FS 101040:
Protocols for surveys of *Norovirus* (NoV) contamination in oysters and fresh produce on retail sale and in the commercial catering environment

Authors
Cefas: David Lees, James Lowther
Fera: Nigel Cook, Martin D'Agostino
Public Health England: David James Allen, Nicola Elviss
University of Liverpool: Miren Iturriza Gomara, Sarah J O'Brien, Steven Williams
Project Lead Contractor: Sarah J O'Brien, University of Liverpool

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1. Background

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

Noroviruses (NoV) 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 NoV 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 NoV that is transmitted through contaminated food vary quite widely. Through a series of linked studies we are seeking to answer the following major research questions:

a) How much NoV 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 so far, we intend to conduct fieldwork in three crucial areas – first to determine the prevalence of NoV 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 NoV found is likely to be infectious or not and thirdly to determine the prevalence of NoV contamination of the catering environment. These are essential data items for being able to conduct a quantitative microbiological risk assessment.

The FSA's policy objective is to develop an evidence based approach to managing risks from, controlling the burden of, foodborne NoV disease as described in the FSA forward evidence plan 2012 and the FSA Science and Evidence strategy 2010-2015. Armed with the information generated from the research, the Food Standards Agency should be better equipped to formulate risk mitigation strategies and develop improved targeted risk management tools.

The survey protocols within this document detail the approaches to be used within this study for shellfish (oysters), fresh produce (lettuce and raspberries) and the catering environment.

2. Shellfish (Oysters)

This shellfish protocol sets out the proposed approach to sampling oysters at the retail level in the UK to assess *Norovirus* contamination. Oyster sampling and analysis is a component of FSA project 101040 which aims to ascertain the amount of the total *Norovirus* illness burden in the UK which can be attributed to a food borne route of transmission. The intention is to conduct the study at the point of sale (i.e. at the retail level) to ensure that products tested are representative of those consumed by the UK consumer.

2.1. Methods:

2.1.1. Survey design

The primary aim of this survey is to assess the level of food borne *Norovirus* risk posed by different higher risk food groups, including oysters, throughout the year. Though the study is not required to attribute the different levels of risk to product origins within each food group, where possible it is important to obtain this information so that biases in the sampling plan can be identified and adjusted for in the final modelling and analyses. The proposed approach to sampling for the oyster survey is set out below.

2.1.1.1. Market Research

The below considerations on survey design were informed by a comprehensive practical evaluation of the purchase routes for oysters available to the UK consumer. This evaluation was undertaken by partner Stericycle during the first year of the project through phone interview, and sometimes physical visits, to identified outlets. This market research was collected in 11 selected cities/regions of the UK to ensure adequate and representative coverage. The regions selected and the summary of findings is given as Appendix 1. Essentially, outlets directly available to consumers of oysters could be subdivided into: supermarkets and similar general stores; fishmongers; restaurants (including oysters bars etc serving prepared food); direct sales (particularly direct internet sales); and wholesale. The possibility of sample acquisition (for oysters) was explored at each of the identified outlets. For oysters issues arising at the outlets included: availability of supply (oysters not available at the time of enquiry but may become available); time of year (oysters only stocked at certain times of the year); oysters not routinely stocked (but could be pre-ordered); oysters not available to purchase and take away (a problem in most restaurants). Regarding information on the origin of oysters Stericycle sought sight of the oyster ‘health mark’ which contains all the necessary traceability information, and retention of which at the retail level for 60 days is a legal requirement. This is particularly important for oysters since, in general, they are not a packaged product and therefore do not include this information on the wrapping material. However, this proved problematic with only 17 of 200 outlets able to provide sight of the health mark. Various reasons were given including: the need to consult a senior manager; lack of familiarity with the health mark or where it could be found; unwillingness to

take the time to find the health mark, the health mark already having been discarded with the original packaging. This was not anticipated and draws into question the assumption that accurate origin of oysters would be readily available from the details on the health mark. It may be possible to improve cooperation on this aspect through provision of an explanatory letter from the FSA explaining the objectives of the study and seeking cooperation of the retailer. A revised draft of this letter is awaited from the FSA prior to testing this at retailers to see if it improves cooperation regarding supply of the health mark. If not it should be noted that, for the large majority of oyster samples, verified information on origin will not be available. However, in this case, information on origin will be requested and any verbal information given will be recorded as well as any obvious written information from, for example, restaurant menus, oyster boxes from which samples are taken, fishmonger tags, etc. An additional issue that has arisen through this exercise is the reluctance of restaurants to permit ‘take away’ orders of oysters. Again it is possible that the FSA letter may improve cooperation and this will be tested as soon as the letter is available. This is important for oysters since it is considered that a significant portion of consumption is undertaken at restaurants. However, if the letter does not improve cooperation it may be necessary to consider an alternative approach. In this case we propose to instead sample at a stage removed from restaurants i.e. at the wholesale supply stage. In some cases wholesale suppliers are also available to the general public (e.g. Billingsgate fish market) in other cases we will approach specialist wholesalers for oysters (e.g. M&J Seafoods) direct and seek cooperation with the project. If this is necessary we will adjust the frequency of sampling at wholesale to a level consistent with the approximate volume of consumption anticipated to be through the restaurant route.

The market research gathered during the above exercise has provided a firm foundation for the practical arrangements for sample collection as described below.

