen raspberries. *No Cq values above 40 were recorded by the thermocycler.

		Before freezing		After freezing	
Virus	Sample	4ºC + RNase buffer	80ºC + RNase 1	4ºC + RNase buffer	80ºC + RNase 1
GI.3	1	30.0	>40*	29.8	>40
		30.2	>40	29.8	>40
	2	30.6	>40	29.8	>40
		31.3	>40	29.9	>40
	3	30.6	>40	29.1	>40
		30.5	>40	29.3	>40
GII.4	1	28.1	>40	26.8	37.0
		28.7	>40	26.7	37.9
	2	28.7	>40	27.7	35.6
		28.9	>40	27.7	>40
	3	27.8	37.9	27.4	36.7
		27.8	>40	27.4	35.6

Before freezing, a measurable ΔCq was only observed from one replicate of hNoV GII.4-spiked berries. Heating of the fresh raspberry extracts to 80ºC then RNase treatment resulted in undetectable hNoV in all other replicates. After freezing, similar results were obtained for all hNoV GI.3-spiked berries. ΔCqs could however be obtained from most replicates of hNoV GII.4-spiked berries; around 8-10 ΔCq was observed. This could have been the result of improved recovery of virus from the frozen fruit, as seen by the lower Cq values compared to fresh berry samples. It could not be concluded from these result that freezing of raspberries had any measurable effect on hNoV capsid integrity. Then successful application of the CIA in this experiment does provide a demonstration of its applicability to the analysis of raspberries within the project.

4.2.4 CIA Discussion

4.2.4.1 Capsid Integrity Assay

The results of a blind inter-laboratory trial have shown that the CIA method is readily transferrable between laboratories. Results have shown that the assay only requires comparison between an unheated control and a heated sample, RNase-digested. For the first time a comprehensive range of hNoV genogroups and genotypes have been compared simultaneously. The GII.1 sample used in this study appeared to possess a more heat resistant capsid. The reasons for this are not known although the sample matrix did not confer a dilutable protective effect, and the RT-qPCR resulted in a product of the expected size when analysed directly by LOC. It may be

that this effect is owing to virus bound stool specific matrix components e.g. fats, protein, antibody, gastric mucin etc. or that the sample already contains RNP. The CIA cannot distinguish differences associated with matrix effects or the particular strain. These data suggest that the temperature of the CIA may require increasing to 85-90°C in PBS in order to observe capsid RNA exposure in all samples.

The capsid stability of GII.4 hNoVs was retained following three freeze thaw cycles in PBS suggesting that freezing or samples may be useful for the archiving of samples during the attribution survey. Surprisingly no significant capsid exposure occurred upon freezing in water. This result was found owing to an increased capsid stability in water (pH 5.2) that could be regained by diluting the sample in PBS (pH7.2). This was considered most likely a pH effect and was supported by the results of stability studies in seawater (pH 8.0), in comparison to water and PBS.

All the CIA data showed the persistence of RNase resistant RT-qPCR (RNP) signals following heat treatment. As expected, addition of artificially prepared RNP (using heat and RNase treatment) to hNoV present in stool samples reduced apparent RNA exposure – (data not shown). Potentially RT-qPCR signals resulting from RNP might compromise the analysis of survey data since they do not represent infectious particles; however, the natural occurrence of RNPs remains unknown. Attempts to model this by accelerated storage at 37ºC for 48h failed to show any evidence for capsid degradation and RNP formation. The stability trials in PBS, water and seawater (which were performed in that order chronologically) suggested that the ageing of stool samples was accompanied by higher Cq values and reduced capsid integrity (i.e. more RNP). The GI.6 sample used in this study completely lost its RT-qPCR signal upon storage at Fera and also at LFR without evidence for residual RNP RT-qPCR signal. Overall this might suggest that RNP is not persistent but an intermediate in virus degradation.

4.2.4.2 Capsid integrity and UV treatment

UV treatment is commonly used as a final step in the treatment of sewage effluent before discharge, and UV disinfected virus may therefore contribute to RT-qPCR positive results in bivalve shellfish samples. UV treatment resulting in a 2 log reduction in infectivity for FRNA bacteriophage is considered an effective dose. The results of the application of the CIA for MS2 and hNoV from a UV treated virus mix were similar and showed that the capsid integrity of neither MS2 nor hNoV was affected by UV treatment (sufficient to produce a 2 log reduction in MS2 infectivity). Therefore, if UV treatment similarly affects hNoV infectivity (unknown, owing to the lack of an established infectivity assays for hNoV), the CIA cannot be used to distinguish UV inactivated and viable hNoV in shellfish.

4.2.4.3 Application of the CIA to Shellfish extract

Although the potentially problematic inclusion of digestion with proteinase K (as in the ISO 15216 method) was found to be not essential for virus extraction, the application of the CIA to shellfish extract was not successful for hNoV (although the available data does show greater RNA exposure for spiked MS2) using the magnetic extraction protocol). Two significant problems were identified. The first of these is associated with the formation of a precipitate upon heating at 80°C that subsequently reduces extraction efficiency. This is a common problem with measuring virus inactivation in food matrices that is usually reduced by centrifugation. However, the addition of the centrifugation step used in these experiments was not helpful and led to further reductions in recovery. The second problem appears to be associated with protection of the hNoV capsid by the shellfish matrix, likely due to the acidic nature of shellfish digestive tissues (although a more specific protection mechanism associated with shellfish extract cannot be excluded). Attempts to remove this protective effect by neutralising shellfish extract were made by using 10X PBS instead of water as diluent, however the pH of extract produced using this buffer was still <7.0.

Before the application of the CIA to naturally-contaminated and bio-accumulated shellfish could be assessed, the CIA protocol required further modification. In particular, further studies were required to:

- Neutralise the pH of the shellfish extract and investigate whether this reduces the protective effect of the matrix.
- Minimise of the effect of precipitates on hNoV recovery

4.2.4.4 Optimisation of the CIA for Shellfish extract

Following the above conclusions LFR investigated the use of an alternative extraction method on the application of the CIA to shellfish extract samples. The results showed that the optimal protocol for performing the CIA using shellfish extract requires alkaline pH and solid phase (Qiagen) extraction.

Although the original intention was to modify the pH of shellfish extract samples, sent by Cefas, the samples unexpectedly underwent a natural pH change from pH 6.0 to pH 8.8 during transit. The reason for this is unknown but might have resulted from the acidification of extract owing to the dissolution of CO_2 within extracts from the dry ice used in shipment. Dissolution of CO_2 within samples may lower the pH of extracts, allowing solubilisation of shellfish tissue calcium carbonate. Subsequent evaporation of CO_2 on arrival at LFR might then have allowed the extract pH to rise. Experimentally it was found that adjustment of the pH of shellfish extract required the addition of 1M Tris pH 8.8. The use of such concentrated buffer was presumably

owing to the high protein content and very strong buffering capacity of the shellfish extract. There is no suggestion that the change of pH would happen where shellfish are sold in shops and presented on ice. Shellfish extract is acidic (pH 5.9) and this stabilises the capsid as expected for an enteric virus. The extract is highly proteinaceous with a strong buffering capacity so the pH is unlikely to change on ice.

The use of alkaline pH samples improved virus extraction efficiency and capsid RNA exposure in the spiked samples (Tables 4 and 5) in comparison with control samples kept at 4ºC. This may not be surprising in relation to the reported acidic isoelectric point of hNoV VLPs (virus-like-particles) ranging from pH 5.5 - 6.9 (Goodridge *et al*., 2004). Additionally, it is well known that hNoV capsid VLPs are less stable at alkaline pH (Jiang, 1992; Ausar *et al*., 2006; Cuellar *et al*., 2010, Shoemaker *et al*., 2010). Studies at alkaline pH would therefore tend to promote capsid disaggregation, decreasing any virus clumping and also promoting capsid disassembly. The data supports the hypothesis that pH critically affects hNoV capsid stability and that pH for the CIA should be at least pH 7.2 or greater.

Recovery of spiked mengovirus was significantly increased when spiked into previously heated shellfish gut extract samples in 3/5 samples and not significantly different in 2/5. These findings contrast with the findings obtained using magnetic bead based extraction in which spike recovery was reduced in 12/13 samples (above).

Recovery of indigenous hNoV GII was not significantly different following heat treatment (80°C for 2 minutes) from any of the 6 shellfish gut extracts. One possible explanation for the apparent differences between the results of the magnetic extraction method and the solid phase method is that the heat treatment step of the CIA increases protein denaturation and precipitation. Precipitates may interfere with the magnetic bead method by trapping solids, however these precipitates may be removed in the solid-phase (Qiagen) method since this method incorporates a centrifugal filtration and binding step that both filters extract and binds RNA to a silica based column. Direct comparative data would be useful in confirming these data.

Based upon the recovery of indigenous GII and spiked mengovirus there was no evidence that the CIA heat treatment step resulted in reduced RNA recovery either when heat treatment was applied before extraction (indigenous GII) or spiked in preheated samples (mengo). This is a very important consideration since loss of recovery of RNA following heat treatment might be mistaken for apparent capsid RNA exposure.

Norovirus capsid RNA exposure following heat treatment at 80°C for 2 minutes was observed for GII.1 and GII.4 hNoV spiked samples (even without the addition of RNase) when spiked in shellfish extract. In general, the recovery of spiked GII.1 and GII.4 varied significantly between the different extracts (Cq SD 2.6, data not shown).

This variance might have implications for the assessment of hNoV load in shellfish extracts. Total amounts of RNA exposure varied between extracts and was only slightly improved by the RNase step, suggesting that high levels of endogenous RNase activity remained in the extract following the heat treatment.
The observed RNA exposure of both the spiked GI and GII samples within SF5 shellfish gut extracts following heat treatment was not accompanied by obvious exposure of indigenous GII hNoV RNA as seen in clinical samples even though the Cq of the indigenous GII signal was relatively low (Cq = 32-34). However, owing to the limitations qPCR a 50% difference in intact particles compared with RNP can only correspond to 1 Cq value and therefore this limits the sensitivity of the CIA. In order for the CIA to effectively measure capsid integrity the population must be predominantly intact. Alternatively, the absence of exposure of indigenous GII may result from additional protection of the capsid by the shellfish matrix. It is worth noting that the hNoV spikes are taken from samples already present in a stool matrix and is therefore difficult to envisage a more representative spiking material.

The results of these experiments showed that the CIA can be applied to shellfish extract using an alkaline sample buffer and the Qiagen extraction method. Further studies are required to investigate the application of this modified assay using more samples and determine the prevalence of intact hNoV capsids within naturally contaminated shellfish gut extracts. However owing to the limitations of the CIA and RT-qPCR significant exposure will require the testing of relatively low Cq (35) samples in order to attach statistical significance to the results.

4.2.4.5 Application of the CIA to berries and leafy greens

Data obtained by Fera showed that the CIA could be applied to fresh and frozen raspberries when spiked with human GII.4 hNoV.

4.2.4.6 Analytical considerations

In the absence of standards, the accuracy of any analytical method cannot be determined. The assessment of significant RNA exposure i.e. the presence of intact, potentially infectious, particles is most readily determined by comparing duplicate analysis and triplicate PCR for 80°C 2 minutes heat treated + RNase samples compared with duplicate unheated controls. A statistical approach to assessing the significance of the results is shown in the standard protocol below.

A limitation of the CIA assay is that samples must be predominantly intact in order to provide a precise estimate of RNA exposure. If only 50% of particles are intact and the remainder RNP, then this can only result in a difference of 1 Cq value. We can therefore estimate that a significant difference of 3 Cq values between a heat + RNase test sample and a control would require that 87.5% of the RNA population is

derived from intact particles. RNP typically appears as a small proportion (1%) of heat inactivated particles and most likely can only represent a small proportion of any intact particles present. Although the CIA may appear insensitive, in the event that an applicable hNoV tissue culture system was available, (and permissive for all hNoVs) and assuming typical particle to pfu ratios of 10-10,000:1 then only 10% - 0.01% of the total infective virus population could be detected in any event.

Based upon the outcomes of these studies a standard protocol was prepared (Appendix 4). Further studies are required to independently assess this protocol and evaluate data from naturally contaminated samples.

4.3 Application of VPg Magnetic Immuno0Capture RT-qPCR to CEN Methods

4.3.1 Introduction

This report provides preliminary data on the development of a novel assay using VPg magnetic immuno-capture PCR as a novel method for measuring capsid and genomic integrity. This assay was the subject of a feasibility study for the FSA strategic call submitted to the FSA by LFR in 2012.

A limitation of the CIA as a molecular assay for infectivity is its inability to detect genomic degradation, this is important since single genomic lesions inactivate virus particles. A simple approach to the problem of identifying genomic degradation might be to design full length RT-PCR reactions to amplify whole genomes, however PCR efficiency decreases with fragment length and although such reactions have been previously described they are currently insensitive (Kostela *et al*. 2008) and not compatible with the ISO 15216 methods. An alternative approach might investigate the use of novel RT approaches capable of efficient full length cDNA synthesis, followed by small fragment RT-qPCR reactions targeted at the 5' end of the genome. This would ensure that the resulting amplicon is derived from full length cDNA and include a more efficient detection step compared with full length amplification. Although this approach is feasible, and has been previously demonstrated (Wolf *et al*., 2009) it has the disadvantage in that it would require new consensus RT-qPCR assays targeted at the 5' end of the genome and control templates for copy number determination. Such assays would then require extensive validation in conjunction with the already developed ISO reactions. Alternatively, the potential to use 5' RNA hybridisation capture followed by ISO RT-qPCR could allow detection of genomes of a minimum size of 5000b since the ISO RT-qPCR target is located 5,000 bases downstream of the 5' end. This approach (and 5'RT-qPCR assays) is limited by sequence variation within hNoVs at the 5' end of the virus. We have therefore investigated an alternative hNoV RNA capture system capable of isolating full length norovirus RNA from intact particles using a novel approach that is potentially

applicable to all noroviruses. This approach simultaneously detects an intact capsid with the largest size intact genome detectable by the ISO methods following the heat treatment of intact particles.

Noroviruses like certain other RNA viruses possess a covalently attached protein at the 5’ end of the virus known as VPg. This 15 KDa protein is considered to be involved in transcription initiation and binds transcription factor eIF3 (Daughenbaugh *et al*., 2003). Purification of full length RNA by conventional methods necessarily co-purifies this protein (albeit probably denatured) owing to its covalent bonding to the 5’ end of the viral RNA. Intriguingly this protein exhibits a very high level of sequence conservation within human GI and GII noroviruses (68% overall aa identity) and the hNoV surrogate mouse norovirus MNV-1 (54% overall aa identity) but less homology with the alternative surrogate feline calicivirus (18% overall aa identity).

A sequence alignment comparing VPg from MNV-1 and human GI Norwalk norovirus is shown below (Daughenbaugh *et al*., 2006) and shows a very high level of sequence conservation particularly at the N and C termini.

Figure 17: MNV-1 VPg and NV VPg share significant amino acid sequence identity. Sequences encoding MNV-1 VPg and NV VPg were aligned with ClustalW. Asterisks indicate identical residues. Semicolons are conservative substitutions. Amino acid numbers correspond to the position in the ORF1 polyprotein. Reproduced from Daughenbaugh *et al.,* **2006**

```
MNV-1  GK-KGKNKKGRGRPG---VFRTRGLTDEEYDEFKKRRESRGGKYSIDDYLADRER-   921
NV     GKNKGKTKKGRGRKNNYNAFSRRGLSDEEYEEYKKIREEKNGNYSIQEYLEDRQRY  1018
       ** *** ******      *  ***:****:*:** ** : *:***::** **:*

MNV-1  EEELLERDE---------EEAIFGDGFGLKATRRSRKAERAKLGLVSGGDIRARKPIDWN   972
NV     EEELAEVQAGGDGGIGETEMEIRHRVFYKSKSKKHQQEQRRQLGLVTGSDIRKRKPIDWT  1078
       **** * :          *  *    *    ::: :: :* :****:* *** ******

MNV-1 VVGPSWADDDRQVDYGEKINFE   994
NV    PPKNEWADDDREVDYNEKINFE  1100
           ****** *** ******
```

In this study we have raised polyclonal antibodies against conserved VPg peptide sequences and developed magnetic immuno-capture reagents for VPg-linked norovirus RNA in conjunction with detection using the existing ISO hNoV PCR reactions. The use of magnetic RNA binding reagents should minimise the degree of modification to the ISO/TS 15216 method required as the ISO method already recommends (non-specific) magnetic RNA binding reagents for RNA extraction. This approach therefore potentially allows detection of near full length viral RNA following heat treatment of intact virus particles whilst still allowing detection using the already developed and well-characterised ISO RT-qPCR assays. Since VPg linked RNA is only located within intact virus capsid such signals can theoretically only be obtained following heat treatment of intact capsid particles.

One potential difficulty of this approach is that proteinase K is used in the ISO virus extraction method for shellfish (but not other foodstuffs) could cleave VPg from the genome. This could possibly prevent the use of this approach however results from CIA studies reported in 1.2.3.9 showed that proteinase K treatment is not required absolutely for successful virus extraction from shellfish suggesting that modification to the ISO method is feasible. Another possible limitation of the VPg approach may occur for UV inactivated virus particles in that although UV damage to RNA resulting in strand cleavage will be detectable, inactivation owing solely to base dimerization may not.

Using the VPg immuno-capture PCR approach in conjunction with temperature treatment (to expose VPg linked RNA) it should be possible to detect norovirus RNA genomes that are a minimum of 5000 nucleotides in length and that are derived from virus particles that have intact capsids. Detection of such material will provide strong evidence of viability. Additionally, evidence that this approach can be applied using antibodies to selected VPg peptides is already present in the literature (Margis *et al*., 1993; Weitz *et al*., 1986).

4.3.2 Methods

Database searching for conserved peptides was based upon the sequence alignment shown above and optimised for GII and GII.4 hNoV using the BLAST facility available at PubMed.

Polyclonal antibodies to each of three identified conserved peptides were raised in rabbits and protein A purified (Pierce). Since the relative affinity of the antibodies for different peptides and hNoVs was not known equal volume mixtures of antisera were pooled and used for preliminary analysis. Binding of pooled sera used protein A magnetic beads in accordance with manufacturers' instructions (Life sciences). PCR following immuno-capture used 5ul magnetic bead solution directly in RT-qPCR and the CEN protocol.

Human norovirus positive stool samples were kindly supplied by Public Health England (PHE). Unless otherwise stated all experiments were performed at least in duplicate with triplicate RT-qPCR.

Table 8: hNoV stool samples provided by PHE

Stool sample	Norovirus genotype
NVREFFS0001	GI.3
NVREFFS0002	GI.6
NVREFFS0003	GI.7
NVREFFS0004	GII.1
NVREFFS0005	GII.4
NVREFFS0006	GII.5

4.3.3 Results

4.3.3.1 Identification of conserved sequences

Sequence alignment and BLAST searches identified three conserved hNoV peptide regions as:

WADDDREVDYNEKINFE 17aa (NV 1083-1100 above)
LGLVTGSDIRKRKPIDW 17 aa (NV 1058-1075 as above)
GLSDEEYDEYKRIREERNG 19 aa (NV 985-1004 as above)

Searches showed that these peptides were 80 - 100% conserved between human GI and GII noroviruses and in the selected GII samples used in this study. Although the peptides were also highly conserved amongst GI sequences in general, no homologies were identified for the selected GI samples used in this study owing to an absence of available sequence for non-structural proteins within the NCBI database.

4.3.3.2 Development of VPg immuno-capture

Trial experiments have resulted in the development of a starting protocol for the preparation of beads and their use in VPg immuno-capture using dilute (1/100) pooled antisera. Beads were washed and tested to ensure the absence of RNase activity. Control beads without antibody were prepared and treated in the same manner.

4.3.3.3 Testing of VPg immuno-capture PCR

Testing of VPg immuno-capture PCR used 0.1% hNoV dilute stool samples and tested VPg binding following heat treatment at 80ºC for 2 minutes either in the presence or absence of VPg antibody beads. Similar experiments were also performed with whole RNA preparations extracted from the GII.4 sample. Results of preliminary duplicate experiments to test VPg immuno-capture PCR are shown in Table I for the GII isolates used in this study.

Table 9: Results of VPg magnetic immuno-capture PCR using dilute 0.1% hNoV stool samples and purified GII.4 RNA.

	GII.4				GII.1		GII.5	
	Experiment 1		Experiment 2		Experiment 2		Experiment 2	
	Mean	σ	Mean	σ	Mean	σ	Mean	σ
80ºC + VPg Ab Beads	22.36	0.34	25.25	0.97	>38		42.72	0.47
80ºC + Beads control	28.67	0.49	28.71	0.99	>38		30.48	1.22
ΔCq	6.32		3.46				5.75	
RNA + VPg Ab Beads	18.21	1.10						
RNA + Beads control	20.26	0.33						
ΔCq	2.14							
RNA + Ab Beads + RNase I	>38							

Both GII.4 and GII.5 dilute stool samples showed significantly greater binding in the presence of VPg antibody beads than to beads without antibody in all experiments following heat treatment at 80ºC for 2 minutes. Background ΔCq values independent of VPg antibody resulting from non-specific binding was also observed. The GII.1 sample did not show either specific or non-specific binding in the duplicate experiments. Data for purified RNA showed binding to the beads irrespective of the presence of antibody. Bound RNA was completely digested by RNase I showing that the residual Cq values obtained for GII.4 and GII.5 resulted from non-specific virus binding.

4.3.4 VPg Discussion

Preliminary results to date have demonstrated the feasibility of applying VPg immuno-capture to detection of near full length RNA from intact virus particles. Significant differences have been obtained for two GII hNoV isolates demonstrating proof of principle. This method and approach is novel and could be patented.

Available data shows that the VPg immuno-capture method does not bind purified RNA presumably owing to conformational differences between heated and guanidinium denatured epitopes (resulting from the RNA extraction procedure). Additionally, some background binding is evident in the absence of heat treatment, whether this is owing to non-specific binding or RNP within the sample is unknown currently. Intriguingly the GII.I isolate that showed increased temperature resistance in the CIA (Part 1) has failed to bind VPg antibodies in immuno-capture PCR (as might be expected) but also failed to exhibit any non-specific binding suggesting that the virus surface properties of this sample may be different to others perhaps owing to bound stool specific matrix components e.g. fats, protein, antibody, gastric mucin etc. further experiments are required to confirm this data.

The method is potentially quick and simple only requiring heat-treatment followed by VPg immuno-capture PCR and direct RT-qPCR using the capture beads. However,

at present the technique has not been fully tested or optimised and further work is required to:

- Obtain more repeat data and extend studies to more GII and GI samples
- Consider the inclusion of RNase inhibitors
- Investigate optimal antibody selection for capture
- Reduce or eliminate non-specific binding to increase sensitivity and specificity
- Trial and compare the assay using shellfish or berry extract and the ISO methods

In summary these studies have demonstrated proof of principle of a unique assay that simultaneously measures capsid and genomic integrity for the first time. Although it has been levelled that this experimental work is hard to judge because of a lack of details of the exact set-up, it has simply not been possible to provide any further details on antibody production since this was obtained commercially. Further pre-competitive studies are required to develop and evaluate this technology before it is likely that it can be progressed through commercialisation. There is some consensus from peer reviewers of this study that further assessment of the VPg assay to detect norovirus would be an interesting avenue of research to explore.

5 Work Package 3: Acquisition of data on norovirus contamination levels in oysters at retail[4]

5.1 Summary

A one year survey of oysters collected from the point-of-sale to the consumer was carried out from March 2015 – March 2016. A total of 630 samples, originating in five different EU Member States, were collected from 21 regions across the UK using a randomised sampling plan, and tested for norovirus using a method compliant with ISO 15216-1, in addition to *Escherichia coli* as the statutory indicator of hygiene status. Viability testing and Next generation Sequencing (NGS) analysis of a subset of survey samples was also carried out.

Norovirus RNA was detected in a high proportion of samples (68.7%), with a strong winter seasonality noted. Some statistically significant differences in prevalence and levels in oysters from different countries was noted, with samples originating in the Netherlands showing lower prevalence and levels than those from either the UK or Ireland. Overall, levels detected in positive samples were considerably lower than seen in a previous survey of oysters from UK production areas. Investigation of potential contributing factors to this pattern of results was carried out. Application of normalisation factors to the data from the two studies based on both the numbers of norovirus illness reports received by national surveillance systems, and the national average environmental temperatures during the two study periods resulted in a much closer agreement between the two data sets.

The large majority of samples (76.5%) contained no detectable *E.coli*, however in a small number of samples (2.4%) levels above the statutory end product standard (230 MPN/100g) were detected.

5.2 Introduction

Filter-feeding bivalve molluscan shellfish such as oysters, mussels and clams can concentrate microorganisms, including viruses such as norovirus, present in their growing waters as a result of contamination with sewage. The risk to human health from the consumption of such animals, especially when eaten raw or lightly cooked, is well established, with a considerable number of reports of outbreaks in the literature (reviewed in Bellou *et al.*, 2013). Although there is currently good data on the prevalence and levels of norovirus in oysters collected from UK harvesting areas

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[4] This work is published: Lowther JA, Gustar NE, Powell AL, O'Brien S, Lees DN. A One-Year Survey of Norovirus in UK Oysters Collected at the Point of Sale. Food Environ Virol. 2018; 10(3): 278-287.

(Lowther *et al*., 2012), there is no corresponding data for oysters at the point-of-sale in the UK.

WP3 of the NoVAS project aimed to address this critical data gap through a one-year survey of oysters collected at the point-of-sale in the United Kingdom, with the samples analysed using the most up-to-date molecular methods for detection and quantification of norovirus RNA. The data collected was fed into WP6, the overall assessment of foodborne norovirus in the UK.

5.3 Materials and Methods

5.3.1 Sampling Plan

The survey design was informed by a comprehensive practical evaluation of the purchase routes for oysters available to the UK consumer. This evaluation was undertaken by Stericycle during the first phase of the project through phone interviews with and physical visits to identified vendors. This market research was conducted in 21 selected cities/regions of the UK, 10 in England (Devon, Falmouth, Hartlepool, London, Liverpool, Leigh-on-Sea, Manchester, Newcastle, Reading, Southampton), 5 in Scotland (Aberdeen, Dundee/Forfar, Edinburgh, Glasgow, Highlands), 4 in Wales (Aberystwyth, Bangor, Cardiff, Tenby) and 2 in Northern Ireland (Belfast, Londonderry), selected to provide a broad geographical spread of sampling locations. Vendors directly available to consumers of oysters were subdivided into the following types: supermarkets (and similar general stores); fishmongers (including individual stalls within large fish markets); restaurants (including oyster bars etc. serving prepared food); online sales; wholesalers. A total of 373 vendors across these different vendor types were identified across the 21 areas. Following an initial pilot study, a sampling approach was designed to maximise availability of both samples and associated identification marks, namely, direct contact by Stericycle with all vendors explaining the study and seeking cooperation, provision of an explanatory letter from the FSA explaining the objectives of the study and seeking cooperation of the vendor, and the pre-ordering of all samples to ensure availability and cooperation of the vendor.

After reviewing available information on oyster production and consumption in the UK the project steering group felt that the considerable data gaps relating to product destination within the UK and consumer buying patterns found made designing a structured survey challenging. It was therefore agreed that a randomised survey would be most appropriate and provide the most representative coverage of the UK market. A sampling plan was drawn up aiming to obtain a total of 630 oyster samples over a one year period (from 16th March 2015 to 15th March 2016), with monthly targets of 26 samples in the truncated months of March 2015 and March 2016, and

53 or 52 samples alternatively for the months April 2015 to February 2016 with the aim to avoid any introduction of seasonal bias by maintaining a consistent level of sampling by month. For each month, the vendors targeted were selected randomly from a subset of the list of all 373 vendors (supermarkets, fishmongers, restaurants, online sales, wholesalers) with no weighting by region. A list of alternative sampling locations (again selected at random) was made available for each region in the event that oysters were not available at the selected vendor or cooperation was refused. Over the course of the survey any shortfall in sample numbers collected in a given month was compensated by the addition of extra samples (selected at random from the same region for logistical reasons) in the sampling schedule for the following month.

5.3.2 Sample Collection

Samples were pre-ordered through direct contact with the vendor, then collected by Stericycle auditors (sampling officers) from 9am on Monday until 3pm on Thursday inclusive. Samples sourced from online sales were accepted if they arrived with the auditors during the same period. Within each vendor, sample collection was limited to native, Pacific, or other oyster species, sold as either ambient, chilled, or frozen. Animals sold live were sampled as the whole animal. To avoid possible contamination by food handlers live animals in restaurants were obtained before shucking by restaurant staff. Cooked, pasteurized, smoked, or otherwise processed oysters were not sampled. Where multiple products or batches of the same product were available, one was picked at random by the sampler. A sample consisted of individual animals from the same batch (same origin and production date).

Given sufficient availability samples consisted of 25 oysters (with a minimum number of 12 oysters required for a valid sample). At the point of sampling, full sample details including date, time, vendor name and address, product types available, whether product sampled was selected at random, sample type, sample temperature at the point of sale (ambient, fresh, frozen), sample origin/identification mark were recorded by the auditor. If identification mark information was not available at the time of sampling the auditor followed this up with a competent person in the vendor (e.g. the manager) by phone in order to acquire this information. At least 2 attempts were made to acquire the information. A high resolution digital photograph of the sample packaging and identification mark (if available) was taken. This information with accompanying photographs was then e-mailed to the Stericycle project co-ordinator for collation in a sample database.

Samples were packaged in temperature controlled Coleman food boxes with cool packs according to the well-established “Cefas Protocol for sampling and transport of shellfish for the purpose of Official Control Monitoring of classified shellfish production areas under Regulation EC 854/2004” and despatched to Cefas via

overnight courier service to arrive at the laboratory by 10am Tuesday to Friday inclusive. In addition, on despatch to the laboratory, each sample was accompanied by a sample submission form including the Stericycle unique sample identifier (supplied to the auditors in advance), the oyster species, the date and time of collection, the storage temperature of the sample at the collection point and the date and time of despatch. Details of the vendor and the origin of the oysters were not included such that the sample testing was carried out blind.

5.3.3 Sample Receipt

Upon receipt at the laboratory each sample was processed according to standard Cefas microbiological SOPs. The sample temperature was taken and recorded; along with other sample information provided on the sample submission form. Samples were unpacked, cool packs defrosted and the Coleman boxes cleaned and filled with necessary cool packs, address labels etc. ready for return to the auditors.

If the sample temperature on receipt was >18°C, fewer than 10 live animals were available, or the condition of the sample was otherwise unsatisfactory, samples were not tested, and replacement samples were collected. In addition, if the sample temperature on receipt was >10°C, fewer than 20 live animals were available, or a period of >48 hours had elapsed between sample collection and receipt at the laboratory, samples were analysed for norovirus only (not norovirus and *E.coli*); under these circumstances replacement samples were not sought.

5.3.4 Detection and quantification of norovirus

Oyster samples were tested for norovirus according to methods compliant with ISO 15216-1; Microbiology of food and animal feed -- Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR -- Part 1: Method for quantification.

5.3.4.1 Virus extraction

For each sample, 10 oysters were selected. The digestive tissues (stomach and digestive diverticula) of these oysters were excised, pooled, and then finely chopped using a razor blade. A 2g subsample of chopped digestive tissues was transferred to a clean tube and in addition a 1g subsample was retained at -20°C for viability analysis. Ten µl of mengovirus vMC0 tissue culture supernatant was added to the 2g subsample as a within-sample virus/RNA extraction process control. Homogenates were prepared by adding 2 ml of a 100 µg/ml Proteinase K solution to the digestive tissues. This was then incubated at 37°C with shaking at 320 rpm for a duration of 1 hour, and subsequently incubated at 60°C for a duration of 15 min. Finally, the sample was centrifuged at 3000 x g for 5 min, the soluble portion (homogenate)

retained for downstream testing and the pellet discarded. Homogenates were stored at 4ºC prior to testing.

5.3.4.2 RNA extraction

Total RNA was extracted from 500 µl of shellfish homogenate using a NucliSENS® miniMAG extraction machine and NucliSENS® magnetic extraction reagents (BioMerieux) following the manufacturer’s instructions (eluting in 100 µl elution buffer). A negative (water only) extraction control sample was also prepared and tested in parallel with each set of samples extracted. Eluted RNA was stored at -20°C until required.

5.3.4.3 One-step qRT-PCR

For GI, QNIF4 and NV1LCR primers, and TM9 probe were used. For GII, QNIF2 and COG2R primers, and QNIFS probe were used. For mengovirus the mengo 110 and mengo 209 primers, and the mengo 147 probe were used. For both norovirus genogroup-specific assays, 3 aliquots of 5 µl sample or extraction control RNA was tested in 25µl total volume with one-step reaction mix prepared using the RNA Ultrasense® one-step qRT-PCR system (Invitrogen) (final concentrations of 1x Reaction Mix, 500 nM forward and 900 nM reverse primers, and 250 nM probe, plus 0.5 µl Rox and 1.25 µl Enzyme Mix per reaction). For mengovirus two aliquots of 5 µl cDNA were used. Amplification was performed using the following cycling parameters; 55°C for 60 minutes, 95°C for 5 minutes, and then 45 cycles of 95°C for 15 seconds, 60°C for 1 minute and 65°C for 1 minute on an Mx3005P real-time PCR machine (Stratagene).

5.3.4.4 qRT-PCR controls and quantification

For each target virus, three wells containing nuclease free H2O and the above qPCR reaction mixes were included on each plate as a negative control. All samples were assessed for extraction efficiency by comparison of sample Ct values for mengovirus with a standard curve generated from the process control material. Samples were in addition assessed for RT-PCR efficiency/inhibition using RNA external controls. Briefly, a 1µl volume containing a high concentration of GI or GII RNA sequences (produced by in vitro transcription from the control plasmid) was added to an aliquot of sample RNA in addition to a 5µl aliquot of water in a separate well. The percentage RT-PCR efficiency for each sample and each genogroup was determined by comparing the Ct values for the sample RNA plus external control RNA with that for the water plus external control RNA. Quantification followed the principles outlined in ISO TS/15216-1. Log dilution series (range 1x105 to 1x101 copies/µl) of linear dsDNA molecules carrying the GI and GII target sequences were included on each qRT-PCR plate to generate a standard curve. The slope and r2

values of the standard curve were assessed for acceptability according to the parameters detailed in ISO 15216-1. Where the curve was acceptable (slope between -3.1 and -3.6, r2 >0.99), for each qRT-PCR replicate for the sample RNA under test a quantity in copies/µl was determined. Negative replicates were given a quantity of zero. The average quantities from the three replicates in each norovirus genogroup-specific qRT-PCR assay were calculated to give an overall quantity in detectable genome copies/g digestive gland. Results were not adjusted for losses during processing or RT-PCR inhibition. Samples were retested if extraction or (RT-)PCR efficiencies fell below action thresholds (1% and 25% respectively) determined as part of the CEN/ISO method standardisation exercise, where positive (RT-)PCR controls indicated reagent failure, or for any positive sample where the negative extraction or PCR controls showed contamination.

5.3.4.5 Statistical Analysis

For statistical analysis and calculation of geometric means as presented in this WP positive results of <100 copies/g (the limit of quantification of the assay) were scored at 50, and not detected samples were scored at 20. Scores for GI and GII were combined prior to analysis. In this way samples that were not detected for both genogroups scored 40 copies/g, and this figure should be considered a baseline for levels. For analysis in WP6 uncensored quantities were used for positive samples and not detected results were scored as zero. Confidence intervals (95%) for datasets were calculated as the geometric mean ± 2x the geometric standard deviation; at the lower end these are censored at 40 copies/g where the calculated value was less than this. Due to the large number of censored values in the dataset, non-parametric statistical tests were used throughout.

5.3.5 Detection and quantification of *Escherichia coli*

Oyster samples were tested for *E. coli* according to ISO/TS 16649-3; Microbiology of food and animal feeding stuffs -- Horizontal method for the enumeration of beta-glucuronidase-positive *Escherichia coli* -- Part 3: Most probable number technique using 5-bromo-4-chloro-3-indolyl-beta-D-glucuronide. Whole animal homogenates were prepared from the flesh and intravalvular fluid of 10 oysters and assayed using a most-probable-number (MPN) method. Results are expressed per 100g of shellfish flesh and intravalvular fluid.

In the case of any sample *E. coli* result exceeding 230 MPN per 100g shellfish flesh, these results were reported on the same working day to named contacts at the FSA by email.

5.3.6 Capsid Integrity Assay

The Capsid Integrity Assay (CIA) as described in Appendix 4 was applied to all retail survey samples where results for either norovirus genogroup were >500 copies/g. These relatively high levels are required for the CIA as quantitative results in the untreated samples are required to determine a ΔCq value, while compared with the standard detection methodology, the sensitivity of the CIA is lower as an effectively 5x smaller quantity of shellfish digestive tissues are subjected to extraction. For each sample only the genogroup(s) with results above this threshold were tested.

5.3.7 Determination of ssRNA virus viability ratios using bacteriophage

Considering the possible limitations of the CIA an alternative approach to estimation of viability using bacteriophage was also utilised to gain insight into the potential for shellfish samples to contain viable virus. The method development and analysis was not funded by the NoVAS project however the FSA kindly gave permission for the method to be applied to the NoVAS samples. With agreement of the FSA the method and data is presented here since it may provide useful information and assist interpretation of the NoVAS samples.

Viability ratios for a subset of retail survey samples (selected at random from the entire set) were calculated by a comparison of PCR-detectable and viable levels of levivirus genogroup II F-RNA bacteriophage ("GA phage").

PCR-detectable levels of GA phage were determined using qRT-PCR using the same RNA extracts and methods as used for detection and quantification of norovirus, substituting GA-specific primers and probes (Wolf *et al*., 2008).

To determine viable levels of GA phage, a double overlay plaque assay (Doré *et al*., 2000), with modifications to enable use of digestive tissues instead of whole animals, was used to grow and enumerate total viable F-RNA bacteriophage (genogroups I, II, III and IV levivirus/allolevivirus) from each sample. Where the sample was positive for total F-RNA bacteriophage, GA phage was specifically enumerated by transfer of plaques to Hybond N+ nylon membranes as described in Flannery *et al*. 2013, followed by hybridisation using a GA-specific digoxygenin-labelled oligonucleotide probe (Beekwilder *et al*., 1996). Results were expressed in pfu/g (plaque forming units per gram of digestive tissues).

The viability ratio was determined by dividing the viable level of GA phage by the PCR-detectable level of GA phage and expressing as a percentage. Where GA phage was detected by qRT-PCR but not using the viability method a ratio was calculated by applying a censored value of 1.5 pfu/g digestive tissues for the viable level (the limit of detection of the assay). In these cases the viability ratio determined was regarded as a maximum. Where the viable level was greater than the PCR-

detectable level the ratio was capped at 100%. Where GA phage was not detected by either qRT-PCR or the viability method, a ratio could not be determined.

5.3.8 Next Generation Sequencing (NGS)

An RNA metagenomic sequencing approach was applied to a subset of retail survey samples with the aim of firstly independently confirming the presence of norovirus in samples, and secondly of attempting to characterise the diversity of norovirus present. Independent confirmation is possible since the metagenomic approach does not utilise the qRT-PCR amplification primers used in the primary analysis. However, both confirmation and characterisation of diversity are dependent on the sensitivity of detection that can be achieved using a metagenomics approach which was known to be a limiting factor, in comparison to qRT-PCR, prior to the commencement of the study.

RNA extracts from samples with high norovirus levels as determined by qRT-PCR were subjected to an initial quality screen for concentrations of total RNA using nucleic acid fluorimetry (using a ThermoFisher Qubit fluorometer). Those samples with sufficient levels of total RNA for downstream applications were then subjected to ribosomal RNA depletion using the Ribo-Zero Gold Epidemiology Kit (Illumina) to enable sequencing capacity to be focused on more informative parts of the RNA metagenome.

rRNA depleted samples were subjected to DNase treatment using DNA-free™ (Life Technologies), then library preparation with the ScriptSeq™ v2 RNA-Seq Library Prep Kit (Illumina). Prepared libraries were then loaded onto a MiSeq v3 600 cycle cartridge (Illumina), and subjected to RNA metagenomic sequencing using the MiSeq NGS platform (Illumina).

Post-run, data generated for each sample was subject to quality control and bioinformatic analysis using a pipeline comprising analysis software for quality control (FastQC, Trim Galore!), read mapping (bwa mem, Qualimap), sequence assembly (SPAdes) and taxonomic classification (Diamond/Centrifuge, Pavian). To specifically check for norovirus sequences, the full set of sequence contigs generated from each sample were blasted against a set of 38,249 norovirus sequences downloaded from NCBI on the 9th March 2017 using blast+ software.

See More on Next Generation Sequencing in this chapter for more detail and discussion on NGS.

5.4 Results/Discussion[5]

Over the course of the one-year survey 16th March 2015 to 15th March 2016 a total of 646 oyster samples were received by the Cefas laboratory, of which 630 were subjected to laboratory testing for norovirus (the original target number). The other 16 samples were rejected primarily due to elevated temperatures on arrival (>18°C) or insufficient numbers of live animals (<10).

Following completion of all analysis details of the vendor where the sample was purchased (name, address and vendor type) and any accompanying details on the origin of the oysters as recovered by the Stericycle auditor were matched to tested samples. This was possible in 602 cases (95.5% of samples tested during the survey). In a further 22 cases, it was possible for Stericycle to extrapolate the details of the vendor from the unique sample identifier included on the sample submission form. For the remaining 6 samples (0.95% of the total), Stericycle were not able to identify the vendor from where the sample was purchased. Of the 624 samples where the vendor was identified, 407 were collected from fishmongers (65.2%), 65 from supermarkets (10.4%), 65 from restaurants (10.4%), 62 from online sales (9.9%) and 25 from wholesalers (4.0%).

In 492 cases (78.1% of the total samples), the dispatch centre from which the oysters originated could be identified as a result of information collected by the auditor (this identification was supported by a photograph of the identification mark or other identifying labels/packaging in 378 cases). Oysters originated from a total of 33 different dispatch centres in 5 different EU Member States; the number of samples per dispatch centre varied from 1 up to 61. A large proportion of the samples (376 - 76.4% of samples where the dispatch centre was identified) were from one of a group of ten dispatch centres. Of the 492 samples with identified dispatch centres, 434 samples (88.2%) originated in the UK, 29 (5.9%) from the Netherlands, 25 (5.1%) from Ireland, 3 (0.6%) from France and 1 (0.2%) from Spain. Of the samples from the UK, 291 came from dispatch centres in England, 132 from Scotland, 6 from Northern Ireland, 4 from Jersey and 1 from Wales. A total of 175 samples (35.6% of all samples where dispatch centre details were available) originated from dispatch centres in a single English county, Essex.