2.1.1.2. Potential Approaches to Sampling

In designing the sampling framework to address the project goals, two possible approaches could be used: 1) a structured survey that selects outlets at random (but weighted by their proportion of the market share) and within which batches are selected at random but based on their origin weighted by the quantity supplied to the UK public by that origin (i.e. batches from sources comprising a large amount of the market share would be sampled more than those comprising a small amount of market share), or, 2) a randomised survey where both outlets and batches within outlets are selected at random. In order to ensure consistency and prevent biases in the final analyses it is important that the same sampling approach is taken for all of the food products included in the survey.

2.1.1.3. Sampling considerations

A review of available information on oyster production and consumption in the UK (Appendix 2) highlighted several data gaps relating to product destination within the UK and consumer buying patterns. Though similar information has not been formally compiled on the other food groups to be tested in the survey, the project steering group felt that similar data gaps were likely to exist. These data gaps make a structured survey challenging unless reliable data can be obtained at the point of sale. Preliminary market research data collected by Stericycle showed that in many cases information on the origin of oysters and other products was difficult to obtain. Additionally, as anonymity of the origin and outlets of product testing positive for *Norovirus* cannot be guaranteed under FSA commissioning rules, and the project staff have no legal basis on which to demand samples and information relating to their origins, the project steering group felt that compliance in the survey could not be assured, thus making it difficult to preselect batches from different sources for testing. In addition to these limitations, the available funding and capacity of the laboratories limits the number of samples that can be tested each month to 45 to 53 samples for each food product. These relatively small sizes limit the power to adjust for differences between the strata used in a structured survey and could actually result in biases being introduced.

2.1.1.4. Final study design

Based on the constraints described above, the project steering group agreed that a randomised survey would be most appropriate in addressing the project's primary aim. Discussion was held as to whether to bias sampling to particular months in the year, and though available data on monthly oyster production, imports and *Norovirus* levels showed distinct seasonal trends, this data was not available for the other food groups. Consequently a constant sample size per month was deemed most appropriate in order to prevent the occurrence of biases between food groups.

After careful consideration it was decided that the survey should obtain a total of 45 to 53 samples per month (depending on food group) from the different outlets identified as selling the food products of interest selected at random from a predefined study population across the 11 study regions. Mail order outlets will be treated as a region in their own right and include a number of retailers representative of their overall contribution to national consumption (i.e. if 25% of trade is via mail order trade then these outlets will form 25% of the overall population). Where outlets decline to take part in the study this will be recorded and they will be removed from the sampling frame and another outlet will be selected at random. This study design assumes that the number of outlets in each region and outlet type is proportional to the demand and therefore the amount of product consumed. This study design has the potential to under represent wholesalers and large fish markets, which will only feature once in the sampling frame despite selling large quantities of the product available to consumers. However, it is assumed that the point of sale for the majority of product from these sources will be via smaller outlets, which will comprise a larger

proportion of the sampling frame. Should there be a lack of compliance when sampling restaurants it may be necessary to over sample markets and wholesalers to compensate for the inability to assess the contribution made by restaurants to overall oyster consumption volumes.

2.1.1.5. Compiling the sampling frame

Following completion of the market research SCRL will compile a final list of all available outlets for oysters in each of the 11 regions. Since data on the volume of sales from each outlet or outlet type is not available, our proposed approach is to select sampling locations on a random basis from this list. This makes the assumption that market forces will close supply outlets with insufficient volume flow and hence we will only be sampling commercially viable sales volumes. Randomisation of sampling site selection across the UK list will adjust for oversupply of outlets in some regions compared with others. We consider that, in the absence of structured data on sales volume by outlet, this is the best way to avoid introduction of sampling bias.

A list of alternative sampling locations (again selected at random) will be available in the event that oysters are not available at the selected outlet or cooperation is refused. This approach will be kept under review in the event that it proves impractical and hazards obtaining sufficient samples. In this event an alternative approach will be substituted following consultation with the project board. Since mail order outlets are available irrespective of region we will assign a percentage of the sampling effort to this outlet type and then sub select suppliers for sampling at random using the above approach. The percentage allocated will be determined following consultation with the project board and utilising any available information from stakeholders.

For restaurants we will follow the above approach except that, in the event that they become significantly under represented through unwillingness to allow ‘take away’ orders, we will compensate by oversampling wholesale suppliers to restaurants. In this case we may need to approach significant specialist wholesale suppliers direct and seek cooperation with sampling.

2.1.1.6. Within outlet sampling approach

On arrival at an outlet a sampler may be faced with several batches and product types from which to sample. Only native and pacific oysters sold as ambient, chilled, frozen, and whole, on the half shell or shucked should be sampled. Cooked, pasteurized, or smoked oysters and oyster block should not be considered. Where multiple products are available, one should be picked at random by the sampler. Where multiple batches of the selected product are available, again the sampler should select one at random. Where multiple product types and batches are available at an outlet details of these should where possible be recorded by the sampler. A sample should, in general, consist of individual animals from the same

batch (same origin and production date). However, if a supplier offers to make up a sample from different batches, because insufficient animals are available from any individual batch, then this is acceptable as a sample. In this case the details of all contributing batches should be recorded.