Of the 630 samples tested, 613 (97.3%) were identified by experienced staff as Pacific oysters *Crassostrea gigas* upon receipt at the laboratory, with the remaining 17 (2.7%) identified as native oysters *Ostrea edulis*. For one sample there was a discrepancy between the species identified in the laboratory (*Crassostrea gigas*) with the species identified on the photograph of the identification mark. In this case, where the identification mark photo provided was for the venus clam *Venerupis*

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[5] The data file for Work Package 3 can be viewed in Excel format (see Appendix 5)

japonica, it seems highly probable that the vendor provided the auditor with the identification mark for the wrong sample; details recorded from this identification mark and six other cases where e.g. packing dates on the identification mark were incompatible with the tested sample, were removed from the database and are not considered in the totals above. In six further cases samples identified as *Crassostrea gigas* in the laboratory were recorded as different species by the Stericycle auditor (4 x *Ostrea edulis*, 1 x *Crassostrea virginica*, 1 x *Mytilus edulis*), but no photographs of identification marks were available to investigate the root cause of these cases, and it is not possible to rule out a simple error by the auditor. There is however a possibility of mislabelling or mistakes by the producer, supplier or vendor in some of these cases.

According to auditors, 624 (99.0%) of the samples tested were sold chilled, with the remaining 6 sold at ambient temperature. None of the tested samples was frozen.

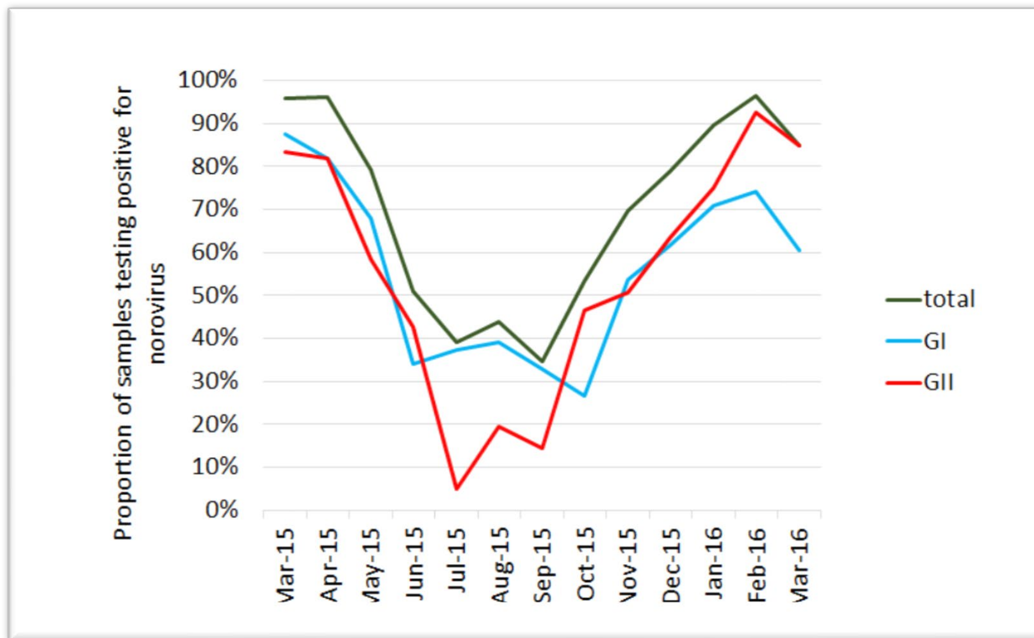
Four hundred and fifty two of the 630 samples (71.7%) were tested for both norovirus and *E.coli* with the remaining 178 samples (28.3%) being tested for norovirus only. In these cases *E.coli* testing was not carried out primarily due to insufficient live animals in the sample to conduct both tests (<20), or elevated temperatures on arrival (>10°C). Due to collection failures and corresponding increases in the sampling burden the following month, the numbers of samples collected in the 11 whole months of the survey (April 2015 to February 2016) varied between 41 (August 2015) and 69 (November 2015) compared with the original monthly target of 52 or 53 samples.

5.4.1 Noroviruses

5.4.1.1 Study results

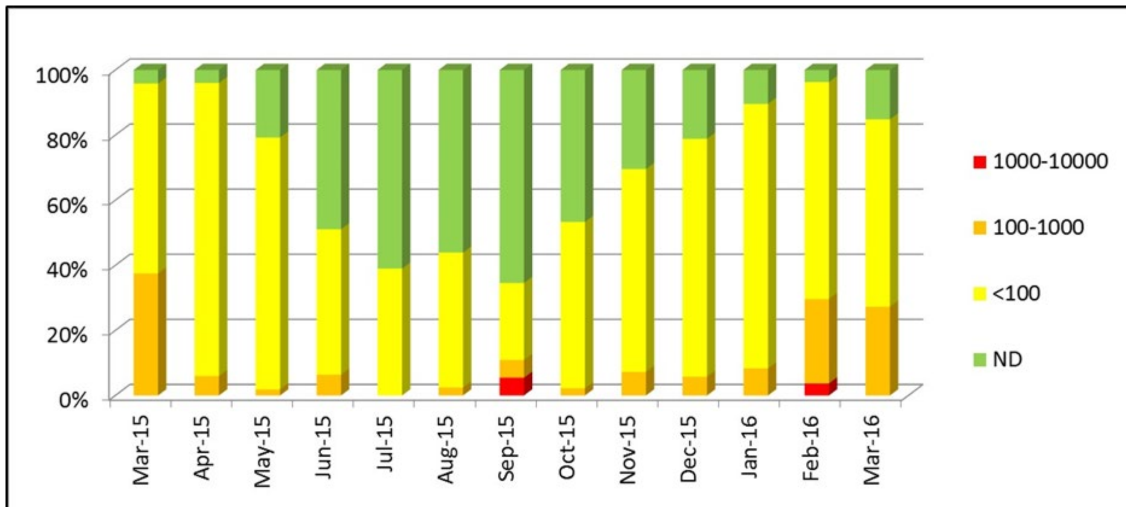
Of 630 samples tested, 433 (68.7%) were positive for norovirus RNA. Of these, 99 samples (15.7%) were positive for GI only, 88 (14.0%) were positive for GII only and 246 (39.0%) were positive for both GI and GII. A clear seasonality was observed with 79.7% of samples collected in the months October-March positive compared with 57.0% in the months April-September. This difference was found to be statistically significant (Fisher's exact test; $p < 0.0001$). The highest and lowest monthly prevalences were recorded in February 2016 (96.3%) and September 2015 (34.6%) respectively (Figure 18).

Figure 18: Month-by-month prevalence of norovirus RNA in survey samples



In the majority of samples testing positive the levels recorded were below the limit of quantification of the assay (100 copies/g) for both norovirus GI and GII. In total 61 samples produced results of >100 copies/g for one or both genogroups, representing 9.7% of total samples (14.1% of positive samples). Of these 61 samples, 7 produced results of >100 copies/g for both genogroups, 2 for GI only and 52 for GII only. The highest monthly incidence of samples giving results >100 copies/g was March 2015 (37.5%). Over the course of the survey, 5 samples (0.8% of total samples) produced results for GI and GII combined of >1000 copies/g; 3 of these samples were collected in September 2015, and 2 in February 2016. The highest levels recorded in individual samples were 586 copies/g for GI and 1802 copies/g for GII. Norovirus levels were higher during the winter period with a geometric mean level of 87 copies/g in the months October-March compared with 65 copies/g in samples collected from April-September. This difference was found to be statistically significant using the Kruskal-Wallis test ($p < 0.001$).

Figure 19 shows the proportions of samples giving norovirus results in different quantity brackets on a month by month basis throughout the survey.



5.4.1.2 Comparison with the production area study

The prevalence of norovirus RNA in oyster samples recorded in this survey (the “retail survey”) was 68.7%. This figure is similar but slightly lower than the prevalence (76.2%) found in a previous two-year survey (2009-2011) of oysters from UK production areas (the “PA survey”; Lowther *et al*., 2012). A similar seasonality with increased prevalences and levels in the winter months was noted in both surveys. However, the overall levels of norovirus recorded in the retail survey were considerably lower than in the PA survey. In the PA survey, 36.5% of total samples contained levels >100 copies/g for one or both norovirus genogroups (cf. 9.7% in the retail survey), combined levels of >1000 copies/g were found in 14.6% of samples (cf. 0.8% in the retail survey), and combined levels of >10000 copies/g were found in 1.1% of samples (none recorded in the retail survey). Geometric means for all results were 76 copies/g and 159 copies/g for the retail and PA surveys respectively. This difference was found to be statistically significant (Kruskal-Wallis test; $p < 0.001$).

Possible underlying causes for this pattern of results include:

- **Risk reduction measures by Food Business Operators (FBOs)**: The two surveys targeted oysters at different stages of the food chain; at the point of production and the point of sale to the customer. Post-production, oysters in the UK are routinely subject to commercial purification (depuration) prior to sale which may reduce virus levels. However, conventional depuration treatments have not been found particularly effective at removing norovirus. Enhanced depuration methods using longer periods and higher temperatures may reduce detectable viral loads (Lees *et al*., 2010). However, there is an absence of data on both the application of such techniques in commercial depuration and on their effectiveness for virus

reduction. In the absence of paired (pre- and post-depuration) samples it is not possible to investigate the contribution of this factor using the data collected in the surveys. An alternative risk reduction strategy used by some FBOs is the use of norovirus testing on raw products to inform decisions on choice of supply for processing and marketing. Whilst this strategy could be effective, depending on the decision matrix, data is not available on the degree of implementation by FBOs and hence whether this contributed to the generally low norovirus levels seen in retail samples compared with the previous PA study.

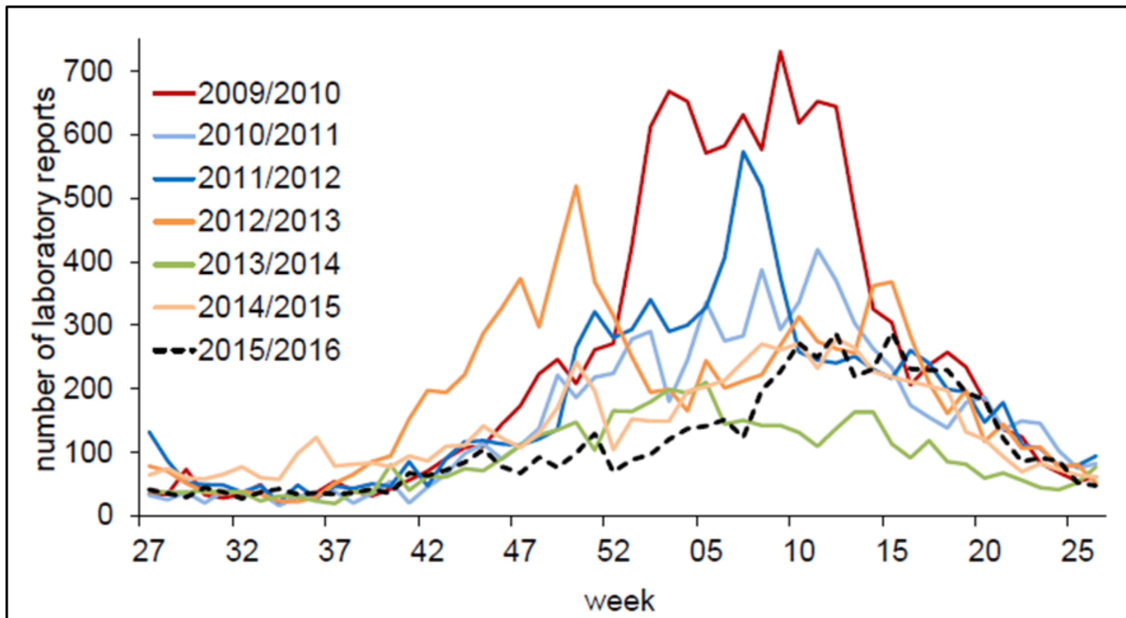
- **Representativeness of samples**: It is possible that the PA survey was not representative of the volumes of oysters placed on the UK market. The selection of sites for the PA survey was meant to provide a representative selection of production areas with different risk profiles and a good geographical spread, and was not designed to represent production volumes or market share. There are several possible contributing factors to this element; in the PA survey no samples from outside the UK were tested while in the current retail survey 11.8% of samples with dispatch centre details were supplied by dispatch centres outside the UK. Meanwhile the proportion of samples originating in Scotland is approximately two times higher in the retail survey (30.7%) than it was in the PA survey (15.4%). In the current retail survey levels from non-UK samples were found to be lower than in UK samples, and levels from Scottish-supplied samples were found to be lower than those from elsewhere in the UK (see below). Corroborating this, in the PA survey sites in Scotland also tended to have lower norovirus levels than those from other parts of the UK (however, it should be noted that in >20% of samples no origin details were recovered, and where details for the dispatch centre were available it is also possible that the area of production was not local to that dispatch centre).

Finally the PA study used official representative sampling points (RMPs) which are selected to reflect the point of worst case contamination within the classified area. Hence it is possible that the commercial harvest, which may be from anywhere within the classified area, may be less contaminated than the RMP. Without details on the actual site of production for the majority of samples it is not possible to examine this possibility.

- **Variation in norovirus shedding rates in the general population**: Sewage treatment is known to only reduce norovirus by a limited extent (Campos & Lees, 2014). Consequently, a key factor influencing norovirus contamination in filter-feeding shellfish impacted by sewage discharges will be the degree of virus infection, and hence the degree of virus shedding in faeces, in the population contributing to the sewage inputs. During this study unusually low levels of norovirus were observed in the general population during the winter of 2015-16, particularly during the months November to January, compared with unusually high levels during the

winters of 2009-10 and 2010-11 (Figure 20). These differences are particularly significant and hence judged likely to impact on the levels seen in oysters.

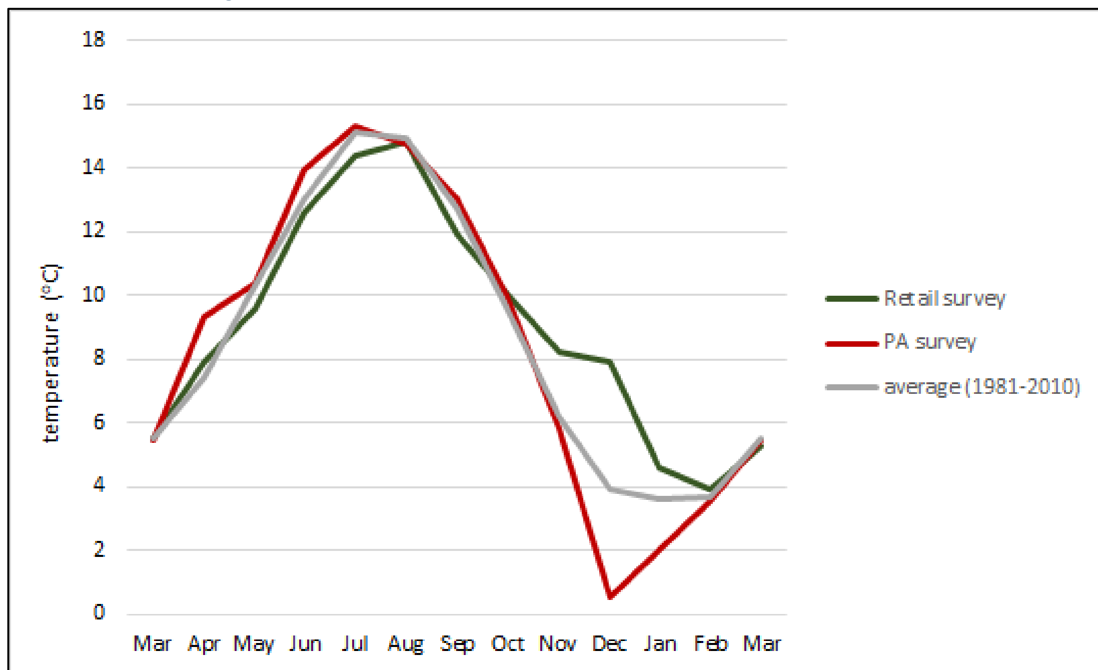
Figure 19: Seasonal comparison of laboratory reports of NoV in the community (England and Wales).



Reproduced from the website of Public Health England. PA survey ran from week 18 in 2009 until week 17 in 2011, retail survey ran from week 12 in 2015 until week 11 in 2016.

• **Variation in environmental temperatures**: Shellfish are poikilothermic (Gosling, 2008), and their metabolic rate, and hence the degree of contaminant uptake and particularly removal, is significantly influenced by the temperature of their environment. Analysis of data from the PA survey indicated a significant relationship between environmental temperatures and norovirus levels in UK oysters (Lowther *et al*., 2012), with lower temperatures linked to higher norovirus levels. In this study environmental temperatures in the UK were unusually high during the winter of 2015-16, particularly during the months November to January, compared with unusually low temperatures during the PA study winters of 2009-10 and 2010-11 (Figure 21). The difference is significant and considered likely to potentially impact results – particular during the higher risk winter periods.

Figure 20: Monthly mean air temperatures for the UK.



Temperatures shown for each month of the period of the retail survey (March 2015 to March 2016), alongside averages for the equivalent calendar month during the PA survey period (May 2009 – April 2011) and during the years 1981-2010. Data from the UK Met Office website.

Of the above factors potentially influencing the variation seen between contamination levels in the PA study and in this study, it is only possible to perform further analysis on the impact of general population norovirus shedding rates and environmental temperatures since data is not available for the other factors. To further investigate these possible contributing elements, month-by-month normalisation factors were determined using PHE data on illness reports and Met Office data on national average monthly temperatures.

For **illness reports**, the normalisation factor was determined as:-

(average illness reports per day for the relevant calendar month in the period of the PA survey [May 2009 – Apr 2011])

÷

(average illness reports per day for the month in question)

Such that where illness reports for a given month were lower than the average for that calendar month in 2009-2011, the normalisation factor was >1. For example, in April 2015, the average number of illness reports per day was 30.27, compared with the average for April during the PA survey of 34.05 reports per day. The normalisation factor for April 2015 was therefore 34.05/30.27 = 1.12.

For **temperatures**, the normalisation factor was determined as:-

(20 - the long term time series average temperature for the relevant calendar month [1981-2010])

÷

(20 - the recorded monthly UK average temperature for the month in question)

Such that where the UK average air temperature for a given month was higher than the long term average for that calendar month in 1981-2010, the normalisation factor was >1. For example, in April 2015, the UK average temperature was 7.9°C, compared with a long term average for April of 7.4°C. The normalisation factor for April 2015 was therefore (20-7.4)/(20-7.9) = 1.04.

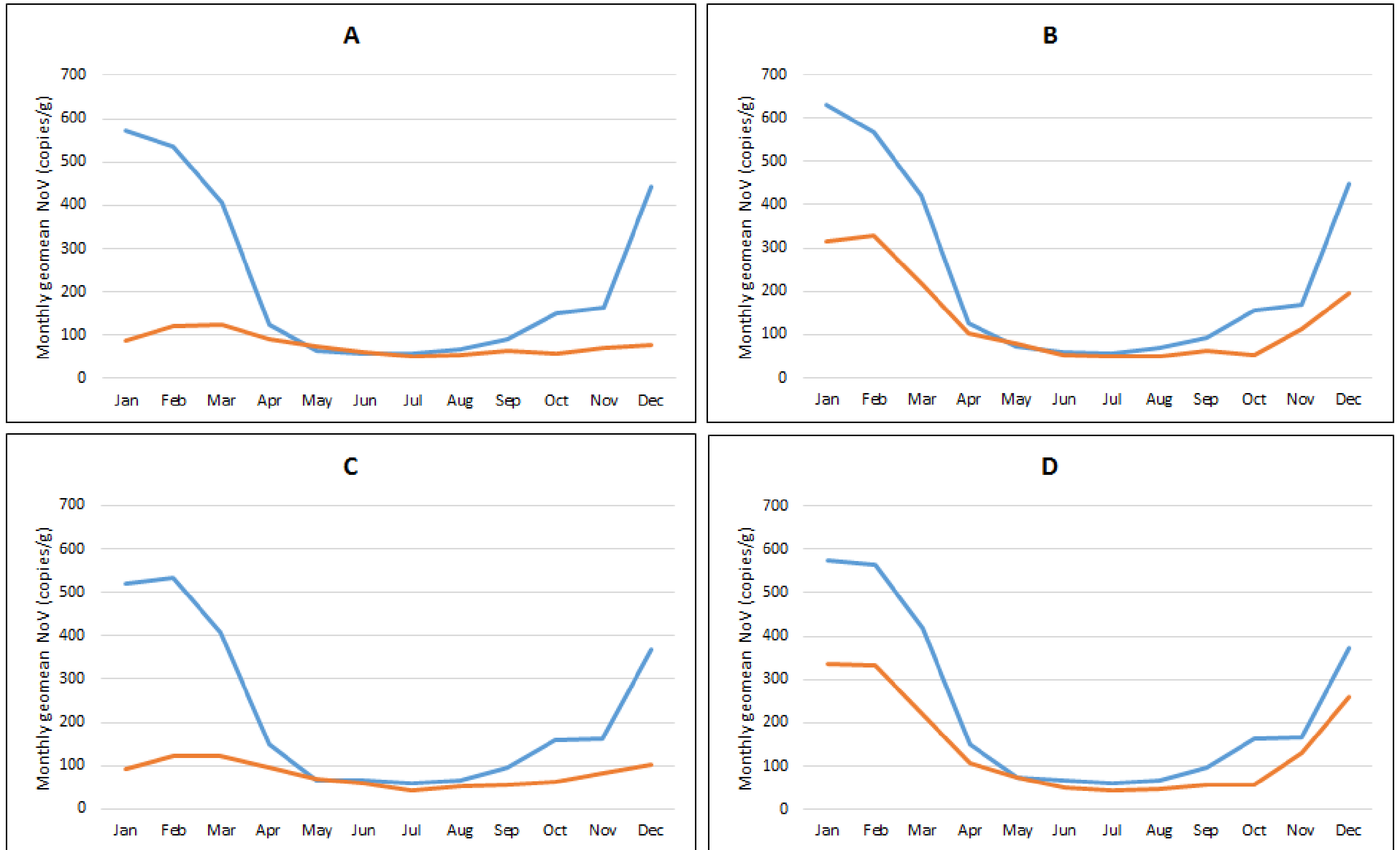
Normalisation factors calculated in this way were applied to the geometric mean norovirus levels recorded for each month of both the retail and PA surveys. For both surveys an average level for each calendar month was calculated.

Application of the normalisation factors based on illness reports resulted in a notable improvement in correspondence in results by calendar month between the two surveys (see Figure 22). Levels for each month in the two surveys are plotted against each other in Figure 23 alongside lines of best fit and equality; for data normalised according to illness reports (Figures 22-B and 23-B) the slope of the line of best fit (0.4723) is considerably closer to equality and the correlation is considerably closer to total (r2 = 0.9506) than for non-normalised data (Figures 22-A and 22-B; slope = 0.0887 and r2 = 0.5384).

Application of the normalisation factors for temperature in isolation yielded only a modest improvement in agreement between the results of the two studies (Figures 22-C and 23-C). However, application of both the illness and temperature-based normalisation factors in combination produced the best line of best fit overall (Figures 22-C and 23-C; slope = 0.5626 and r2 = 0.9576).

This analysis indicates that much of the difference in the norovirus levels between the retail and PA surveys can be attributed to the different levels of norovirus in the community between the two study periods, with some portion of the remaining difference explained by the differing temperatures, particularly during the early part of winter. Nevertheless, even normalising using these factors together results in levels in the retail study on average ~56% as high as during the PA survey, suggesting other factors as discussed above also contributed to the different pattern of results.

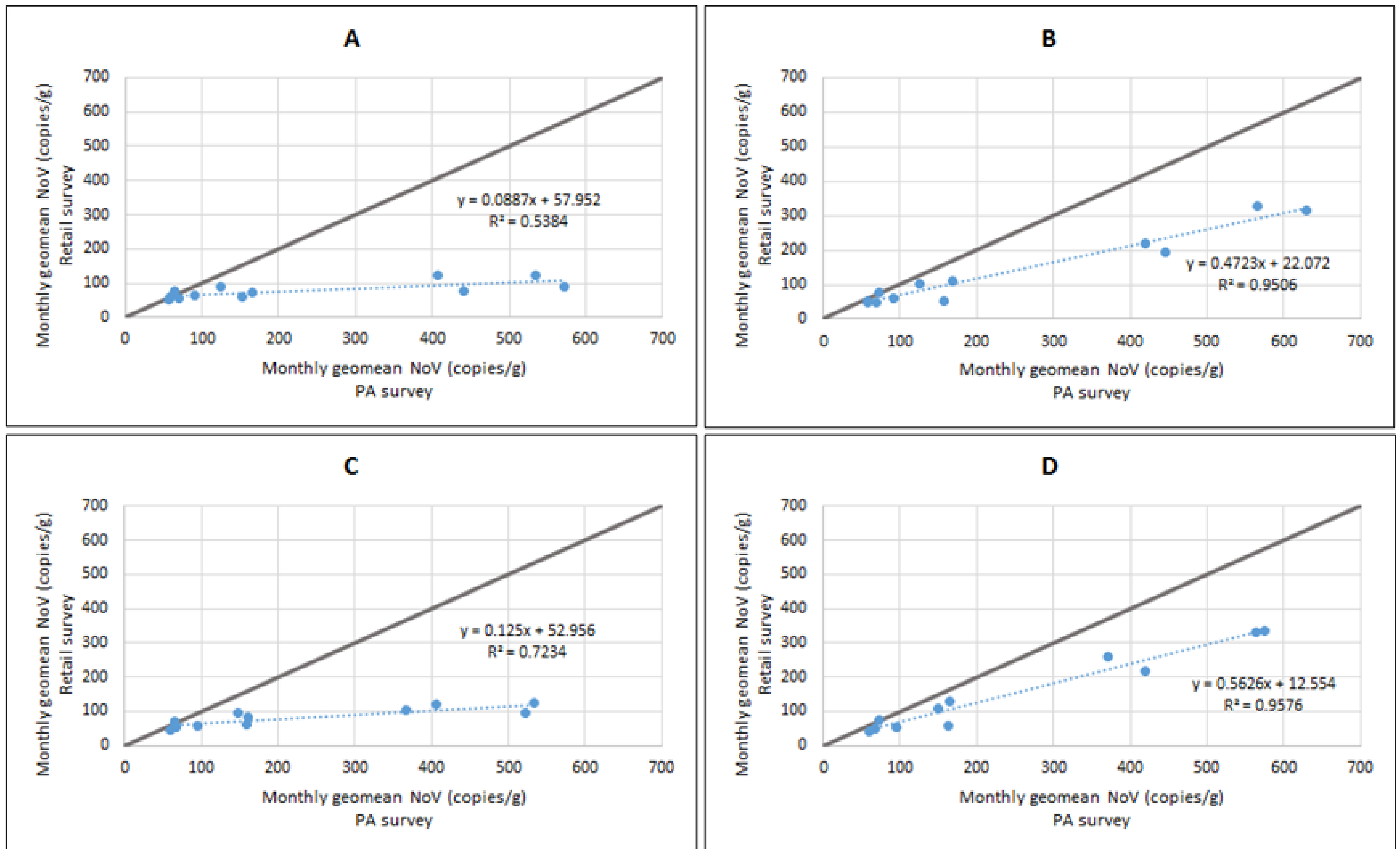
Figure 21: Normalisation of monthly geometric mean NoV levels.



In all sections the geometric mean levels for each calendar month in the retail survey are shown in orange, and the levels for the PA study are shown in Blue.

A; no normalisation factors applied.
B; normalisation factors for illness reports applied.
C; normalisation factors for temperature applied.
D; normalisation factors for illness reports and temperature applied.

Figure 22: Agreement of monthly results from the retail and PA surveys Data points are for individual calendar months. Lines of best fit and equality are shown in blue (dashed) and grey respectively.



A; no normalisation factors applied.
B; normalisation factors for illness reports applied.
C; normalisation factors for temperature applied.
D; normalisation factors for illness reports and temperature applied.

5.4.1.3 Norovirus in oysters originating in different countries

It is a legal requirement within the EU (Anon, 2004) to include an identification mark for each package of bivalve shellfish showing the approval number of the dispatch centre and the member state in which it is situated. The identification mark however is not required to detail the production area where the shellfish originated - this information is recorded on the registration document retained by the dispatch centre. However, it is not uncommon for establishments to include information on the origin of the shellfish on the identification mark or packaging. Of 492 samples with dispatch centre information, additional information on at least the country of origin of the oysters was available in 238 cases. In each case the country of origin was the same as the member state in which the dispatch centre was located. Of 215 samples where the country of origin was identified as the UK, in 141 cases the UK constituent

country/Crown dependency (England, Scotland etc.) where the oysters originated was further identifiable. In 131 of these cases (92.9%) this was the same as the UK constituent country/Crown dependency in which the dispatch centre was situated, the exception being a small number of samples of oysters grown in Scotland or Jersey but supplied by a dispatch centre in England. Considering this observed congruence between the geographical location of the dispatch centre and the actual origin of the oysters supplied, it was considered reasonable to analyse data by geographical origin using dispatch centre location as a proxy.

Prevalences of norovirus detection and geometric mean levels of norovirus for samples originating in different EU Member States, as well as for the different UK constituent countries/Crown dependencies are shown in Table 10.

Table 10: Norovirus results by country of origin

Country of origin[a]	Number of samples	NoV results	
		Prevalence (% positive)	Geometric mean (copies/g); 95% confidence interval in parentheses)
UK (all)	434	71.7%	78 (40-277)
England	*291*	*71.1%*	*82 (40-319)*
Scotland	*132*	*71.2%*	*71 (40-201)*
Northern Ireland	*6*	*100.0%*	*79 (54-114)*
Jersey	*4*	*75.0%*	*73 (40-173)*
Wales	*1*	*100.0%*	*100 (n/a)*
Netherlands	29	31.0%	49 (40-91)
Republic of Ireland	25	84.0%	69 (40-120)
France	3	33.3%	48 (40-92)
Spain	1	100.0%	275 (n/a)

a = country of origin determined as the country in which the dispatch centre was located

It is important to note that this study cannot provide a systematic comparison of oyster production in different countries, as by definition only oysters available to the UK consumer were tested, and these may not be representative of the national production, especially for countries other than the UK itself. In addition, the low number of samples from certain regions complicates statistical analysis. Nevertheless, some statistically significant observations could be made. Overall prevalence and levels of norovirus were lower in samples originating outside the UK (55.2% of samples positive, geometric mean of 58 copies/g), than in samples from the UK (71.7% positive, geometric mean of 78 copies/g). These differences were found to be statistically significant (Fisher's exact test, $p = 0.0144$; Kruskal-Wallis test, $p < 0.001$). Further subdivision of non-UK samples to enable country-by-country

analysis showed that for oysters from the Netherlands both prevalence and levels were significantly lower than for the UK (Fisher's exact test, $p < 0.0001$; Kruskal-Wallis test, $p < 0.0001$). Prevalence and levels for oysters from the Republic of Ireland were not significantly different from those for the UK, but were significantly higher than those for the Netherlands (Fisher's exact test, $p = 0.0001$; Kruskal-Wallis test, $p = 0.0081$). No apparent seasonal bias in collection dates for samples from the three countries were found to explain these differences (no significant difference was found between the proportions of samples collected during the winter months October-March using Fisher's exact test). Statistical analysis of norovirus results for samples from France and Spain was not carried out due to the small number of samples.

Within the UK, in England and Scotland (the two constituent countries/Crown dependencies that provided more than 10 samples), recorded prevalences were almost identical (71.1% and 71.2% respectively). However, norovirus levels in Scotland were somewhat lower than in England. This difference was statistically significant (Kruskal-Wallis test, $p = 0.034$).

A large proportion of English samples (175 samples; 60.1%) originated from dispatch centres in a single county, Essex. However, norovirus prevalence and levels for these samples were not significantly different from those in other parts of England.

5.4.1.4 Norovirus in oysters collected from different vendor types

The prevalences of norovirus detection and geometric mean levels of norovirus, and the origin of oysters (where determination was possible from the identification mark) within samples collected from the five different vendor types targeted in the study is shown in Table 11.

Table 11: Norovirus results and origin of oysters for samples collected from different vendor types

Vendor type	Number of samples	NoV results		Origin of Oysters[a]			
		Prevalence (% positive)	Geometric mean (copies/g); 95% confidence interval in parentheses)	England	Scotland	Other UK	Non-UK
Fishmonger	407	67.8%	75 (40-269)	67.2%	16.4%	1.5%	14.9%
Online sales	62	64.5%	81 (40-364)	85.4%	9.8%	2.4%	2.4%
Restaurant	65	73.8%	75 (40-197)	37.5%	37.5%	10.4%	14.6%
Supermarket	65	66.2%	65 (40-137)	1.8%	98.2%	0.0%	0.0%
Wholesaler	25	84.0%	101 (40-391)	94.1%	0.0%	0.0%	5.9%

a = percentages are calculated only including samples where origin was determined using the identification label

No statistically significant differences in prevalences between the different vendor types were apparent however differences in norovirus levels were found to be significant between wholesalers and supermarkets (Kruskal-Wallis test, $p = 0.0031$) and wholesalers and fishmongers ($p = 0.0100$) but not between any other combination of vendor types. Although oysters from England predominated amongst samples collected from Fishmongers, percentages of oysters from Scotland were notably elevated within samples collected from restaurants and particularly supermarkets, where only one sample out of 57 of known origin originated in a dispatch centre outside Scotland. This is not influenced by the locations of the supermarkets since only 50.9% of these samples were collected in Scotland. The predominance of product from Scotland was evident in all 3 supermarket brands targeted during the survey – this finding therefore presumably reflects the purchasing policy of supermarkets.

5.4.1.5 Comparison of the location of collection and norovirus content

Prevalences and geometric mean levels of norovirus by sampling location were compared to establish whether norovirus content was linked to geographic point of sale. A comparison of samples collected in the four constituent countries of the United Kingdom is shown in Table 12.

Table 12: Norovirus results and origin of oysters for samples collected in different UK countries

Location of purchase	Number of samples	NoV results		Origin of Oysters[a]			
		Prevalence (% positive)	(copies/g); 95% confidence interval in parentheses)	England	Scotland	Other UK	Non-UK
England	410	68.3%	77 (40-277)	73.1%	16.4%	1.2%	9.4%
Scotland	151	62.9%	65 (40-189)	11.3%	67.0%	0.0%	21.7%
Wales	50	84.0%	96 (23-231)	76.3%	13.2%	2.6%	7.9%
Northern Ireland	19	84.2%	84 (40-202)	0.0%	0.0%	100.0%	0.0%

a = percentages are calculated only including samples where origin was determined using the identification label

Statistically significant differences in prevalences were observed between samples collected in England and Wales (Fisher's exact test, $p = 0.0221$) and Scotland and Wales ($p = 0.0052$), while levels in samples collected in Scotland were significantly lower than in any of the three other countries (Kruskal-Wallis test, $p \leq 0.0142$). In addition levels in samples collected in England were significantly lower than in those collected in Wales ($p = 0.0119$).

Lower norovirus levels in samples collected in Scotland may reflect the increased percentages of samples originating either from Scotland, or from outside the UK. In the former case a small degree of sampling bias may have contributed to the large number of samples originating from Scotland as a comparatively high number of samples collected in Scotland were sourced from supermarkets (22.5% compared for example with 5.6% of samples collected in England), and Scottish oysters predominated in samples collected from supermarkets throughout the UK. However the predominance of Scottish oysters amongst samples collected in Scotland was also noted amongst samples collected from other vendor types (54.5% compared with 10.3% Scottish oysters amongst samples collected in non-supermarket vendors in England).

5.4.2 E.coli

Of the 452 samples tested for *E.coli*, the bacterium was not detected (<18 MPN/100g) in 346 cases (76.5%). In 11 samples (2.4%), levels in excess of the EU legal end product standard (230 MPN/100g; Anon, 2005) were detected. In these cases, the FSA were informed on the same working day that the result became available. All 11 of these samples were collected between March and September 2015, with the highest monthly incidence of 5 samples >230 MPN/100g in July 2015, representing 15.2% of the samples collected in that month. In one sample a level in excess of the upper limit of quantification of the *E.coli* assay (>18000 MPN/100g) was recorded from a sample collected on 15 July 2015.

No association with vendor types/ origins or elevated norovirus levels was noted for samples with >230 MPN/100g.

In comparison with the PA survey, levels of *E.coli* recorded in this study were very low. No *E.coli* was detected in the majority of the samples, while results over the A classification and end product standard were rare. In the PA survey by contrast, *E.coli* proportions were 14.3% undetected and 40.0% >230 MPN/100g. Although other factors may have contributed this difference is likely to be largely the result of the well-established high efficacy of standard depuration conditions for the removal of *E.coli* bacteria (Dore & Lees, 1995). Since the removal of *E.coli* is a good proxy for other bacterial pathogens derived from sewage contamination (Lees *et al*., 2010), this demonstrates the contribution to public health of the classification and depuration regulations for protection from bacterial illness. This finding is supported by the low numbers of bacterial infections associated with consumption of oysters in the UK (Lees, 2000).

The small number of results of >230 MPN/100g, including one result of >18000 MPN/100g, indicates that despite the high level of adherence to the legal standards, problems can nevertheless occur. The root cause of the high *E.coli* levels detected in some samples is not known, but could conceivably be linked to problems post-harvest, during transportation, or at the point-of-sale.

5.4.3 Capsid Integrity Assay

The CIA was applied to all retail survey samples where results for either norovirus genogroup were >500 copies/g. For each sample only the genogroup(s) with results above this threshold were tested. In total 2 samples were tested for both GI and GII and 9 samples were tested for GII only using the CIA assay (see Table 13). In the majority of cases it was not possible to determine a ΔCq value for these samples due to qRT-PCR replicates in the untreated subsamples providing negative results or results equivalent to <1 copy/µl. However, in four cases (all GII) it was possible to determine a minimum ΔCq value and correspondingly a lower limit for the exposed virus qRT-PCR target (>38% in all cases). It was not possible to determine an upper limit to the ΔCq value and exposed virus qRT-PCR target for any samples, due to qRT-PCR replicates in the treated subsamples providing negative results or results equivalent to <1 copy/µl.

Interpretation of the data is difficult due to the small number of samples where the CIA could determine a ΔCq value. However, in those cases where a result was determined, a significant proportion of the qRT-PCR target was determined to be sensitive to heat/RNase exposure, and therefore possibly derived from viable virus. This data, whilst limited, suggests that for the samples tested (those containing >500 norovirus copies/g) the presence of viable virus cannot be excluded based on the CIA assay.

Table 13: Application of the CIA to survey samples with high norovirus levels

Sample number	NoV genogroup	qRT-PCR result (copies/g)	Capsid Integrity Assay result
15-606	GII	688	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
15-1844	GII	1802	ΔCq ≥ 2.39, Exposed virus qRT-PCR target ≥ 73.30%
15-1855	GII	566	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
15-1858	GII	965	ΔCq ≥ 1.29, Exposed virus qRT-PCR target ≥ 41.71%
15-1895	GII	705	ΔCq ≥ 1.05, Exposed virus qRT-PCR target ≥ 38.58%
15-1896	GII	1127	ΔCq ≥ 1.45, Exposed virus qRT-PCR target ≥ 48.44%
16-394	GII	507	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
16-416	GII	587	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
16-446	GII	833	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
16-195	GI	545	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
"	GII	904	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
16-204	GI	586	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable
"	GII	1144	ΔCq not quantifiable, Exposed virus qRT-PCR target not quantifiable

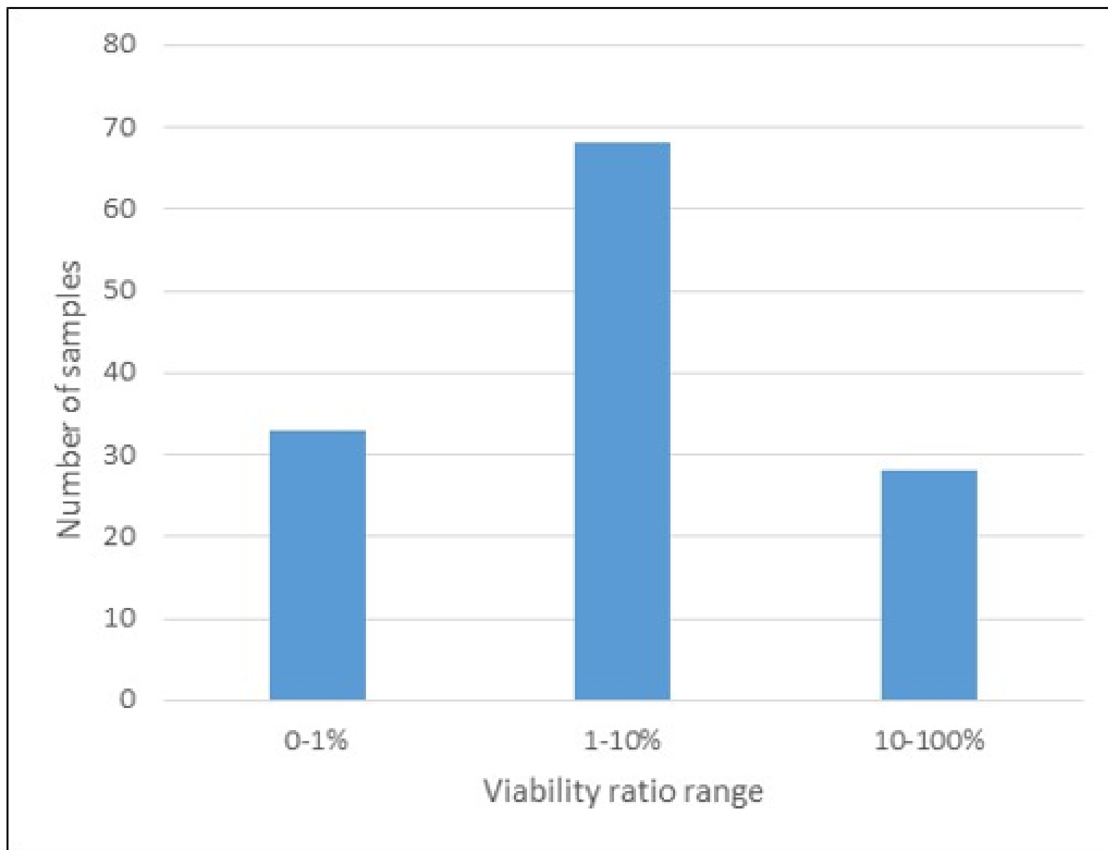
5.4.4 Determination of ssRNA virus viability ratios using bacteriophage

Considering the limited data available from the CIA assay further analysis was performed using a novel bacteriophage viability assay. This analysis was not funded by the NoVAS project (see materials and methods), however the data is presented here since it may assist interpretation of the retail study data for shellfish and has contributed towards WP6. Of the 630 samples tested for norovirus, extra data to enable calculation of viability ratios was generated in 153 cases (24.3%). Of these samples, 128 (83.7%) were positive for GA phage by qRT-PCR, with the highest level recorded 4137 copies/g. Sixteen samples were negative for both norovirus and GA phage by qRT-PCR; of the remainder, in 114 cases (83.2%) PCR-detectable levels of GA were greater than norovirus, while a statistically significant positive correlation ($p<0.0001$) between levels of the two viruses was found, indicating the general suitability of GA phage as an indicator of norovirus.

Viable total F-RNA bacteriophage was detected in 73 samples (47.7%), however, viable GA phage was only identified in 52 samples (34.0%) following hybridisation (the remaining samples presumably containing other genogroups of levivirus/allolevivirus). The highest level of viable GA phage recorded was 104 pfu/g and the geometric mean of all positive samples was 9.3 pfu/g.

Of the 153 samples tested for both viable and PCR-detectable GA phage, a viability ratio could be calculated in 129 cases (84.3%). In the other cases the sample was negative for both PCR-detectable and viable GA phage. Of the 129 ratios calculated, 77 were calculated using a censored vale of 1.5 pfu/g for viable GA phage (as the sample was negative for this parameter), and 1 was capped at 100% due to higher levels of viable than PCR-detectable GA phage. Calculated ratios ranged from 0.07% to 100% (discounting the single sample capped at 100% the highest ratio calculated was 82.63%). The median value of the viability ratios was 3.27%. The distribution of the calculated ratios in the ranges 0-1%, 1-10% and 10-100% is shown in Figure 24.

Figure 23: Number of samples in different ranges of ssRNA virus viability ratios



5.4.5 More on Next Generation Sequencing (NGS)

As featured previously in this chapter, an RNA metagenomic sequencing approach was applied to a subset of retail survey samples with the aim of firstly independently confirming the presence of norovirus in samples, and secondly of attempting to characterise the diversity of norovirus present.
Total RNA concentrations were determined for the 10 retail survey samples (all Pacific oysters) with the highest levels of norovirus. In three cases the total RNA concentration was insufficient for downstream applications, however in the remaining 7 cases the sample RNA was further subject to rRNA depletion, DNase treatment, library preparation, RNA metagenomic sequencing and bioinformatic analysis as described above.

In all samples the majority of sequences in the RNA metagenome mapped to Crassostrea sp. (the oyster genus including Pacific oysters), with smaller numbers of sequences mapped to other eukaryote taxa, bacteria and viruses. Following specific blasting for norovirus sequences, a single fragment of norovirus sequence was identified in the RNA metagenome of four separate samples.

15-1858
391b fragment with ~98.5% identity to Kaohsiung strain (GII.6; GenBank accession number KM267740.1 [partial genome sequence]), nucleotides 3246-3636, corresponding to nucleotides 6673-7063 of 0907-26 strain (GII.6; GenBank accession number KU935739.1 [whole genome sequence]), which spans the junction of ORF2 (encoding the major capsid protein) and ORF3 (encoding the minor capsid protein).

16-195
104b fragment with ~95.2% identity to Groningen strain (GI.6; GenBank accession number LN854564.1), nucleotides 3152-3255, which falls within ORF1 (encoding the polyprotein). This part of ORF1 encodes the part of the polyprotein that becomes VPg after cleavage.

16-204
185b fragment with ~96.8% identity to Musashimurayama strain (GII.P7/GII.7; GenBank accession number KJ196295.1), nucleotides 1701-1885, which falls within ORF1. This part of ORF1 encodes the part of the polyprotein that becomes p41 (the virus protease) after cleavage.

16-446
60b fragment with 100% identity to Shanghai strain (GII.17; GenBank accession number KT380915.1), nucleotides 2885-2944, which falls within ORF1. This part of ORF1 encodes the part of the polyprotein that becomes VPg after cleavage.

None of these fragments encodes an area of the genome targeted by the qRT-PCR or RT-PCR primers in use at Cefas establishing that the sequences detected are not a consequence of laboratory contamination by PCR products. The results independently confirm the presence of norovirus detected by RT-PCR in these samples and offer some insights into the diversity of norovirus strains found in environmentally contaminated oysters. However, as anticipated the limited sensitivity of the methodology used combined with the relatively low concentrations of norovirus detected in the retail survey samples means that only limited numbers of norovirus sequences could be identified. Benchtop technology that can deliver increased read numbers (and hence greater capacity to identify rare components within the RNA metagenome) compared to the MiSeq platform is likely to become more affordable in the near future however in the interim, sequencing of conventional RT-PCR amplicons using NGS platforms may be a preferable option for generating data on norovirus diversity in food samples with low to moderate levels of contamination.

5.4.5.1 Expectations and Limitations of NGS

At the inception of the NoVAS project, NGS (i.e. metagenomics) technology was becoming increasingly accessible and the capability of these methods to identify under-represented sequences in complex samples was increasingly apparent. As such, the NoVAS Consortium felt it timely and scientifically worthwhile to include a small number of aims within the study that would address the utility of NGS/metagenomics methods, in a complimentary approach alongside using established PCR/Sanger sequencing-based approaches, for virus detection and characterisation from food and environmental samples.

From these studies, some noteworthy observations were made in the course of the project, for example those presented above; a metagenomics approach being used to interrogate Pacific oysters previously demonstrated to contain high levels of norovirus (>100 copies/g). Use of metagenomics provided confirmation of the presence of norovirus RNA in the specimen, and provided information on virus genotypes present in the oyster. Further, in Appendix 6, we present data demonstrating the use of massively parallel sequencing technology to investigate outbreak (transmission) links between clinical samples collected during an outbreak investigation. This approach enhanced the investigation by providing complete virus genomes and identifying minority virus variants – neither of which would be obtained using current standard methods based on Sanger sequencing.

However, the work undertaken during the NoVAS project which explored use of NGS (metagenomics) approaches for detection and characterisation of norovirus was impeded by limitations in the technology that fail to overcome the inherent challenges of recovering viral signals from food and environmental samples, as these tend to contain low levels of virus in complex matrices.

Limited sensitivity of amplification-free NGS methodologies hinders success of these methods. In both the studies on oysters and on stool specimens, success was, at least in-part, dependent upon high norovirus loads in the primary sample. Only oysters with >830 copies/g successfully yielded sequence data, and stool specimens with low Cq values (<28) yielded adequate genome coverage. From these data, it is clear that there remain significant limitations in the sensitivity of amplification-free NGS methods, but that this is not the only limiting factor. It is notable that two oysters with viral loads >1000 copies/g did not yield data, and similarly, one stool sample with Cq of 14 yielded <55% genome coverage.

Whilst the limits in sensitivity of the amplification-free technology observed here may be overcome by employing technologies that exploit an amplification step, thereby increasing the amount of template available for sequencing, this in turn creates undesirable limitations. Any amplification step will introduce bias in the nucleic acids amplified and resulting libraries are unlikely to be truly representative of the

population of RNA viruses in the original sample. The work presented in Appendix 6 demonstrates the advantages of using amplification-free methods in combination with a mucin based capture method to effectively remove large proportions of non-norovirus sequences and in effect increasing the norovirus sequence representation (metagenome data form the oyster samples exemplified the problem, with the majority of the sequences obtained belonging to the oyster). In addition, through greater sequencing depth achieved using HiSeq it was also possible to reveal minority variants that are present within the transmission network – a level of resolution not possible to observe with Sanger-based approaches and that would may be lost where a pre-amplification step is employed prior to massively parallel sequencing.

For this reason, we intentionally selected amplification-free methods for this project. The rationale being that any NGS-based method developed would need to discriminate sequence differences to a high level, particularly where outbreak investigations intend to use such methods to resolve transmission networks, for example, that might implicate a food handler. The results of this pilot suggest that the sensitivity of the methods is insufficient to obtain such level of sequence discrimination from foods or environmental surfaces that contain small amounts of viral particles.

Based on the results from small-scale studies conducted as part of the NoVAS Project, there is clearly high potential benefits from implementing NGS approaches in outbreak investigations. However, until improvements in sensitivity of these methods are made, standardised PCR-based approaches for virus detection and characterisation will remain the standard in the field. Furthermore, aside from the technical limitations of these technologies, the economic and time cost of the wet-lab and bioinformatics associated with these approaches remains prohibitively high for implementation in the routine outbreak investigation work of public health laboratories. Further developments in these technologies that enhance sensitivity, reduce cost and processing time, and yield data more rapidly will be needed in order to bring these methods closer to the front-line of public health outbreak investigations.

5.5 Conclusion

The retail survey described here is the first systematic study of norovirus in oysters collected at the point-of-sale in the UK, and one of the largest studies of its kind to date worldwide. Norovirus RNA was detected in 68.7% of samples tested, comparable with the prevalence found in a previous survey carried out using the same methods on oysters from UK production areas (76.2%; Lowther *et al*., 2012). The prevalence described here is considerably higher than recorded in surveys of norovirus in bivalve shellfish collected at the point-of-sale in some other countries, for example the United States (3.9%; Woods and Burkhardt, 2010), France (9%;

Schaeffer *et al*., 2013) and Thailand (12.3%: Kittigul *et al*., 2016), however comparatively frequent detection of norovirus has been reported in shellfish from production areas in Ireland (37.1%; Flannery *et al*., 2009), Italy (51.5%, Suffredini *et al*., 2014) and Spain (52.4%; Polo *et al*., 2015). Although the majority of samples were found to be positive, levels exceeding 100 norovirus copies/g were found in only a relatively small percentage of samples (9.7%). Analysis of these results in the context of overall risk to consumers from oysters at the point of retail sale is complicated by uncertainty on the variability of risk factors from year to year. During the year of the survey some significant potential risk factors, in particularly the number of outbreaks occurring in the general population and hence the likely extent of virus shedding into shellfish production areas, was low compared with previous studies. However, it is also possible that Food Business Operator risk management interventions (such as virus testing) may have impacted the low virus levels seen at retail. These findings highlight the general difficulty in comparing data sets collected in different time-frames. Direct comparison, within the same time period, of levels in production areas with those seen at retail would assist assessment of the contribution made by producer practices. Examination of the sample details for the 630 samples collected during the study has produced some novel observations on oyster supply within the UK.

The findings illustrate the predominance of a relatively small number of dispatch centres, and provide some evidence of differential norovirus risks (as judged by PCR analysis) in oysters from different geographical origins, or purchased in different vendor types. The very high number of samples compliant with the *E.coli* end product standard indicates the good compliance with current regulatory requirements in the UK oyster supply chain and the consequential probable low risk from bacterial pathogens such as salmonella. Metagenomic sequencing independently confirmed the presence of norovirus in a selection of positive samples with evidence of sequence diversity. Novel assays to determine putative virus viability were applied to a sub-selection of samples. Viability ratios of >1% were determined for the majority of analysed samples using both the CIA and the ssRNA virus viability ratio approach (using bacteriophage).

6 Work Package 4: Prevalence of norovirus in fresh produce sold at retail

6.1 Summary

One thousand one hundred and fifty two samples of fresh produce (comprising [6]568 lettuce, 310 fresh raspberries and 274 frozen raspberries) were collected and analysed for norovirus (1146 for *E. coli*), to acquire UK prevalence data to be used within the NoVAS study. Of 568 samples of lettuce, norovirus RT-PCR signals were obtained from 79 (13.9%) samples; replicate RT-PCR signals (giving a greater indication of the presence of norovirus) were obtained from 30 (5.3%) lettuce samples. Eight lettuce samples (out of 573 analysed; 1.4%) tested positive for *E. coli*. Most (24/30) lettuce samples which tested positive for norovirus (replicate RT-PCR signals) were grown in the UK and contained norovirus GI (19/24). Twenty one of the UK-grown samples were collected from lettuce on sale between May and August 2015.

Thirty seven / 310 (11.9%) samples of fresh raspberries gave norovirus RT-PCR signals; 7 samples (2.3%) gave replicate RT-PCR results. Most (6/7) of the positively-testing fresh raspberry samples in the NoVAS survey were imported, but no predominance of a genogroup, or any seasonality, was observed. Thirty four of the 274 (~12.6%) samples of frozen raspberries gave norovirus positive results; 10 samples (3.6%) gave replicate RT-PCR results. The country of origin of the positively-testing frozen raspberries samples was not identified in most (7/10) instances. None of the fresh (0/317) fresh and (0/256) frozen raspberries samples tested positive for *E. coli*.

6.2 Introduction

One of the key aims of the NoVAS project is to investigate the contribution of norovirus-contaminated food to the burden of disease caused by this viral agent in the UK. To assist this, the project planned to acquire representative data on contamination with norovirus in berry fruit and salad vegetables, since these commodities have been prominently implicated in several outbreaks of norovirus gastroenteritis globally (Advisory Committee on the Microbiological safety of Food, 2015). The European Food Safety Authority (EFSA Panel on Biological Hazards (BIOHAZ), 2013), risk ranked the combination of leafy green vegetables, eaten raw as salads, together with *Salmonella* spp. and norovirus, as of highest importance for human cases of infection originating from food of non-animal origin in the EU. EFSA

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[6] This work is published: Cook N, Williams L, D'Agostino M. Prevalence of Norovirus in produce sold at retail in the United Kingdom. Food Microbiol. 2019; 79: 85-89.

also ranked raspberries / *Salmonella* / norovirus and strawberries / *Salmonella* / norovirus as being the 4th and 6th respectively most often linked to foodborne human cases originating from food of non-animal origin in the EU (EFSA Panel on Biological Hazards (BIOHAZ), 2013).

Currently, there are no data on prevalence of norovirus within berry fruit and leafy green vegetables sold at retail in the UK, which is a key evidence gap regarding the extent of foodborne virus contamination (ACMSF, 2015). To acquire representative data, WP4 aimed to collect and analyse samples of lettuce, fresh raspberries and frozen raspberries as key examples of leafy green vegetables and berry fruits respectively. The number of samples testing positive may indicate the level of exposure of the UK population to norovirus from consumption of these fresh produce items.

6.3 How the survey was conducted

The survey was performed between 1st March 2015 and 7th April 2016, and was conducted according to the procedures detailed in Appendix 7.

6.3.1 Survey Design

Fresh produce samples were taken from retail premises from the 4 United Kingdom countries: England, Northern Ireland, Scotland, and Wales. The number of samples taken from each country were planned to proportionately represent the population of each country compared to the total UK population. Samples were taken from four regions in England (Devon, London, Manchester and Southampton), 3 regions in Scotland (Aberdeen, Dundee and Glasgow), and 2 regions each in Northern Ireland (Belfast and Londonderry) and Wales (Bangor and Cardiff). It was not possible given the resources of the project to cover all UK regions; however, as the regions chosen represent major population centres within each of the countries, it is considered that the survey gave as representative overview of the UK as possible within the given constraints.

The samples were taken from 4 categories of outlets: Wholesalers (including suppliers of catering establishments and restaurants), Supermarkets, Markets (including farmers' markets, stalls, pick-your-own and on-line stores), and Small Retailers (e.g. convenience stores).The sampling plan attempted to reflect the higher market share that supermarkets have for food purchases in the UK (information from Defra's Food Statistics Pocketbook, 2014). According to this source, 82% of food purchases are made from the top 10 supermarkets. It was possible to approximate this for the English regions, since due to the large population a large number of samples were taken. For Northern Ireland, Scotland and Wales, it was not possible to precisely reflect this proportion, but more samples were taken from supermarkets

than from the other outlet types in these countries. Frozen berries were not sampled from markets as it is unlikely frozen produce will be on sale at such outlets.

No weighting was given to UK-produced versus imported produce, as the relative levels of availability of each are not known.

6.3.2 Sampling

Sampling was conducted between March 2015 and April 2016 and was performed by SRCL. Analysis of samples was performed “blind”. SRCL samplers redacted product information and attached a unique identifier before sending samples to the Fera. The full details were stored on SRCL’s database along with the unique identifier number and provided to the laboratory post-analysis. At least 100 g of lettuce heads and at least 100 g of each raspberry category were taken at randomly selected sampling points. Only samples of open leafed lettuce (e.g. not Iceberg or any lettuce with a similar closed leaf appearance and not ready-to-eat bagged lettuce) were collected. Lettuces of this type were considered most likely, due to the loose nature of their leafy heads, to retain viruses that may have contaminated them at primary production. One thousand twelve hundred and eighty nine samples were collected in total: 631 samples of lettuce, 346 samples of fresh raspberries, and 312 samples of frozen raspberries. Approximately 90 samples of fresh produce were collected each month. Immediately upon collection, samples were placed in cool boxes and dispatched to Fera Science Ltd (Fera).

6.3.3 Sample receipt

Immediately upon receipt at Fera, all samples were examined for suitability; samples were rejected if unsuitable (e.g. wrong type or degraded). From each accepted sample, two portions of 25-30g were taken and immediately spiked with 106 GE mengovirus vMC0 as sample process control virus (SPCV; Ruhanya *et al*., 2015). One portion was analysed immediately, the other stored (at 4oC for lettuce and at -20°C for raspberries). Another portion of 25-30g was taken from each sample but not spiked with mengovirus (this was used for *E. coli* analysis, see 6.3.8 below).

6.3.4 Sample treatment

Viruses and nucleic acids were extracted from each mengovirus-spiked sample following the method of Anonymous (2013). Briefly, virus was eluted from the food surfaces by immersion in alkaline buffer containing beef extract, then precipitated by flocculation and centrifugation. Nucleic acids were extracted from the flocculate by commercial kit (NucliSens, Biomerieux). A final volume of 100 µl nucleic acid extract was obtained from each sample.

6.3.5 Detection of Norovirus and mengovirus

Detection of norovirus GI and GII was performed on the mengovirus-spiked sample portions following the protocol described in (Anonymous (2013). Detection of mengovirus vMC0 was likewise performed following the protocol described in (Anonymous 2013). 5 □l aliquots of both neat extract and 10-1 dilution were analysed by RT-PCR in duplicate for norovirus, but only the 10-1 dilution was analysed in duplicate for mengovirus.

6.3.6 Interpretation of RT-PCR results

All amplification data were converted to logarithmic plots using a Bio-Rad CFX Manager version 3.1 program, with the cycle threshold set manually. Analysis was considered to have failed if no external amplification control (EAC) signal was observed, if the recovery of the process control was less than 1%, or if RT-PCR signals were observed in the amplification negative control. If analysis of a sample failed, the stored mengovirus-spiked sample was taken for analysis. All samples from which RT-PCR analysis for norovirus produced a signal, even in only one replicate assay, were recorded. To facilitate a more rigorous interpretation of the data (see Discussion), all samples from which replicate RT-PCR norovirus signals were obtained, were also recorded.

6.3.7 Capsid Integrity Assay

Norovirus positive-testing samples were stored at -20°C for later capsid integrity testing. The capsid integrity testing was performed only on samples where analysis of the neat or the 10^{-1} dilution of the nucleic acid extract for either norovirus GI or norovirus GII had produced Cq values <40 in duplicate, as it was considered that these had the best likelihood of producing robust results. The capsid integrity assay was performed as described in the chapter for Work Package 2.

6.3.8 Detection of Escherichia coli

Detection of *E. coli* was performed on the unspiked sample portions by the PHE Food, Water and Environmental Laboratory, York, using standard culture methods, based on Anonymous (2001) and Anonymous (2005).

6.4 Results[7]

The results of the survey are summarised in the tables below. Some data from SRCL was not available.

In total, 1289 samples were received (Table 14). 90 samples were rejected immediately upon receipt (Table 15), for several reasons including late delivery and poor condition (e.g. excessive liquid in raspberry samples). From the accepted samples, it was not possible to send 33 samples for *E. coli* testing, as there was insufficient sample to perform all the necessary tests. Forty seven RT-PCR based analyses for norovirus were considered to have failed, when recovery of the sample process control virus (SPCV) was below 1 % after 2 successive tests. Finally, 1152 samples were tested for norovirus, and 1146 were tested for *E. coli*. Norovirus RT-PCR signals were detected in 13.0% of samples, and *E. coli* was detected in 0.7%. In several samples, RT-PCR signals were observed only in one replicate assay. Replicate (either within one test or over two tests) norovirus RT-PCR signals were obtained from 4.1% samples. In the early stages of the survey, if a sample portion produced an RT-PCR signal, the second portion (spiked with mengovirus) was analysed in a second test. Later in the survey however time and resource did not permit analysis of the second spiked portion.

Table 14: Summary of overall results

Samples Received	Samples Rejected	Norovirus Analyses failed	Norovirus Analyses completed	Norovirus RT-PCR signals* detected	Replicate Norovirus RT-PCR signals detected	*E. coli* analyses completed	*E. coli* detected
1289	90	47	1152	150 (GI 75) (GII 94) (GI & GII 19)	47 (GI 33) (GII 19) (GI & GII 5)	1146	8

*** Samples where both singlicate signals only, and replicate signals, were observed**

Table 15: Summary of rejected samples

Type	Late delivery	Poor condition	Wrong type	No Paperwork
Lettuce	24	0	11	1
Fresh raspberries	13	15	0	0
Frozen raspberries	6	20	0	0

[7] The data file for Work Package 4 can be viewed in Excel format (see Appendix 8)

Table 16: Samples analysed, by country. * to nearest round number

Country	% of UK population	Total (% of total*)	Lettuce (% of total*) 568	Fresh raspberries (% of total*) 310	Frozen raspberries (% of total*) 274
England	84	982 (85)	493 (86)	254 (82)	235 (86)
Northern Ireland	3	18 (2)	7 (1)	8 (3)	3 (1)
Scotland	8	106 (9)	52 (9)	29 (9)	25 (9)
Wales	5	46 (4)	16 (3)	19 (6)	11 (4)

Table 17: Samples analysed, by outlet category

Outlet category	% of market share*	Total	Lettuce	Fresh rasps	Frozen rasps
Markets	NA	35	23	12	0
Small retailers	NA	50	31	13	6
Supermarkets	82	1021 (89)†	487 (86)†	276 (89)†	258 (94)†
Wholesalers	NA	33	18	7	8
Not identified	-	13	9	2	2

*** Information from DEFRA's Food Statistics Pocketbook, 2014**
NA: not available
† % of total samples taken

Table 18 shows the summary of results obtained from analysis of the lettuce samples. Norovirus RT-PCR signals were detected in 13.9% samples, and *E. coli* was detected in 1.4%. Norovirus GI RT-PCR signals were detected from 7.4% lettuce samples, GII signals in 7.9%, and GI and GII signals were detected together in 1.4% lettuce samples. Replicate norovirus RT-PCR signals were detected from 5.3% lettuce samples, replicate GI signals in 3.9%, replicate GII signals in 1.6%, and replicate GI/GII signals were detected together in 0.2% lettuce samples.

Table 18: Summary of lettuce results

Total samples received	Norovirus Analyses completed	Norovirus RT-PCR signals* detected	Replicate Norovirus RT-PCR signals detected	E. coli analyses completed	E. coli detected
631	568	79 (GI 42) (GII 45) (GI & GII 8)	30 (GI 22) (GII 9) (GI & GII 1)	573	8

***Samples where both singlicate signals only, and replicate signals, were observed.**

Table 19 shows the summary of results obtained from analysis of the fresh raspberry samples. Norovirus RT-PCR signals were detected in 11.9% samples, but no *E. coli* was detected in any sample. Norovirus GI RT-PCR signals were detected in 5.8% fresh raspberry samples, GII signals from 8.4%, and GI and GII signals were detected together in 2.3%, fresh raspberry samples. Replicate norovirus RT-PCR signals were detected in 2.3% fresh raspberry samples. Replicate norovirus GI RT-PCR signals were detected in 0.9% samples, replicate GII signals from 1.6% samples, and replicate GI and GII signals were detected together in 0.3% fresh raspberry samples.

Table 19: Summary of fresh raspberries results

Total samples received	Norovirus Analyses completed	Norovirus RT-PCR signals* detected	Replicate Norovirus RT-PCR signals detected	*E. coli* analyses completed	*E. coli* detected
346	310	37 (GI 18) (GII 26) (GI & GII 7)	7 (GI 3) (GII 5) (GI & GII 1)	317	0

***Samples where both singlicate signals only, and replicate signals, were observed.**

Table 20 shows the summary of results obtained from analysis of the frozen raspberry samples. Norovirus RT-PCR signals were detected from 12.4% samples, but no *E. coli* was detected in any samples. Norovirus GI RT-PCR signals were detected from 6.2% frozen raspberry samples, GII signals from 8.0% frozen raspberry samples, and GI and GII signals were detected together in 1.8% frozen raspberry samples. Replicate norovirus RT-PCR signals were detected in 3.6% frozen raspberry samples. Replicate norovirus GI RT-PCR signals were detected in 2.9% samples, replicate GII signals from 1.8% samples, and replicate GI and GII signals were detected together in 1.1% frozen raspberry samples.

Table 20: Summary of frozen raspberries results

Total samples received	Norovirus Analyses completed	Norovirus RT-PCR signals* detected	Replicate Norovirus RT-PCR signals detected	*E. coli* analyses completed	*E. coli* detected
312	274	34 (GI 17) (GII 22) (GI & GII 5)	10 (GI 8) (GII 5) (GI & GII 3)	256	0

***Samples where both singlicate signals only, and replicate signals, were observed.**

Table 21 shows the summary of the samples analysed, by origin. Norovirus RT-PCR signals were detected in 14.9% of UK lettuce samples, 13.1% imported lettuce samples, and 3.6% of lettuce samples of which the origin was unknown. Replicate norovirus RT-PCR signals were detected in 6.1% of UK lettuce samples, and 4.1% imported lettuce samples. Norovirus RT-PCR signals were detected in 13.3% of UK fresh raspberry samples, 11.3% imported fresh raspberry samples, and 11.1% of fresh raspberry samples of which the origin was unknown. Replicate norovirus RT-PCR signals were detected in 1.0% of UK fresh raspberry samples, and 3.0 % imported fresh raspberry samples. Norovirus RT-PCR signals were detected in 11.8% of UK frozen raspberry samples, 13.3% imported frozen raspberry samples, 13.5% frozen raspberry samples sourced from various countries, and 11.8% of frozen raspberry samples of which the origin was unknown. Replicate norovirus RT-PCR signals were detected in 1.5% of UK frozen raspberry samples, 4.4% imported frozen raspberry samples, 5.8% frozen raspberry samples sourced from various countries, and 3.6% of frozen raspberry samples of which the origin was unknown.

Table 21: Samples analysed, by origin

	UK	Imported	Various countries	Not known
Lettuce	395 (59)* (24)†	145 (19)* (6)†	0	28 (1)* (0)†
Fresh raspberry	98 (13)* (1)†	203 (23)* (6)†	0	9 (1)* (0)†
Frozen raspberry	67 (8)* (1)†	45 (6)* (2)†	52 (7)* (3)†	110 (13)* (4)†
Total	560 (80)* (26)†	393 (48)* (14)†	52 (7)* (3)†	147 (15)* (4)†

*** Samples where both singlicate signals only, and replicate signals, were observed.**

† Replicate RT-PCR signals observed.

Table 22 shows the summary of the samples analysed, by outlet type. Norovirus RT-PCR signals were detected in 17.4 % of lettuce samples obtained from markets, in 3.3 % of lettuce samples obtained from small retailers, in 14.4 % of lettuce samples obtained from supermarkets, and in 22.2 % of lettuce samples obtained from wholesalers. Replicate norovirus RT-PCR signals were detected in 13.0% of lettuce samples obtained from markets, in 5.3% of lettuce samples obtained from supermarkets, and in 5.6% of lettuce samples obtained from wholesalers. Norovirus RT-PCR signals were detected in 8.3 % of fresh raspberry samples obtained from markets, in 7.7% of fresh raspberry samples obtained from small retailers, in 11.9 % of fresh raspberry samples obtained from supermarkets, and in 28.6% of fresh raspberry samples obtained from wholesalers. Replicate norovirus RT-PCR signals were detected in 7.7% of fresh raspberry samples obtained from small retailers, in 1.8% of fresh raspberry samples obtained from supermarkets, and in 14.2% of fresh raspberry samples obtained from wholesalers. Norovirus RT-PCR signals were detected in 33.3% of frozen raspberry samples obtained from small retailers, and in 12.5% of frozen raspberry samples obtained from supermarkets. Replicate norovirus RT-PCR signals were detected in 16.7% of frozen raspberry samples obtained from small retailers, and in 3.5% of frozen raspberry samples obtained from supermarkets.

Table 22: Samples analysed, by outlet type.

	Markets	Small retailers	Supermarkets	Wholesalers	Not identified
Lettuce	23 (4)* (3)†	31 (1)* (0)†	487 (70)* (26)†	18 (4)* (1)†	9 (0)* (0)†
Fresh raspberry	12 (1)* (0)†	13 (1)* (1)†	276 (33)* (5)†	7 (2)* (1)†	2 (0)* (0)†
Frozen raspberry	0	6 (2)* (1)†	258 (32)* (9)†	8 (0)* (0)†	2 (0)* (0)†
Total	35 (5)* (3)†	50 (4)* (2)†	1021 (135)* (40)†	33 (6)* (2)†	13 (0)* (0)†

*** Samples where both singlicate signals only, and replicate signals, were observed.**
† Replicate RT-PCR signals observed.

Table 23: Lettuce samples in which replicate RT-PCR signals were observed

Date of collection	Region	Outlet type	Country of origin	Norovirus
14/04/2015	Southampton	Market	UK	GII
11/05/2015	London	Supermarket	Spain	GI
11/05/2015	London	Supermarket	UK	GII
12/05/2015	Devon	Supermarket	UK	GII
12/05/2015	London	Supermarket	UK	GI
26/05/2015	Manchester	Supermarket	UK	GII
08/06/2015	Manchester	Supermarket	UK	GI
09/06/2015	Southampton	Wholesaler	UK	GI
10/06/2015	Manchester	Supermarket	UK	GI
10/06/2015	Devon	Market	UK	GI
15/06/2015	London	Supermarket	UK	GI
27/06/2015	London	Supermarket	UK	GI
27/07/2015	Manchester	Supermarket	UK	GI
28/07/2015	London	Market	UK	GI
28/07/2015	Glasgow	Supermarket	UK	GI
28/07/2015	Cardiff	Supermarket	UK	GI
28/07/2015	Cardiff	Supermarket	UK	GI
29/07/2015	Southampton	Supermarket	UK	GI
29/07/2015	Devon	Supermarket	UK	GI
29/07/2015	Devon	Supermarket	UK	GI/GII
04/08/2015	London	Supermarket	UK	GI
05/08/2015	Manchester	Supermarket	UK	GI
11/08/2015	Devon	Supermarket	UK	GI
23/11/2015	London	Supermarket	Spain	GII
08/12/2015	Devon	Supermarket	UK	GII
02/02/2016	Devon	Supermarket	UK	GI
10/02/2016	Manchester	Supermarket	Spain	GI
02/03/2016	Devon	Supermarket	Spain	GII
07/03/2016	London	Supermarket	Spain	GII
22/03/2016	Southampton	Supermarket	Spain	GII

Table 24: Fresh raspberry samples in which replicate RT-PCR signals were observed

Date of collection	Region	Outlet type	Country of origin	Norovirus
29/04/2015	Dundee	Wholesaler	Spain	GI/GII
12/05/2015	Devon	Supermarket	Spain	GII
12/05/2015	Southampton	Supermarket	Morocco	GI
30/07/2015	Belfast	Small retailer	UK	GII
08/02/2016	London	Supermarket	Morocco	GI
07/03/2016	Manchester	Market	Spain	GII
07/03/2016	Glasgow	Small retailer	Spain	GII

Table 25: Frozen raspberry samples in which replicate RT-PCR signals were observed

Date of collection	Region	Outlet type	Country of origin	Norovirus
28/04/2015	Devon	Supermarket	Various	GI/GII
28/04/2015	Devon	Supermarket	Serbia	GI
29/04/2015	Manchester	Supermarket	Serbia	GI
29/04/2015	Manchester	Small retailer	UK	GI/GII
05/05/2016	Devon	Supermarket	Various	GI
13/05/2015	London	Supermarket	NA	GI/GII
13/07/2015	Manchester	Supermarket	NA	GII
28/07/2015	Manchester	Supermarket	Various	GI
30/07/2015	Dundee	Supermarket	NA	GI
09/11/2015	London	Supermarket	NA	GII

NA = Information not available.

Table 26: Samples selected for Capsid Integrity testing

Produce Type	GI Cq value*		GII Cq value*	
	Neat[n]	-1[n]	Neat[n]	-1[n]
Lettuce	37.08† 39.92†	NS NS	NS NS	39.92 NS
Lettuce	NS NS	37.73 NS	34.14 34.43	38.61 41.62
Frozen raspberries	37.58 44.70	NS NS	31.24 31.20	42.27 34.71
Lettuce	NS NS	NS NS	30.46 30.56	36.23 38.97
Lettuce	32.32 32.11	35.84 36.21	NS NS	NS NS
Lettuce	NS NS	NS NS	36.85 36.68	NS NS
Lettuce	NS NS	NS NS	37.04 35.64	NS NS
Fresh raspberries	42.08 NS	NS NS	35.38 37.03	NS NS
Lettuce	NS NS	40.41 NS	NS NS	38.32 33.01

*** Cq values obtained during survey. Sample nucleic acid extracts were tested in duplicate**
[n] Nucleic acid extract
† Singlicate results from repeated tests (Test 1 and Test2)
NS: No signal.

Table 27: Results of Capsid Integrity tests on selected samples

Fera Sample Reference	Sub-Sample 1 Treated		Sub-Sample 1 Untreated		Sub-Sample 2 Treated		Sub-Sample 2 Untreated	
	Neat[n]	10^{-1n}	Neat[n]	10^{-1n}	Neat[n]	10^{-1n}	Neat[n]	10^{-1n}
179	34.14†	39.4	33.18	NS	35.64	37.22	33.74	45.93
	34.43	38.21	34.81	NS	33.28	NS	33.18	NS
	34.07	39.44	34.97	NS	35.53	NS	34.31	NS
180	38.12	NS	35.19	36.90	38.27	45.6	37.20	NS
	37.26	NS	NS	NS	NS	NS	35.08	MS
	34.14	NS	NS	NS	NS	NS	NS	NS
196	37.67	NS	NS	NS	41.17	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
214	38.21	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
272	NS	NS	NS	NS	44.00	NS	NS	NS
	NS	NS	NS	NS	37.94	NS	NS	NS
	NS	NS	NS	NS		NS	NS	NS
846	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
1118	39.25	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
1127	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS
1211	NS	NS	NS	NS	NS	NS	38.48	NS
	NS	NS	NS	NS	NS	NS	NS	NS
	NS	NS	NS	NS	NS	NS	NS	NS

[n]Nucleic acid extract
[1]Capsid integrity analysis was performed using norovirus GI assay.
[2] Capsid integrity analysis was performed using norovirus GII assay.
† Cq values. Sample nucleic acid extracts were tested in triplicate. Where less than three Cqs are given it signifies that not all replicate RT-PCRs produced a signal.
NS: No signal.

Table 28 contains details of the fresh produce samples in which *E. coli* was detected. All the samples that E. coli was detected in were lettuce; the bacterium was not detected in any fresh or frozen raspberry sample. The United Kingdom was the origin of all the *E. coli* positive samples. Five of the lettuce samples were purchased from supermarkets, 2 from markets, and 1 from a wholesaler. Four samples were purchased in Southampton, 3 in London, and 1 in Manchester. In one *E. coli* positive lettuce sample, a norovirus GI RT-PCR signal was detected.

Table 28: Details of *E. coli* positive samples

Produce type	Date collected	Retailer type	Region	Origin	CFU g^{-1}	Norovirus RT-PCR signal detected
Lettuce	14/07/2015	Wholesaler	Southampton	UK	60	No
Lettuce	27/07/2015	Supermarket	London	UK	1370	Yes*
Lettuce	03/08/2015	Market	Southampton	UK	10	No
Lettuce	04/08/2015	Market	Southampton	UK	10	No
Lettuce	24/08/2015	Supermarket	Manchester	UK	10	No
Lettuce	24/08/2015	Supermarket	London	UK	10	Yes†
Lettuce	08/09/2015	Supermarket	London	UK	40	No
Lettuce	10/11/2015	Supermarket	Southampton	UK	40	No

*** GI signals obtained from this sample.**
† GII signal obtained from this sample.

6.5 Discussion

The relative numbers of samples in each UK country in this study closely reflect the relative proportions of the UK population. Similarly, the preponderance of samples from supermarkets reflects the relative market share of this type of outlet compared to markets, small retailers and wholesalers. The samples may thus be regarded as as-representative as possible of the consumption of the produce types in the UK at the time of the study, given the available information.

The norovirus RT-PCR signals obtained in this study need to be interpreted with care. The external amplification controls (EACs), prescribed in ISO 15216 to be used in the RT-PCR sample analysis, are derived from actual norovirus sequences, and the primers used for amplification of the EACs are the same as those which were used to amplify the sequences from norovirus strains. Likewise, the same probe is used for detection of the amplicons of the EAC and amplicons of any norovirus strains extracted from the sample. Thus, the EAC RT-PCR signals produced by the GI and GII EACs are identical to the signals which are produced by amplification of norovirus sequences from actual viruses. The EAC amplicons might be distinguished from norovirus GI amplicons by sequencing, but in no instance when sequencing of the norovirus amplicons was performed was confirmation obtained that they were derived from actual viruses and not from contaminating EAC. It is difficult to obtain sequence information from such small (~90 bp) nucleotide fragments (Baert *et al*. (2011) and Stals *et al*. (2011) also reported that they were unable to obtain sequence information for the majority of norovirus amplicons they obtained in their studies). The EACs were first described in Le Guyader *et al*. (2009), who inserted a sequence facilitating recognition by the BamHI restriction enzyme into the EAC sequence. Thus it should be possible to distinguish true norovirus signals from contaminating EAC signals by cloning the amplicons and digestion with BamHI, but

time and resource did not allow this within the study. Meanwhile, a more rigorous interpretation of the data, reducing uncertainty due to any possible cross-contamination with EACs, can be performed by classifying only those samples which yielded replicate RT-PCR signals, as norovirus-positive.

No previous surveys of fresh produce for norovirus have been conducted in the UK. There have however been a limited number of surveys in other countries (EFSA BIOHAZ Panel (EFSA Panel on Biological Hazards), 2014a,b). In the NoVAS study, RT-PCR analysis indicated that 30/568 (5.3%) of lettuce samples were norovirus positive. Mattison *et al*. (2010) sampled 641 samples of lettuce sold in supermarkets in Canada, and found 181 positive for norovirus (28.2%). Kokkinos *et al*. (2012), analysed lettuce samples at point of sale in three European countries, and found 2/149 (1.3%) samples to be norovirus GI positive and 1/126 (0.8%) to be norovirus GII positive. The prevalence of norovirus in lettuce recorded in the NoVAS survey is intermediate between these two sets of findings. Most (24/30) lettuce samples which tested positive for norovirus were grown in the UK. Also notable is the observation that most of the positively testing UK lettuce samples contained norovirus GI, and displayed some seasonality in that 21 of the UK-grown samples were collected from lettuce on sale between May and August 2015. It would be interesting to see whether this pattern is reflected in any reports of norovirus infection around this period. All the *E. coli*-positive samples were UK-grown lettuce, and 6/8 were obtained within the same period.

In the NoVAS study, RT-PCR analysis indicated that 7/310 (12.3%) of fresh raspberry samples were norovirus positive. Baert *et al*. (2011) found 10/150 (6.7%) samples of fresh berry fruits obtained from food companies in France were norovirus positive, and in the study of Stals *et al*. (2011), 4/10 fresh raspberry samples obtained from a processing company in Belgium tested positive for norovirus. However, when Maunula *et al*. (2013) analysed 60 samples of fresh raspberries at point of sale in 4 European countries, no norovirus positive samples were identified. Most (6/7) of the positively-testing fresh raspberry samples in the NoVAS survey were imported from identified countries, and no predominance of a genogroup, or any seasonality, was observed.

In the NoVAS study, RT-PCR analysis indicated that 10/270 (3.6%) of frozen raspberry samples were norovirus positive. Maunula *et al*. (2013) examined 39 frozen raspberry samples from point of sale, but no norovirus positive samples were identified. Sarkiviki *et al*. (2012) analysed 14 samples of frozen raspberries implicated in an outbreak in Finland and detected norovirus in 2 (14.3%) samples, and Mäde *et al*. (2013) analysed 11 samples of frozen raspberries implicated in an outbreak in Germany and found 7 (63.6%) to be positive. In the NoVAS survey, the country of origin of the positively-testing frozen raspberry samples was not identified in most (7/10) instances. In three samples, the raspberries had been grown in several unstated countries, and in 4 samples no information was available on the

origin of the fruit. This likely reflects the nature of the supply chain of this commodity, with products being sourced from various locations.

Until further prevalence data is acquired by further national or international studies, it is difficult to comment on how the norovirus prevalence in UK retail fresh produce in this study precisely compares with the global situation.

Two attempts were made to sequence the positive fresh produce samples from Work Package 4. In the first, several WP4 sample nucleic acid extracts were sent to PHE Colindale, who found the results of their sequencing to be inconclusive. The second attempt was performed by Fera, in the form of NGS of selected WP4 samples, and the report for this can be found in Appendix 9. You may wish to refer back to the section on NGS in the chapter above for Work Package 3 for details on some of the issues found with sequencing low yield food and stool samples.

RT-PCR detection of norovirus does not indicate per se that the virus was infectious. Nine norovirus positive samples were selected for capsid integrity tests, which might have allowed more information on potential infectivity to be acquired. Only from Subsample 2 of sample 179 could a ∆Cq be obtained by comparison of all 3 replicate RT-PCRs from the neat extracts of the treated and untreated subsamples. For untreated Subsample 2, the average Cq from the neat extract was 33.74 and the average Cq from the neat extract of treated Subsample 2 was 34.81. The ∆Cq was therefore 1.07. This may signify that in this subsample ~40% of the GII virus particles present had intact capsids and therefore had the potential to be infectious, but otherwise the result is inconclusive as it was not observed with the other subsample. Likewise all other results were inconclusive, as either no signal was obtained, or numbers of Cq-producing replicates were not the same between treatments, or no ∆Cq was observed. It is likely that the Cq values were too low (corresponding to low numbers of norovirus particles) to allow the capsid integrity assay to be performed effectively. However, the production of norovirus RT-PCR signals on reanalysis of seven samples may be taken as confirmation of the original result, and strongly indicative of the presence of norovirus in these samples. Effectively, although the CIA results were inconclusive – meaning it is difficult to say whether the detection of RNA indicates that products are infectious or not (and therefore likely to make the consumer ill or not) – the data acquired does provide evidence that there was contamination of produce by norovirus, and therefore there was an element of risk to public health at the time. The magnitude of this risk is estimated using the risk model in Work Package 6.

7 Work Package 5: Prevalence of norovirus in the catering environment in outbreak and non-outbreak premises

7.1 Summary

We conducted a prevalence survey of norovirus in the catering environment in outbreak and non-outbreak premises, in collaboration with Environmental Health Officers. This will allow us to assess the contribution of food handlers to contamination of the catering environment.

Overall, 11% (30/252) of the premises sampled yielded at least one norovirus positive sample (environmental, and/or hand swab), and 2.5% of the swabs were positive for norovirus in total. The proportion of premises in which norovirus was detected in the Greater London (GL) area was 21% compared to 4% in the North West (NW).

The proportion of norovirus positive premises was not significantly different during outbreak investigation or routine surveillance sampling: 25% (4/16) compared to 18% (44/247).

In this study, bacterial indicators did not provide an effective indicator for norovirus. A four-fold higher number of premises had bacterial indicator organisms isolated (118) compared to those with a norovirus being detected (30).

The overall prevalence of norovirus in the catering establishment was lower than expected, but it must be taken into consideration that norovirus is a seasonal infection, and that there is wide year to year variation in the overall prevalence of norovirus disease. Norovirus surveillance data collected by PHE demonstrated low levels of norovirus disease and norovirus outbreaks in the UK during the study period, therefore the results of this study must be interpreted in the context of a low incidence of norovirus illness in the UK.