2.1.2. Pilot study

It is proposed to undertake a pilot sampling exercise prior to initiation of the main survey. The objectives of this practical exercise are firstly to ascertain whether, in practise, it will be possible in sufficient locations to gain sight of the oyster health mark. The FSA study explanation letter will be utilised and it should be possible from this exercise to gauge likely cooperation rates. Secondly the pilot will enable trial of the practical arrangements for sampling and for sample transport. It is proposed that each of the auditors contracted by Stericycle to take samples from each of the 11 locations during the survey will be asked to collect and submit one sample of oysters for laboratory analysis at Cefas following the protocols detailed below. This will increase familiarity with the procedures in the survey protocol and enable any issues to be identified in advance of the full survey. This aspect of the sampling protocol awaits confirmation from FSA.

2.1.3. Sampling

2.1.3.1. Sample sizes and sampling plan

The sampling phase of the survey will run for 12 consecutive months (March 2015 – February 2016). A total of 630 samples will be collected with Stericycle targeting an average of 52-53 samples per month. Any shortfall in samples numbers by month will be made up in the following month. The aim is to avoid any introduction of seasonal bias by maintaining a consistent level of sampling by month.

2.1.3.2. Sampling officer training

Each auditor will undergo thorough training provided by the Stericycle Project Manager. This training will take the form of visual training documentation containing strict guidelines on collection/handling of samples at the point of sale, packing of samples for shipment, completion of required paperwork, and organising collection/timing of deliveries.

2.1.3.3. Sampling collection and transportation

Each auditor will be informed by Stericycle at the beginning of each month of the survey period which products they should be purchasing in which week of the following month; this is to minimise the risk of any auditors being unavailable on the weeks that they are allocated. This will enable the Stericycle auditors to advise of any holidays or any changes to the scheduling, prior to the week the samples should be retrieved. This plan will be supplemented and updated with any information on identified new outlets as the study progresses. Each week throughout the sampling, Stericycle will send out a reminder via email to each of the auditors confirming the samples that should be retrieved the following week. Samples shall be collected by

Stericycle auditors after 12pm on Monday to Thursday inclusive (excluding samples sourced from mail-order suppliers, where deliveries shall be accepted at any time from 9am on Monday until 3pm on Thursday). Samples shall consist of 30 fresh or frozen oysters. Whole animals, meats on the half shell and shucked meats shall be included if appropriate however samples of cooked or processed (e.g. cold-smoked) oysters will not be included. Auditors shall not make samples up to 30 oysters by mixing animals from different batches at the point-of-sale unless this is offered by the supplier (see 2.1.6). Samples will be 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”. Auditors will take a photograph of all packed samples immediately prior to shipment so that any damage to the samples during transit can be identified.

Samples will be sent to the testing laboratory via overnight courier service (addressed to specific microbiology laboratory contacts with colour-coded labels to ensure samples are not mixed with other sample streams at the laboratory), to arrive at the laboratory by 9am Tuesday to Friday inclusive.

Following shipment the auditor will update the Stericycle Project manager who will contact Cefas and inform them of the shipments made and the expected time of arrival. The Stericycle Project manager will track all shipments and check for successful and timely delivery and will follow up promptly should any issues arise.

These arrangements will be kept under review through monthly teleconferences between Cefas and Stericycle to review the previous months sampling, the practicality of the arrangements, and whether any adjustments are necessary to meet the project objectives.

2.1.3.4. Sample information

At the point of sampling, full sample details including date, time, outlet name and address, willingness to comply, product types available, whether product sampled was selected at random, sample type, sample condition (ambient, fresh, frozen), sample origin/health mark (if possible) will be recorded by the auditor. A high resolution digital photograph of the sample packaging shall be taken. This information with accompanying photographs shall then be e-mailed to the Stericycle project co-ordinator for inclusion in the sample database. During the sample collection period the database shall be updated on a daily basis.

In addition, on despatch to the laboratory, each sample will be 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.

2.1.3.5. Sample receipt

Upon receipt at the laboratory samples will be booked into the Cefas microbiology system according to the accredited standard operating procedures (SOPs). Each sample will be given a unique Cefas microbiology sample number which will be used throughout processing. The sample temperature will be taken and recorded; along with other sample information provided on the sample submission form, in the Cefas shellfish microbiology and shellfish virology sample books. If shucked meats, or meats on the half shell are provided this shall be noted. Samples will be unpacked, cool packs defrosted and the Coleman boxes cleaned and filled with necessary cool packs, address labels etc. ready for return to the auditors using Stericycle's courier account. Completed sample submission forms, cross-referenced with the Cefas microbiology sample number, will be retained in a dedicated folder.

2.2. Microbiological testing

Any mud adhering to the surface of the shells will be washed off prior to initiating testing. For samples collected from the point-of-sale, microbiological testing will be initiated within 24 hours of the time of collection. Testing of samples from mail-order suppliers will be initiated within 48 hours of the time of collection (determined as the time of receipt of these samples by the auditors). Oysters will be tested provided the temperature upon receipt is below 8°C, that samples collected frozen have not defrosted in transit or vice versa and that the condition of the animals received is good and suitable for testing. In the event that any samples arrive in an unacceptable condition Cefas will inform Stericycle who will work with auditors to promptly arrange a replacement sample.

2.2.1.1. Detection of Norovirus

Oyster samples will be tested for *Norovirus* according to methods compliant with ISO TS/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. Detailed methods are given in the generic protocol "Quantitative detection of *Norovirus* and hepatitis A virus in bivalve molluscan shellfish" included as Appendix 3.

Virus extraction. For each sample, at least 10 oysters are selected. The digestive glands (stomach and digestive diverticula) of these oysters are excised, pooled, and then finely chopped using a razor blade. A 2g subsample of chopped glands is transferred to a clean tube then 10µl of mengo virus vMC0 tissue culture supernatant is added as a within-sample virus/RNA extraction process control. The remaining glands are retained at -20°C for viability analysis. Homogenates are prepared by adding 2 ml of a 100 µg/ml Proteinase K solution to the glands. This is 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 is centrifuged at 3000

x g for 5 min., the soluble portion (homogenate) retained for downstream testing and the pellet discarded. Homogenates are stored at 4ºC prior to testing.

RNA Extraction. Total RNA is 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 is also prepared and tested in parallel with each set of samples extracted. Eluted RNA is stored at -20°C until required.

One-step qRT-PCR. For GI, QNIF4 and NV1LCR primers, and TM9 probe will be used. For GII, QNIF2 and COG2R primers, and QNIFS probe will be used. Mengo virus primers and probes are as described by Pinto et al., (2009). For both *Norovirus* genogroup-specific assays, 3 aliquots of 5 µl sample or extraction control RNA are 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 mengo virus two aliquots of 5 µl cDNA are used. Amplification is 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).

qRT-PCR controls and quantification. Wells containing nuclease free H_2O and the above qPCR reaction mixes will be included on each plate as a negative control. All samples will be assessed for extraction efficiency by comparison of sample C_t values for mengo virus with a standard curve generated from the process control material. Samples are 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) is 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 is determined by comparing the C_t values for the sample RNA plus external control RNA with that for the water plus external control RNA. Quantification follows the principles outlined in ISO TS/15216-1. For each sample RNA, log dilution series (range $1x10^5$ to $1x10^1$ copies/µl) of linear dsDNA molecules carrying the GI and GII target sequences are included on each qRT-PCR plate to generate a standard curve; this dilution series also serves as a PCR positive control. For each qRT-PCR replicate for the sample under test a quantity in copies/µl is determined using the corresponding standard curve. Negative replicates are given a quantity of zero. The average quantities from the three replicates in each *Norovirus* genogroup-specific qRT-PCR assay are calculated to give an overall quantity in detectable genome copies/g digestive gland. Results are not adjusted for losses during processing or RT-PCR inhibition. Samples will be retested if extraction or (RT-)PCR efficiencies fall

below action thresholds (1% and 25% respectively) determined as part of the CEN/ISO method standardisation exercise, where positive (RT-)PCR controls indicate reagent failure, or for any positive sample where the negative extraction or PCR controls shows contamination.

2.2.1.2. Assessment of virus viability

The project includes a component of analytical work aimed at assessing the likely viability of *Norovirus* detected in the sample. The project intention was to utilise the capsid integrity assay to make this assessment. However, evaluation of this method has demonstrated significant issues in its application to oyster samples. It is therefore not yet clear whether it will be possible to apply this assay to oyster samples in this study. Consequently, it is proposed to archive at -20°C residual sample digestive glands following initial processing. This material can subsequently be utilised for possible future testing using either the capsid integrity assay or an alternative approach to assessment of potential virus viability. In the event that viability assessment methods become available during the study samples may also be tested fresh to avoid uncertainties over the effect of freezing on virus viability.

2.2.1.3. Detection of Escherichia coli

Oyster samples will be 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 are 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.

2.3. Quality Assurance (QA) and Quality Control (QC)

All procedures and practices at Stericycle are accredited to IS0 9001 quality assurance standards by the United Kingdom Accreditation Service (UKAS).

The *Norovirus* and *E. coli* tests used during this study are accredited to ISO 17025 quality assurance standards by UKAS. In addition to the controls built in to the test methods, procedures for analyst training, equipment maintenance, quality control of reagents etc. are included in the laboratory SOPs.

2.4. Data handling and reporting

Sample details as submitted by the auditors to Stericycle will be stored on a secured database, held on the Stericycle main and back up servers. The Stericycle service propositions provide services to many large International and Global clients which involve hosting/maintaining a large amount of data/databases, as such Stericycle have developed robust measures to ensure data is held securely.

All laboratory test data generated at Cefas will be subjected to double checking as part of the laboratory SOPs to ensure accuracy and fidelity. All sample details and results will be stored in a secured, backed-up database.

In the case of any sample *E. coli* result exceeding 230 MPN per 100g shellfish flesh, these results will be reported on the same working day to named contacts at the FSA by email. FSA will be responsible for supplying email contact details of responsible officers and for putting in place email forwarding arrangements in the event of 'out of office' notifications.

Upon receipt, the FSA officers receiving the report will consult with the FSA incidents team; in the event that FSA decides to investigate results for any individual sample then they shall contact Stericycle using pre-arranged contact points for additional sample details not held on the Cefas database for reasons of testing anonymity.

Results for all samples for *E. coli*, *Norovirus* GI and GII, and associated details including the Stericycle unique sample identifier, will, at a period of no more than 9 weeks, be combined into a report and e-mailed from Cefas to the above FSA contacts. Upon receipt, the FSA officers shall consult internally and contact Stericycle for more information if required as above.