7.2 Aims of the Study

This study aimed to investigate the following hypotheses:

(a) Contamination of the kitchen environment with norovirus will be higher in premises that have recently reported a foodborne norovirus outbreak than those that have not;

(b) The levels of environmental contamination are likely to be seasonal, with greater levels of contamination being detected in the winter months (November to March);

(c) In food handler associated outbreaks the viruses in the environment will exhibit the same sequence types as viruses found in faecal samples from food workers and affected consumers.

7.3 Materials and Methods

A prevalence survey was carried out in catering premises across North West and South East England (Comprising the Greater London area). Catering premises were defined as a commercial or voluntary organisation that prepares and serve food to the final consumer. This included restaurants, public houses, cafes, takeaways, hotels, guesthouses, and caterers, but excluded passenger carrying ships that travel outside the UK, private houses, mobile retailers, manufacturers and suppliers.

7.3.1 Surveillance sampling

A detailed study protocol was previously submitted (Appendix 7). In summary, sampling was carried out by Local Authority (LA) Environmental Health Officers (EHOs) undertaking routine inspections of catering premises across the London Boroughs (London Borough of Southwark, Royal Borough of Greenwich, London Borough of City of London, London Borough of Tower Hamlets and London Borough of Ealing) and in the North West of England LAs representative from each of the four Food Liaison groups in the North West agreed to participate (Sefton for Cheshire and Merseyside, Allerdale for Cumbria, Fylde for Lancashire and Salford for Greater Manchester).

The sampling was carried out at monthly intervals, and covers one 15 months between January 2015 and April 2016. Premises were selected at random to represent the food hygiene rating scores and premise types that are represented in their areas.

Inspections were recorded using the current UK Food Surveillance System (UKFSS), as done routinely by LAs. During inspection visits EHOs took two types of swabs from food and hand contact surfaces as routinely used for microbiological indicators of hygiene and including food preparation surfaces such as chopping boards, kitchen worktops, refrigerator/chiller handles, kitchen sink taps, staff toilet handles etc. (see Appendix 10) surface, those, bacteriological SpongeSicle™ swabs; and virology swabs for the detection of norovirus RNA. A standardised form was used during inspection visits to collect the information required (see Appendix 10).

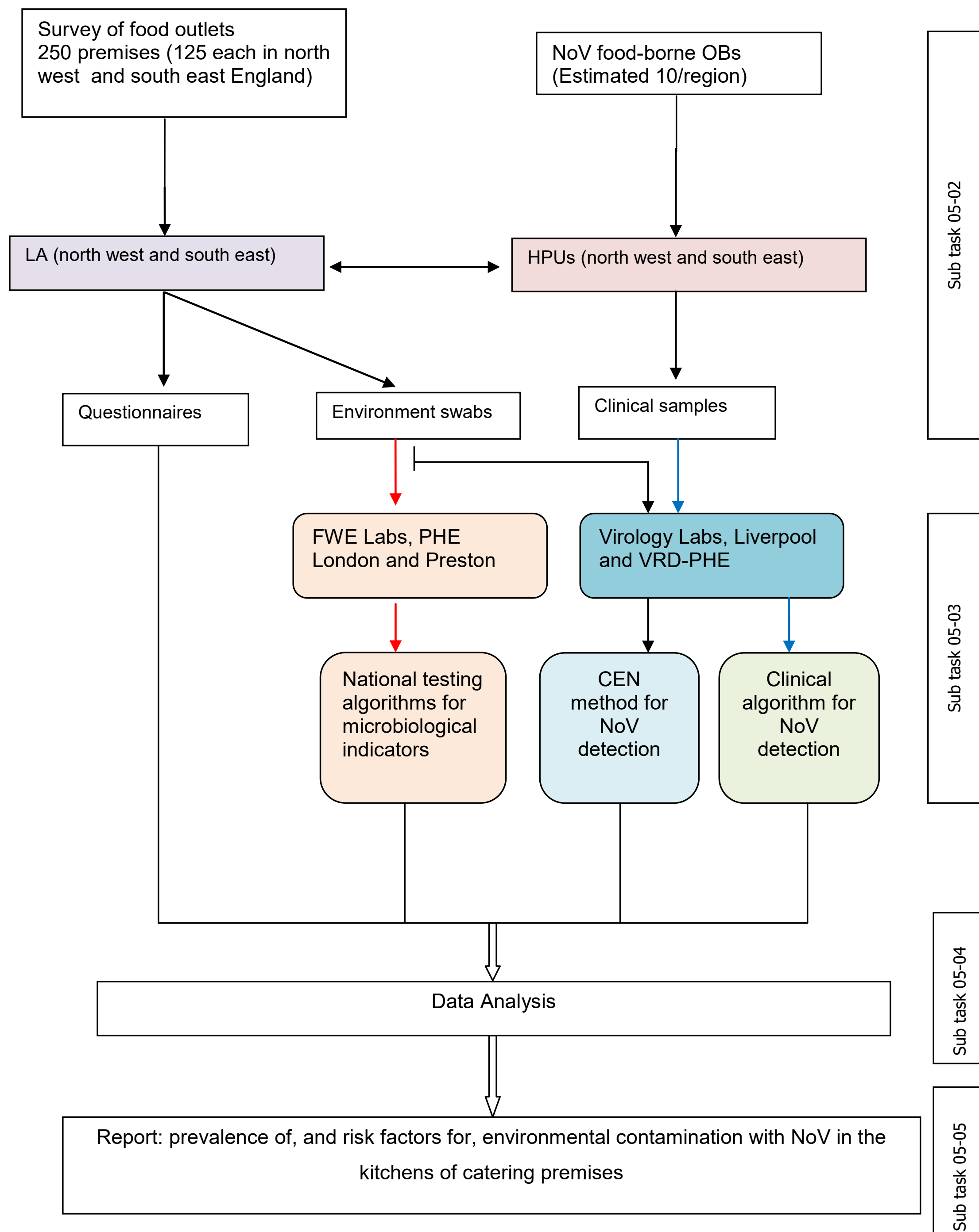
7.3.2 Sampling during outbreaks

The prevalence survey was supplemented by including premises that were being investigated because of a foodborne norovirus outbreak. An outbreak was defined as either (a) two or more people from more than one household who are thought to have a common exposure to proven norovirus infection or (b) clinically on the basis of Kaplan's criteria. Foodborne norovirus outbreaks were defined according to the following criteria:

- The outbreak is a point source outbreak.
- There is a common food exposure – meal/buffet lunch/wedding breakfast.
- Foodborne transmission is the only or predominant transmission pathway identified by investigators.
- The cases do not have any other common exposure that could explain the outbreak apart from the consumption of food.
- The outbreak is not known to be the result of a guest or member of staff vomiting in a public area.

In outbreak premises, in addition to environmental swabs, stool sample from members of staff were collected where possible via the appropriate Environmental Health Department. Where possible clinical specimens associated with outbreaks of norovirus in catering establishments in the North West of England and the South East were submitted to the respective lead Public Health Laboratory for norovirus detection, Preston and London, respectively. Norovirus positive outbreak case specimens associated with catering establishments were referred for genotyping and strain characterisation (see Figure 25).

Figure 24: Sampling and testing algorithm



7.3.3 Virology: Norovirus testing

The method was described in detail in the WP5 study protocol (Appendix 7), and briefly consists of the following steps; Upon arrival to the virology laboratory (Enteric Virus Unit [EVU], Public Health England [PHE], London or Clinical Virology, at the Royal Liverpool and Broadgreen University Hospital Trust [RLBUHT] Liverpool), swabs were immersed in lysis buffer with an external control (EC) added; samples were then stored refrigerated (+4°C to +8°C) until processing. Total nucleic acid was

extracted from the entire sample using magnetic silica beads and an automated extractor system. Positive and negative controls were included in each run.

The results were interpreted qualitatively only.

- Samples with cycle threshold (Cq) values below the cut-off in either of the norovirus-specific assays (Cq40) were considered positive.

- Samples with Cq values above the cut-off in both of the norovirus-specific assays were considered negative only if the EC RT-PCR results are within the expected Cq range.

- Samples with Cq values above the cut-off in either of the norovirus-specific assays in which EC RT-PCR results are not within the expected Cq range (suggesting inhibitory samples) were retested in a 1 in 10 dilution from the RNA extract (as all sample was extracted at once, it was possible to re-extract the nucleic acid) and reported accordingly. If the sample remained inhibitory in the dilution the results were reported as invalid test.

Positive samples derived from the kitchen/premises environment or food handler hand swabs or form clinical samples were genotyped using the current reference genotyping standard methods. In brief, cDNA was synthesised by performing a reverse transcription reaction using random hexamers. Subsequently, genotyping was carried out by amplification and sequencing of a fragments of the norovirus capsid S domain. Sequences were analysed by alignment against a database containing representative sequences of all known genotypes and assigned to a genotype based on >80% amino acid homology to a given reference sequence genotype. For outbreak tracking, when two or more samples were of the same genotype, amplification, 100% homology between norovirus strains within the capsid hyper variable region (P2 domain) indicated a common source and <100% identity indicated more than one potential source. Note that environmental samples are usually associated with low viral loads and that genotyping PCRs are inherently less sensitive than the detection RT-PCR. If amplicons were not obtained in the genotyping PCR, cDNA was retested in the detection PCR to confirm positivity. When results were concordant, they were determined to be “untypable”, if upon retesting the results were discordant with the original result, the results were considered equivocal.

7.3.4 Bacteriological testing

SpongeSicle™ swabs with 10 ml neutralising buffer were supplied by the PHE Food, Water and Environmental Microbiology Service for LAs to collect bacteriological swabs. Sampling Officers could collect samples from a defined template area (10 x 10 cm) or from a random area at the sampling point and method of sampling recorded on the sample request form submitted with the samples.

For random or non-template swabs, results will be presented as colony forming units (cfu) per swab. Samples were enumerated for:

- Coagulase positive *Staphylococci*, including *Staphylococcus aureus*
- *Escherichia coli*
- Enterobacteriaceae

For template area swabs (10 x 10 cm), results were represented as cfu per cm2. Samples were enumerated for:

- Aerobic colony count
- Coagulase positive *Staphylococci*, including *Staphylococcus aureus*
- *Escherichia coli*
- Enterobacteriaceae

Results for bacteriological swabs were interpreted as shown in Table 29.

Table 29: Interpretation for swabs collected for bacteriological testing

Sample Type	Test	Interpretation		
		CONSIDERED SATISFACTORY	CONSIDERED BORDERLINE	CONSIDERED UNSATISFACTORY
Measured Area (CFU per cm2)	Aerobic colony count	<100	$\geq 10^2$ - $<10^3$	$\geq 10^3$
	Escherichia coli	<2	-	≥ 2
	Enterobacteriaceae	<2	-	≥ 2
	Coagulase positive Staphylococci	<2	-	≥ 2
Random Area (CFU per swab)	*Escherichia coli*	<200	-	≥ 200
	Enterobacteriaceae	<200	-	≥ 200
	Coagulase positive Staphylococci	<200	-	≥ 200

The threshold for swabs is based on the PHE standard method for detection in swabs. A volume of 100 ml of diluent was added to the swab and the swab was homogenized using a Stomacher (Don Whitley Scientific). A volume of 0.5 ml of the homogenized, diluted sample was spread plated on to the selective agar plates giving a limit of detection (LOD) of 200 cfu per swab. As the sample was diluted 200 fold, the detection of a single colony on a plate is equivalent of 200 colonies per swab. This method is based on PHE Food, Water and Environmental Microbiology Service Standard Operating Procedure FNES4 and is the methodology used across the service for all microbiological public health swab samples collected by local authorities in England.

This includes the following calculation:

$$\text{Count} = \frac{C}{v\,(n1 + 0.1n2)d} \times n3$$

where: -

C is the sum of colonies on all plates counted;

v is the volume applied to each plate;

n1 is the number of plates counted at the first dilution;

n2 is the number of plates counted at the second dilution;

n3 is the original volume of neat suspension (i.e. 10 for swab, 500 or 100 for other samples);

d is the dilution from which the first count was obtained e.g.10-2 is 0.01;

Interpretation was based on the assumption that the surface sampled was clean and free of contamination, therefore the presence of any bacterial contamination was scored as unsatisfactory.

7.4 Results

7.4.1 Premises sampled

From a total of 256 premises sampled across the two regions, 252 met the study inclusion criteria, 123 in the South East (SE; greater London) and 129 in the North West (NW; greater Manchester). Of the premises visited, 236 were sampled for surveillance purposes, and 16 were sampled as part of an outbreak investigation (Table 30).

Overall 2,038 swabs were submitted for norovirus testing, with an average of 8 per premise (range 2 to 23) and a median of 7. The number of swabs submitted for outbreak investigation ranged from 10 to 23 with an average and a median of and 13 samples.

Table 30: Characteristics and number of premises sampled

	SE	NW	TOTAL
Total number of eligible premises sampled	123	129	252
Total Number of virology swabs referred	1,088	950	2,038
Surface swabs	831	705	1,536
Hand Swabs	257	245	502
Premises sampled for surveillance	117	119	236
Premises sampled for outbreak investigation	6	10	16

Sampling was carried out between January 2015 and April 2016 from premises across the food hygiene ratings (Table 31; Figure 26). Mean sampling was 127 samples/month, median 132 (range 51-205); with mean and median in the NW and the SE or 63 average and 60 median (range 41-111) and 68 and 61 (range 10-116), respectively. A detailed description of premises types included in the survey is shown in Appendix 11.

Figure 25: Distribution sampling by month in the NW and SE regions

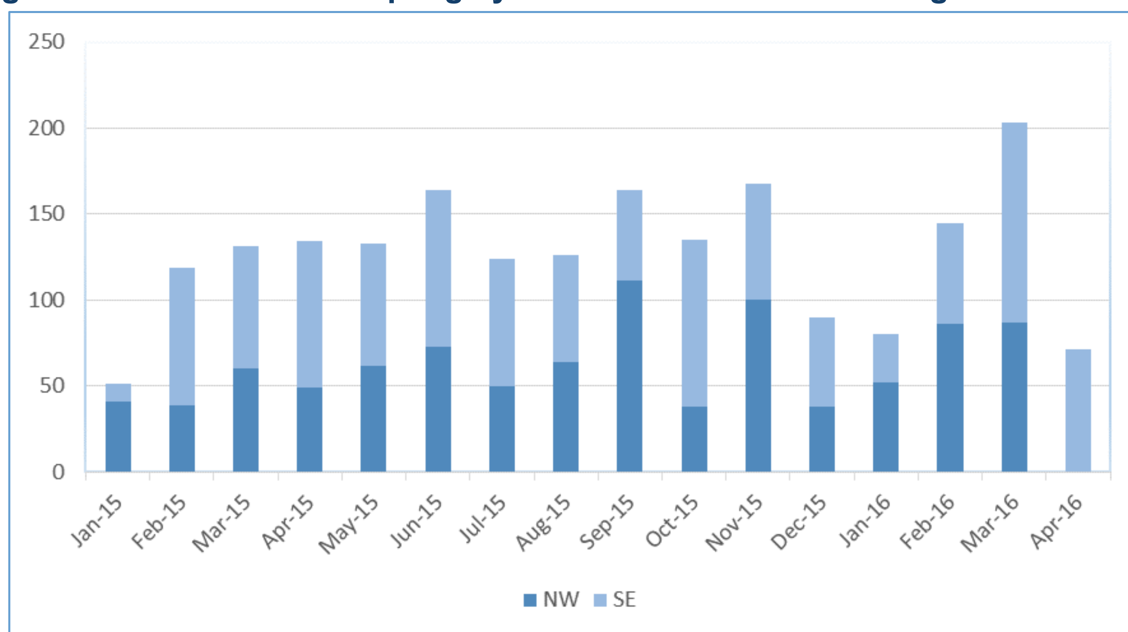


Table 31: Distribution of premises and samples by food region and food hygiene rating

	Food Hygiene Rating							
	0	1	2	3	4	5	Not available	Total No
Number of premises (SE)	18	19	19	17	18	34	2	127
Number of samples (SE)	168	162	171	141	145	283	18	1088
Number of premises (NW)	0	9	8	17	18	44	33	129
Number of samples (NW)	0	60	50	112	111	303	314	950
Total Premises	18	28	27	34	36	78	35	256
Total Samples	168	222	221	253	256	586	332	2038

The Food Hygiene Rating Scheme (FHRS) in England, Wales and Northern Ireland helps consumers choose where to eat out or shop for food by giving them information about the hygiene standards in restaurants, takeaways and food shops. The following web-link provides further information on the scheme: www.food.gov.uk/business-industry/hygieneratings

7.4.2 Virology results

Norovirus positive samples were detected throughout the study period, with a peak in the norovirus positivity rate and attributable to sampling in the SE in April 2016 (Figure 27) (the last month of the study period) and coinciding with increased detection of norovirus in the national surveillance. In total 30 premises (12%: 27[22%]) and 3 (2%) in the SE and the NW, respectively, had norovirus detected throughout the study period (from outbreak and non-outbreak samples) with a total of 50 swabs being positive (Table 32).

Table 32: Norovirus positivity

	SE	NW	TOTAL
Norovirus positive premises	27/123	3/129	30/252
Norovirus positive samples	45/1088	5/950	50/2038
Norovirus undetermined samples (inhibitory)	19[8]	16[9]	35
Positive Surface Swabs	32/831	3/705	35/1536
Positive hand swabs	13/257	2/245	15/502

[8] From 6 different premises
[9] From 5 different premises

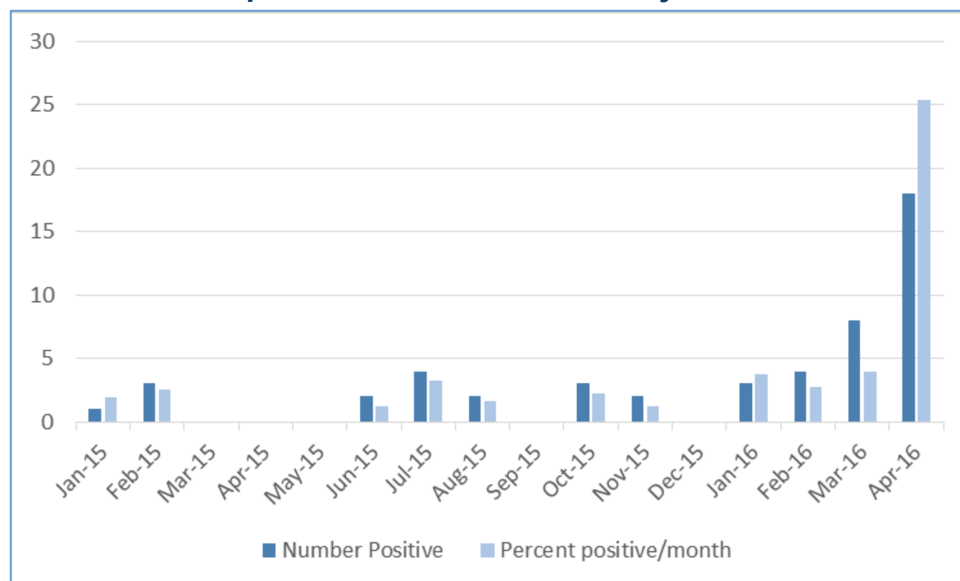
The percentage of positive norovirus premises (on or more positive swabs) was lower among those premises with FHR of 5, compared to all lower FHR combined, risk ratio 0.36 [95%CI 0.14-0.89]. No differences were seen in positivity rate between premises with FHR between 0 and 4), although the numbers are too small for meaningful statistical analysis between each group. Among positive premises the proportion of that were positives was similar between FHR 0 to 5 (Table 33), suggesting similar degree of contamination, and that more intensive swabbing in some premises compared to others has little impact on the detection of positive premises.

Positivity rate per month was relatively low throughout the study, and no positive samples were detected in 5 months, whilst the median positivity rate was 1.8%. There was a significant increase in the proportion of positive swabs in April 2016 compared with the previous 15 months of the study (p<0.001 with either chi-square with Yates correction or Fishers's exact test). During the last moth of the study, 18 swabs were positive out of a total of 71 screened (25%), all in the SE (Figure 27).

Table 33: Norovirus positive swabs by Food hygiene rating

	Food Hygiene Rating					
	0	**1**	**2**	**3**	**4**	**5**
Number positive premises	2/18	3/28	2/27	7/34	8/36	5/78
Percentage of total	11.1%	10.7%	7.4%	20.6%	22.2%	6.4%
Number positive swabs	5	3	5	13	9	15
Percent positive	3.0	1.4	2.3	5.1	3.5	2.6

Figure 26: Detection of positive norovirus swabs by month



7.4.3 Norovirus characterisation

Attempts to recover sequence data from all norovirus-positive swab samples yielded genotyping results in 6 (20%). In total one GI.6 and five GII.4 norovirus sequences were identified in four swabs from the SE and one from the NW.

7.4.3.1 South East

All four samples were from non-outbreak premises. Three were collected on food handler hands, and one was taken from a food preparation surface. These premises represent a range of Food Hygiene Rating Scores (1x rating 1; 2x rating 3; & 1x rating 4). The samples were collected across four different London LAs (Greenwich, Ealing, City of London and Sutton), and represent a café, two restaurants and a takeaway.

Two sequences had significant similarity to the GII.4/Sydney2012 genocluster, one had similarity to the GII.4/Den Haag/2006 genocluster, and one sequence could not be clearly assigned to a genocluster due to the quality of the sequence.

7.4.3.2 North West

Sequence was recovered from one positive surveillance sample collected in the North West, which indicated detection of a GII.4 norovirus, although the sequence data quality did not allow for genocluster assignment.

Following an outbreak event in the North West in summer 2015, environmental and clinical samples were collected (Smith *et al.* 2017). A GI norovirus was detected in

ten clinical samples, of which 8 could be further characterised as GI-6, including a stool specimen from a food handler. Norovirus characterisation of the positive environmental swab was not possible.

The ability to genotype of norovirus positive samples with low viral loads is the main limitation to linking environmental samples with cases in suspected foodborne outbreaks. Although this is less of a challenge when clinical samples are available, current methods are not always successful and may also have some inherent biases. We have as part of this work package piloted the use of massively parallel sequencing methods for outbreak investigation (see Appendix 6).

7.4.4 Bacteriology results

Bacteriology results were obtained for 229 premises, 119 in the NW and 110 in the SE. Overall 51.5% of the premises (118) yielded at least one unsatisfactory result, with no difference in failure rates were seen between the two regions or by FHRS. The vast majority of the unsatisfactory results were due to counts of Enterobacteriaceae being over the acceptable limits (Table 34). Fourteen of these premises also failed the E. coli counts and two failed the coagulase positive Staphylococci counts in addition to failing on the Enterobacteriaceae counts, and eight premises failed only on coagulase positive Staphylococci counts. No associations were found between norovirus positivity and bacteriology indicators, or between bacteriology indicators and hygiene ratings. In total, 12 premises (7 in the SE and 5 in the NW) had norovirus detected and had an unsatisfactory result for at least one bacterial indicator test.

No associations were found between norovirus positivity and bacteriology indicators, or between bacteriology indicators and hygiene ratings.

7.4.4.1 Why Do Bacteriological Testing?

Bacteriological indicators have traditionally been used as a general indicator for environmental contamination, with *E. coli* contamination particularly being associated with faecal material and a key example of this is in the classification of shellfish beds. Opinion on bacteriological indicators has been mixed with some researchers identifying strong associations demonstrating that they are not associated with the presence of pathogens. As a culture for norovirus is not currently a possibility, laboratories are reliant on molecular technologies for its detection. The low levels of norovirus contamination in the environment and the potential for inhibitory substances being introduced to assays (e.g. cleaning disinfectant residues) make detection more challenging. Within the NoVAS study Work Package 5 there were inhibitory samples identified from the food preparation environment samples collected. As bacteria have more robust culture pathways, the ability to use them as a proxy for norovirus would be useful, but the assumption that they are an appropriate proxy has not been broadly tested in the food preparation environment.

The other reason for using bacteriological indicators in Work package 5 was because they are the routine approach used by Environmental Health Officers (EHOs) in determining the efficacy of cleaning and establishing hygiene practices at a premises. This helped provide an incentive for the EHO participation in the study as they gained useful results from their sampling visit. Interpretative criteria established by Public Health England are routinely used by EHOs.

Microbiological testing and hygiene practices at a premises are only part of the criteria used to establish the Food Hygiene Rating Scheme score and so the two results are not likely to be comparable. Other premises details are determined by the EHO to establish the score given including confidence in management and the premises infrastructure.

Table 34: Summary of bacteriological sampling and results

	SE	NW	TOTAL
Number of eligible premises	123	129	**252**
Number of premises sampled for bacteriological indicators	110	119	**229**
Number of bacteriological swabs referred	416	533	**949**
Premises sampled for surveillance	117	119	**236**
Premises sampled for outbreak investigation	6	10	**16**
Bacteriology results			
Number of premises with unsatisfactory bacteriology swab results	64	66	**130**
Number of samples with unsatisfactory bacteriology swab results	117	111	**228**
Number of samples with an unsatisfactory ACC result	6	12	**18**
Number of samples with an unsatisfactory coagulase positive *Staphylococci* result	2	8	**10**
Number of samples with an unsatisfactory *E. coli* result	7	7	**14**
Number of samples with an unsatisfactory Enterobacteriaceae result	104	84	**188**
Number of premises with two or more bacteriological test results being reported as unsatisfactory	11	16	**27**

7.5 Discussion

Overall, 11% (30/252) of the premises sampled yielded at least one norovirus positive sample (environmental, and/or hand swab), and 2.5% of the swabs were positive for norovirus in total, which was higher than published data, 4.2% of establishments and 1.7% of the environmental samples were positive in a previous study in the Netherlands (Boxman *et al*. 2011), but lower than the 40% positivity rate previously detected in a pilot study in the London area (Iturriza-Gomara *et al.* unpublished data). The proportion of premises in which norovirus was detected in the South East was 21% compared to 4% in the North West. Differences in the proportion of positive samples between the SE and the NW are difficult to interpret;

these were not associated with testing protocols or laboratory differences, as demonstrated in a head to head comparison of methodologies (Appendix 12).

It is possible that differences seen may be associated with potential local differences in norovirus epidemiology at any given time. No differences were seen between the number of kitchen staff between positive and negative premises or between regions. The mean and median number of staff per norovirus positive premise were 4 and 2 range (1-19) respectively in the NW and 3.2 and 2 (range 1-15), respectively in the SE compared to 5.2 and 3 (range 1-31) in the NW, and 4.2 and 3 (range 1-28) in the SE among the norovirus negative premises. It may be speculated that population density could play a role in differences in norovirus prevalence between densely populated areas such as London in comparison to some of the more sparsely populated areas sampled in the NW, however, surveillance data which is biased primarily towards capturing health and social care associated outbreaks does not allow us to explore this further at present. The only comparable available data from the study in the Netherlands also found no association between number of kitchen staff and norovirus detection rates, but a positive association with population density and norovirus detection rate in the environment (Boxman *et al*. 2011).

Amongst the norovirus positive swab samples, 30% (15/50) were from food handler swabs and 70% (35/50) were from other swabs collected from environmental sources. Overall, the food handler hand swabs had a positivity rate of 3% (15/502) and the other environmental sampling points had a positivity rate of 2% (35/1536). The origin of the contamination is difficult to ascertain and evidence exists of transmission of contamination between surfaces and food and between contaminated hands and food and surfaces (Rönnqvist, M *et al*. 2014; Sharps *et al*. 2012; Stals A 2013).

Contrary to our hypothesis, and findings in the study in The Netherlands (Boxman *et al.* 2011) the proportion of norovirus positive premises was not significantly different during outbreak investigation compared to routine surveillance sampling: 25% (4/16): 33% (2/6) in the SE and 2% (2/10) in the NW) compared to 18% (44/247): 33% (43/128) in the SE and 0.8% (1/119) in the NW, respectively. One major difference between the our study and that of Boxman *et al*. is that we specifically excluded premises in health and social care institutions given that they constitute a population at high risk of norovirus infection and outbreaks and are not representative of the general population. This difference may also account at least in part for this discrepancy and the availability of a larger number of premises associated with norovirus outbreaks in the study in The Netherlands

Among the outbreaks, positive samples from affected consumers and a food handler were identified in one single outbreak. Although no virus sequence that allowed strain characterisation was obtainable form the single environmental positive swab associated with this outbreak, further epidemiological investigation determined prior

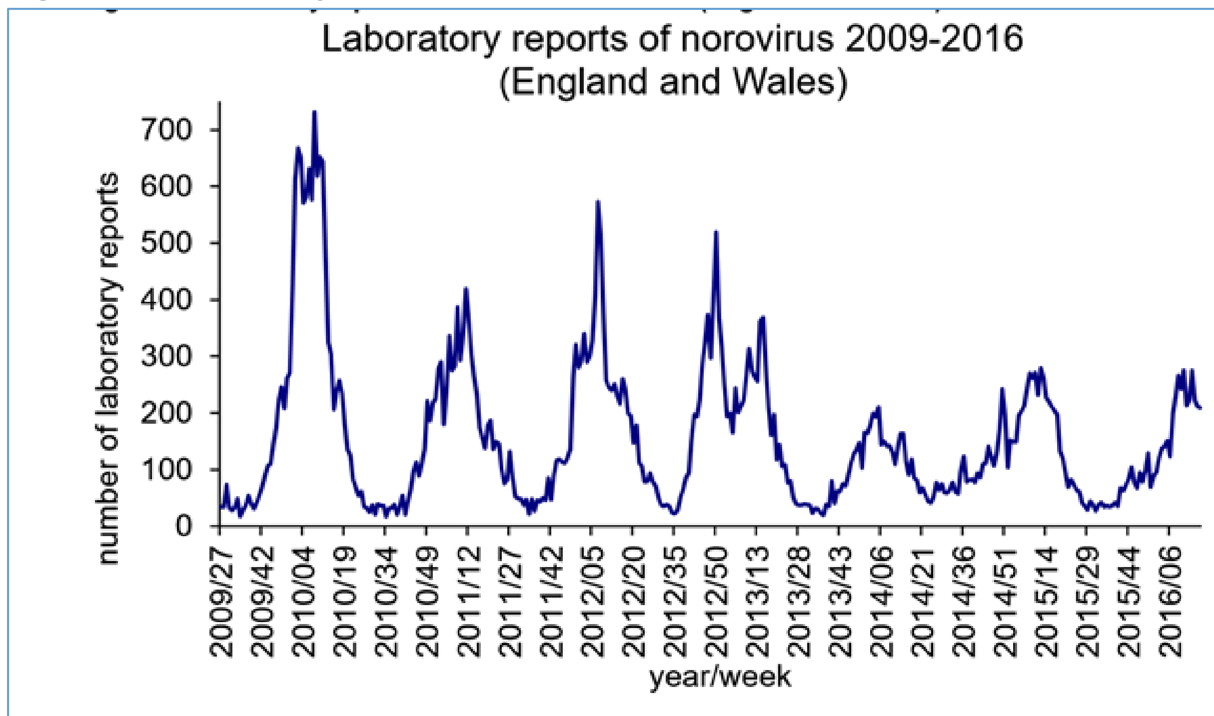
illness of the food handler as the most plausible source of the outbreak (Smith *et al*. 2017).

We were unable demonstrate a clear seasonal distribution of positivity rates in this study. We did nevertheless observe a significant increase in the rate of positive swabs detected during the last month of the study, and this coincided with an increase in the overall reporting of norovirus outbreaks to the national surveillance system. Extension of the sampling period would have been needed in order to confirm attribution of this increase to the seasonal variation in incidence of norovirus infections in the population.

In this study, bacterial indicators did not provide an effective indicator for norovirus. A four-fold higher number of premises had bacterial indicator organisms isolated (118) compared to those with a norovirus being detected (30). There has been a tradition of using bacteria as a proxy for viruses due to limitations in culture-based detection for targets such as norovirus. The correlation between the results for these two very different organisms has been an issue of longstanding debate, with Lees (2000) highlighting that *E. coli* is not an appropriate indicator for viral contamination in bivalve shellfish. This is combined with direct evidence from outbreak settings that the presence of bacteria in samples does not correlate with the presence of viral pathogens (Koopmans and Duizer 2004).

This study has various limitations. The overall prevalence of norovirus in the catering establishment was lower than expected (20% of premises were expected to be positive) from the limited data available upon which our sample size estimations were based. Therefore, our study was insufficiently powered to prove or disprove some of our hypotheses. It must be taken into consideration that norovirus is a seasonal infection, and that there is wide year to year variation in the overall prevalence of norovirus disease. Norovirus surveillance data collected by PHE demonstrated low levels of norovirus disease and norovirus outbreaks in the UK during the study period (Figure 28), therefore the results of this study must be interpreted in the context of a low incidence of norovirus illness in the UK.

Figure 27: Laboratory reports of norovirus 2009-2016



It is likely that at times of higher norovirus circulation, and more frequent outbreaks, differences between premises associated with outbreaks and those that were not may become more evident than in a season with a low base line level. In order to assess seasonal variations in the rates of norovirus positivity of food premises, longer term and sustained surveillance is necessary to overcome biases introduced by the natural year to year variations seen in norovirus circulation in the population.

Another limitation is the well-recognised difficulty in identifying norovirus outbreaks associated to food premises. It is generally acknowledged that norovirus outbreaks or acquisition of norovirus infection from catering/food establishments is likely to be grossly underreported, as such cases typically come to light only when they involve functions or gathering in which the connection with the event is more easily made, this is exemplified by the outbreak in the NW associated with a wedding party, briefly described in this report and for which a detailed description of the investigation and finding has been published (Smith et al. 2017). We cannot rule out onward transmission in those establishments that were not associated with a reported outbreak, and this is a major limitation imposed by the limitations of the surveillance systems available. In practice this is a difficult to overcome limitation, which may require innovative approaches to surveillance, involving self-reporting and social media, for data capture in well-defined populations that can be traced and linked to food consumption behaviours.

8 Work Package 6: Estimating the disease burden from foodborne norovirus in the UK, a microsimulation based quantitative microbial risk assessment

8.1 Summary

In this chapter we have used the data generated earlier in this report to estimate the burden of foodborne norovirus in the UK through a Quantitative Microbial Risk Assessment (QMRA). However, in order to do this, we had to overcome a series of challenges not normally considered within QMRA studies. The first challenge was that we were interested in the combined risk from multiple food categories. The second challenge was that norovirus is actually a highly diverse pathogen with little or no cross-immunity between many strain types. Additionally, there is currently no easy measure of norovirus infectivity and so not all detected gene copies represent infectious virus particles. Also of concern is the fact that foodborne transmission is just one of several competing transmission pathways for the virus. A systematic review undertaken to help guide the development of our risk assessment of QMRA studies of foodborne norovirus found no prior studies of relevance and certainly none that would help us overcome these challenges.

In order to address these challenges, we were not able to use standard QMRA approaches and so developed a novel microsimulation modelling approach to QMRA in which we were able to address many of the challenges described above. This microsimulation approach basically models risk at the level of the individual and simulates event histories at the individual rather than the population level. Exposure data were derived either from other work packages within the NoVAS project, from the literature or from expert consultation.

Risk per meal in susceptible individuals was highest for oysters with a mean risk of 6.78/1000meals or about one infection in every 150 meals, for lettuce 1/12000 meals, for raspberries one in every 9000 meals. Meals eaten out or takeaways had an intermediate risk of about one in 1200 meals. Annual risk accounting for intrinsic resistance but not acquired resistance was 23.76 infections per 1000 py (person years) with catered foods accounting for 74.2% of infections (43.7.0% from foods eaten out and 30.5% from takeaways), lettuce for 19.5%, raspberries for 2.9%, and oysters for 2.7% of all foodborne norovirus infections.

When including adjustments for immunity due to prior infection the estimates of clinically relevant norovirus infections drops substantially, though the exact figure is critically dependent on the assumed duration of immunity to homotypic strains after a single infection which is currently not known with certainty. In the final analysis we modelled duration of immunity using PERT distribution with min and mod = 6 months and max =24 months. Using this approach, our preferred estimate of the annual

incidence of symptomatic foodborne norovirus to be about 7.65 episodes /1000py (person years). Using different assumptions for duration of immunity would give a range of 0.99 to 10.78 illness/1000py. This would equate to approximately 500,000 foodborne noroviruses illnesses annually in the UK (range 65,000 to 700,000). Assuming that the IID2 estimate of total norovirus disease burden of 47 episodes/1000py is correct, this would suggest that the proportion of all norovirus illness due to foodborne disease is about 16% with a range in the range 2.1 to 22.9%. However, this does not mean that even if all foodborne transmission was blocked that total norovirus infections would fall by this amount or even at all.

Accounting for intrinsic resistance and prior immunity risk per 1000 meals would be 1.64 (0.20 – 2.23) for oysters, 0.02 (0.003 – 0.03) for lettuce, 0.03 (0.003 – 0.04) for raspberries, 0.19 (0.02– 0.26) for meals eaten out and 0.20 (0.03 – 0.27) for takeaways. Annual risk of illness/1000py for the food groups would be about 14,000 cases associated with oysters annually (0.21 (0.03-0.29) illnesses/1000py), 98,000 cases for lettuce (1.49 (0.19-2.03) /1000py), 16,000 for raspberries (0.22 (0.03-0.30) /1000py), 220,000 from eating out (3.34 (0.42-4.55) /1000py) and 150,000 from takeaways (2.34 (0.29-3.18) /1000py). These estimates are consistent with what would be suggested from the few epidemiological studies available to-date.

In the sensitivity analyses the most critical variables were the amount of virus on food or food handlers' hands, though estimates of the proportion of virus viable and for catering the number of times food is touched also had a significant impact on exposure. For annual risk the duration of immunity had the biggest impact as mentioned above, but also estimates of exposure and the community incidence of infection also had an impact. The models also suggested that eliminating norovirus from the food chain would not necessarily reduce the total burden of disease in the community and under certain assumptions may even marginally increase disease burden. This suggestion is consistent with empirical evidence in other contexts.

There is a real need for more research into the epidemiology of norovirus infections and particularly into the intensity of the infection pressure from multiple transmission pathways not just for foodborne transmission. The role of food in the epidemiology of norovirus cannot be fully understood in isolation from these other pathways but needs to be part of a holistic understanding on norovirus transmission.

8.2 Introduction

The aim of this chapter is to synthesise the outputs of previous chapters so that an estimate of the total burden of foodborne disease in the UK can be made.

Noroviruses (NoV) are the most common cause of infectious intestinal disease in the UK (Tam *et al*, 2011; Tam *et al*. 2012), yet there remain many unanswered questions as to its epidemiology and transmission through the food chain. Foodborne outbreaks of norovirus are well described in the literature. In a review of such outbreaks in the US, the authors identified almost 3000 foodborne outbreaks between the years 2001 to 2008 (Hall *et al.* 2012). Of those outbreaks where it was possible to attribute a food source, infected food handlers were considered the primary cause in 53% and were thought to have contributed to a further 29%. Leafy vegetables were the single most common food type (33%), with fruits/nuts (16%), and molluscs (13%) also making important contributions. Further discussion of the epidemiology of outbreaks appears in earlier chapters of this report.

Although we do have strong evidence of the cause of outbreaks of norovirus we know much less about the epidemiology of sporadic disease. Given that only a relatively small proportion of total norovirus disease burden is associated with known outbreaks (www.gov.uk/government/collections/norovirus-guidance-data-and-analysis compared to estimated annual numbers from IID2 (Tam *et al*. 2012), it is would be a mistake to extrapolate only from outbreaks to total disease burden. Although there have been some case control studies of sporadic disease reported in the literature ((de Wit *et al*. 2003; Fretz *et al*. 2005; Phillips *et al*. 2011), these do not allow an adequate quantification of foodborne disease burden for sporadic infections.

The past 20 years has seen a substantial growth in the use of quantitative microbial risk assessment (QMRA) as an efficient tool to estimate risk of infection (WHO 2016). Essentially QMRA consists of four key stages; problem formulation, exposure assessments, health effects and risk characterisation. In this study our problem formulation was to determine the disease burden from foodborne norovirus infections in the UK. In this study the target foods were oysters, leafy salads/lettuce, raspberries and catered meals. Consideration was also given to estimating foodborne transmission within the home where there was an infected food handler but given the high efficiency of within home transmission from direct person to person spread it was considered that foodborne transmission in that context would not add to intra-family spread and be extremely difficult to quantify. Exposure assessment consists of estimating the amount of infectious virus to which consumers are exposed through the various foods under investigation and the previous chapters in this report essentially provide most of this information. The health effects part of the risk assessment consists of identifying an appropriate dose-response model to determine the risk of infection given known exposures from the exposure assessment. Finally the risk characterisation is the synthesis of exposure

assessment and risk assessment in order to produce a quantitative estimate of risk/disease assessment. This is the primary function of this chapter.

Compared to prior QMRA studies, however, this study has considerable additional challenges. Firstly, because we are interested in foodborne transmission, this means we will have to incorporate exposure assessment from multiple foods simultaneously rather than just a single food source or transmission pathway. Secondly, noroviruses are not a homogenous group of microorganisms but represent a highly diverse collection of different genotypes and subtypes with little or no cross-immunity between the different strains (Parra *et al*. 2017). These two observations alone undermine the standard approaches to QMRA which are generally about a single pathogen in a single transmission pathway. Thirdly, in the absence of any adequate culture method, counts of norovirus in food are based on quantitative PCR which measures gene copies and not necessarily infectious virus. Consequently, human dose response studies may not be directly applicable to risk assessment of norovirus in the food chain because of subsequent inactivation. Finally, in the real world foodborne transmission is just one of several and probably not the major transmission pathway for norovirus and so any risk assessment must be adjusted for the presence of these competing pathways.

These challenges raised in the previous paragraph have been central to the complexity of undertaking this work. In this chapter we initially undertook a systematic review of QMRA studies of norovirus in food in order to determine whether previous authors have adequately overcome these challenges. The rest of the chapter then goes onto describe our development of a QMRA based model that overcomes these challenges and produces estimates of the total burden of foodborne norovirus infections in the UK. In doing this we have had to develop a novel microsimulation model that incorporates variation in exposure between individuals, summation of risk across multiple pathways, multiple repeat exposures to norovirus in an individual along with periods of immunity post infection. This model also included estimates of non-foodborne exposure.

8.3 Systematic Review

The systematic review presented here was undertaken early in the course of the NoVAS project in order to inform subsequent the subsequent risk assessments undertaken as part of the project. Our aim was to review those studies that had undertaken QMRA on food products that would be relevant to the UK context. We were particularly interested in how studies combined risk over several foods and how any studies dealt with the issue of loss of infectivity of virus in the environment and food chain.

8.3.1 Methods

Both SCOPUS and Ovid (Medline) databases were searched up until the end of 2013.[10] We used very broad search terms: ("risk assessment" OR QMRA OR "Disease burden") AND Norovirus using both free text and MESH headings. Any study that undertook a quantitative risk assessment of disease risk to humans from norovirus in a commercially available food likely to be consumed in Europe or North America were included. We explicitly excluded any study from a low-income country. Studies in all languages were included.