At the end of the survey period, upon completion of all laboratory testing, the Stericycle database shall be forwarded to Cefas to enable addition of full sample details into the Cefas database, and their incorporation alongside microbiological test results into the WP3 report. In addition all sample details including microbiological test results shall be forwarded in an agreed format to the WP6 leaders (University of East Anglia) for contribution to the quantitative microbial risk assessment.

FSA will be responsible for informing all suppliers of samples of their individual results at the completion of the study, or at other appropriate intervals, in line with FSA requirements in the Guideline for Undertaking Analytical Surveys (2014). Stericyle and Cefas will cooperate to provide FSA with the information they require to undertake this reporting on request from FSA. FSA will also be responsible for any further stakeholder communication or engagement as required by the FSA survey rules. Cefas and Stericyle will cooperate with FSA in this regard as requested.

3. Fresh produce (Raspberries and Lettuce)

There have been several outbreaks of *Norovirus* gastroenteritis in which epidemiological investigation has implicated contaminated berry fruit or leafy green vegetables as the vehicle for transmission of the virus. A limited number of surveys have been conducted to examine the prevalence of *Norovirus* in these foodstuffs at retail. No similar survey has been conducted in the United Kingdom, and consequently there is no information available on the extent of exposure of the community in the UK to *Norovirus* through consumption of berry fruit and leafy green vegetables. The planned survey will analyse samples of lettuce and raspberries for *Norovirus*, in order to assess the contribution of these fresh produce items to the burden of *norovirus* gastroenteritis in the UK. Concomitantly, the samples will be analysed for *E. coli*, to determine whether any correlation might exist between *Norovirus* presence and the presence of this commonly used indicator of faecal pollution.

This study aims to address the following questions:

(a) What, if any, is the extent of *Norovirus* contamination of berry fruit sold at retail in the UK?;
(b) What, if any, is the extent of *Norovirus* contamination of leafy green vegetables sold at retail in the UK?;
(c) Are detected Noroviruses fully encapsidated, and thus have the potential to be infectious? (Dependant on the outcome of WP2)
(d) Can any correlation be observed between the presence of *Norovirus* and the presence of *E. coli* in berry fruit and leafy green vegetables?

3.1. Methods:

3.1.1. Survey design

The survey will involve the collection of samples of2 types of lettuce (Webb and Romaine). These were chosen as they were considered most likely, due to the loose nature of their leafy heads, to retain viruses that may have contaminated them at primary production through contact with contaminated water. Two categories of raspberries will be sampled: fresh and frozen. Samples will be analysed for *Norovirus* GI and GII, and for E. coli, by standard methods. Samples positive for *Norovirus* will be reanalysed by a variant of the standard method which incorporates the capsid integrity assay (depends on results of WP2).

3.1.2. Pilot study

To be discussed.

3.1.3. Sampling

3.1.3.1. Sample sizes and sampling plan

In the absence of information on relative levels of consumption by the UK populace, all 4 produce types will be considered as being consumed in equal numbers, and therefore identical numbers of samples will be analysed.
No weighting will be given to UK-produced versus imported produce, as the relative levels of consumption of each are not know, and whether the produce is home-grown or imported is not relevant to the contribution of lettuce and raspberries per se to the burden of *Norovirus* infection.

The samples will be 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). In the absence of information regarding the relative levels of purchasing of fresh produce items from these categories in the UK, it will be considered that items are obtained equally from each outlet type, and therefore the number of samples taken from each will be identical in each region (except Belfast where more samples will be taken from supermarkets than from the other outlet types, to allow overall sample numbers from Northern Ireland to be proportionate to the total UK population).

The samples will be taken from 4 United Kingdom countries: England, Northern Ireland, Scotland, and Wales. The overall number of samples taken from each country will be in proportion to the overall numbers taken in the survey, as the population of each country is in proportion to the total UK population.

Table X shows the number of samples to be taken, in total and by region and outlet type.

One head of lettuce (each type) and at least 100 g of each raspberry category will be taken at each sampling point.

Table 1: Numbers of lettuce and raspberries to be sampled

Location & outlet type					Total samples to be purchased across 13 months				
Country	Estimated Population	% of sampling	Region	Outlet type	Lettuce (WEBB)	Lettuce (ROMAINE)	Raspberry (fresh)	Raspberry (Frozen)	Totals
NORTHERN IRELAND	1,800,000	3%	BELFAST	WHOLESALERS	1	1	1	1	4
				SUPERMARKETS	2	2	2	2	8
				MARKETS	1	1	1	1	4

				SMALL RETAILERS	1	1	1	1	4
			LONDONDERRY	WHOLESALERS	1	1	1	1	4
				SUPERMARKETS	1	1	1	1	4
				MARKETS	1	1	1	1	4
				SMALL RETAILERS	1	1	1	1	4
SCOTLAND	5,300,000	8%	ABERDEEN	WHOLESALERS	2	2	2	2	8
				SUPERMARKETS	2	2	2	2	8
				MARKETS	2	2	2	2	8
				SMALL RETAILERS	2	2	2	2	8
			DUNDEE	WHOLESALERS	2	2	2	2	8
				SUPERMARKETS	2	2	2	2	8
				MARKETS	2	2	2	2	8
				SMALL RETAILERS	2	2	2	2	8
			GLASGOW	WHOLESALERS	2	2	2	2	8
				SUPERMARKETS	2	2	2	2	8
				MARKETS	2	2	2	2	8
				SMALL RETAILERS	2	2	2	2	8
WALES	3,100,000	5%	BANGOR	WHOLESALERS	1	1	1	1	4
				SUPERMARKETS	2	2	2	2	8
				MARKETS	2	2	2	2	8
				SMALL RETAILERS	2	2	2	2	8