8.3.2 Results

A total of 241 papers was identified after exclusion of duplicates (figure 29) of which the full text was retrieved on two papers (Barker *et al*. 2013; Mara and Sleigh 2010). One paper was subsequently excluded as it was an analysis of home grown lettuce should the home use re-cycled grey water and not lettuce that would be commercially available (Barker *et al*. 2013). The other was excluded as its primary focus was on risk associated with the use of wastewater in irrigation in low income countries (Mara and Sleigh 2010). No papers were identified that satisfied the inclusion criteria. There were several norovirus risk assessments in the literature looking at risks associated with potable water and recreational water contact but none of relevance to the NoVAS project.

Although not satisfying the inclusion criteria we did review the two excluded papers, the model around greywater reuse was quite complex and included estimates of the frequency of bathing or showering among family members and the amount of faeces on underwear and how much would appear in waste water for irrigation (Barker *et al*. 2013). This study made no adjustment for acquired immunity but did attempt to adjust for loss of infectivity assuming (incorrectly) that norovirus infectivity declined in proportion to *E. coli* counts. The paper by Mara and Sleigh (2010) also did not account for loss of infectivity in the food chain only making an estimate of viral

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[10] Note that papers published since 2013 have been summarised in section 10 of this report.

concentration in waste-water based on *E. coli* counts. This study also did not account for acquired immunity from prior infections.

8.3.3 Conclusions

Formal Quantitative Risk Assessments of commercially available food products relevant to the European market had not been reported in the literature prior to the start of the NoVAS project. In particular, we could find no adequate prior studies that addressed the issues of loss of viability of norovirus in the environment or through the food chain, no study addressed the issue of the diversity of genotypes in food or the impact of acquired immunity due to prior infection. In order to undertake an adequate risk assessment to determine disease burden from foodborne norovirus we will have to develop methods and approaches ourselves.

Figure 28: PRISMA flow diagram

PRISMA 2009 Flow Diagram

Identification

Records identified through database searching (n = 257)

Additional records identified through other sources (n = 0)

Records after duplicates removed (n = 241)

Screening

Records screened (n = 241)

Records excluded (n =239)

Eligibility

Full-text articles assessed for eligibility (n = 2)

Full-text articles excluded, with reasons (n = 2)

Included

Studies included in qualitative synthesis (n = 0)

Studies included in quantitative synthesis (meta-analysis) (n = 0)

From: Moher D, Liberati A, Tetzlaff J, Altman DG, The PRISMA Group (2009). *Preferred Reporting Items for Systematic Reviews and Meta-Analyses: The PRISMA Statement.* PLoS Med 6(7): e1000097. doi:10.1371/journal.pmed1000097

For more information, visit www.prisma-statement.org.

8.4 Methods and Justification

8.4.1 Model Overview

The mathematical model presented here is a novel application of a microsimulation-based Quantitative Microbial Risk Assessment (QMRA). This microsimulation model was constructed in @Risk 7.5™. It was our original intention to undertake this risk analysis using Bayesian QMRA. However, recent concerns about extrapolating from daily to annual risks in QMRA and the need to include multiple transmission pathways necessitated the use of a microsimulation approach. All prior QMRA studies that have produced annualised estimates have used one of two approaches to extrapolate from daily to annual risks (equations 1 or 2 below). The calculated annual risks from these two approaches can give very different estimates (Karavarsamis and Hamilton 2010). We have shown that both these approaches may give biased results (Appendix 13). As discussed in Appendix 13 the sources of the bias in both these approaches comes from the assumptions underpinning both equation 1 (that daily risk in an individual is constant throughout the year) and equation 2 (that daily exposure and risk are entirely random) are naive.

Equation 1:

$$P_T = 1 - (1 - p)^{365}$$

Equation 2:

$$P_K = 1 - \prod_{k=1}^{365} (1 - p_k)$$

In order to overcome these sources of bias we used a microsimulation model. Microsimulation models differ from other simulation models in that they simulate individual event histories (Rutter *et al*. 2011). In other words, rather than modelling based on population level variables the model selects a hypothetical individual and then determines exposure characteristics for that individual, models the risk for that individual over the course of a year before repeating for another notional individual. Consequently, Microsimulation substantially increases the number of random nodes from less than 10 in most QMRA to several thousands. The increased computational demands with Bayesian QMRA compared to Monte Carlo QMRA and the very large number of nodes running into several thousand would require far more computational power than available. Although the epidemiology of norovirus infection in humans does show significant seasonality, we were unable to identify data on seasonality in food consumption to enable seasonality to be included in our models.

The model was developed to study the annual disease risks associated with each of the following food pathways in isolation and combined: oysters, lettuce, raspberries, and catered foods both meals eaten out and takeaways as these were considered to be the main foodborne transmission pathways. Consideration was given to the

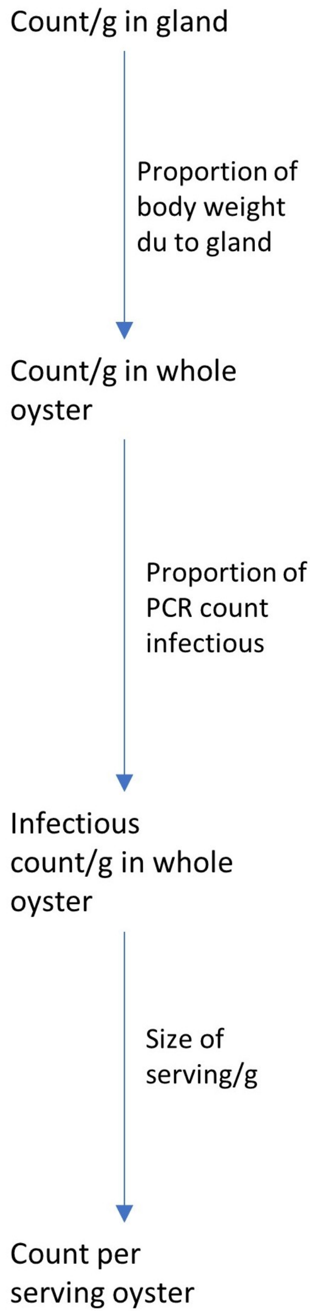
inclusion of risk of foodborne transmission within the home from infected domestic food handlers. However, given that transmission of norovirus is so effective within the household context even in the absence of foodborne transmission that foodborne transmission would not provide a substantive additional risk and was not included (Heun *et al*. 1987; Gastañaduy *et al*. 2015; Marsh *et al*. 2018).

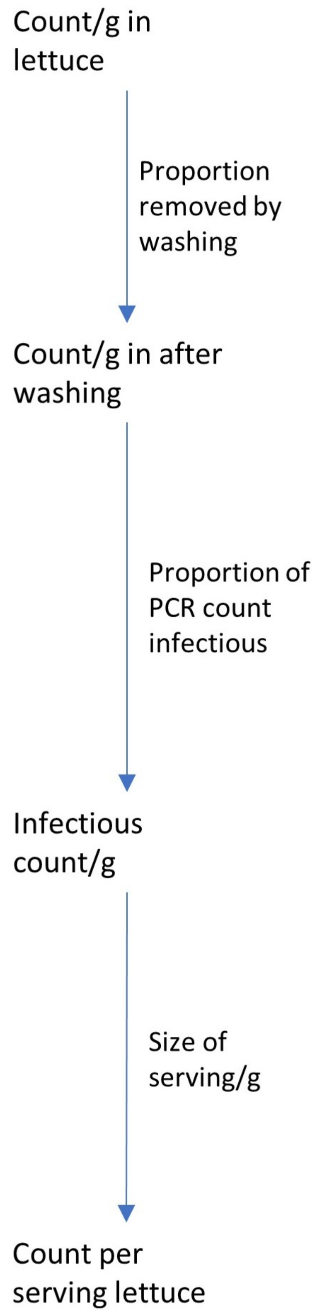
The model was effectively two related models. The first model generated estimated exposures of viable virus per serving or meal in each of the food groups. These generated estimates were then copied over into the risk model, which was able to estimate both risk per meal and annual risk. In addition, the risk model incorporated a SIR (Susceptible-Infected-Resistant) element which adjusted the annual risk to account for immunity from prior exposure.

All parameter values used in the model are described in Table 35. Where necessary additional description will be given in the methods section to explain their derivation or justify their choice.

Figures 30, 31 and 32 set out the structure of the model in diagrammatic form. Figures 30a and 30b illustrate the exposure model which generates the exposure count of infectious Norovirus per serving or meal. These exposure counts are then used in the risk model (figure 31) to generate total daily and annual foodborne risk. Figure 32 is the part of the model that takes the daily risk of infection and estimates the daily and annual risk of illness (symptomatic infection) adjusted for acquired immunity due to prior infection with homotypic strains of norovirus.

Figure 29a: Exposure models for oysters, lettuce and raspberries





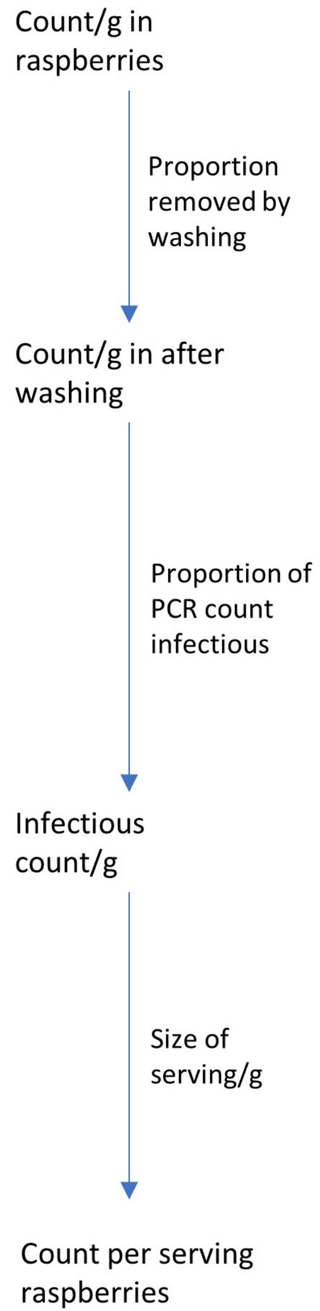
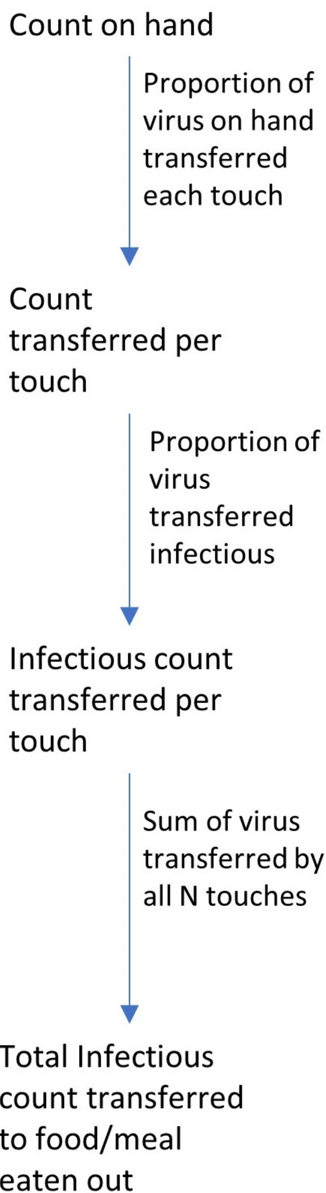


Figure 30b: Exposure models for catered food (meals eaten out and takeaways)



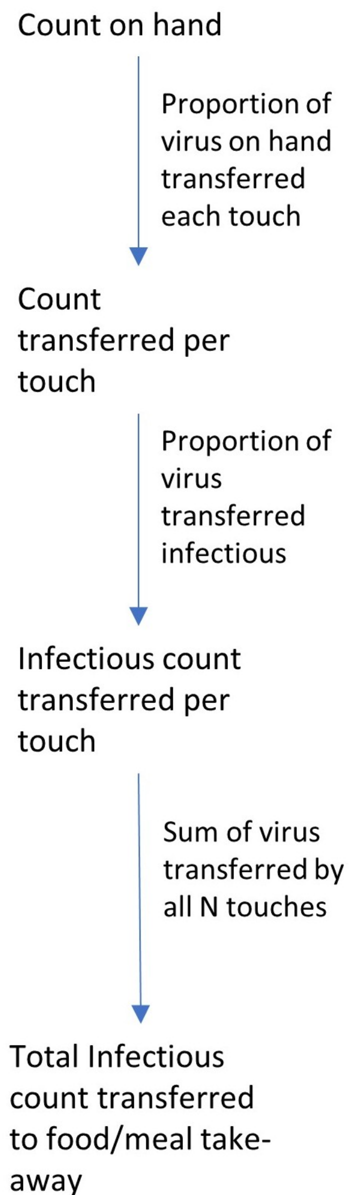


Figure 30: Microsimulation QMRA model estimating daily and annual risk of foodborne norovirus with no adjustment for acquired immunity from prior infection

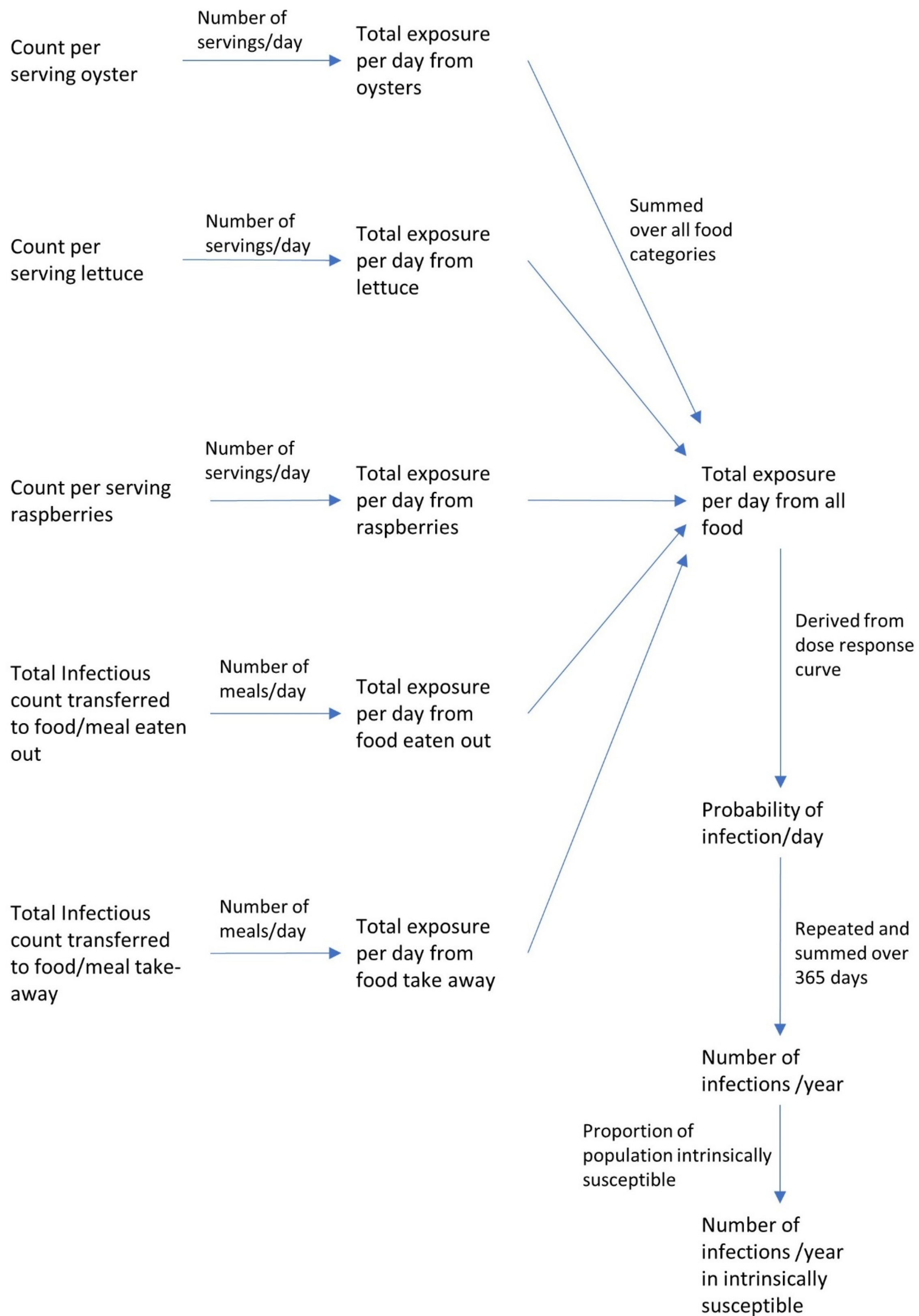


Figure 31: Microsimulation QMRA model estimating daily and annual risk of foodborne norovirus with adjustment for acquired immunity from prior infection

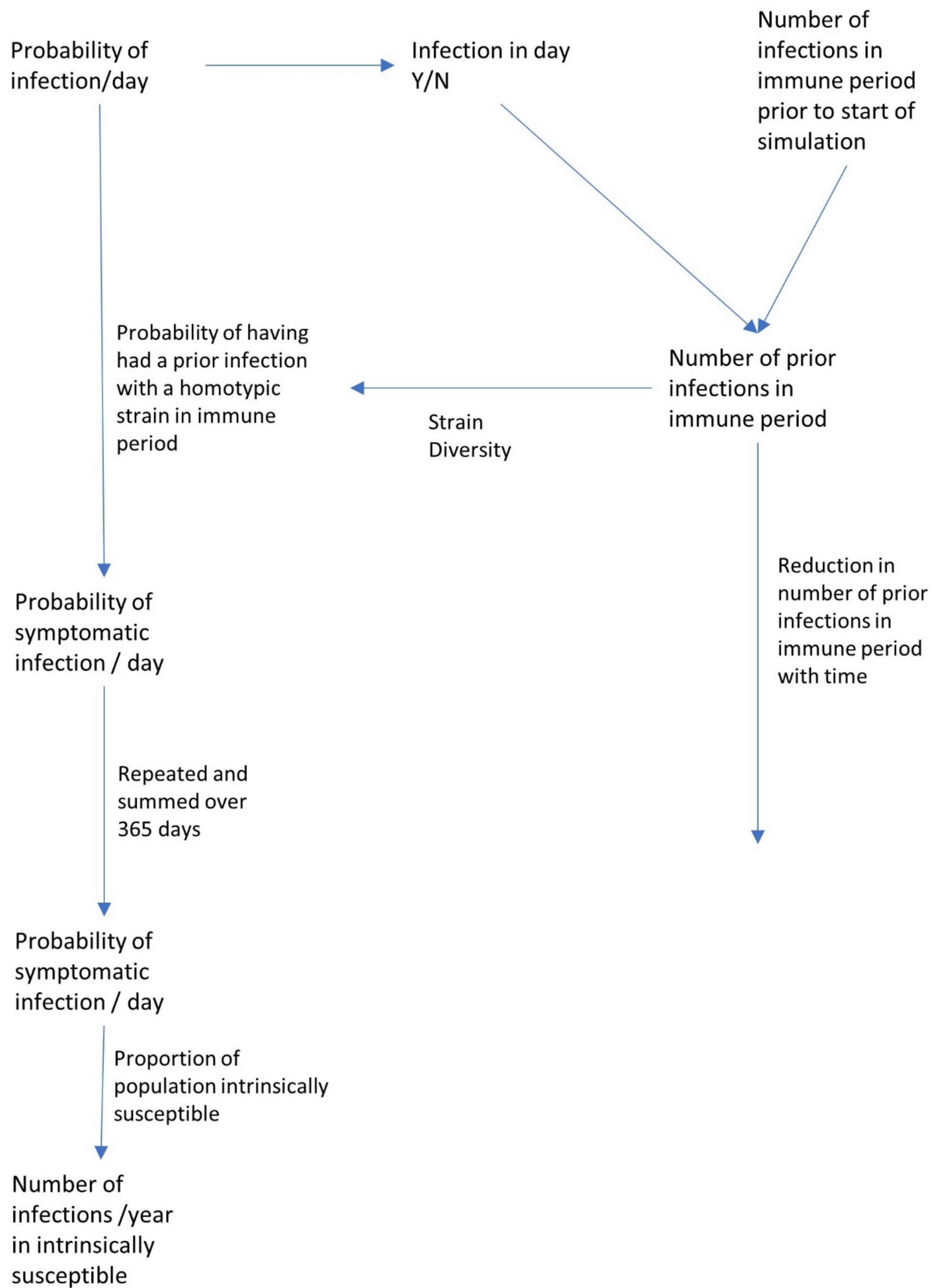


Table 35: Parameters used in subsequent models

Function	Equation Form	Parameter	Value	Reference
Probability of Person being "Intrinsically sensitive" for NoV	Binomial Distribution	**Prob**	0.75	Text 8.4.5
Number of Oyster meals consumed/py	Poisson Distribution	**λ**	0.1369	Text 8.4.6
Probability of Oysters NoV positive	Proportion	**P**	0.6873	Text 8.4.2
Oyster Meal Size (g)	Truncated Normal Distribution	**μ** **σ** **Min** **Max**	37.22 14.67 20 60	Text 8.4.2
Oyster Dig. Gland NoV Concentration (counts per g)	Log10normal Distribution	**μ of Log_{10}** **σ Log_{10}**	1.2675 0.7621	Text 8.4.2
Dig. Gland proportion of whole Oyster	Shifted Gamma Distribution	**Shape** **Scale** **Shift**	2.2114 0.02135 0.13896	Text 8.4.2
Number of Lettuce meals consumed/py	Poisson Distribution	**λ**	72.1	Text 8.4.6
Lettuce Meal Size (g)	Truncated Normal Distribution	**μ** **σ** **min** **max**	24.50 16.13 1.50 135.0	Text 8.4.3
Lettuce NoV Concentration (counts per g)	Log10linear	**Log_{10}Intercept** **Log_{10}Slope**	-23.245 24.217	Text 8.4.3

Function	Equation Form	Parameter	Value	Reference
Probability of lettuce being washed before consumption	Binary	**P**	0.8704	Barker et al. 2013
Proportion of Lettuce NoV removed by washing	Log10 PERT Distribution	**Log_{10}Min** **Log_{10}Mod** **Log_{10}Max**	0.1 1 2	Barker et al. 2013
Number of Raspberry meals consumed/py	Poisson Distribution	**λ**	7.99	Text 8.4.6
Raspberry Meal Size (g)	Truncated Normal Distribution	**M** **σ** **min** **max**	50.98 40.29 0.6 237.0	Text 8.4.3
Raspberry NoV Concentration (counts per g)	Log10linear	**Log_{10}Intercept** **Log_{10}Slope**	-28.105 28.929	Text 8.4.3
Probability of raspberries being washed before consumption	Binary	**P**	0.8704	Barker et al. 2013
Proportion of Raspberry NoV removed by washing	Log10 PERT Distribution	**Log_{10}Min** **Log_{10}Mod** **Log_{10}Max**	0.1 1 2	Barker et al. 2013
Number of catered meals eaten out consumed/y	Poisson Distribution	**λ**	16.48	Text 8.4.6
Number of times preparer touches food, meals eaten out	Poisson Distribution	**λ**	7.8	Stals et al. 2015
Number of NoV counts on hands, meals eaten out	Log10linear	**Log_{10}Intercept** **Log_{10}Slope**	-88.38 91.06	Text 8.4.4

Function	Equation Form	Parameter	Value	Reference
Proportion of NoV counts transferred from hands to food, meals eaten out	Generalized Beta Distribution	**α** **β** Min Max	0.76 1.04 0.026 0.46	Stals et al. 2015
Proportion of food cooked, post handling meals eaten out	Binomial Distribution	Prob	0.33	Text 8.4.4
Number of catered meals, take away, consumed/y	Poisson Distribution	**λ**	12.36	Text 8.4.6
Number of times preparer touches food, take away meals	Poisson Distribution	**λ**	7.8	Stals et al. 2015
Number of NoV counts on hands, takeaways	Log10linear	**Log_{10}Intercept** **Log_{10}Slope**	-88.38 91.06	Text 8.4.4
Proportion of NoV counts transferred from hands to food, takeaways	Generalized Beta Distribution	**α** **β** Min Max	0.76 1.04 0.026 0.46	Stals et al. 2015
Proportion of food cooked, post handling takeaways	Binomial Distribution	Prob	0.33	Text 8.4.4
Proportion of transferred virus derived from direct faecal contamination	PERT	**Min** **Mod** **Max**	0 0.2 1	Text 8.4.4
Proportion of gene copies representing infectious virus	Truncated Log_{10}Normal Distribution	**μ of Log_{10}** **σ of Log_1** **Max Log_{10}**	-1.5196 0.6776 0.0	Text 8.4.2

Function	Equation Form	Parameter	Value	Reference
Norovirus dose response	Approximate Beta-Poisson	**α** **β**	0.349 357.1	Van Abel et al. 2016
Duration of acquired immunity post infection	PERT	**Min** **Mod** **Max**	6 6 24	Text 8.4.7
Carriage rate of norovirus in asymptomatic individuals		**Percent**	16	Amar *et al*. 2007
Duration of excretion of norovirus post infection in days			16.4	Milbrath *et al*. (2013)
Diversity of norovirus in England and Wales	Hunter Gaston Index	***D***	0.5609	Text 8.4.7
Symptomatic incidence in UK			47/1000 person years	Tam *et al.* 2012

8.4.2 Estimating dose per meal for oysters

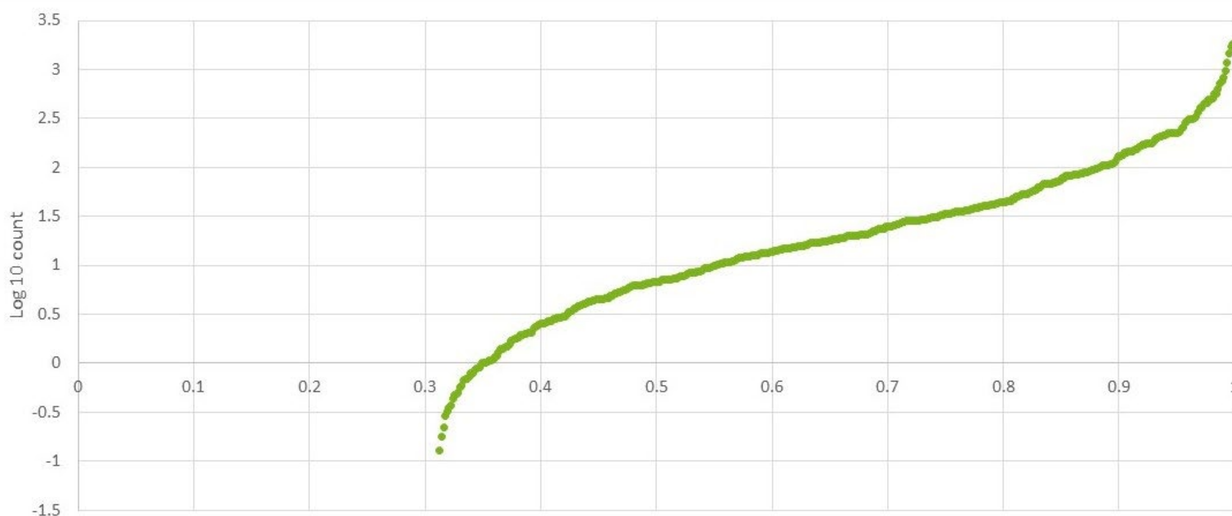
For oysters the basic model of viable virus is given in Equation 3:

Equation 3: $$D_o = (G \times Z) \times V \times S$$

where D_o = the final dose per meal, G = the concentration of virus in the gland in gene copies, Z = the proportion of the oyster flesh composed of the oyster gland, V = the proportion of the virus in gene copies that represent viable virus and S = the size of the meal. None of these parameters represent single values but are drawn at random from distributions as given in Table 35.

The inverse cumulative density function of the estimated counts of virus in the oyster glands in gene copies per g is illustrated in Figure 33 (data collected within NoVAS Chapter 5). Gene copy counts are calculated *a posteriori* using a typical DNA standard curve as in the ISO method in all positives. This was done even when those counts were below the limit where quantitative results would typically be given for oysters and all subsequent food groups in this risk assessment.

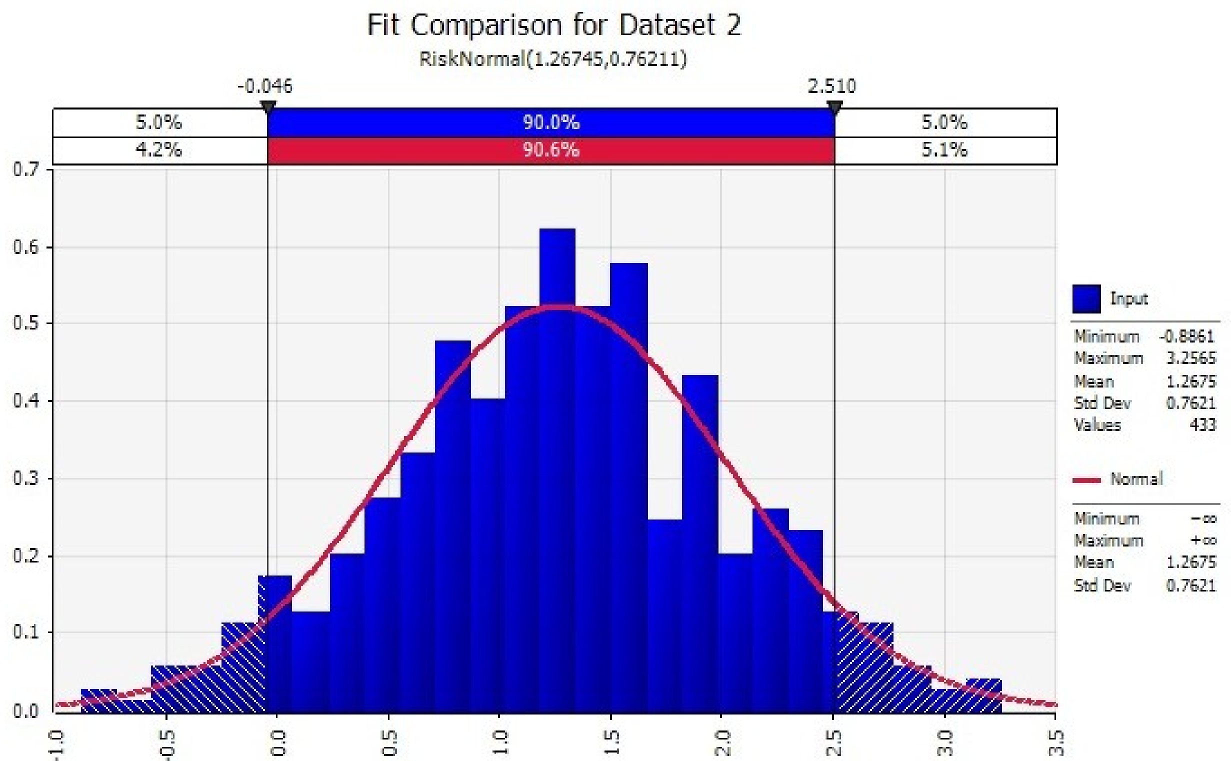
Figure 32: Inverse cumulative density function of estimated gene copies of norovirus in oyster glands



It can be seen that this distribution represents a standard distribution in positive samples but that about 30% are completely negative. Consequently, the distribution of estimated gene copy counts does not follow a single distribution. Rather it can be thought of as a Bernoulli distribution with a negative or positive outcome and for the samples with positive outcomes, the counts follow a log normal distribution, better seen in Figure 34. This distribution is not diagnostic of aggregates. It is consistent with the presence of aggregates. Indeed, this distribution can be approximately modelled by Poisson distribution λ=1.1 for the number of aggregates and the mean

size of aggregates being 11 and log standard deviation being Log10(10)/2, though this distribution was not used in the QMRA analysis.

Figure 33: Distribution of Log gene copy counts in oyster glands where initial tests are positive



The proportion of virus in gene copies that represent viable virus in (V) in Equation 3 in oysters as measured by gene copies that represents infectious virus was obtained from Cefas based on relative viability of a F+RNA bacteriophage GA provided by Cefas based on work in oysters (Lowther *et al*. 2019). The distribution was based on an early set of data eventually used in the above paper and fitted used the distribution fitting function of @Risk. Censored data was replaced with a value equal to the cut off value for each censored sample. Although estimates for virus infectivity were made within the NoVAS study using capsid integrity assays, recent research with both murine norovirus and with human virus in enteroids has shown that capsid integrity assays over-estimate norovirus infectivity, often substantially so (Rönnqvist *et al*. 2014; Farkas *et al*. 2018). The value Z is comes from data provided by Cefas as part of the NoVAS project. Stals *et al.* (2015) gives an estimate for the proportion of PCR signal that represents infectious virus that is somewhat lower than used here. However, Stals *et al.* (2015) based their estimate on results of studies in that estimated decline in infectivity in laboratory studies post inoculation. We consider this approach to underestimate infectivity as not only will infectivity decline after inoculation but so will PCR copy numbers. The benefit of the approach used here is that it directly measures the proportion of the PCR gene copies that represent infectious virus in a relevant food sample.

Data on the size of the meal/servings (S) of oysters came from the National Diet and Nutrition Survey and relevant data were provided by the Food Standards Agency (www.gov.uk/government/collections/national-diet-and-nutrition-survey). Data was in adults and included data from years one to six of the surveys.

8.4.3 Estimating dose per meal for lettuce and raspberries

The model for both lettuce and raspberries is represented by Equation 4:

Equation 4: $$D_p = G \times (1 - R) \times V \times S$$

where D_p = the final dose per meal, *G* = the concentration of virus in the purchased lettuce or raspberry, *R* = the proportion of virus removed by washing, *V* = the proportion of the virus in gene copies that represent viable virus and *S* = the size of the meal. Following Barker *et al*. 2013, the same value *R* was used for both lettuce and raspberries. As for oysters, none of these parameters represent single values but are drawn at random from distributions as given in Table 35. Note that the proportion of virus removed by washing is itself a function of the proportion of virus removed when washed and whether or not the produce is actually washed. In the absence of adequate studies of viability of virus on produce on sale we used the same estimates as for the oysters. That such an adjustment was reasonable comes from recent studies showing a one log reduction in virus infectivity within one day of inoculation of salad (Esseili *et al*. 2015; Esseili *et al*. 2016). The data from within the NoVAS study on the gene copy counts on both food products are shown in figures 35 and 36. Unlike the situation with oysters there were insufficient positive data to be confident about any distribution so both were modelled as simple log linear distributions with probability as the x variable modelled as a uniform distribution with the range zero to one. The values below the limit of detection are extrapolated from the available data.

Figure 34: Inverse cumulative density function of estimated gene copies of norovirus in lettuce

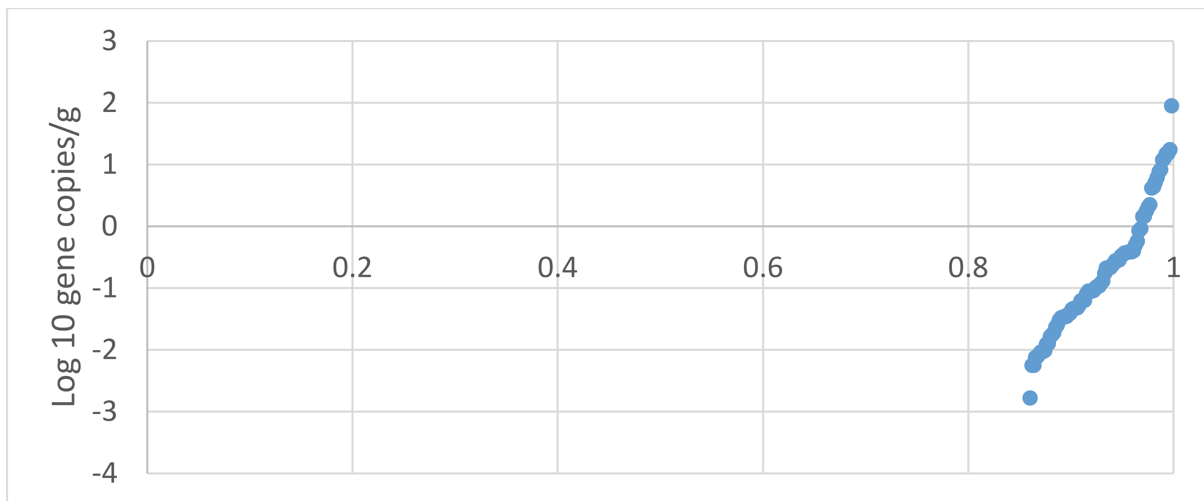
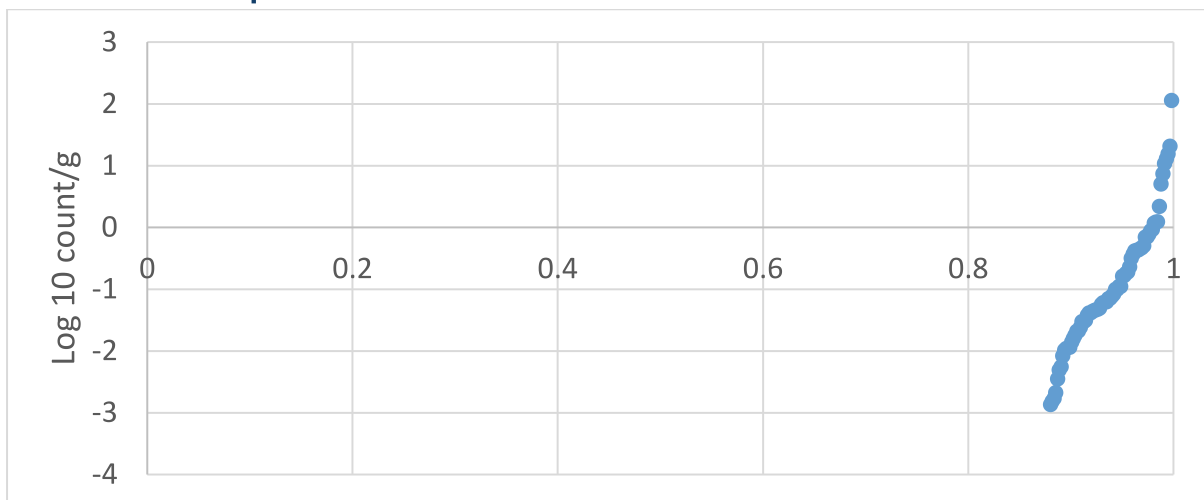


Figure 35: Inverse cumulative density function of estimated gene copies of norovirus in raspberries



Data on the size of the meal/servings of leafy salad and raspberries came from the National Diet and Nutrition Survey and relevant data were provided by the Food Standards Agency from the first six yearly surveys (www.gov.uk/government/collections/national-diet-and-nutrition-survey).

8.4.4 Estimating dose per catered meals

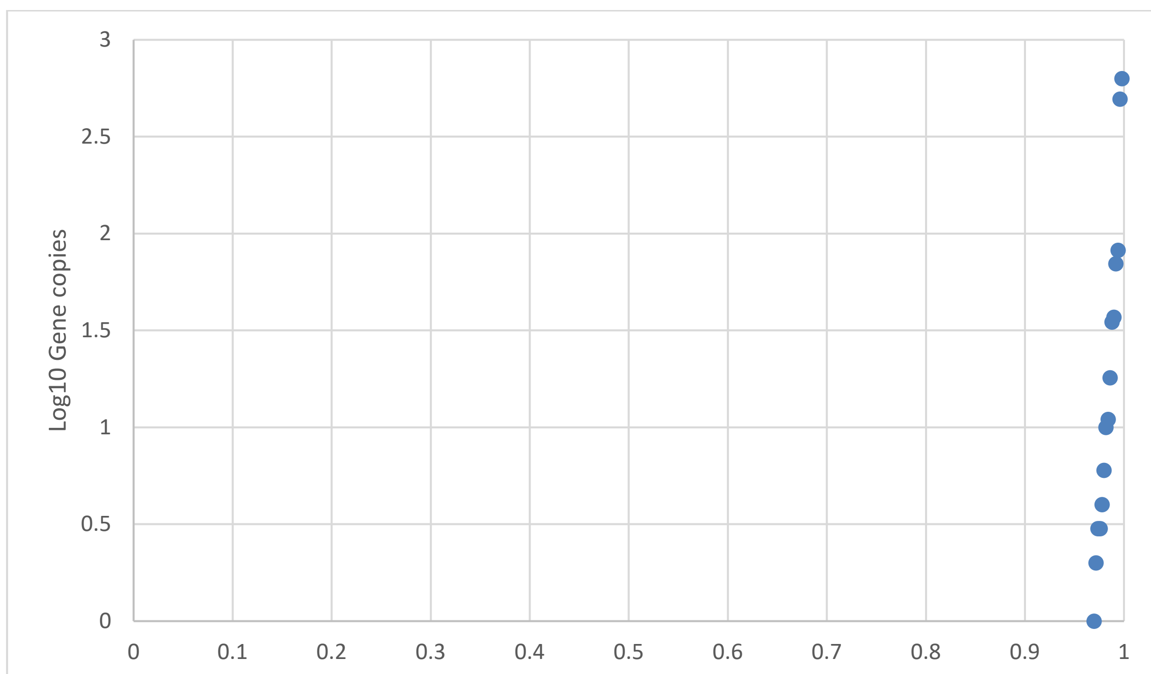
By catered meals it is meant both meals eaten out and take away meals eaten at home. Whilst many of the assumptions in the model are the same for both categories, we have modelled them separately in case further information becomes available that enables separate assumptions on contamination to be made. The basic model to estimating exposure dose in catered meals is substantially more complex than the models for oysters, lettuce and raspberries. There have been prior

risk modelling studies on sandwich production that have produced very large models with a considerable number of assumptions. Many of these assumptions were used to produce estimates of norovirus contamination of the hands of food handlers (Stals *et al*. 2015).

Within NoVAS we have the advantage of having data on hand contamination of food handlers (Chapter 7). In that chapter results were interpreted only qualitatively, because previous experience showed that hand and environmental swabs contain low viral loads and positive results are typically around the limit of detection of the assay. Such very low counts would be expected to have low relative precision and so are not typically reported. However qualitative results cannot be included in any risk assessment, and in order to use these data we estimated viral loads a posteriori, by plotting the CT values obtained on positive hand swabs against an average standard curve drawn from the values obtained in 10 PCR assays run in the course of WP5, that included the standard cDNAs a described in the ISO method to calculate the copies of DNA per microliter, and correcting for the dilution factor to express the total copy numbers present in the swab (multiplying by 50 as this was the total elution volume for each swab). Even though individual counts in low count results would have low precision, combined results across multiple samples would have sufficient accuracy to generate a reasonable distribution.

Using this a posteriori approach we were able to estimate the counts on hand shown in figure 37.

Figure 36: Inverse cumulative density function of estimated gene copies of norovirus recovered from hands



The model for catered meals is represented by Equation 5.

Equation 5:

$$D_c = \sum_{i=0}^{n} G \times T \times V \times (1 - H)$$

where D_c = the final dose per meal, n = the number of times the meal is touched by the hand of the food handler, G = the concentration of virus on the food handler's hand (NoVAS Work Package 5, chapter 7), T = the proportion of virus transferred from the hand to the food at each touch, V = the proportion of the virus in gene copies that represent viable virus at each touch and H = whether the food touched will be subsequently heat treated to inactivate any transferred infectious virus. The parameters for this part of the model are given in Table 35. The key parameters n and H are derived from papers focussing on risk models for sandwich production rather than catering models generally so giving uncertainties in this part of the model, which will be addressed in the sensitivity analyses.