			CARDIFF	WHOLESALERS	2	2	2	2	8
				SUPERMARKETS	2	2	2	2	8
				MARKETS	2	2	2	2	8
				SMALL RETAILERS	2	2	2	2	8
ENGLAND	53,900,000	84%	LONDON	WHOLESALERS	16	16	16	16	64
				SUPERMARKETS	16	16	16	16	64
				MARKETS	16	16	16	16	64
				SMALL RETAILERS	16	16	16	16	64
			DEVON	WHOLESALERS	12	12	12	12	48
				SUPERMARKETS	16	16	16	16	64
				MARKETS	16	16	16	16	64
				SMALL RETAILERS	16	16	16	16	64
			NEWCASTLE	WHOLESALERS	16	16	16	16	64
				SUPERMARKETS	16	16	16	16	64
				MARKETS	16	16	16	16	64
				SMALL RETAILERS	16	16	16	16	64
			SOUTHAMPTON	WHOLESALERS	16	16	16	16	64
				SUPERMARKETS	16	16	16	16	64
				MARKETS	16	16	16	16	64
				SMALL RETAILERS	16	16	16	16	64
TOTALS	64,100,000	100 %	11	4	300	300	300	300	1200

3.1.3.2. Sampling collection and transportation

Samples will be transported from sampling point to the analytical laboratory under refrigeration. (For Northern Ireland it may be necessary to transport frozen raspberries on dry ice).

3.1.3.3. Sample information

All labels from each sample will be collected and retained.

3.1.3.4. Sample receipt and photographs

Samples must be received at the analytical laboratory by Thursday 5 pm.

3.2. Microbiological testing

3.2.1.1. Detection of Norovirus

Detection of *Norovirus* will be performed following the protocol described in ISO/TS 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. International Organization for Standardization, Geneva, Switzerland. An additional calibration of the quantitative RTPCR assays using RNA standards will be performed.

3.2.1.2. Capsid Integrity Assay

Dependant on the successful outcome of experimental work currently being carried out in Fera with regard to applying the capsid integrity assay to soft fruits, it is our intention to perform this assay on all NoV-positive samples during the survey period.

3.2.1.3. Detection of Escherichia coli

Analysis shall be performed by standard methods. Results will be reported to Fera no later than 10 working days after receipt of the samples by Public Health England (PHE).

3.3. Quality Assurance (QA) and Quality Control (QC)

QA / QC will be documented in worksheets relevant to the virus detection methods being undertaken during the survey. The QC data will include information such as the Sample reference number, the analyst, date of sampling, storage conditions, condition of sample upon arrival etc. The QC of media / reagents will also be documented to ensure expiry dates are strictly adhered to and storage conditions of reagents are complied with according to manufacturer's instructions. Temperatures of refrigerators and freezers used for the storage of reagents and samples are checked daily with UKAS accredited temperature readers. Electronic balances are checked each time before use using weights accredited by UKAS and records kept in a logbook for future reference.

3.4. Data handling and reporting

All samples will be reported as either positive or negative for *Norovirus* GI and / or GII. Where samples are *Norovirus*-positive, numbers of genome copies detected will be given where possible. Results of reanalysis of *Norovirus*-positive samples using the capsid integrity assay will be reported as numbers of intact virus particle equivalents detected above any background count (assumed to be possibly due to ribonucleoprotein complexes).

4. Catering Environment

Prevalence of NoV in the catering environment in outbreak and non-outbreak premises

Recently it has been suggested that NoV genetic diversity and genotype profiles can be used to discriminate between foodborne outbreaks linked with transmission via food-handlers from those associated with food contaminated at source.

Foodborne outbreaks associated with the consumption of shellfish or other foods contaminated with sewage are often associated with multiple strains of NoV, including genotype GII-4, among the individuals implicated in the outbreaks (Gallimore et al, 2005a; Gallimore et al, 2005b), whereas in outbreaks associated with transmission via a food-handler, the same strain is often found in all involved, including the food-handler (Daniels et al, 2000; Sala et al, 2005; Vivancos et al, 2009). Currently there are no UK data on contamination of the catering environment with NoV to provide evidence of the role of the infected food handler in transmission.

In collaboration with Environmental Health Officers (EHOs), we will undertake a prevalence survey of NoV in the catering environment in outbreak and non-outbreak premises. This will allow us to assess the contribution of food handlers to contamination of the catering environment.

This study aims to investigate the following hypotheses:

(a) Contamination of the kitchen environment with NoV will be higher in premises that have recently reported a foodborne NoV 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.

4.1. Methods:

4.1.1. Study design and definitions

We will perform a prevalence survey in catering premises across north west and south east England. Catering premises will be defined as a commercial or voluntary organisation that prepares and serves food to the final consumer. This includes restaurants, public houses, cafes, takeaways, hotels, guesthouses, and caterers. It does not include passenger carrying ships that travel outside the UK, private houses, mobile retailers, manufacturers and suppliers.

4.1.2. Pilot study

In order to assess the feasibility and validate sampling protocols and schedules, referral and communications paths and suitability of the information and data collection forms, a pilot study was conducted in August 2014. Sampling was performed in ten premises in each of the two regions (20 in total) between July and August 2014. The feedback obtained from EHOs and data collected during this pilot have been feed into the design of the standardised form for data collection (Appendix 4). The target of sampling 10 premises per region was achieved in the expected time, and including sampling of one premises linked to a suspected food borne outbreak (see summary in Appendix 5). All environmental samples taken during the pilot study, including those from linked to an outbreak, were negative for the presence of *Norovirus* RNA (note that sampling was carried out in the summer, during a period of very low *Norovirus* activity in the community).

4.1.3. Surveillance Sampling

We have estimated that sampling 250 premises will allow us to detect environmental contamination in the kitchen at a level of 20%, with 95% confidence and an error in the final prevalence estimate of +/- 5%.

The sampling will be carried out at monthly intervals, include a similar number of premises each month (20-22 premises /month ; 10-11 in each of the regions included in the survey) and cover one calendar year to account for the known high risk period for NoV contamination. Premises will be selected at random to represent all food hygiene rating scores (see Appendix 6 for example sampling plan). Each Local Authority will sample across the six scores and across premises types that are represented in their area. National chains will be allowed, however sampling Officers will aim to include a variety of premises and national chains to not exceed 20% of premises sampled.

In London we will work with Local Authority (LA) EHOs undertaking routine inspections of catering premises across the London Boroughs and in North West England, Greater Manchester LAs will respond to support the sampling required for the study. Sampling will be divided between the LAs to ensure the requirements of the study are met. For London, of a total of 23 LAs, agreement to participate in the study has been confirmed for the following LAs: London Borough of Southwark, Royal Borough of Greenwich, London Borough of City of London, London Borough of Tower Hamlets and London Borough of Ealing (see Appendix 7) for breakdown of establishments available for sampling in these LAs). There is interest from additional LAs in the study, and if required, the inclusion of additional LAs can be readily arranged through the Association of London Environmental Health Managers.

For the North West of England local authorities representative from each of the four Food Liaison groups in the North West have agreed to participate specifically Sefton for Cheshire and Merseyside, Allerdale for Cumbria, Fylde for Lancashire and

Salford for Greater Manchester. In addition there are a number of other authorities who are also interested.

Inspections will be recorded using the current UK Food Surveillance System (UKFSS), as done routinely by Local Authorities. The Local Authority will select the sample type as swab and clearly mark in the notes field that this is for the NoVAS study. The UKFSS code will be used to link the bacteriology and virology swabs. The PHE Food, Water and Environmental (FEW) Microbiology Service Laboratory Information Management System, STARLIMS, does allow *Norovirus* to be selected as a test, so viral swabs will be logged onto this system alongside the swabs collected for bacteriological testing and once results are returned by the viral testing labs, results will be uploaded. This will mean that Local Authorities will get a report for both the viral and bacteriological testing, although the viral testing report will not be available in real-time.

Pre-moistened Polystyrene Shaft Viscose Tip Swab in Deionised Water (TS/6-62, Technical Service Consultants Ltd.) and SpongeSicle™ swabs with 10 ml neutralising buffer (SSL-10NB, Sterilab Services) will be supplied by the PHE FWE Microbiology Service for Local Authorities to collect bacteriological swabs alongside viral swabs for this study. Sampling Officers can collect samples from a defined template area or from a random area at the sampling point.

During inspections the following environmental surfaces will be swabbed using the virology swabs:

In the kitchen or food preparation area:

- The refrigerator door handle
- A food preparation surface (e.g. chopping board)
- Kitchen sink tap

In the toilets used by members of staff:

- The inside door handle of the toilet
- The toilet flush

Similar areas of the kitchen will be sampled with the bacteriology swabs, but not the same the exact areas; for the kitchen taps, one tap will be used for bacteriological sampling the other for virology sampling; for the food preparation surfaces, adjacent areas will be sampled using the standard 10cmx10cm square template.

In addition, the hands of up to five kitchen staff (the right hand if the individual is right-handed or the left hand if the individual is left-handed) will be swabbed for

virology testing only. Individual level data about staff will not be collected in order to increase compliance with sampling.

After sampling, all swabs will be sent back to the corresponding region's FWE laboratory; swabs for NoV testing will be then referred to the virology labs (Liverpool or VRD), and those for microbiological indicators will be tested at the FWE labs.

EHOs will complete the short questionnaire provided including details such as the type of premises (cafe, restaurant, pub, gastro-pub, takeaway, hotel etc.), number of covers (if applicable), number of staff, number of sinks and wash basins for hand-washing in the kitchen, number of toilets designated for staff, type of hand drier. In addition details on food hygiene rating score, confidence in management systems or business food safety culture scores will be recorded (see Appendix 4).

4.1.4. Sample sizes and sampling plan

We will supplement the prevalence survey by including premises that are being investigated because of a foodborne NoV outbreak (estimated to be up to 10 a year per region).