H has a Bernoulli distribution that is 1 if the food touched will be subsequently heated and 0 if not. We were unable to find any data on the proportion of touches that would be before or after cooking in catering establishments. Duret and collegues (2017) assumed that “The food serving includes three ingredients, one of the ingredients is cooked”. In the absence of other data, we consequently assocummed that on average 1/3 of all touches preceed a final coloking step or 2/3 of touches of of food ready to eat without further heat treatment.

In determining the proportion of transferred virus that is viable we were conscious that some of the virus on the food handlers hand would have been transferred from food where there would have been loss of infectivity (which we assumed was the same as for oysters) and some from direct faecal contamination that we assumed may be fully viable. Consequently, we estimated likely infectivity with varying estimates of the virus on the hand being transferred from food or faeces. We modelled this estimate as a PERT distribution that that could have a minimum of 0 (No virus was derived from food) or 1 (all virus was derived from food). The mod value of the PERT distribution was estimated to be 0.2 as 20% was the next highest decile from the proportion of asymptomatic people excreting norovirus (Amar *et al*. 2007).   We used the same proportion of gene copies that represent infectious virus when not direct from human faeces as for the other food categories above.

8.4.5 Dose response model

There is a considerable number of different dose-response models that have been proposed for norovirus (van Abel *et al*. 2016). In a recent review van Abel and colleagues have divided those models that were based on exposure studies where the authors did and did not process the inocula used in human dosing studies in such a way as to break up aggregates of virus. Given our observation that the distribution of viral counts in oysters suggests that virus is present in aggregates, we prefer the models derived from aggregate inocula. Whilst there were insufficient data in NoVAS to be confident about the presence of aggregates in salads and lettuce, it seems reasonable to assume that viruses were also present in aggregates in these food products. Therefore, the $_2F_1$ hypergeometric model would also be appropriate for aggregated virus, but as shown by van Abel, the more computationally simple Approximate Beta Poisson model (Equation 6) produces results very similar to those of the $_2F_1$ hypergeometric model as can be seen in figure 1 in the van Abel paper, even though it was not based on a study using aggregated innocula. It can also be seen from van Abel's figure 1 that the variation is risk with the disaggregated models give massively different estimates of risk at low doses (>2,5 log greater), though the aggregated models give very similar risk estimates to each other. The model used in our analyses (BP_AGI in the van Abel paper), although a disaggregated model, gives risk estimates that are very close to the mean estimates of the different aggregated models at both low and high dose.

Equation 6: $$P_{infection} \approx 1 - \left(1 + \frac{dose}{\beta}\right)^{-\alpha}$$

Where dose = total daily dose and the other parameters are given in Table 35. This is the approximate Beta Poisson model which was determined using a Maximum likelihood estimation of the dataset from Atmar *et al*. (2013) (van Abel *et al*. 2017).

Not all individuals are susceptible to norovirus even if they have no prior exposure. Such intrinsic resistance is due to genetic variation in the host. Lindesmith (2003) and colleagues found that 29% of their study population were secretor positive and were not susceptible to Norwalk (norovirus GI). But secretor status does not only affect risk from GI strains. Although the Lindesmith (2003) study was using a GI norovirus there is variation in intrinsic resistance to other noroviruses including GII.4 (Nordgren *et al*. 2016). The situation is even more complex than this and varying host genetic factors seem to have different and currently unpredictable impacts on susceptibility differences to different norovirus types and sub-types (Rodríguez-Díaz *et al*. 2017). Consequently, we have used the same assumption value of intrinsic resistance for all noroviruses until better predictions of host resistance become clear.

8.4.6 Calculating risk per meal, per day and annually

When the count per meal model was run this generated 10,000 estimates of counts of infectious virus particles for each of the five food categories. Risk per meal was simply calculated using the dose response model and a random selection of the distribution of counts per relevant food categories produced by the exposure model. Following the advice of the European Food Standards Agency (2012) we summed gene copy counts in a single sample across both genotypes.

In order to estimate daily consumption of meals, we used the data National Diet and Nutrition Survey as provided by the Food Standards Agency (www.gov.uk/government/collections/national-diet-and-nutrition-survey) to estimate the number of meals consumed per day (using all adult data from study years 1 to 6. In this data 0.15% of the study population reported having a single meal of oysters during the four-day follow-up. So, the population mean number of meals in the four-day period is 1*0.15/100. This is then multiplied by 365/4 to give the annual figure in Table 35.

The food consumption data for lettuce and raspberries was slightly more complex as number of meals was not given but rather the meal/portion size and the average amount consumed over the four-day period. For lettuce 44% of people reported eating lettuce in the four-day period, the mean weight consumed per day in those eating lettuce was 10.99 g and the mean portion size was 24.50 g. The mean number of meals consumed per day in those eating lettuce was 0.449 (10.99/24.5) and in the population as a whole 0.198. This latter figure was then multiplied by 365 to give the annual number of meals consumed in Table 35. This was repeated for raspberries but the proportion of participants eating raspberries was just 4.8%, portion sizes were 50.98g and the mean amount eaten per day was 23.26g.

The number of meals out were derived from Adams *et al*. (2015). This gave the proportion of people who reported eating out (27.1%) or buying a take-away to eat at home at least one per week (21.1%). So that 72.9% of adults did not eat a meal out, and 78.9% did not buy a take away meal in a one-week period. Assuming that the number of meals eaten in a week is Poisson distributed, then the probability of zero meals per week is given by Equation 7.

Equation 7: $$P\ (X = 0) = e^{-\lambda}\frac{\lambda^0}{0!}$$

Or more simply $e^{-\lambda}$ given that λ^0 and 0! are both equal to 1. For a probability of 0 = to 0.729 this would be solved as λ= 0.316 (eat out) and for 0.789 λ= 0.237. In the Poisson distribution λ equates to the mean so the mean number of meals eaten out per person week is 0.316 and take away meals eaten at home 0.237. Multiplying these values by 365/7 give the mean annual number of meals eaten out or taken away as in Table 35.

In prior QMRA studies that have produced annual estimates of risk one of two equations have been used to extrapolate from daily to annual risk Equations 8 and 9.

Equation 8:

$$P_T = 1 - (1 - p)^{365}$$

Equation 9:

$$P_K = 1 - \prod_{k=1}^{365}(1 - p_k)$$

Karavarsamis and Hamilton (2010) argued that the assumptions underpinning Equation 8 were naïve and argued for Equation 9 which they claimed to be the gold standard. We have shown that in fact both equations are naïve and lead to systematic biases. Indeed, recent guidance from the World Health Organization has highlighted the difficulties in annualising risk (WHO 2016). We have proposed microsimulation as a less biased approach in determining annual risk. This is discussed in more detail in Appendix 13. There is however, an additional issue in that norovirus is actually a complex species with multiple strains (Parra *et al*. 2017). Both of the above equations essentially calculate the probability of one or more infections per year. For many pathogens this is probably a reasonable approximation, but with multiple strains repeat illness with norovirus are not unknown. Consequently, we summed the daily risks over the 365 days of the year to get the annual risk. This is further discussed in the following section.

In the microsimulation model we run the model for 10,000 times for a notional year (365 days). We then sum the infections over the year. The basic simulation is illustrated in the following steps (Table 36).

Table 36: Basic steps in the Microsimulation model

Step		
1	For all food categories separately	Randomly generate the number of meals likely to be consumed each year using Poisson model
2	For all food categories separately	Estimate mean number of meals eaten each day Step1/365
3	For each of 365 days for each food category separately	Randomly estimate number of meals on that day using Poisson model with λ=mean from step 2.
4	For each of 365 days for each food category separately	For each meal quantified in step 3, randomly choose a count value from the file generated by the exposure per meal model described above
5	For each of 365 days for each food category separately	Sum the exposure for all meals within each category for that day
6	For each day but all categories	Sum the total exposures for across all food categories for that day
7		Estimate risk on that day on exposure counts for each food category individually and for all categories summed together using dose response model
8		Repeat steps 3 to 6 for 365 times
9		Sum risks over the “year” for each food category and for all foods together
10		Repeat steps 1 to 9 10,000 times.

8.4.7 Adjusting for acquired immunity from prior infection

When including the SIR element in the model we generated an estimate of the background incidence of infection. At this point it is important to reiterate the difference between infection and illness. In the way we are using the term in this paper illness refers to symptomatic illness whereas infection may or may not be symptomatic and may or may not contribute to disease burden. Whilst almost certainly of critical importance in the epidemiology of norovirus it is actually very difficult to get good estimates of the incidence of asymptomatic infections. We do have good prevalence data from a reanalysis of samples from the initial IID study (Amar *et al*. 2007). In this study it was found that the mean prevalence of carriage of norovirus in asymptomatic cases was 16% across all age groups, though the

prevalence varied by age with children under 1 year having a prevalence of 31% and people in the 50 to 59 year age group this was just 5%. Milbrath *et al*. (2013) undertook a review of all studies of the duration of excretion of norovirus post infection and found that the reported duration of excretion was variable but that the mean duration of excretion was 16.4 days in otherwise health adult individuals. In the absence of strong evidence otherwise we assumed the duration of excretion was the same in asymptomatic as in symptomatic individuals. The incidence of any disease is the prevalence divided by the duration. This gives the annual incidence of asymptomatic infection as 365*0.16/16.4. (3.56 asymptomatic infections per person year). Adding the incidence from IID2 gives 3.61 infections per year.

In order to incorporate the SIR model into the microsimulation model we built into the model an estimate of the number of previous infections likely to have been experienced by each notional individual during a period equal to the stated duration of immunity. This was done at the start of the "year" but then recalculated on a daily basis. Each day there was a probability of loss of immunity to one of those prior infections with a probability = Number of prior infections /Duration of immunity in days. The probability of a new infection in a day was the background number of infections occurring in a community/365. The probability of loss of immunity to one of the prior infection was given by the number of prior infections in the immune period/duration of immunity in days.

However, to incorporate the SIR model into the microsimulation model a knowledge of the duration of immunity to norovirus infections is required. Previous studies of norovirus have assumed that immunity last for just 6 months (Lane 2014). However, it is also commonly stated that immunity lasts for between 6 and 24 months (Robilotti *et al*. 2015). One study has even suggested that immunity to the homotypic challenge is much longer at 4 years, though we are not convinced by the analyses in this paper (Simmons *et al*. 2013). For all basic analyses we modelled duration of immunity as a random PERT distribution with the Min=6, Mod=6 and Max=24 in order to give preference to the 6-month value but also to allow uncertainty up to 24 months. The PERT distribution is a smoothed version of the triangular distribution. Most prior studies have considered that an infection within the model provides immunity to all strains of the pathogen. For norovirus this is manifestly not the case. There are many different strains of norovirus and there is often little or no cross immunity between the different types and sub-types (Parra *et al*. 2017). We have already raised this above when considering the usual methods of annualising risk in QMRA. In order to account for multiple strains, we used the Hunter Gaston equation, which we first developed some 30 years ago for the differentiation of different strains in microbial typing schemes (Hunter and Gaston 1988). This equation (10) describes the probability that two strains randomly sampled from the same population would be of the same type.

Equation 10:

$$D = 1 - \frac{1}{N(N-1)} \sum_{j=1}^{S} n_j(n_j - 1)$$

Where *N* is the total number of strains in the sample population, *S* the total number of different types and n_j the number of strains in the jth type. The dataset on which the value of D was calculated is that given by Gallimore *et al*. (2007) which reports on the diversity of norovirus strains in England and Wales from 2003 to 2006.

To determine the probability that any new infection is the same type as one or more or those responsible for a prior infection in the period of immunity and to which a person should be immune we used Equation 11.

Equation 11: $$P = 1 - D^n$$

Where *D* is the Hunter-Gaston index and *n* is the number of prior infections in the immune period.

8.4.8 Reporting uncertainty

Most Quantitative Microbial Risk studies present uncertainty in terms of the variance of the outcome measure being assessed. However, in a microsimulation model as presented here this is not really of value. Each run generates simulated data on 10,000 individuals. We present the mean and standard deviation and then the 5th, 50th and 95th percentile values. These represent the distribution in individual risk in the population. But in terms of population risk it is the mean value that is primarily important. We present the 90% credible intervals in the mean values based on 5 repeat runs. Although this gives an indication of the uncertainty in the models using the stated assumptions these should not be considered to represent the actual uncertainty in the final values.

In the model described here, the main sources of uncertainty are driven by uncertainty in the parameters used. Some of that uncertainty will be due to statistical variation in the parameter (e.g. counts of virus in food samples) but a substantial amount of uncertainty would be due to inadequate knowledge of the likely values of any input parameters. Whilst we initially intended to use Bayesian methods the model became far too complex for the statistical power available to us. In the end we opted to use the most likely minimum and maximum values of that input parameter that had most impact on annual risk. As will be seen below this was the maximum and minimum likely durations of acquired immunity post infection. Care should be taken, however, not to interpret this as any statistically defined range.

8.5 Results

The results of the analyses are presented in five sections. The first section is concerned with modelling the exposure in terms of viable virus per meal. The second section presents the results of the basic risk model with results presented as risk per meal in intrinsically susceptible individuals as well as risk in the general population. The third section introduces the results from the epidemic model that accounts for immunity from prior exposure. The fourth section presents the results of the key sensitivity analyses and the final section the conclusions and synthesis.

8.5.1 Virus exposure per meal

The summary results of the exposure model showing the mean, standard deviation and key percentiles for the model outputs (exposure per meal) over 5 independent runs are shown in Table 37. It can be seen that there is some variability in counts from model run to model run and that this is more marked for the produce and oyster counts than for the catered meals. The count of virus in oysters was substantially higher than in the other food groups. From the available data the counts are clearly asymmetric and skewed to the right. This would explain the relatively high relative standard deviations. In the following tables The Mean, Std Dev and the %ile values can be considered to represent the spread of probabilities in individual meals/people whereas the Mean of means, Std Dev of means and credible intervals represent the mean and variation in repeated runs estimating the population mean.

Table 37: Results of five runs meal exposure model predicting counts of viable norovirus per meal

Food	Count/ meal	Run 1	Run 2	Run 3	Run 4	Run 5	Mean of means	Std De of means	90% credible intervals
Oyster	Mean	10.68	10.24	10.36	9.97	9.68	10.19	0.38	9.56-10.81
	Std Dev	88.42	79.45	84.63	81.96	80.38			
	5th%ile	0	0	0	0	0			
	50th%ile	0.25	0.24	0.25	0.25	0.26			
	95th%ile	33.20	33.29	34.96	31.63	33.12			
Lettuce	Mean	0.09	0.08	0.08	0.10	0.10	0.09	0.01	0.08-0.10
	Std Dev	1.59	1.53	1.14	1.73	1.97			
	5th%ile	6.89E-24	6.62E-24	7.24E-24	7.79E-24	7.68E-24			
	50th%ile	6.15E-13	6.37E-13	6.6E-13	6E-13	6.43E-13			
	95th%ile	0.06	0.05	0.06	0.05	0.05			
Raspberries	Mean	0.14	0.11	0.08	0.16	0.11	0.12	0.03	0.07-0.17
	Std Dev	2.36	2.01	1.22	3.31	1.49			
	5th%ile	3.52E-28	3.27E-28	4E-28	3.77E-28	3.59E-28			
	50th%ile	3.3E-15	3.67E-15	4.07E-15	4.15E-15	3.99E-15			

Food	Count/ meal	Run 1	Run 2	Run 3	Run 4	Run 5	Mean of means	Std De of means	90% credible intervals
	95th%ile	0.05	0.04	0.04	0.04	0.04			
Eat out or take away	Mean	0.86	0.81	0.88	0.87	0.80	0.84	0.04	0.78-0.90
	Std Dev	5.32	4.93	5.50	5.23	4.81			
	5th%ile	1.85E-51	5.22E-52	2.22E-51	5.14E-51	2.82E-52			
	50th%ile	1.43E-11	1.29E-11	1.01E-11	1.71E-11	1.34E-11			
	95th%ile	2.63	2.91	2.84	3.07	2.81			

8.5.2 Risk results

The results of five runs of the risk model are presented in Tables 38 and 39. The risk of infection per meal is shown in those intrinsically susceptible table is shown in Table 38 meal whilst the risk per 1000-person-years (1000py) is shown in Table 39.

By far the highest risk of infection per meal is from oysters with a mean risk of 6.78/1000meals or about one infection in every 150 meals. By contrast the risk per meal for the other food groups were substantially lower. The risk per meal for lettuce was about one in every 12000 meals, for raspberries this was one in every 9000 meals. Meals eaten out or takeaways had an intermediate risk of about one in infection in every 1200 meals. Assuming that 25% of people are intrinsically resistant then the risks of infection per meal/serving of oysters, lettuce, raspberries and catered meals would be about one infection per 200, 16000, 12000 and 1700 meals respectively. These estimates are unadjusted for immunity due to prior infection and so represent infection and not necessarily illness.

The summary data for estimated annual risks is shown in Table 39 and the distribution of these annual risks in the population are shown in Figure 38. The annual risks are adjusted for intrinsic resistance but not for any acquired immunity. The total annual infection rate for all four food groups together is high at 23.76 infections per 1000py. This is high, about one half of the total number of illnesses estimated to be due to norovirus in IID2 (47 infections/1000py) (Tam et al. 2012). Although the risk per meal for oysters is very high the annual risk from consuming oysters is actually very small contributing about 2.7% of all estimated infections as the frequency of consumption is very low. The major contribution to foodborne norovirus comes from catered foods. Summed together both meals eaten out and take away foods account for 74.2% of all foodborne norovirus (43.7% from foods eaten out and 30.5% from takeaways). The single food product responsible for most infections is lettuce 19.5% of infections. Raspberries account for only 2.9%.

Table 38: Estimated risk per 1000 meals for each of the food groups in intrinsically susceptible people and not accounting for prior immunity[a]

Food	Risks/ 1000 meals	Run 1	Run 2	Run 3	Run 4	Run 5	Mean of means	Std De of means	90% credible intervals
Oyster	Mean	6.89	6.87	6.86	6.63	6.66	6.78	0.13	6.57-7.00
	Std Dev	28.64	27.96	27.65	27.35	26.32			
	5th%ile	0	0	0	0	0			
	50th%ile	0.24	0.24	0.24	0.24	0.26			
	95th%ile	30.55	30.63	32.07	29.19	30.48			
Lettuce	Mean	0.09	0.07	0.08	0.09	0.09	0.08	0.01	0.07-0.10
	Std Dev	1.40	1.31	1.05	1.52	1.65			
	5th%ile	0	0	0	0	0			
	50th%ile	0	0	0	0	0			
	95th%ile	0.05	0.05	0.06	0.05	0.05			
Raspberries	Mean	0.12	0.10	0.08	0.14	0.10	0.11	0.03	0.07-0.15
	Std Dev	1.97	1.74	1.10	2.58	1.36			
	5th%ile	0	0	0	0	0			
	50th%ile	0	0	0	0	0			
	95th%ile	0.05	0.04	0.04	0.04	0.04			
Eat out	Mean	0.80	0.75	0.81	0.80	0.74	0.78	0.03	0.73-0.83
	Std Dev	4.71	4.38	4.78	4.59	4.30			
	5th%ile	0	0	0	0	0			
	50th%ile	1.4E-11	1.27E-11	9.99E-12	1.67E-11	1.32E-11			
	95th%ile	2.56	2.83	2.76	2.98	2.73			
Take away	Mean	0.81	0.84	0.84	0.83	0.85	0.83	0.02	0.81-0.86
	Std Dev	4.62	4.74	4.80	4.81	4.89			
	5th%ile	0	0	0	0	0			
	50th%ile	2.32E-11	1.38E-11	1.51E-11	1.41E-11	1.6E-11			
	95th%ile	2.96	3.02	3.24	3.17	3.11			

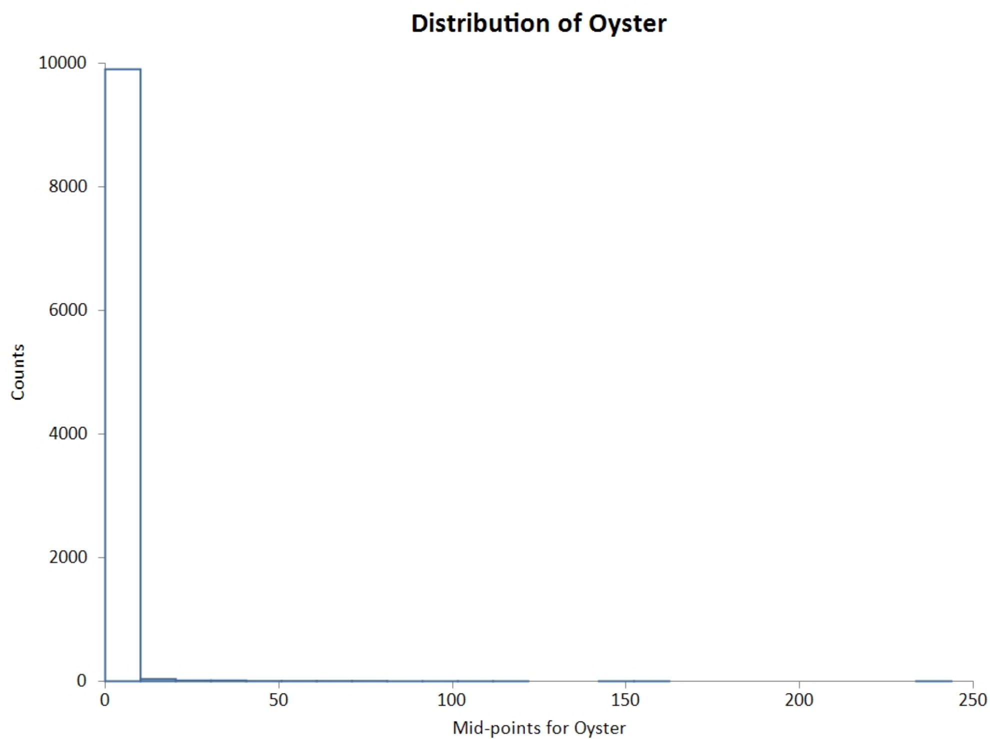
[a] The measures of variation for each run can be thought of as the variation in risk per 1000 meals for an individual (e.g. for oysters in run 1 only 5% of people would have a risk of 29/1000 or greater).

Table 39: Estimated annual risk for each of the food groups and combined using stated primary assumptions in 1000 person years accounting for intrinsic sensitivity but not acquired immunitya

Food	Risk/ 1000py	Run 1	Run2	Run3	Run4	Run5	Mean of means	Std De of means	90% credible intervals
Oyster	Mean	0.74	0.75	0.72	0.49	0.56	0.65	0.12	0.46-0.85
	Std Dev	9.54	9.85	10.43	5.96	6.21			
	5th%ile	0	0.00	0.00	0.00	0.00			
	50th%ile	0	0.00	0.00	0.00	0.00			
	95th%ile	0	0.00	0.00	0.00	0.00			
Lettuce	Mean	4.88	4.22	4.30	4.87	4.85	4.62	0.33	4.07-5.17
	Std Dev	11.19	10.78	8.26	11.39	12.15			
	5th%ile	0	0.00	0.00	0.00	0.00			
	50th%ile	1.25	1.05	1.25	1.33	1.25			
	95th%ile	26.03	15.36	20.44	21.18	18.71			
Raspberries	Mean	0.77	0.63	0.44	0.96	0.67	0.69	0.19	0.38-1.01
	Std Dev	4.95	4.37	2.54	7.06	3.56			
	5th%ile	0.00	0.00	0.00	0.00	0.00			
	50th%ile	0.00	0.00	0.00	0.00	0.00			
	95th%ile	2.67	1.87	1.70	2.57	2.19			
Eat out	Mean	10.15	10.67	10.40	10.21	10.48	10.38	0.21	10.04-10.72
	Std Dev	17.86	18.67	17.97	18.21	18.72			
	5th%ile	0.00	0.00	0.00	0.00	0.00			
	50th%ile	1.64	1.72	1.79	1.71	1.97			
	95th%ile	49.39	51.77	49.67	48.60	51.80			
Take away	Mean	7.56	6.97	7.30	7.53	6.88	7.25	0.31	6.73-7.77
	Std Dev	15.77	14.04	15.14	15.33	13.88			
	5th%ile	0.00	0.00	0.00	0.00	0.00			
	50th%ile	0.45	0.49	0.63	0.62	0.57			
	95th%ile	42.72	36.06	36.84	39.13	36.32			
Combined	Mean	24.16	23.43	23.36	24.23	23.60	23.76	0.41	23.08-24.43
	Std Dev	30.03	30.46	29.65	30.56	29.60			
	5th%ile	0.00	0.00	0.00	0.00	0.00			
	50th%ile	13.39	12.88	13.57	13.14	13.30			
	95th%ile	85.54	85.88	80.72	85.12	84.52			

[a] The measures of variation for each run can be thought of as the variation in risk for 1000 people over the course of one year (e.g. for lettuce in run 1 5% of people would have a risk of 60/1000 infections or greater per year).

Figure 37: Distribution of annual risk of infection in the different food groups individually and combined
(Note x and y-axes are not all to the same scale). The x-axes represent estimated number of infections per 1000 person years and y-axes the number of times the model generated that risk out of 10,000 iterations. In these histograms the count represents the number of times "counts" that an estimated annual risk fell into the range of possible risks covered by that bar. The x axis shows the mid points as annual number of infections/1000py



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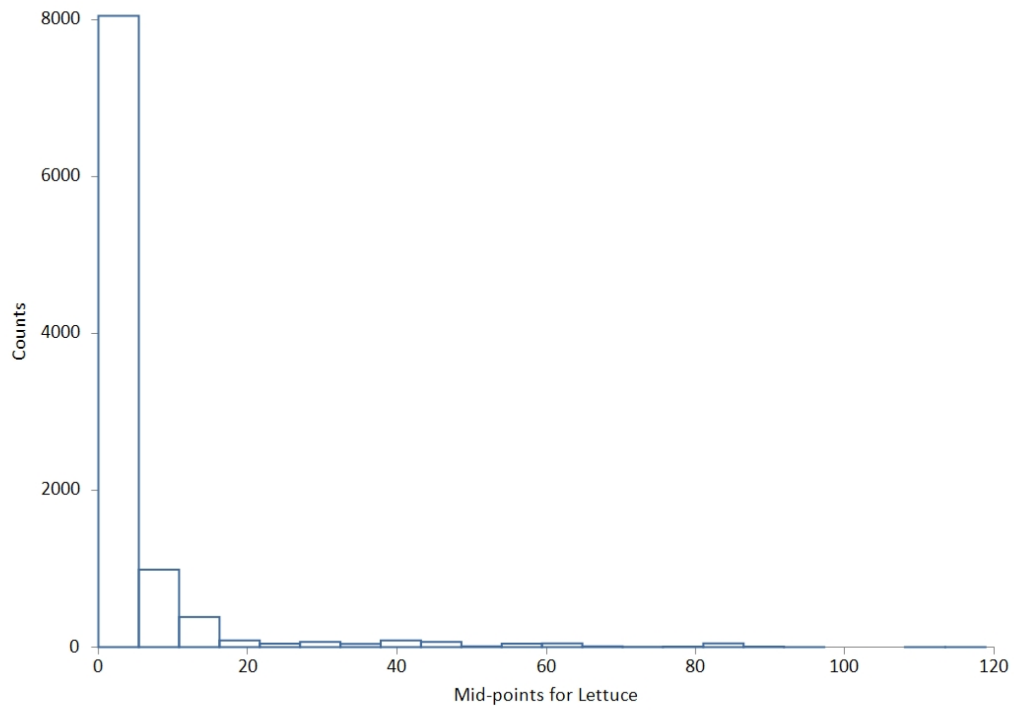

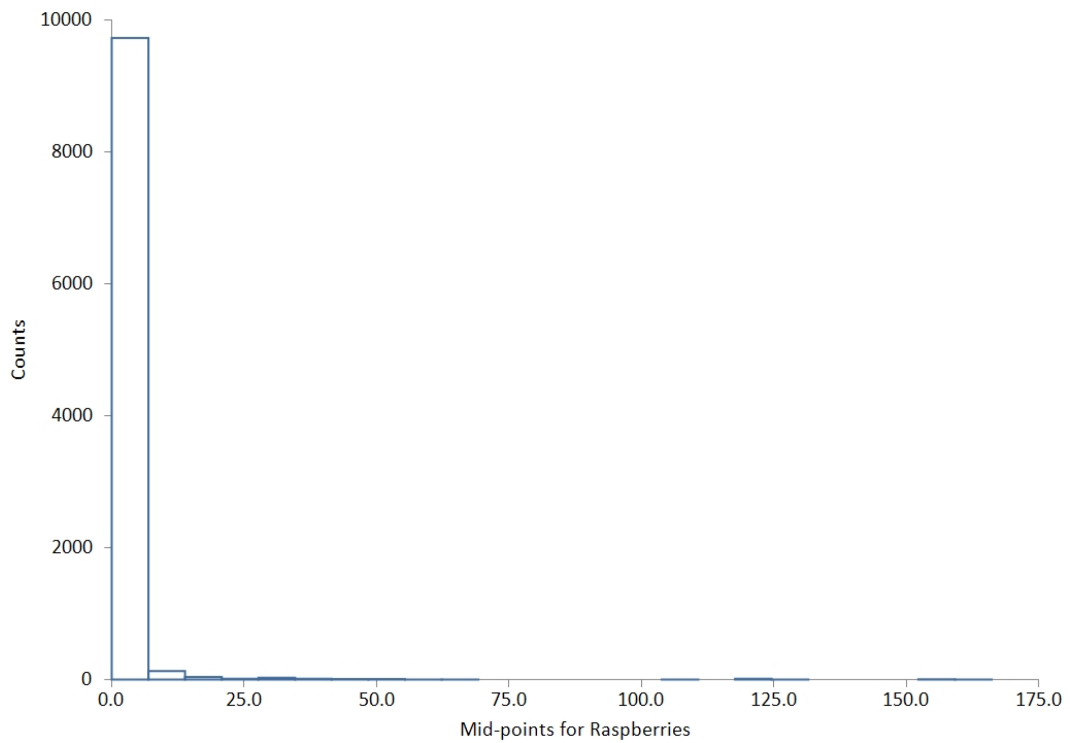

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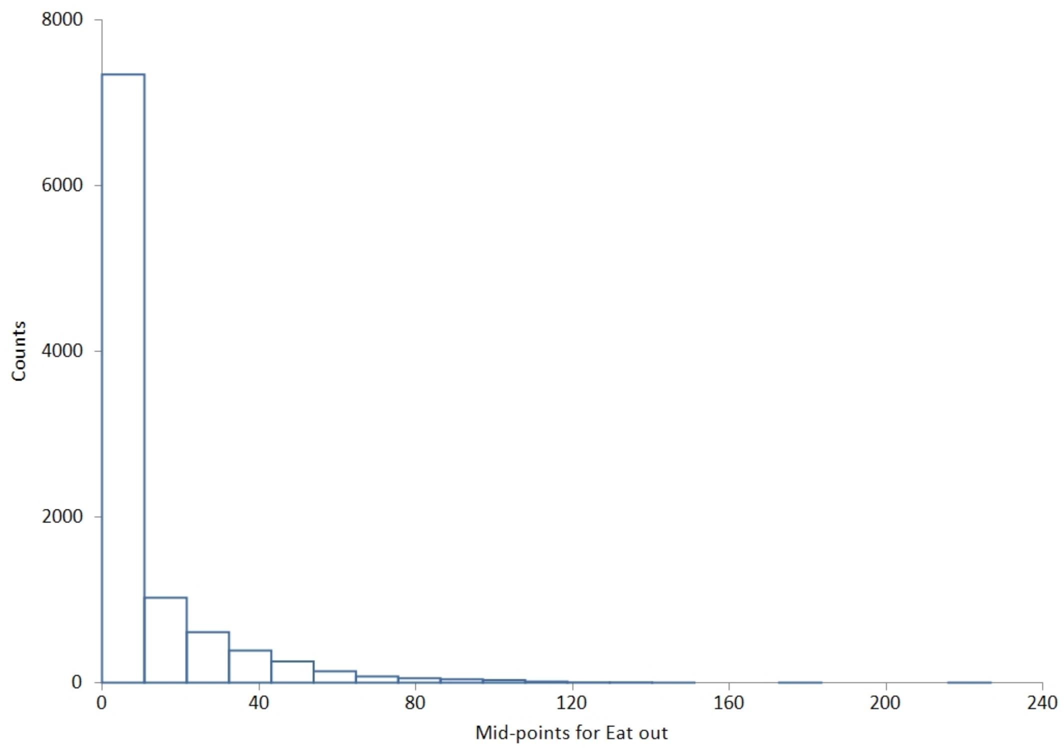

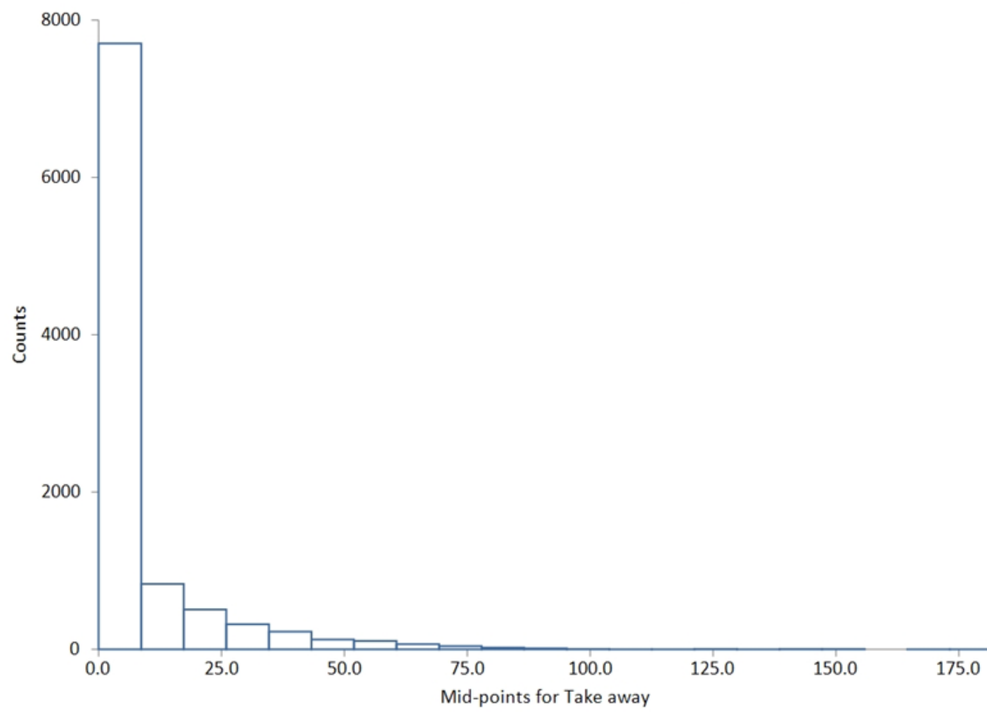

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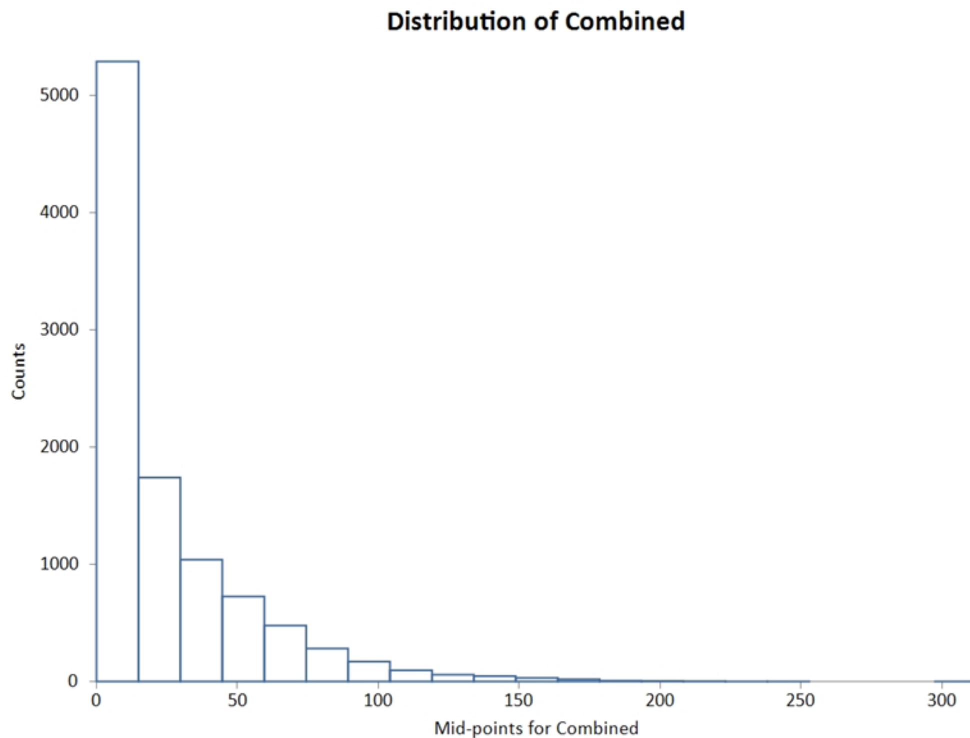


8.5.3 Epidemic model – accounting for multiple prior infections

In Table 40, it is shown that once prior infections are accounted for in an epidemic model then the predicted rates of symptomatic illness falls by about two thirds compared to that predicted in Table 39. The attack rate is now just 7.65 from 23.76 episodes/1000py. Given the importance of multiple food pathways in generating prior immunity only the data for the combined foodborne risk are presented.

Table 40: Predicted infection rates from all five food categories combined both adjusted to account for intrinsic resistance and either adjusted or not for acquired immunity due to prior infection

Food	Risk/ 1000py	Run 1	Run2	Run3	Run4	Run5	Mean of means	Std De of means	90% credible intervals
Combined	Mean	24.16	23.43	23.36	24.23	23.60	23.76	0.41	23.08-24.43
	Std Dev	30.03	30.46	29.65	30.56	29.60			
	5th%ile	0.00	0.00	0.00	0.00	0.00			
	50th%ile	13.39	12.88	13.57	13.14	13.30			
	95th%ile	85.54	85.88	80.72	85.12	84.52			
Combined Adjusted	Mean	7.92	7.62	7.46	7.73	7.54	7.65	0.18	7.36-7.95
	Std Dev	13.59	14.40	13.04	13.66	13.19			
	5th%ile	0.00	0.00	0.00	0.00	0.00			
	50th%ile	2.69	2.48	2.68	2.70	2.62			

Food	Risk/ 1000py	Run 1	Run2	Run3	Run4	Run5	Mean of means	Std De of means	90% credible intervals
	95th%ile	33.75	32.84	31.12	33.57	32.35			

Assuming the same ratio between adjusted and unadjusted estimates for the combined annual risk applies to the food specific risks this would give estimated risk of illness/1000py of 0.21 for oysters, 1.49 for lettuce, 0.22 for raspberries and 5.68 for catered meals (3.34 for meals eaten out and 2.34 for take away meals). Risks of illness/1000meals after accounting for intrinsic resistance and acquired immunity would be about 1.64 for oysters, 0.02 for lettuce, 0.03 for raspberries and 0.20 for catered meals.

8.5.4 Sensitivity analyses

Whereas most of the input variables in the model were based on empirically obtained data several were based on expert judgement or extrapolated from sources that may not be directly applicable to the context of this model.

Within the viral exposure model the catered food was prone to particular uncertainties that we could not better define especially in relation to the number of times food is touched by hand and whether the finished product will be heated treated after these touches. Also, the proportion of virus on hand transferred to the swab and to a range of foods with each touch is also not known with certainty. In an experimental study Grove *et al.* (2015) reported a transfer of about 1.1% from hands to lettuce after deliberate inoculation of volunteers' hands which is rather low compared to the estimates used in this model which are derived from Stals *et al*. (2015). However, our estimates of virus on hands came from swabbing and only a proportion of the virus present of the hand would have been recovered by the swab, so our choice of the Stals parameters are reasonable.

Another key uncertainty that impacted on the produce and catered foods was the viability of the virus. We have good experimental data on virus viability in oysters but whether this is directly transferable to salads is not known. Our basic model assumes the same viability in produce as in oysters but it may be that the more severe environments of the ocean and oyster gut increase the loss of viability relative to what may be seen in fruit and salads.

In the risk model major areas of uncertainty reside around the duration of immunity, the amount of exposure to viable virus in food and also to the background infection rate in the community. One particular issue is regarding the difference in high incidence years and low incidence years, especially given that the surveys within the NoVAS project were undertaken in a relatively low incidence year.

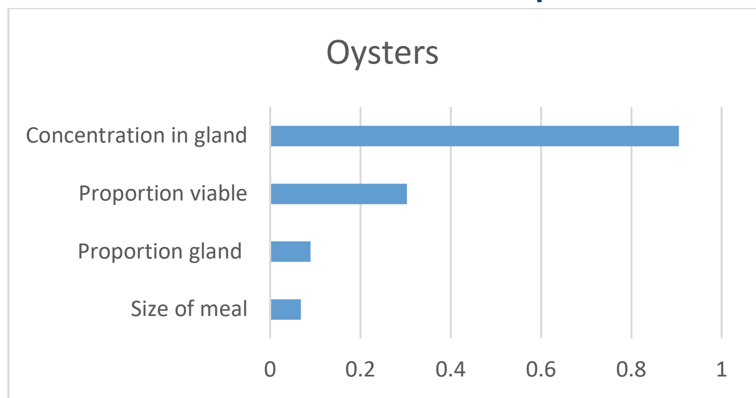
Figure 35 shows tornado plots of the correlation coefficients of the key input variables for the key food groups. A plot is not shown for takeaways, as the model at this stage is identical to the eat-out model. It can be seen that in all cases the highest correlation between counts in meals and inputs is with counts on the raw product or, in the case of catered food, with counts on the food handlers' hands. In addition, we modelled the potential impact on risk per meal assuming 100% viability in lettuce, raspberries and catered food. The impact of changing frequencies of touching catered meals on risk were also modelled (Table 41). The assumption around proportion of virus on food being viable has a very large impact on exposure and on risk per meal. Changing assumptions around handling in catered food has an impact but this is not as great. Given that in these analyses, the models for exposure per meal for both meals eaten out and takeaways are identical we only present sensitivity analyses for meals eaten out.

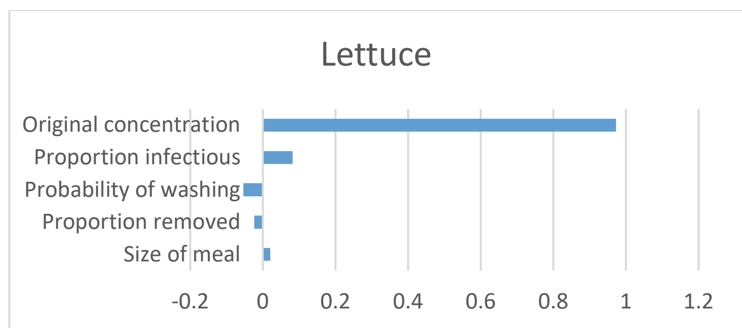
The largest source of uncertainty in this part of the model represents uncertainty over the proportion of detected virus likely to be viable in all food groups. As discussed above we have good empirical data for oysters on the proportion of virus gene copies likely to be infections but whether or not this is applicable to the other food groups is unclear. If all virus on the other food groups were viable then this would substantially increase the risk per meal by about a factor of 10. However recent research showing a one log decline in infectious murine norovirus just one day after inoculation onto post-harvest lettuce gives credence to our use of the same infectivity factor (Esseili et al. 2015; Esseili et al. 2016).

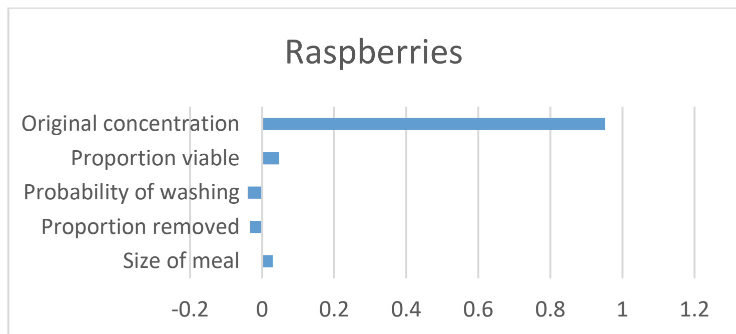
The other area of greatest uncertainty is the number of times catered food is touched, what proportion of virus on hand is transferred to food with each touch and whether or not that food is heated after that touch. Whilst the parameters used were taken from the literature those studies were primarily focussed on sandwich preparation and it is not clear how they apply to other catered foods. The dose per meal and risk per meal both increase, or decrease, in line with these parameters. For example, a halving of the proportion of virus transferred by touch roughly halves the dose and risk per meal. This is an area worthy of further study to improve the input parameters in this part of the model. It is likely, however, that sandwich preparation required more physical touching than other foods, which are also more likely to be subject to further cooking. Whilst there is a number of studies that have investigated food handling behaviour in the catering industry, we were only able to identify studies that have investigated sandwich production (Stals *et al*. 2015). Studies of food handlers' behaviour in the wider industry have not recorded the number of times food is touched (Green *et al*. 2005). Whilst it is likely to be the case that handling of sandwiches is more intensive than most other food products we used this value for full meals, even though each food item in a meal will be touched less, there are several food items and several courses in a single restaurant meal. In addition, we could not find data on the frequencies of different types of restaurant meals or takeaways consumed in the UK. It is likely to be the case that some types

of catered meal have no direct touch or only before cooking, such as fish and chips. These meals are unlikely to pose a risk from norovirus and so our estimate for risk from catered foods should be considered the worst-case scenario.

Figure 38: Tornado plots showing Spearman correlation (x-axes) between viable counts of norovirus on food and model outputs







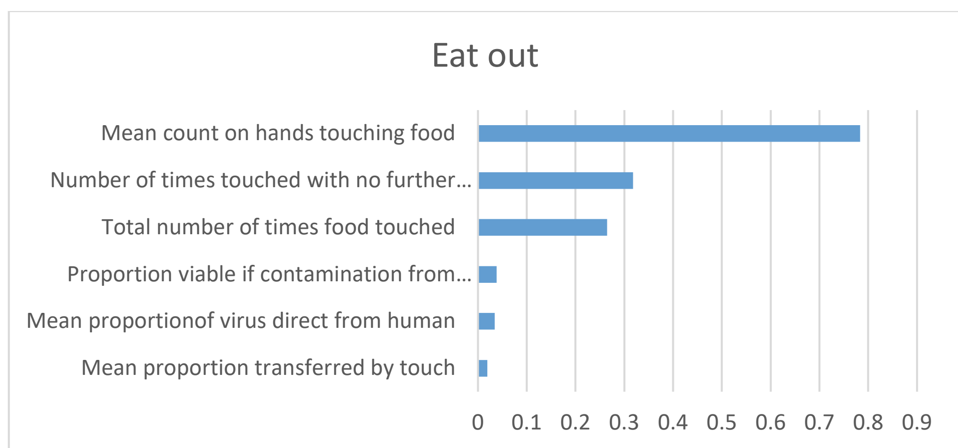


Table 41: Sensitivity analysis of key uncertain assumptions on dose and risk per meal using the run with median risk

Food	Model	Mean	Std Deviation	5% Perc	50% Perc	95% Perc
Dose per meal						
Lettuce	Base	0.10	1.97	7.68E-24	6.43E-13	0.05
Lettuce	All virus viable	1.22	10.97	2.72E-22	2.27E-11	1.69
Raspberries	Base	0.11	1.49	3.59E-28	3.99E-15	0.04
Raspberries	All virus viable	1.36	13.66	1.30E-26	1.36E-13	1.59
Eat out	Base	0.80	4.81	2.82E-52	1.34E-11	2.81
Eat out	All virus viable	2.66	13.80	0	7.02E-11	10.26
Eat out	Halve touches	0.46	3.63	0	4.41E-24	0.34
Eat out	Halve proportion transferred per touch	0.41	2.43	0	1.22E-11	1.41
Risk per 1000 meals						
Lettuce	Base	0.09	1.65	0	0	0.05
Lettuce	All virus viable	1.03E-03	8.14E-03	0	2.22E-14	1.64E-03
Raspberries	Base	0.10	1.36	0	0	0.04
Raspberries	All virus viable	1.11E-03	9.16E-03	0	0	1.55E-03
Eat out	Base	0.74	4.30	0	1.32E-11	2.73
Eat out	All virus viable	2.30E-03	1.13E-02	0	6.86E-14	9.83E-03
Eat out	Halve touches	4.23E-04	3.27E-03	0	0	3.28E-04
Eat out	Halve proportion transferred per touch	3.95E-04	2.27E-03	0	1.20E-14	1.38E-03

Table 42: Sensitivity analyses of risk model where key areas of uncertainty are modelled

Risk per 1000 py	Mean	Std Deviation	5%ile	50%ile	95%ile
No adjustment for prior exposure					
Basic risk	23.60	29.60	0	13.30	84.52
6 month duration of immunity	23.80	30.10	0	13.25	83.45
24 month duration of immunity	24.18	32.14	0	13.30	85.61
Double estimate of background infection rate	23.53	30.06	0	13.34	83.91
Double viral load in food	45.54	56.12	0	26.73	160.22
Double background incidence and viral load in food	44.76	54.70	0	26.18	154.10
Adjustment for prior exposure					
Basic risk	7.92	13.59	0	2.69	33.75
6 month duration of immunity	10.78	16.93	0	4.62	43.36
24 month duration of immunity	0.99	2.78	0	0.16	4.32
Double estimate of background infection rate	2.58	6.03	0	0.54	11.85
Double viral load in food	14.10	23.32	0	5.27	60.38
Double background incidence and viral load in food	4.89	11.15	0	1.05	22.32

The results of the sensitivity analyses around the annual foodborne risks are shown in Table 42. It is clear that the largest impact on prevalence of foodborne illness in this model is the duration of immunity where increasing the estimated duration of immunity period from 6 to 24 months leads to a reduction in the estimated annual

incidence of symptomatic foodborne norovirus illnesses by about a factor of 10 (from 10.78 to 0.99 illnesses/1000py. As can be seen in the second half of Table 42, increasing exposure by doubling the estimated counts in food almost doubles the estimated annual risk (from 7.92 to 14.10 illnesses per 1000py) after accounting for the effect of prior immunity. Whereas doubling the estimate of the background prevalence of asymptomatic illness as may be expected in an epidemic year more than halves the estimate of foodborne illnesses provided the amount of virus in food remains constant (from 7.92 to 2.58 illnesses per 1000py). Given that human norovirus only replicates in the human host it is likely that incidence of infection and exposure increase or decrease together. In other words, counts of norovirus in food are highest when norovirus infections are at a peak. In Table 42 it can be seen that in epidemic years (background incidence doubles and exposure via food doubles) the incidence of foodborne illness will actually decline slightly (from 7.92 to 4.89 illnesses/1000py).

The issue of the duration of immunity is more problematic given the large impact it has in the model on annual risk of infection. A Food Standards Agency Funded Study (Lane 2014) used a six-month duration of immunity but, as discussed above there has been recent studies suggesting that strain specific immunity may be much longer and possibly lasting several years (Simmons *et al*. 2014). We are not convinced by this latter study, in part because we do not think it adequately accounts for the very likely high background exposure rates that asymptomatic carriage rates would suggest so leading to high frequency of reinfections and maintenance of immunity. Although with the current state of knowledge we are not able to state the exact duration of immunity, we consider 6 months to be a plausible lower limit and 24 months the upper limit. As discussed above the use of the PERT distribution provides some variation in our assumptions with a mean duration of immunity of about 9 months. In the event we consider the models using 6 and 24 months immunity to give the spread of uncertainty in the annual risk of illness (0.99 to 10.78 illness/1000py).

Applying this uncertainty ranges to the point estimated annual risks derived previously would give estimated risk of illness/1000py of 0.21 (0.03 – 0.29) for oysters, 1.49 (0.19 – 2.03) for lettuce, 0.22 (0.03 – 0.30) for raspberries and 5.68 (0.71 – 7.73) for catered meals (3.34 (0.42 – 4.55) for meals eaten out and 2.34 (0.29 – 3.18) for take away meals). After accounting for intrinsic resistance and acquired immunity, risks of illness/1000meals would be 1.64 (0.20 – 2.23) for oysters, 0.02 (0.003 – 0.03) for lettuce, 0.03 (0.003 – 0.04) for raspberries, 0.19 (0.02– 0.26) for meals eaten out and 0.20 (0.03 – 0.27) for takeaways.

8.5.5 Synthesis

The analyses presented above suggests an incidence of foodborne norovirus infections is about 23.76 infections /1000 py. After accounting for adjusting for the probability of acquired immunity from prior infection the incidence of symptomatic would be 7.65 illnesses /1000py with a range of 0.99 to 10.78. Given the IID2 estimate of total norovirus disease burden of 47 episodes/1000py, this would suggest that the proportion of norovirus illness due to foodborne disease is about 16% with a range in the range 2.1 to 22.9. However as discussed in the IID2 report the estimate of total disease burden from that study may be biased because of incomplete submission of stool samples and reporting fatigue, meaning that the true incidence might be higher than the estimated incidence. If this is the case, then the proportion of total disease burden that is foodborne would be lower than suggested above. Nevertheless, with current population levels in the UK (66.04 million) this would equate to about 500,000 foodborne noroviruses annually (range 65,000 to 700,000).

Given the importance of combining exposure from all foods to develop a single estimate of exposure it is not possible to use this model to derive exact estimates of disease burden from individual food groups. However, it is safe to assume that food specific disease burdens would be in roughly the same proportion as infection rates in the model that does not account for immunity due to prior infection as given in Table 39. So among the estimated 7.65 episodes of foodborne illnesses /1000py (or 500,000 infections annually) there would be about 14,000 cases associated with oysters annually (0.21 illnesses/1000py), 98,000 for lettuce (1.49/1000py), 16,000 for raspberries (0.22/1000py), 220,000 from eating out (3.34/1000py) and 150,000 from takeaways (2.34/1000py). So, the major contribution to disease burden was catered food (eating out and takeaways combined), though lettuce is a very close second.

These should not be taken to imply that if foodborne spread was reduced or even eliminated then total disease would fall or even that incidence of norovirus would decline at all. The relationship between exposure and disease risk may not always be monotonic in that disease risk does not always rise in line with exposure and may even decrease. This can be seen in part in Table 42 where the scenario of high exposure years where the total incidence and foodborne exposure doubles is actually associated with a lower risk of foodborne illness. Such perverse impacts of changing exposure on total disease has been shown in other circumstances (Swift and Hunter2004; Frost et al. 2005) and would explain the apparent protective effect of consuming lettuce on asymptomatic carriage of norovirus found in IID1 (Phillips et al. 2011). This is clearly an issue that needs further investigation.

8.5.6 Validation

In order to validate the model presented here we sought to compare our results with the empirical findings of epidemiological studies. There have actually been very few such studies reported in the literature. One such was a study of self-reported illness in consumers eating oysters (Lowther et al. 2010). The authors reported that an overall attack rate of 0.21% or 2.1 episodes per 1000 meals consumed, though if one batch with a particularly high attack rate was excluded the attack rate was only 0.08% or 0.8 episodes per 1000 meals. In determining these overall attack rates the authors simply summed all affected people and all estimates of meals eaten across all batches of oysters. In order to better account for batch to batch variation in attack rates and the different batch sizes, we used a random effects pooled analysis of the data in the Lowther paper and estimated that the overall attack rate was 1.0 episodes per 1000 meals (95%CI 0.35 – 2.0). Our estimate of risk per meal was in intrinsically susceptible individuals with no adjustment for prior immunity was about 6.8 episodes per 1000 meals. Adjusting for intrinsic resistance and acquired immunity would give an estimate of about 1.64 illnesses per 1000 meals, very close to the estimated overall risk from the paper by Lowther et al. (2010) especially when considering that this was based on self-reported illnesses and so would have a degree of under-ascertainment.

For a more general epidemiological estimate of foodborne norovirus infections from the literature we identified three case control studies that have estimated risk factors in the general population (de Wit et al. 2003; Fretz et al. 2005; Phillips et al. 2011). Unfortunately, none of these studies presented the risk factors in a way that would allow estimation of pooled risks. However, two of the studies found prior contact with a probable case in over half of illnesses (de Wit et al. 2003; Phillips et al. 2011). The other study from Switzerland found about 39% of cases associated with another probable case prior to illness. Given that it is not always clear if you have been in contact with an infected individual, especially if they are asymptomatic or are trying to hide the fact that are or have recently been sick, such case control studies are likely to under-estimate the proportion of illness attributed to direct person to person transmission. Our findings in this study are compatible with prior epidemiological studies on risk factors for norovirus infections.

Finally, in the review of US outbreaks by Hall et al (2012), 53% of outbreaks were due to infected food handlers, and so likely to be in catered foods, and 33% of outbreaks were with leafy green vegetables. These findings are very close to what would be predicted by our results.

8.6 Discussion

We have presented an estimate of foodborne norovirus infection in the UK using a Quantitative Microbial Risk Assessment (QMRA) approach. Accounting for intrinsic resistance and acquired immunity, our preferred estimate is that foodborne norovirus accounts for 23.76 infections/1000py and 7.65 episodes of illness /1000py or about 500,000 of illness cases a year in the UK. However, largely depending on estimates of the duration of immunity to homologous strains of norovirus post-infection this estimate could be in the range 0.99 and 10.78 illnesses/1000py. The estimated risks from oysters (0.21 infections/1000py and 14,000 cases annually), lettuce (1.49 and 98,000), raspberries (0.22 and 16,000), eating out (3.34 and 220,000) and takeaways (2.34 and 150,000). These figures should not be taken as an indication of the reduction in total norovirus illness that could be achieved if it was possible to totally eradicate norovirus from the food chain. Using the assumptions in the models we have run, eradicating norovirus from food may have little if any impact on the total disease burden.

In developing this estimate of foodborne norovirus illness, we have had to make a number of modifications of the standard QMRA approach, in part due to developments in our understanding of the difficulties in extrapolating from daily risks to annual disease burden and in part due to recent advances in our understanding of the epidemiology of norovirus. Of key relevance has been the increased understanding of the difficulties in determining annual risk from daily risk. We have shown that the two currently used approaches are based on flawed assumptions in that daily risk is either assumed to be constant or entirely random irrespective how similar a person’s individual exposure is from one day to another. In using a microsimulation approach, we have been able to deal with this issue and develop a less biased estimate of annual risk. The other big advantage of the microsimulation approach is that it is easier to include exposures from multiple sources into a single risk calculation, something that was essential in this study. The final advantage of the microsimulation model was that it allowed us to include the SIR epidemic model within the microsimulation model. The primary disadvantage in the extra demand on computing power.

There remain, however, a number of areas where the current state of knowledge remains uncertain and where further primary research is needed in order to improve the model. Given the impact that the assumptions of the duration of immunity have on the final estimate of risk this is certainly one of the most important areas to understand. Nevertheless, as Havelaar et al. (2009) pointed out not to account for immunity runs the risk of overestimating the impact on public health. In making their arguments in support of their conclusions Havelaar et al. (2009) referred to the findings that although seroprevalence is very high in developing countries, clinical Campylobacter infections are rare over two years of age. For norovirus we recently showed that in Africa carriage rates of norovirus in asymptomatic people frequently

exceed the rate in symptomatic children (Kabue et al. 2016). Such findings are consistent with asymptomatic infections in partially immune individuals. As discussed above previous studies have generally assumed just six months (Lane 2014), though there is actually some significant uncertainly around this figure (Simmons et al. 2013). In our final model we used a PERT distribution that still gave six months as the most likely duration of immunity, but allowed durations of immunity up to 24 months. We consider this to be a reasonable assumption and would be compatible with a relatively short duration of immunity on first exposure but, on repeated exposure, this tends to be longer.

It needs to be asked at this point why do we need to adjust for prior immunity when the dose response model used in this study was derived from clinical illness in human volunteers (Atmar *et al*. 2013)? All human dosing studies have been undertaken using a single strain of norovirus GI (van Abel *et al*. 2016). Although this strain was once common it is much less so now and indeed antibody levels to all GI strains have declined dramatically since the 1960s (van Beek *et al*. 2016). As such it is likely that population immunity to this strain will be much less than that to currently circulating strains. In this context it may also be unwise to rely on dose response models derived from outbreaks. The number of foodborne norovirus identified varies from one year to another being higher in epidemic years after the appearance of a new strain type. Any dose-response model derived from outbreaks is more likely to be associated with a currently highly active strain to which population immunity is still low and so over-estimate infectivity for the majority of strains.

The other primary area for debate is the frequency of infections that we have used predicted by the presence of asymptomatic carriage found in IID1 (Amar *et al*. 2007). The ratio of asymptomatic to symptomatic infections appears to be very high. Nevertheless, such a high rate of exposure is not that dissimilar for what has been suggested for other enteric pathogens. Monge *et al*. (2018) have recently shown that the incidence of Campylobacter infection vastly exceeds that of clinically reported cases with about 1.61 infections/person-year. Given that norovirus is rather more infectious than Campylobacter, an annual infection rate of over 3 infections/person-year would not be too surprising. A further point in support of this argument comes from the observation that in the case control study undertaken as part of IID1, consumption of lettuce was found to be negatively associated with asymptomatic carriage (Phillips et al. 2011). Indeed, we argued this point as early as 2004 (Swift and Hunter, 2004), when we stated that “It is shown that risk of illness declines with increasing age and that this risk declines most rapidly in those groups at increased exposure. In high exposure groups, the relative risk of illness, compared to a group with lower exposure, also declines with age, eventually becoming less than one. The threshold age at which the relative risk is less than 1, i.e., factor B becomes protective, decreases with higher exposure rates”.

A further complexity with norovirus is the high genetic diversity amongst human strains with often limited or no cross-immunity between types (Parra et al. 2017). We were able to account for this diversity in the model using the Hunter-Gaston diversity index (Hunter and Gaston, 1988) and consider this to be the first such use of this equation to modelling immunity in the context of QMRA where there are multiple strains in circulation that do not have complete cross immunity.

Based on our analyses there several areas where further research is needed to improve the estimates of foodborne disease burden from norovirus. It should come as no surprise given the discussion above that we consider the two most important areas for further research to be into immunity to norovirus and the actual level of the background level infection in the community. Recent research has highlighted the importance of the competition between host acquired immunity and the evolving strains of norovirus (Parra et al. 2017). However, we are still not clear about the actual duration of acquired immunity and the degree of cross immunity between different strains of the virus. In our models we have relied on a binary understanding of immunity, people are either immune or not. It may be the case that immunity to norovirus is more analogue in that after an infection immunity declines gradually with the possibility that such immunity can be overcome with lower and lower doses over time. Should this prove to be the case then the implication for risk estimates could be substantial. The related issue of the background incidence of infection needs a study like that by Monge et al. (2018) done for Campylobacter but for norovirus.

Given that norovirus is not yet easily culturable all dose response curves are based on gene copy numbers that may or may not represent infectious viral particles. In NoVAS we did work on capsid integrity assay in order estimate the proportion of gene copies that represent viable virus. However, studies using murine norovirus or human norovirus culture in human intestinal enteroids over the last few years it has been clear that the capsid integrity assay substantially overestimates norovirus infectivity (Rönnqvist et al. 2014; Farkas et al. 2018). In determining infectivity for this study we used data on viability of a F+RNA bacteriophage GA provided by CEFAS based on work in oysters. In the absence of similar data in produce we then applied this to the other food groups as described above. There is a need to repeat the phage work done on oysters in other food settings.

An issue that has not be possible to address in this model is the impact of seasonality on norovirus disease burden. Norovirus should a highly seasonal variation in illness rates. However, we were not able to include seasonality as we did not have adequate estimates of seasonality in food consumption. Nevertheless, we did show that if, as would be expected, higher exposure coupled with higher background levels of non-foodborne infection would be associated with little change in actual foodborne disease burden.

The other key area for research is in risks within the catering industry. As discussed above the few papers that we identified of relevance to the catering industry were primarily concerned with sandwich production. There is a need for more observational studies in the catering industry around food handling practices, especially how often foods are directly handled and whether that handling is on food that will be subsequently cooked. There is also a need for more research to better understand how much virus is transferred from hand to food with each touch.

There may also be other foods that add to total disease burden from norovirus that are not included in the models. The QMRA conducted in this section was only able to use data generated in the NoVAS project. Although the target foods were chosen based on the proven importance in norovirus and we are not aware of other food products that are likely to contribute substantial additional disease burden. Nevertheless, future research should include analysis of any additional food products identified as being important in norovirus transmission.

8.7 Conclusions

In conclusion we have estimated the total disease burden due to foodborne norovirus in the UK to be about 500,000 cases a year. However, this does not mean that these cases could be prevented if norovirus was eradicated from the food chain. In order to arrive at this estimate we have had to implement a number of novel approaches to the standard QMRA, most importantly were the use of the microsimulation approach in order to deal with the problem of extrapolating annual risk from daily risk and also combining multiple exposure pathways with high probability of multiple infections in the same year into a single risk assessment. The microsimulation model also made it much easier to link with a SIR epidemic model to adjust estimates for immunity from prior exposure. One of the most important sources of uncertainty in the assessment was that different estimates of the duration of immunity had major impacts on the estimated amount of foodborne disease. There is a need for more research to better understand immunity to norovirus and also to improve our knowledge of food handling practices in the catering industry.

9 Individual-based model to investigate the dynamics of norovirus infections in communities

9.1 Introduction

This document outlines the rationale, construction and testing of an individual-based model to investigate the dynamics of norovirus (NoV) infections in communities.

Norovirus is a highly contagious virus with a very low infectious dose and multiple pathways of transmission. It is spread by droplet transmission by the faecal/oral or vomitus/oral routes. The dose required for infection can be as low as 10 particles and the virus is capable of remaining viable in the environment for 50+ days. This means that environmental contamination and fomites generally can act as a source of infection. Millions of virus particles are shed during the acute illness. These virus particles can enter the food chain through contamination or, in the case of filter feeding by shellfish, virus may be concentrated in tissues which are then consumed by humans.

The complexity in transmission means that analysing the relative impacts of different pathways to infection is difficult. Disease transmission is effectively a mixture of environmental exposure and contagious spread. Transmission may depend on the social context in which susceptible and infected individuals come into contact with NoV. Human social contacts over the course of a day can be considered as a series of social networks. These networks may change through time.

We developed an individual-based epidemiological model to investigate the spread of norovirus disease through a community of individuals, which was characterised in terms of membership of social networks (Rushton *et al*, 2019 (in press)). Here we describe this model and outline how it was extended to investigate the relative contribution that consumption of contaminated foods makes to the public health burden at the community scale.

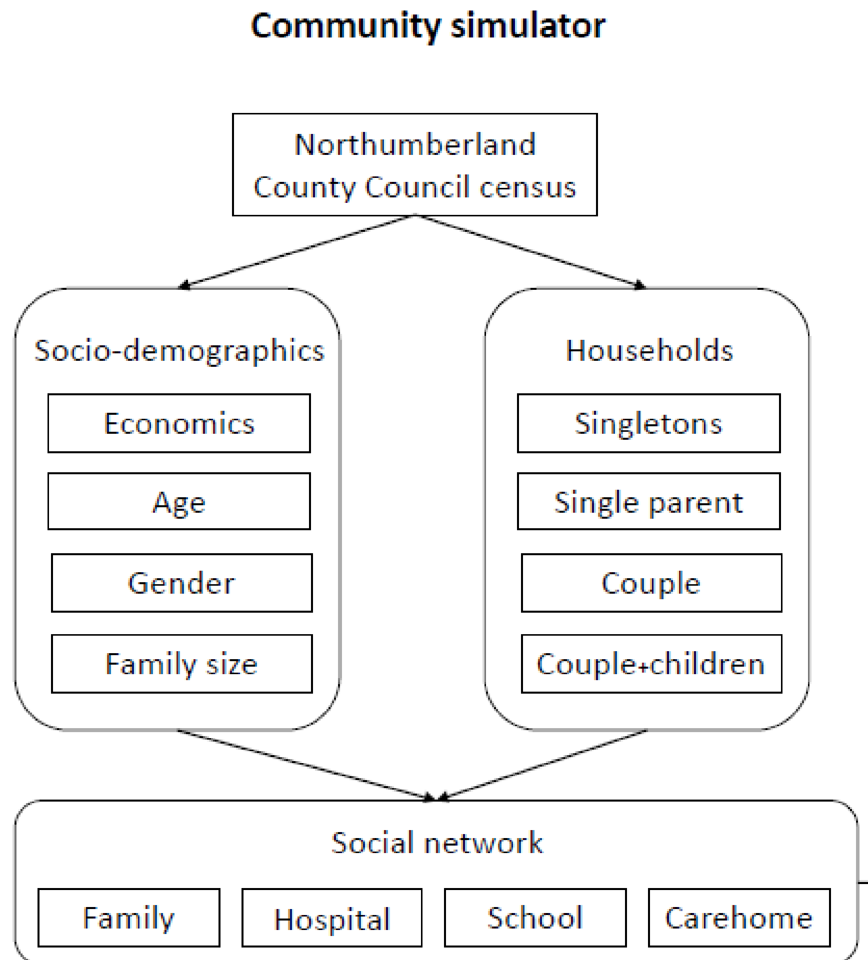
9.2 Methods

9.2.1 The individual-based model

The individual-based model comprised two components and was written in the R programming language (R Core Team, 2016) using custom scripts. The first component was a community simulator (Figure 40), which created a data frame (matrix) of individuals whose age, sex, home and family status (individual, single parent, couple, with or without children) were determined by drawing samples from the appropriate distributions derived from the demographic details of the population in a ward in NE England. The data were collated from tables provided at the ward level for Northumberland by Northumberland County Council. These data describe

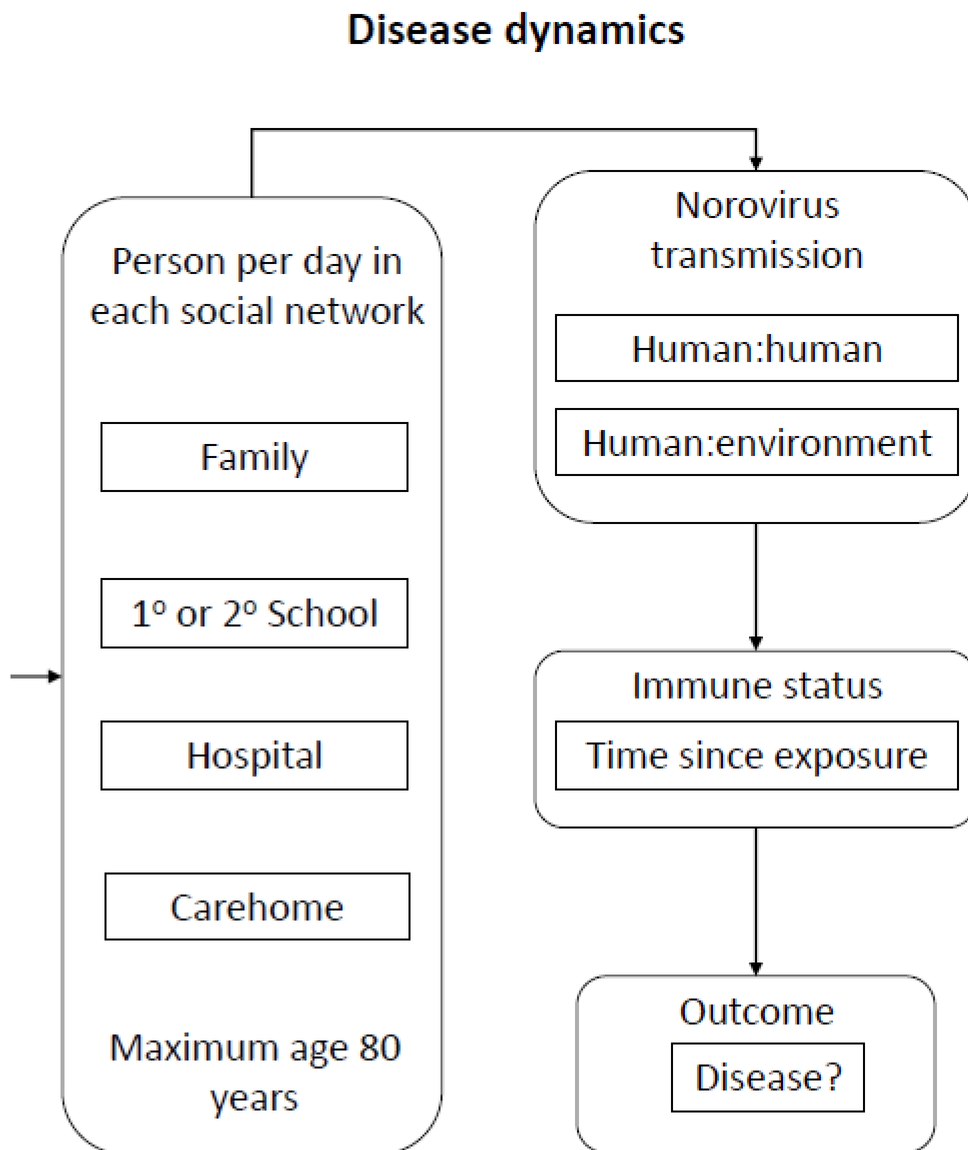
the number of households, the frequency distribution of size of household, number of children, single parent families as well as age. We used these data to generate households for a modelled cohort. In effect we had a digital representation of the population in this community. We also noted the number of primary and secondary schools and hospitals in the community and allocated individuals to each on the basis of age within the chosen community. This model can be parameterised to run in any ward in the UK for which there are equivalent socio-demographic data.

Figure 39: Schematic for the community simulator



The second component of the model was concerned with modelling the dynamics of infection at the individual level within the cohort created in component 1 (Figure 41). Each individual in the cohort was a member of a number of social networks within which disease could spread. These were family, primary and secondary schools, hospitals and care homes. The model was run on a daily time step with individuals undergoing exposure events involving food, fomite contact and contagious contacts with sick individuals in the relevant social networks to which they belonged. Schools were not attended at weekends, children moved from primary school to secondary at age 11; individuals entered (and left) hospitals based on admission statistics and had the option to entered care homes when above the age of 65.

Figure 40: Schematic showing the networks and modelling process for disease transmission



Exposure to NoV took place in three ways; through exposure to a sick individual in a network on a given day; through exposure to an environment in which another individual had been ill previously (environmental contamination) and through consumption of certain foods. Spread of infection within the networks was simulated using a susceptible-infected-recovered (SIR) approach ~ with appropriate transmission coefficients (Beta in the SIR framework) which varied according to setting. The SIR model was originally developed by Kermack and McKendrick (1927, 1932, 1933). It is a compartmental model, which assumes that individuals in a population belong to one of three compartments i.e. susceptible, infected or recovered. The original SIR model is effectively a series of differential equations, which describe the rate of transition of individuals between the three compartments. If the whole population starts off in the susceptible compartment then transition to the infected compartment is determined by the number of infected people and their

ability to transmit to others in the susceptible population (who then become infected). The cases per infectious individual is known as the transmission coefficient. Infected cases then leave their compartment at a rate determined by the recovery rate. The series of equations is usually solved by numerical integration.

The model assumes that all individuals are homogeneous and there is complete population mixing. This is a naïve assumption and does not allow for different transmission pathways or different exposure scenarios that may arise in real communities. Here we used the SIR approach but modelled at the individual level. Each infection and recovery event is determined stochastically at the level of the individual, given the levels of infected individuals and susceptible individuals in the different networks or settings where we anticipate that transmission pathways and disease dynamics will be different. We are also able to vary dose and other parameters associated with transmission and recovery that cannot be included in a typical compartment model setting.

Once exposed the dose of particles an individual received was estimated stochastically and this was used to assess the probability that an individual became ill. The probability of illness was also dependent on the level of immunity of the individual. Prior exposure to NoV led to immunity, which declined exponentially since the last exposure. We assumed that the level of immunity was directly proportional to the probability of illness. Thus the probability of immunity given challenge declined exponentially with time. We then used the dose response model to predict the probability of illness under the assumption that the individual was naïve immunologically. The occurrence of illness following exposure was then determined on the basis of the balance of probabilities of being immune versus that of becoming ill given the dose. If the probability of immunity was greater than the probability of illness then the individual did not become ill. This meant that the illness response to dose was dependent on immunity. Any exposure led to a return to maximum immunity even if illness did not occur. Infection arising from environmental contamination and consumption of oysters were modelled in a similar fashion. This was based on contact with a previously contaminated environment, or consumption of oysters, which was seasonal. Levels of consumption of oysters and their contamination were derived from the literature.

The model was run for a number of years from a starting population that aged progressively and into which new babies were born. On each day the number of cases of illness were recorded for each mode of transmission ~ specifically, contagious contact in family, primary or secondary school, care home and hospital as well as contact with fomites in any of the settings, and the consumption of oysters. We used sensitivity analysis to analysis the relative contribution of different pathways to the burden of disease. In this we varied the transmission coefficients in each setting, environmental contamination risk, environmental dose and the immune period. We used Latin Hypercube Sampling to generate suites of input parameters

across the range of input variable. We then ran the model and recorded cases of illness per day for each transmission pathway. We used generalised linear modelling to investigate how variation in the disease parameters impacted on the burden of illness in the community and calculated standardised estimates of parameters to provide a means of comparing the contribution of each variable to the model outputs for each transmission pathway. In addition we used Partial Least Squares Regression (PLSR) analysis and Variable Influence on Projection (VIP) analysis to quantify the relative contribution of each variable to the baseline infection represented by the intercept.

9.2.2 Extending the individual-based model to include data on oysters, berry fruits and lettuce collected in the NoVAS project

The approach to modelling exposure using data from the NoVAS project was based on analysing risk through breaking down the exposure into three main components for which data had been collected. The components were:

i) the frequency with which meals of particular foods (berry fruits (fresh or frozen), lettuce or oysters) were eaten;
ii) the size of the portion consumed in a meal and,
iii) the levels of contamination in terms of NoV particles per unit weight of foodstuff consumed.

The data shown in Table 35 (Chapter 8) were represented as first and second moments of the distributions identified for each. Typically these were defined as the functional form (e.g. log-normal distribution); first and second moments (mean and standard deviation). Each individual in the population was allocated a number of meals of each food per year based on the population level estimates of food consumption. On any one day the likelihood that the food was consumed was determined stochastically from the number of days in the year, and the number of meals eaten per year by that individual. Having determined whether any food as eaten on a day, the size of the meal and the number of norovirus particles consumed were then determined by sampling from the distributions for meal size and particles per unit weight of food. In the case of oyster consumption the dose of particles varied seasonally and this variation was used to allow for seasonality in the contamination level.

We extended the model by providing every individual with the predicted number of meals of each of the food types per year. These were randomly drawn Poisson deviates sampled from the first and second moments of the distributions of for each food stuff listed in Table 35. Each individual on each day then had a risk of consuming a meal that was effectively the annual number of meals divided by 365.

Variation in the risk posed by eating berries, lettuce and oysters to individuals were included in the model as draws from the appropriate distributions shown in Table 35. In the case of oysters NoV contamination varied seasonally and so particle dose was also adjusted for seasonality. We used harmonic regression to quantify the change in particle contamination with season and used this to predict dose per unit weight of oyster on each day.

To investigate the sensitivity of the model to variations in the consumption of the foodstuffs themselves we used multipliers in the range 0 to 2 to vary the meal size of the foods consumed at any one sitting. We also included a function to adjust for the fact that all particles produced during an infection were not viable. We varied five transmission coefficients in the model. These were transmission in each of the settings: primary school, secondary school, care home hospital, family and environment (fomites). The rationale for having a coefficient for each setting was that the physical and social structures of all of these environments differ. The most obvious of these is the family setting where the force of infection is shown to be high (Miura *et al.* 2016) because of close contact between family members in enclosed environments. School contacts are also highly dynamic (Fournet and Barret, 2014). Parameter ranges used in the Sensitivity Analysis are detailed in Table 43.

Firstly we ran the exposure components of this new model to assess the likely level of exposure to norovirus particles in each of the foodstuffs as expected from the frequency distributions of occurrence of meals, their size and their levels of contamination as analysed and reported in section 8. This effectively allowed us to quantify exposure frequency and particle exposure level. The model was run on a daily time step for a cohort of 10,000 individuals to allow for the temporal pattern of contamination in oysters. We then ran the complete model with the social networks represented by family, school, care home, hospital and environment. This effectively represented the community burden of disease given the different exposure routes and different contaminated food sources.

Table 43: Parameter ranges used in LHS sensitivity analysis

Parameter	Primary-school[a] (probability)	Secondary-school[a] (probability	Hospital[a] (probability)	Family[a] (probability)	Care-home[a] (probability)	Infectious duration[b] (days)	Environmental contamination risk[a]	Environmental viral particle dose[a]	Maximum immune time[c] (days)	Berries	Lettuce	Oysters
Minimum-maximum	0.0001 - 0.005	0.0001 - 0.005	0.0001 - 0.005	0.0001 - 0.25	0.0001 - 0.005	2 -14	0.0001 - 0.0050	10 - 1800	10 - 720	0 - 2	0 - 2	0 - 2

a) Reliable data not available; plausible ranges used.
b) Atmar et al [1]
c) Parrino et al [2]

1 Atmar, R. L. *et al.* Norwalk virus shedding after experimental human infection. *Emerg Infect Dis* **14**, 1553-1557, doi:10.3201/eid1410.080117 (2008).

2 Parrino, T. A., Schreiber, D. S., Trier, J. S., Kapikian, A. Z. & Blacklow, N. R. Clinical Immunity in Acute Gastroenteritis Caused by Norwalk Agent. *New England Journal of Medicine* **297**, 86-89, doi:10.1056/nejm197707142970204 (1977).

We ran this community level model under two scenarios. The first assumed that the population was naïve to previous infection as would occur following the introduction of a new strain into a naïve community. This is equivalent to epidemic spread of a new strain into a population. For the second scenario we investigated the dynamics of cases of illness when the disease was effectively endemic and the population was no longer naïve to the strain. We assumed the strain to be endemic when the cases of illness became stable after two years exposure to it. Since the spread of disease was highly dynamic in the naïve population where spread was epidemic, we modelled the relationship between log-transformed cases of illness per day against time as well as the disease parameters used in each run for the naïve population. For the non-naïve population with endemic disease ~ the number of cases of illnesses per day was more stable and so we did not include time. We used the significance of the parameters to assess importance of variables in the model. We then went on to assess the relative importance of the different parameters in contributing to the burden of disease predicted by the model using PLSR analysis and VIP analysis to demonstrate the relative importance of each variable in determining outcomes from the model following the methodology of Wold *et al.* (2001).

9.2.3 Interpreting VIP scores

Determining the relative importance of different predictor variables in PLSR analyses can be challenging when collinearities exist between the variables. One solution is to calculate VIP scores; for each explanatory variable these are calculated across all the PLSR loadings in the model, as a weighted average. This means that the average of all the VIPs for all the predictor variables in the model is 1.0, and thus predictors with a VIP score higher than 1.0 are more important. Whilst they should not be interpreted as simple proportions (i.e. a VIP of 1.10 does not necessarily indicate that a variable is 10% more important than one with a VIP of 1.00), the higher the VIP score the more important the predictor variable.

9.3 Results

9.3.1 Exposure risk and exposure levels to norovirus

The risk of exposure to NoV through consumption of lettuce was 0.00438 per 1000 person years. The equivalent figures for berry consumption and oyster were 0.000247 and 0 respectively. The mean number of norovirus particles in an exposure event were 11.32 (Standard Deviation (SD) 4.77) for lettuce and 0.372 (SD 2.859) for berries. It was not possible to identify the risk through take away meals as we did not have appropriate levels of particle contamination on the food eaten. Assuming a completely immunologically naïve individual, then this level of contamination would lead to a probability that an individual would be ill of 0.69 and 0.00 respectively. In effect berries pose a limited risk to naïve individuals because they are eaten infrequently whilst lettuce, which is consumed much more frequently, does.

9.3.2 Sensitivity analysis of exposure and transmission parameters on community burden of disease in norovirus naïve and norovirus non-naïve communities

Numbers of infections in the naïve population rose rapidly to a high level and then declined as immunity built up with repeated exposure to the pathogen. The results of the generalised linear model relating log transformed number of cases of illness per day to the model input parameters in the naïve population are shown in Table 44.

Table 44: Regression diagnostics for analyses relating the log transformed cases of illness per day against the disease parameters varied in the sensitivity analyses.
The model was run 30 times for a period of two years with inputs derived from a Latin Hypercube Sampling of the parameter space bounded by the limits in Table 43.
B represents the transmission coefficient in the respective setting (rows2-6); Illness duration in days; environmental contamination risk (probability 0-1); environmental particle dose (numbers of virus particles); maximum immune time in days. Food consumptions multiplier in range 0 to +2

Naive (years 1 to 2)

	Estimate	Standardised Estimate	Std. Error	t value	p-value
Days	-0.0037	-0.6607	<0.001	-183.864	<0.001
Primary school B	-31.3593	-0.0375	4.5693	-6.8631	<0.001
Secondary school B	-38.2049	-0.0456	5.2897	-7.2225	<0.001
Hospital B	-17.6025	-0.0211	3.7087	-4.7463	<0.001
Family B	-0.4460	-0.0272	0.0732	-6.0916	<0.001
Care home	-72.5444	-0.0869	3.7759	-19.2125	<0.001
Illness_duration	-0.0118	-0.0347	0.0018	-6.6472	<0.001
env_contamination_risk	-21.7287	-0.0258	3.6822	-5.901	<0.001
env_particle_dose	0.0001	0.0486	<0.001	8.1296	<0.001
max_immune_time	-0.0027	-0.4647	<0.001	-107.328	<0.001
Berry consumption	-0.0298	-0.0146	0.0093	-3.1868	0.0014
Lettuce consumption_2	-0.1961	-0.0965	0.0090	-21.6696	<0.001
Oyster consumption	-0.0226	-0.0110	0.0121	-1.8728	0.0611

All variables were significant predictors of the log-transformed number of cases of illness per day. The VIP analyses give a better estimate of effect size for each of the variables (Figures 42 and 43). VIP values less than 1 indicate that the variable is having no significant effect on the outcome of interest. Here it is evident that only two of the variables have a VIP value greater than 1 and these are the duration of immunity and the time since the introduction of the new strain. The high significance of the duration of time since introduction of the new strain reflects the rapid decline in cases with time as immunity builds up. In the case of the non-naïve population again

all disease variables that were varied in the sensitivity analysis were significant predictors of the log transformed cases of illness (Table 45).

However, consideration of the VIP analyses (Figures 42 and 43) and the standardised estimates in Tables 44 and 45 demonstrate that only two variables were significant predictors of illness and these were the environmental particle dose and the duration of immunity. The difference between the two statistical approaches to the results is not surprising given the sample size used in each regression. The significant regression coefficient says nothing about the effect size that the variables have on the outcomes and, as such, are of less utility in analysing the sensitivity of the model.

Figure 41: Results of a PLS regression analysis of the contribution of each of the disease parameter variables to the log transformed number of cases per day in the naïve population.
Figures show the regression parameters in (top) and the Variance in Projection (VIP) for each variable bottom. Note that any VIP value of 1 or less indicates no real effect on the outcome of interest.

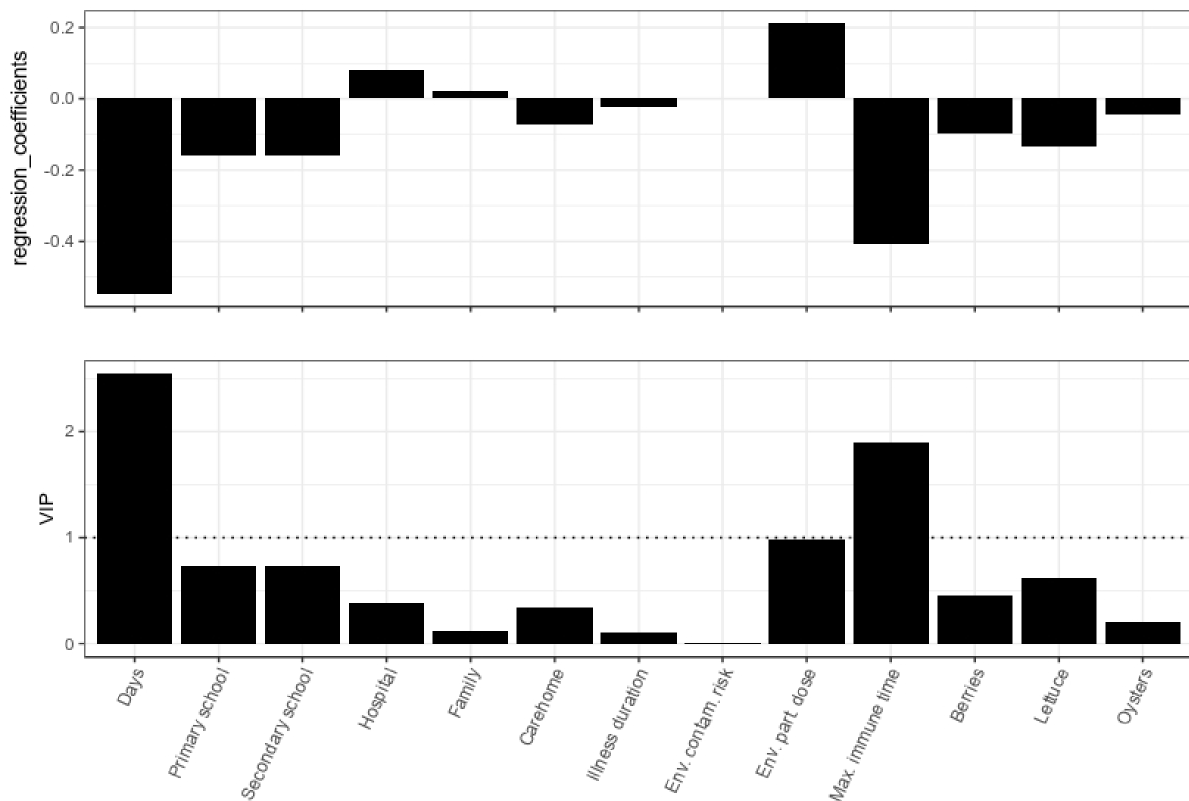


Figure 42: Results of a PLS regression analysis of the contribution of each of the disease parameter variables to the log transformed number of cases per day in the non-naïve population.
Figures show the regression parameters in (top) and the Variance in Projection (VIP) for each variable bottom. Note that any VIP value of 1 or less indicates no real effect on the outcome of interest

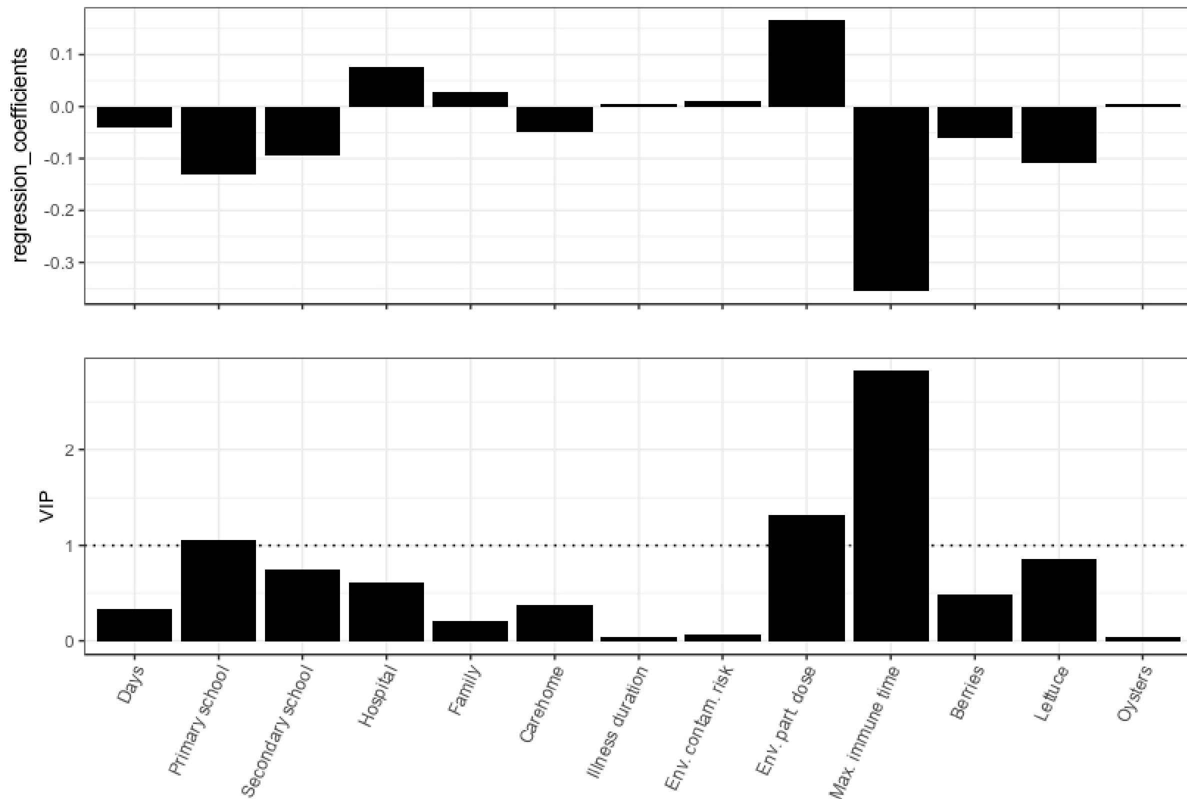


Table 45: Regression diagnostics for analyses relating the log transformed cases of illness per day against the disease parameters varied in the sensitivity analyses.
The model was run 30 times for a period of one year after prior exposure for 2 years with inputs derived from a Latin Hypercube Sampling of the parameter space bounded by the limits in Table 1.
B represents the transmission coefficient in the respective setting (rows2-6); Illness duration in days; environmental contamination risk (probability 0-1); environmental particle dose (numbers of virus particles); maximum immune time in days. Food consumptions multiplier in range 0 to +2.

Non-naive (year 3)

	Estimate	Standardised Estimate	Std. Error	t value	p-value
Days	-0.0007	-0.0926	<0.001	-17.6883	<0.001
Primary school B	-72.1099	-0.1269	4.5262	-15.9316	<0.001
Secondary school B	-5.6211	-0.0099	5.2398	-1.0728	0.2834
Hospital B	-28.5079	-0.0503	3.6737	-7.7599	<0.001

	Estimate	Standardised Estimate	Std. Error	t value	p-value
Family B	-0.0405	-0.0036	0.0725	-0.5581	0.5768
Care home B	-82.9659	-0.1462	3.7403	-22.1816	<0.001
Illness _duration	-0.0076	-0.0328	0.0018	-4.3077	<0.001
env_contamination_risk	-7.6011	-0.0133	3.6475	-2.0839	0.0372
env_particle_dose	<0.001	-0.0173	<0.001	-1.9797	0.0478
max_immune_time	-0.0031	-0.7895	<0.001	-125.1210	<0.001
Berry consumption	0.1356	0.0981	0.0092	14.6613	<0.001
Lettuce consumption	-0.2513	-0.1818	0.0090	-28.0308	<0.001
Oyster consumption	0.1490	0.1067	0.0120	12.4433	<0.001

Table 46: Risk of becoming ill with norovirus per individual per 1,000 person-years in a non-naïve population for the different exposure pathways in the community model

	Mean	SD	CI lower	CI upper
Primary School	0.046957805	0.08657337	0.0159779231	0.07793769
Secondary School	0.040249547	0.11085961	0.0005789458	0.07992015
Hospital	0.006708258	0.03674264	0.0064399276	0.01985644
Family	11.088750252	15.67839275	5.4783094617	16.69919104
Care Home	0.020124774	0.06140644	0.0018492370	0.04209878
Oysters	0.035078443	0.10860757	0.0037862753	0.07394316
Environment	0.040249547	0.12281287	0.0036984741	0.08419757
Berries	10.884712314	22.87038526	2.7006500556	19.06877457
Lettuce	38.319486427	63.10738679	15.7367980921	60.90217476

We used the outputs of the 30 sensitivity analysis runs for the non-naïve population to estimate the attributable burden of disease to each mode of transmission assuming that the range of input parameters used in the model were representative of the range of those that might pertain. We used this to estimate the mean risk of becoming ill from norovirus infection per 1,000 person-years from each of the different exposure and transmission pathways for the NE community that we modelled (Table 46). Risk of illness per 1,000 person-years ranged from around 38 for lettuce, 11 each for berries and family (household) transmission and 0.03 for oysters. It is possible to predict the number of illnesses after setting the various food parameters to zero using an accompanying R Shiny App, which can be found at https://mep-ncl.shinyapps.io/fsa-web.

9.4 Discussion

From the FSA's perspective, the most important results are the extent to which exposure to the three foodstuff leads to a burden of disease. Exposure to norovirus in lettuce, berries and oyster was low with risk below 0.004 in 1,000 person years. In the case of oysters the risk was effectively zero. Particle exposure was negligible for berries, but the risk of infection following consumption of virus particles found on lettuce was high at 0.69. We conclude that consumption of lettuce poses a small risk of disease for individuals. Whilst the particle dose was low the predicted risk of illness from this dose was high. These conclusions are based on the assumption that the individuals exposed in the modelling were naïve and had no immunological experience from exposure to NoV. We went on to investigate spread of a novel strain at the community level incorporating the contagious nature to disease spread as driven by contacts and fomites in the different social networks within the community. We then analysed the subsequent dynamics of disease after the community had been exposed for two years. The net effect of this is to assess the contribution of the different parameters whilst immunity develops in the population and the subsequent dynamics when community level disease effectively becomes stable. The most obvious feature of the results of the community model for a completely naïve population was that cases of illness rose rapidly as the disease spread through the population from an initial source and then declined as individuals became immune to the strain. The dominant process determining the number of cases in the non-naïve population was similar, but time was not a predictor, indicating that following 2 years of exposure to the virus cases of illness stabilised. The relatively stability of cases of illness in the non-naïve population probably reflects an interaction between frequency of exposure through the various mechanisms involving food (of which lettuce posed the highest risk) and a gradual decline in immunity post-exposure. When considered at the community level risks were higher for individual parameters with lettuce having the highest risk. This reflects the fact that we introduced variation in the levels of consumption across all parameters all of the input parameters. It is worth noting that cases of illness directly attributable to consumption of lettuce is not the whole story in the community model as contagion and fomites following an illness event caused by eating contaminated lettuce, will have led to cases being attributed to the direct cause, rather than the indirect effects not attributable directly to the lettuce consumption itself.

The results of the community model and the QMRA model are similar insofar as they indicate that changes in immunity were the major determinant of disease. Understanding the human-NoV immune interaction is critical for assessing the utility of the model and also the spread of disease. Understanding of the mechanisms of immunity to NoV infections in human populations is mixed. On the one-hand, mechanisms by which virus particles bind to host cells are well documented. NoV particles bind to polysaccharides present on the surface of host cells that are related to the histo-blood group antigens (HGBA). Susceptibility to disease is thus partially

associated with blood group, with individuals lacking key polysaccharides showing reduced susceptibility to disease (reviewed in Tan and Jiang 2010). The gene complement of the virus is also well characterised in terms of the open-reading frames (ORFs) that are responsible for the production of enzymes and non-structural proteins as well as the viral capsid and the capsid surface protein that actually binds to the host cells (der Graaf *et al*, 2016; Maher *et al*, 2013). The positions of key amino-acids responsible for the binding to host cells are also documented. Furthermore, the virus is subject to hyper-mutation, where copy failure and amino acid substitutions change the properties of the surface binding proteins (Maher *et al*, 2013). In addition the virus can also undergo recombination where different ORFs recombine in the host (Maher *et al* 2013; de Graaf *et al* 2016), implying that infection of individual host cells is not necessarily a single particle event. Unfortunately whilst the patterns of mutation and recombination have been well characterised, their impacts on host susceptibility and immunity are not well understood. This has impacted on the wider understanding of immunity to norovirus ~ as estimates of the immune period following illness/infection range from 6 months to years (reviewed in Simmons 2013).

Many of the weaknesses of this network modelling approach have been considered in the publication describing the development of the original model (Rushton *et al*, 2019 (in press)). The most important considerations in the original model were the realism of the social network structure and the representation of immunity. The networks were based on those that could occur in a community in North-East England as defined by socio-demographic data collected by the local authority and knowledge of the infrastructure in that community (number of primary schools etc.). These provide basic contact networks but will not have included all contacts between individuals in the community that did occur. We had no means of quantifying links in work settings or those out-with the area. We did not capture all behaviour that will have impacted on the transmission process, At best, our model was conservative of the likely impacts of social mixing and this will have possibly emphasised the significance of foodborne relative to contagious spread.

We consider the assumptions made in our modelling of immunity in more detail as levels of immunity and their underlying mechanisms are likely to be of significance in the context of repeated exposures to contaminated foodstuffs and the maintenance of disease in the community and they were important in both the QMRA and community models. Whilst we conclude that levels of risk through exposure to foodstuffs are low, and that lettuce poses the higher risk compared with fruit and oyster consumption, we believe that the risk posed by food consumption cannot be considered independently of the immunity of the individuals arising from past exposure. Analyses of the epidemiology of other food borne diseases like *Campylobacter* have suggested that immune status of individuals is a factor determining whether or not individuals become ill on exposure to the pathogen (Havelaar, *et al* 2009). This is where we advocate further work. Currently there are

limited data information on exposure histories, illness and food consumption and none linking these to specific strain and host immunity. We believe that understanding host immunity in the context of past exposure is important if we wish to control this disease. There are several key steps to achieving this, for which we suggest further work:

i) Developing a more complete understanding of the role of evolution and selection that leads to the development of new strains

ii) The interaction of strain with host biochemistry

iii) The interaction between host immunity and NoVs strain ~ at what level does strain evolution lead to a change in immunological response?

iv) The role of low-level exposure (e.g. through consumption of lettuce) in changing immune status without necessarily causing disease.

Modelling the mechanistics of NoV evolution is comparatively easy and there is an abundance of data describing changes in the genome in key areas associated with host susceptibility. What is less clear is the link between strain type, and susceptibility ~ or more specifically when are mutations sufficiently accrued to necessitate the recognition of a new strain and epidemic/pandemic spread? Many of these areas could be addressed through a combination of modelling and strain sequence typing linked to measurements of the immunological trends in markers such as norovirus specific Immunoglobulin A (IgA) as could be collected in a case-control or longitudinal cohort study.

Finally, we recognise that NoV illness is highly contagious, the role of network connectivity and fomites are evidently of importance in on-going transmission of NoV infection, but we have few real data with which to investigate the relative importance of foodborne versus contagion and fomites in disease. Whilst targeting sources of food contamination may be a key feature of reducing disease, we cannot say what the impacts of this would be on addressing the issues of the wider burden of disease in the community since this would also require some form of contact/network-tracing.

10 The models in context

10.1 Introduction

The aim of this chapter is to compare the results from the two modelling approaches undertaken in the NoVAS research programme and consider them in the context of the international literature.

10.2 Re-cap of major outcomes from each model

In the QMRA (Chapter 8) the proportion of foodborne transmission of norovirus (NoV) was estimated to be 16% (range 2.1% to 22.9%) (Table 47). Our preferred estimate took into account the fact that a proportion of the population is intrinsically resistant to NoV infection and assumed that the population had been exposed to the virus previously and had, therefore, built up acquired immunity. Our estimate is consistent with the international peer-reviewed literature in which the percentage of norovirus that is estimated to be foodborne ranges from 2.4% to 40% (Table 47).

The estimate of the proportion of NoV transmitted through food in the current study is some ten-fold higher than an earlier UK estimate (O'Brien *et al,* 2016). The earlier UK estimate relied heavily on outbreak data to derive the proportion foodborne and was likely to have been an under-estimate because of under-diagnosis of NoV and under-reporting of NoV cases and outbreaks, in particular because of changes to the outbreak surveillance system that favoured reporting of NoV outbreaks in health and social care settings as opposed to foodborne outbreaks. The capture-recapture study undertaken as part of NoVAS gives an important insight into under-reporting of foodborne outbreaks associated with just one food commodity (namely seafood) (Hardstaff *et al*, 2018). Assuming that this is repeated across other foodborne outbreaks and food commodities it is unsurprising that the earlier UK estimate was likely to have been too low. It should be noted that we could not repeat the capture-recapture for other food commodities because there were no sources of data other than PHE for outbreaks involving other foodstuffs.

The QMRA included catered food in two forms – eating out at a restaurant and eating takeaway food. These are both proxies for food handler-related transmission, which accounted for around 75% of total foodborne transmission (illnesses per 1,000 person-years = 5.68 (range = 0.71 to 7.73). This was followed by lettuce (20% or 1.49 illnesses per 1,000 person-years (range 0.19 to 2.03). Berries and oysters accounted for only around 3% and 3% respectively. The QMRA did not include routes of transmission other than through food or food handling.

Table 47: International estimates of proportion of norovirus burden (NoV) that is foodborne

Year	Country	Estimate of proportion of norovirus burden (NoV) that is foodborne	Method(s) used to estimate proportion of foodborne NoV	Major sources of uncertainty	References
2018	UK	16% (range 2.1% to 22.9%)	Modified QMRA using microsimulation to enable extrapolation of annual risk from daily risk, combining multiple exposure pathways into a single risk assessment and incorporating an SIR epidemic model dealing with acquired immunity	Duration of acquired immunity; Selected foods only; Amount of NoV on food or food handlers' hands; Community incidence of NoV; Norovirus viability; Food handling behaviour; Estimates of exposure; Norovirus transfer from hands to food.	Chapter 8: This Report
2018	UK	35% (range 11% to 55%)	Individual-based modelling of food consumption and indirect transmission pathways, immunity and contagion	Over-simplification of contagious pathways to disease; Simplified model of immunity; Selected foods; Indirect contagious pathways, e.g. takeaways, not modelled because of lack of data.	Chapter 9: This Report
2014	Canada	18% (90% Credibility Intervals (CrI) = 4% to 40%)	Expert Elicitation; Estimates combined via triangular probability distributions	Limited number of experts taking part; Experts lacking an understanding of the broader nature of enteric illness transmission; Issues around phrasing of questions; Survey fatigue.	Butler *et al*, 2015

Year	Country	Estimate of proportion of norovirus burden (NoV) that is foodborne	Method(s) used to estimate proportion of foodborne NoV	Major sources of uncertainty	References
2008-13	France	12-16%	Molecular epidemiological study originating from the Netherlands (see below)	Under-reporting; Under diagnosis;	Van Cauteren *et al*, 2017
1999-2012	Global	14% (range 12% to 16%)	Molecular epidemiological study across three international outbreak surveillance datasets - proportion of outbreaks caused by food determined by genotype and/or genogroup		Verhoef *et al*, 2015
2010	Global	18% (95% Uncertainty Interval = 11 to 30%)	Structured Expert Elicitation	Uncertainty around the proportion of foodborne transmission.	Kirk *et al*, 2015; Hald *et al*, 2016
2010	Australia	18% (95% CrI = 4% to 38%)	Expert Elicitation	Lack of evidence, or the presence of conflicting evidence, relating to the degree of foodborne transmission.	Vally *et al*, 2014
2009	The Netherlands	17% (95% Confidence Interval (CI) = 13% to 28%)	Structured Expert Elicitation - joint probability distributions created by probabilistic inversion.	Experts' individual estimates did not agree in many cases reflecting uncertainty about the proportion of pathogens transmitted by food	Havelaar *et al*, 2008; Verhoef *et al*, 2013
2009	UK	2.4% (range 1.7% to 3.5%)	Used outbreak data to determine % foodborne – based on 61 of 228 (2.7%) norovirus outbreaks identified as foodborne and 1,500 cases in foodborne outbreaks out of a total of 58,855 outbreak cases (2.5%).	Under-diagnosis; Under-reporting of foodborne norovirus outbreaks resulting from changes in surveillance methodology; Under-investigation of norovirus outbreaks outside of healthcare settings;	O'Brien *et al*, 2016

Year	Country	Estimate of proportion of norovirus burden (NoV) that is foodborne	Method(s) used to estimate proportion of foodborne NoV	Major sources of uncertainty	References
				Assumption that outbreak cases reflect epidemiology in the wider community	
2008	Canada	31% (95% CI = 14% to 48%)	Expert Elicitation; Factor Analysis; Cluster Analysis; Monte Carlo simulation using triangular distributions	Considerable variation between experts in their estimated foodborne attributable proportions observed over all diseases, unrelated to the expert's background	Ravel *et al*, 2010
2006	US	26%	Outbreak data - Based on 179 norovirus outbreaks examined by CDC from 2000-2005. Of 13,944 persons ill, 3,628 (26%) were in foodborne outbreaks.	Variable data quality; Representativeness of some data sources; Assumptions about the proportion of illnesses transmitted by food because data often lacking	Scallan *et al*, 2011
2006	Canada	31%	Monte Carlo simulation	Under-reporting; Under-diagnosis; Estimate of the proportion foodborne	Thomas *et al*, 2013
2005	New Zealand	Simulated mean = 39.2% (95% CI = 8 – 64%)	Expert Elicitation - two-pass modification of the Delphi method	Incidence estimates for norovirus; Uncertainties from the expert elicitation process for estimating the proportion of disease due to foodborne transmission; Limited number of people with suitable expertise among New Zealand's small population;	Lake *et al*, 2010

Year	Country	Estimate of proportion of norovirus burden (NoV) that is foodborne	Method(s) used to estimate proportion of foodborne NoV	Major sources of uncertainty	References
				Uncertainty about the actual numbers of cases of foodborne diseases in New Zealand; Factors used to scale up from notifications to total cases were large, and had high variability.	
2000	Australia	25% (95% CrI = 12 – 38%)	Simulation using outbreak data	Under-reporting; Variable data quality; Reliance on outbreak data, which can be very sensitive to outcomes from larger events. This can bias the estimate of the proportion foodborne in either direction	Hall *et al*, 2005
2000	UK	11%	Outbreak data (N = 171 foodborne norovirus outbreaks out of 1592 total outbreaks of norovirus)	Assumption that outbreak cases reflect epidemiology in the wider community; Under-reporting of foodborne outbreaks	Adak *et al*, 2002
1997-2000	France	14%	Estimation of the proportion of NoV infections attributable to foodborne transmission, based on surveillance data, outbreak reports or specific studies on risk factors for infection.	Under-diagnosis; Under-reporting	Vaillant *et al*, 2005

Year	Country	Estimate of proportion of norovirus burden (NoV) that is foodborne	Method(s) used to estimate proportion of foodborne NoV	Major sources of uncertainty	References
1996	US	40%	Based on a report which found that 47% of norovirus-associated acute gastroenteritis outbreaks in the US in which the modes of transmission were known were foodborne.	Assumed that foodborne-associated outbreaks more likely to be reported than outbreaks with other mechanisms of spread, and so proportion lowered to 40%.	Mead *et al*, 1999; Fankhauser *et al*, 1998

The individual-based model (IBM) (Chapter 9) included other potential routes of transmission in addition to selected food items, though catered foods (restaurant meals and takeaways) were excluded. The IBM yielded around 38 illnesses per 1,000 person-years that were attributable to the consumption of lettuce (95% CI = 16 to 61), 11 illnesses per 1,000 person-years that were attributable to consumption of berries (95% CI = 3 to 19) and 0.03 illnesses per 1,000 person-years that were attributable to consumption of oysters (95% CI = 0 to 0.1). Transmission within families was the other main route for illness in the IBM (around 11 cases per 1,000 person-years (95% CI = 5 to 17)). The IBM did not account for intrinsic immunity i.e. the proportion of the population that are not susceptible to NoV because they do not exhibit the histo-blood group antigens that are required for NoV binding. It did, however, take into account acquired immunity. Assuming that the correction factor for intrinsic immunity used in the QMRA is applicable, then this reduces the illnesses per 1,000 person-years from lettuce, berries and oysters in the IBM to 12.1 (95% CI = 5.1 to 19.5), 3.5 (95% CI = 0.1 to 6.1) and 0.01 (95% CI = 0 to 0.03) respectively. Similarly, it reduces family (household) transmission to 3.6 illnesses (95% CI = 1.6 to 5.4) per 1,000 person-years. Taking the IID2 Study incidence estimate of 47 cases per 1,000 person-years as the denominator (Tam *et al*, 2012), this means that foodborne transmission in the IBM was estimated to be around 35% (range = 11% to 55%), which is consistent with the QMRA estimate because the ranges overlap, although it should be noted that the IBM excluded catered food.

Although the estimates from the QMRA and the IBM in terms of illnesses per 1,000 person-years are slightly different (Table 48), the rank order of individual food items in terms of importance is the same - namely lettuce > berries > oysters. This reflects the fact that lettuce is consumed much more often than either berries or oysters.

Comparing the NoVAS results with those of other studies is hampered by the fact that there is little consistency in the way results are reported (Table 48). Various ways are used to express illness burden and there seems to be little standardisation in terms of the denominators used.

Table 48: International Estimates of the Contribution of Various Foods to Norovirus Illness or Infection

Year	Country	Method	Outcome Measure	Food Commodity/ies	Results	Major sources of uncertainty	Reference
2018	UK	Modified QMRA using microsimulation	Illnesses/1000 person years (accounting for intrinsic and prior immunity)	Catered Food Lettuce Berry Fruits Oysters	5.68 (range = 0.71 to 7.73) 1.49 (0.19 to 2.03) 0.22 (0.03 to 0.30) 0.21 (0.03 to 0.29)	Duration of acquired immunity; Selected foods only; Amount of NoV on food or food handlers' hands; Community incidence of NoV; Norovirus viability; Food handling behaviour; Estimates of exposure; Norovirus transfer from hands to food.	Chapter 8: This report
2018	UK	Individual-based modelling	Illnesses/1000 person years (accounting for intrinsic and prior immunity)	Lettuce Berry Fruits Oysters	12.1 (95% CI = 5.1 to 19.5) 3.5 (0.1 to 6.1) 0.01 (0.00 to 0.03)	Over-simplification of contagious pathways to disease; Simplified model of immunity; Selected foods; Indirect contagious pathways, e.g. takeaways, not modelled because of lack of data.	Chapters 9 & 10: This report
Not stated	US	Probabilistic exposure model using stochastic modelling	Percentage of cases of infection over 10,000 servings simulated at different concentration scenarios	Norovirus transfer from contaminated produce as initial source of Contamination in a school food	0.13% when NoV infective particles (NoVP) per serving of lettuce = 10^2 0.14% when NoVP/serving = 10^3	Behaviour of surrogate viruses, e.g. murine norovirus, is not always representative for human NoV strains; Food handler behaviour and practices;	Pérez-Rodríguez *et al*, 2019

Year	Country	Method	Outcome Measure	Food Commodity/ies	Results	Major sources of uncertainty	Reference
				service operation	0.53% when NoVP/serving = 10^4 0.57% when NoVP/serving = 10^5 0.61% when NoVP/serving = 10^6 1.31% when NoVP/serving = 10^7	Number of contacts with produce; Sequence of events in handling produce	
Not stated	Spain	QMRA	Norovirus burden expressed as Disability Adjusted Life Years (DALYs)/person/year)	Lettuce irrigated using waste water from two waste water treatment plants (WWTP)	Mean (95 percentile) WWTP 1 = 1.94×10^3 (2.00×10^3) WWTP2 = 2.99×10^4 (7.47×10^4)	Reduction in viral concentration due to treatment; Viral concentration in raw sewage; Virus ingestion	Gonzales-Gustavson *et al*, 2019
2014	India	QMRA using Monte Carlo simulations (a) Raw, fresh produce	Mean probability of illness per year	Green Peppers Cucumber	36% (95% Credibility Interval = 3% to 81%)	Concentration of pathogens present on produce; Dose-response parameters;	Kundu *et al*, 2018

Year	Country	Method	Outcome Measure	Food Commodity/ies	Results	Major sources of uncertainty	Reference
		without washing (b) Fresh produce with washing and use of disinfection prior to consumption		English cucumber Cantaloupe Cilantro Green Peppers Cucumber English cucumber Cantaloupe Cilantro	11% (1 to 30%) 33% (3 to 77%) 26% (2 to 65%) 46% (4 to 91%) 2% (0 to 8%) 0% (0 to 1%) 1% (0 to 4%) 1% (0 to 3%) 2% (0 to 7%)	Assumptions about *E. coli* to pathogen ratios; Estimates of the amount of product eaten by consumers; Estimates of diarrhoeal illness rates amongst the Indian population	
2011-13	Belgium/ Poland/ Serbia	Quantitative Exposure Assessment	Mean NoV particles consumed per portion	Raspberry puree Individually quick frozen raspberries	0.28 (Baseline scenario) 0.24 (Baseline scenario)	Contamination considered to be heterogeneous; spread on the picked; Irrigation water not included; Number of NoV shedding food pickers has important impact	Jacxsens *et al*, 2017
Not Stated	US	QMRA using Discrete Event Modelling	Expected number of infected and sick customers	Food establishments with symptomatic	Mean number of infected customers = 167.4 (90% Variability Interval	Assumptions related to employee	Duret *et al*, 2017

Year	Country	Method	Outcome Measure	Food Commodity/ies	Results	Major sources of uncertainty	Reference
			per 2,000 servings	food handler working whilst ill	(VI) = 29.0 to 357.7); Mean number of sick customers = 5.2 (90% VI = 0.1 to 17.2)	practices/behaviour and retail setting; Assumptions related to illness and norovirus; Assumptions related to data and statistical analysis; Did not account for immunity associated with prior episodes of norovirus infection or the fact that genetic susceptibility factors of different norovirus strains may differ from what has already been described for the prototype virus	
Not stated	Europe	QMRA	Infection risk per serving	Romaine Lettuce	3×10^{-4} (95% Interval (sic) = 6×10^{-6} to 5×10^{-3})	Dose-response; Extrapolation from of estimated PCR-Detectable Units of contamination to concentrations into ingested dose of infectious organisms; Virus concentrations for potential contamination Points;	Bouwknegt *et al*, 2015

Year	Country	Method	Outcome Measure	Food Commodity/ies	Results	Major sources of uncertainty	Reference
						Structural versus episodic contamination events	
Not stated	Belgium	Quantitative exposure model using Monte Carlo simulation	Mean human NoV infectious particles ± standard deviation per serving	Lettuce in delicatessen sandwiches (Note: best case scenario in which lettuce is the primary source of human NoV)	6.4 ± 0.8	No dose-response model included; Assumptions about food handler behaviour; Limited data on the presence of human NoV on lettuce	Stals *et al*, 2015
Not stated	Ghana	QMRA	Annual probability of norovirus illness	Street Food Salads	3 x 10^{-1}	Estimates of virus concentration; Estimates using irrigation water quality significantly underestimated health risks	Barker *et al*, 2014
Not stated	Australia	QMRA using Monte Carlo simulation for wastewater irrigated produce	Norovirus burden expressed as DALYs/person/ year	Bok Choy Broccoli Cabbage Choy Sum Cucumber	90% Confidence Intervals 1.62 x 10^{-4} to 2.84 x 10^{-3} 7.57 x 10^{-5} to 2.43 x 10^{-3} 3.38 x10^{-4} to 3.88 x 10^{-3}	Virus removal by wastewater stabilization ponds; Other wastewater treatment parameters; Norovirus shedding rates; Produce consumption rates	Mok *et al*, 2014

Year	Country	Method	Outcome Measure	Food Commodity/ies	Results	Major sources of uncertainty	Reference
				Gai Lan Lettuce	3.07×10^{-4} to 2.84×10^{-3} 2.37×10^{-6} to 7.04×10^{-4} 3.39×10^{-4} to 3.81×10^{-3} 4.66×10^{-4} to 4.40×10^{-3}		

10.3 Strengths

We collected representative new data on contamination levels of food commodities on retail sale and on the prevalence of norovirus contamination in catering premises over the course of a year and during outbreaks. The survey of oysters was the on retail sale was the first systematic study at point-of-sale in the UK and one of the largest studies worldwide to date. Although the majority of samples were positive high levels of contamination (exceeding 100 NoV copies/gram) were infrequent. Similarly the survey of fresh produce items on retail sale was the first conducted in the UK. For oysters and for fresh produce we were able to make quantitative estimates of the levels of NoV contamination where found. In the survey of catering premises, which was also a UK first, we could not make quantitative estimates of contamination levels, because of the nature of the testing method used, but were able to determine the presence/absence of virus.

Our modelling approach had a number of strengths. First, we had to find a mechanism to assess the combined risk from multiple food categories. We did this by developing a novel microsimulation modelling approach to QMRA. The microsimulation basically models risk at the level of the individual and simulates event histories at the individual rather than the population level. This meant that we could incorporate multiple foods. We also estimated the annual risk of illness from the daily risk.

Secondly, we used two different modelling methods to estimate the risk from foods – a novel QMRA and an IBM. These two methods generated different, but overlapping, estimates, which gives a measure of confidence in the results. The ranking of foodstuffs from both modelling approaches was identical and showed that the risk of illness from eating contaminated lettuce was greater than from eating contaminated berries or oysters (in that order).

An additional strength of the QMRA was the inclusion of catered foods (restaurant meals and takeaways), which suggested that nearly 50% of foodborne transmission could be accounted for by food handling. The IBM considered routes of transmission in addition to the food pathway and demonstrated the importance of transmission within families (household transmission), which was, in that model, on a par with transmission through eating contaminated berries.

Finally a major strength of both modelling approaches is that the results have been adjusted to account for intrinsic immunity and acquired immunity. This had a major impact on the outputs, reducing the potential impact of NoV illnesses acquired through food substantially. The caveat is that this might not represent the situation when a novel strain emerges to cause another global pandemic.

10.4 Limitations

The major limitations were that the QMRA did not include transmission pathways other than food and the IBM did not include catered food or transmission in places of work. In the case of the IBM there were insufficient data to parameterise the model for these two situations. Nevertheless the findings were consistent in terms of the rank order of risky foods – lettuce > berries > oysters.

An issue that it was not possible to address was the impact of seasonality on NoV disease burden. Norovirus should exhibit a highly seasonal variation in illness rates. However, we were not able to include seasonality as we did not have adequate estimates of seasonality in food consumption.

Another limitation surrounded information about food handling practices in the catering industry. In the QMRA we had to extrapolate from the literature around sandwich production, which might not be representative of catering the industry as a whole. Key knowledge gaps were around how often foods are directly handled and whether that handling is on food that will be subsequently cooked. There was also little information on how much NoV is transferred from hand to food with each touch.

We also attempted to make an assessment of the likelihood that the NoV that we detected on food commodities had infectious potential. This was successful in part. The capsid integrity assay was applied successfully to leafy greens and berry fruit but was unsuccessful when used with oysters, where it was found to be incompatible with the CEN method. We were also unable to account for virus viability when swabbing the catering environment and food handlers' hands because of the destructive nature of the environmental testing method.

The surveys happened to coincide with relatively low incidence of NoV in the population so that the estimates of the prevalence of contamination and the levels of contamination might be underestimates. This will, in turn, have an impact on the outputs of the QMRA and IBM. In those years where population incidence is greater, then the total foodborne disease burden will also be greater. However, we would suggest that the proportion of cases attributable to food would remain similar in high and low incidence years. Finally, we were unable to account for secondary transmission arising after a foodborne contamination incident/outbreak.

10.5 Sources of Uncertainty

These are described in detail in Chapters 8 and 9. To re-cap, in both models (QMRA and IBM) the major source of uncertainty surrounded assumptions about the duration of acquired human immunity. This had by far the biggest impact on the outputs of both models. Other important sources of uncertainty were virus viability, levels of contamination on food/hands/environmental surfaces and the background incidence of NoV.

10.6 Conclusions

The proportion of NoV transmission that is foodborne was estimated to be 16% in the QMRA and 35% in the IBM so somewhere between a fifth and a third of all NoV could be attributed to the foodborne route. Of that foodborne proportion, nearly 75% occurred through contaminated catered food (a proxy for food handling), with contaminated lettuce accounting for around 20% of illness burden, followed by berries and oysters at 3%.

11 Recommendations

11.1 For research

To improve the QMRA and IBM we recommend that further research is needed to:-

1. Understand the proportion of gene copies in food commodities that represent virus with infectious potential. The VPg immuno-capture PCR and direct RT-qPCR using capture beads was not fully tested or optimised in this study and so further work is required to:-

   - Obtain more repeat data and extend studies to more GII and GI samples
   - Consider the inclusion of RNase inhibitors
   - Investigate optimal antibody selection for capture
   - Reduce or eliminate non-specific binding to increase sensitivity and specificity
   - Trial and compare the assay using shellfish or berry extract and the ISO methods.

2. Develop better dose response models for norovirus.

3. Understand the mechanisms of NoV transmission in commercial kitchens including:-

   - Studies of food handler behaviour during food preparation across the range of catered foods.[11]
   - Assessment of how frequently food is handled during preparation, especially when the food will be served directly to a diner with no further cooking.

4. Understand human immunity to norovirus.

5. Obtain data on seasonal consumption patterns to improve exposure assessment.

6. Understand the likely impact of risk management interventions by industry in reducing consumer exposure to norovirus.

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[11] FSA has published research in this area carried out by Ipsos MORI, Food handlers and norovirus transmission: Social science insights (2017) which comprised a literature review followed by structured environmental and behavioural observations, surveys, and in-depth interviews with 32 food establishments: www.food.gov.uk/research/research-projects/food-handlers-and-norovirus-transmission-social-science-insights

11.2 For policy

Given the importance foodborne NoV and that food eaten away from the home or take away food is the likely primary driver of foodborne NoV we recommend that:- The Food Standards Agency maintains its emphasis on prevention of transmission of NoV in catering outlets (e.g. food handler instructions/procedures) to minimise/mitigate the risk of NoV infection from foods eaten away from the home.

12 List of abbreviations and acronyms used

°C	Degrees Centigrade
µl	Micro-litre
Δ	Delta (Difference/Discriminant)
BLAST	The basic local alignment search tool
bp	Base pair
cDNA	Complementary DNA
Cefas	Centre for Environment, Fisheries and Aquaculture Science
CEN	European Committee for Standardization
CFU	Colony Forming Units
CIA	Capsid Integrity Assay
cm	Centimetre
CO2	Carbon Dioxide
Copies/g	Copies per gram
Cq	Cycle Quantification
Ct	Cycle Threshold
DEFRA	Department for Environment Food & Rural Affairs
DNA	Deoxyribonucleic acid
DNase	Deoxyribonucleic
dsDNA	Double-stranded DNA
E.coli	*Escherichia coli*
EAC	External Amplification Control
EC	European Commission or External Control
EFSA	European Food Safety Authority
EHOs	Environmental Health Officers
EU	European Union
EVU	Enteric Virus Unit
FBOs	Food Business Operators
FCV	Feline Calicivirus
Fera	Food and Environment Research Agency
FRNA	F-Specific Ribonucleic acid
FSA	Food Standards Agency
g	Grams
GI	Genogroup type I
GII	Genogroup type II
H2O	Water
HCI	Hydrochloride
HGBA	Histo-Blood Group Antigens
hNoV	Human norovirus
IBM	Individual-Based Model
IgA	Immunoglobulin A
IID2	The second study of Infectious Intestinal Disease in the community
ISO	International Organization for Standardization

KDa	Kilodaltons
LA	Local Authority
LFR	Leatherhead Food Research
LOC	Lab on a chip
Min.	Minute(s)
ml	Millilitre
MNV	Murine norovirus
MPN	Most Probable Number
NCBI	National Centre for Biotechnology Information
NDNS	National Diet and Nutrition Survey
NGS	Next Generation Sequencing
NM	Nanomolar
NoV	Norovirus
NoVs	Noroviruses
NoVAS	Norovirus Attribution Study
NW	North West
ORFs	Open-Reading Frames
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pfu/g	plaque forming units per gram
pH	potential of Hydrogen
PHE	Public Health England
PLSR	Partial Least Squares Regression
PTPY	Per Thousand Person Years
py	person years
QMRA	Quantitative Microbial Risk Assessment
qPCR	Quantitative polymerase chain reaction
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
RFU	Relative fluorescence units
RLBUHT	Royal Liverpool and Broadgreen University Hospital Trust
RMPs	Representative Monitoring/Sampling Points
RNA	Ribonucleic acid
RNase	Ribonuclease
RNP	Ribonuceloprotein
Rpm	Revolutions per minute
rRNA	Ribosomal ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
SD	Standard Deviation
SE	South East
SIR	Susceptible-Infected-Recovered
SOPs	Standard Operating Procedures
SPCV	Sample process control virus
SRCL	Stericycle ExpertSOLUTIONS

ssRNA	Single-stranded ribonucleic acid
UEA	University of East Anglia
UK	United Kingdom of Great Britain and Northern Ireland
UKFSS	United Kingdom Food Surveillance System
UoL	University of Liverpool
UV	Ultra-violet
VIP	Variable Influence on Projection
VLP	Virus like particles
WP	Work Package

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