An outbreak will be defined as either (a) two or more people from more than one household who are thought to have a common exposure to proven NoV infection or (b) clinically on the basis of Kaplan's criteria. Foodborne NoV outbreaks will be defined according to the following criteria:

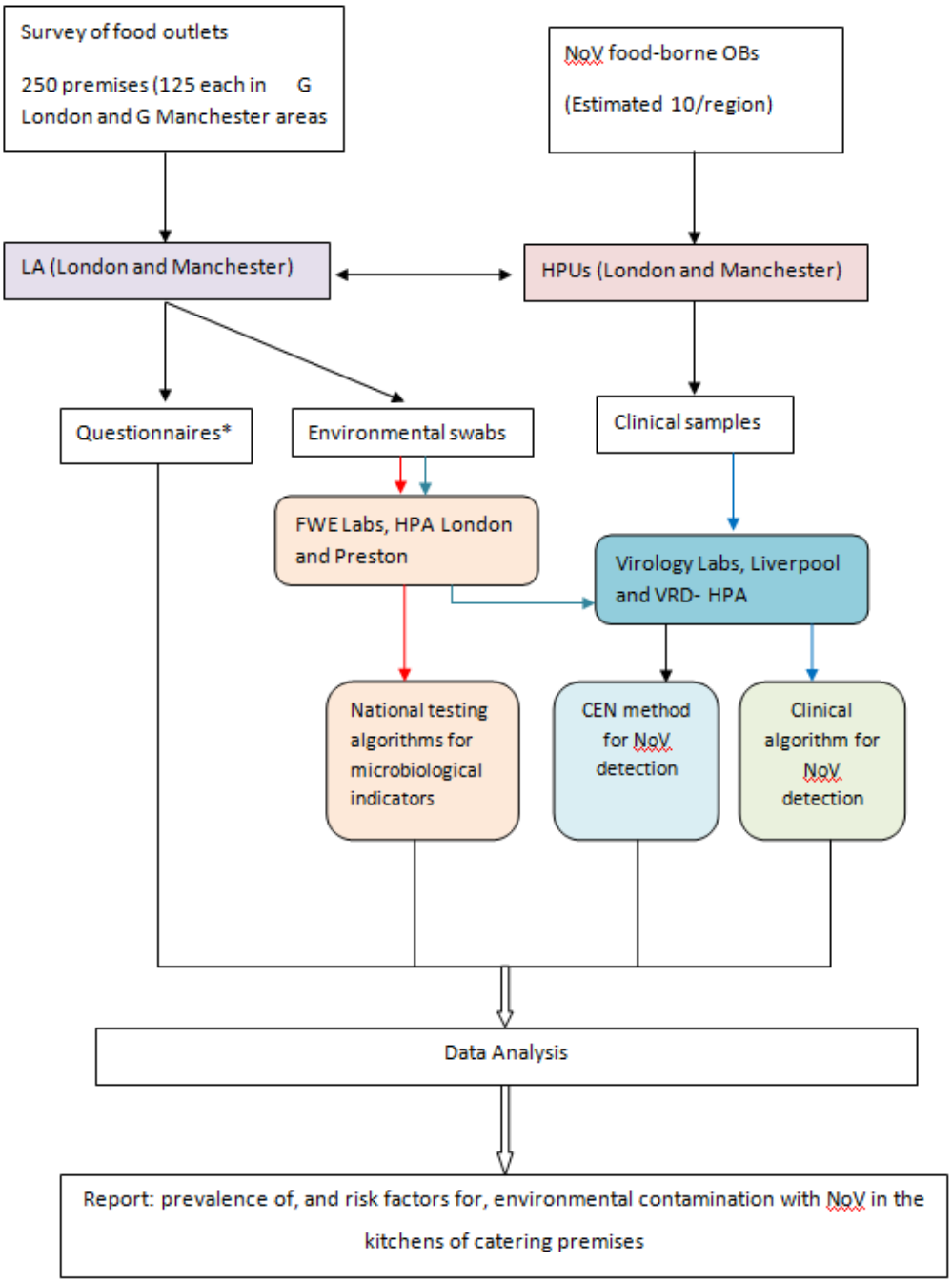
- The outbreak is a point source outbreak.
- There is a common food exposure – meal/buffet lunch/wedding breakfast.
- Foodborne transmission is the only or predominant transmission pathway identified by investigators.
- The cases do not have any other common exposure that could explain the outbreak apart 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, a stool sample from members of staff will be collected via the appropriate Environmental Health Department. where possible clinical specimens associated with outbreaks of NoV in catering establishments in the North West England and London will be submitted to the respective lead Public Health Laboratory for NoV detection. NoV positive outbreak case specimens associated with catering establishments will be referred for genotyping and strain characterization as described below. Outbreak case

genotypes will be compared with NoV genotypes from environmental or food handler positive samples for source attribution.

Outbreak premises will be revisited after cleaning has taken place and samples as in the first visit. Cleaning products and procedures used will be recorded in order to assess their effectiveness in reducing or removing norovirus RNA detection rates. For each outbreak premises, two matched control premises will be selected to be included in the monthly surveillance inspection visits. Matching will be done on food hygiene rating, size (number of covers) and type of premises.

Figure 1: Sampling and testing algorithm



4.1.5. Laboratory Methods

4.1.5.1. Virological testing Sample information

Detection of norovirus RNA in environmental and hand swabs will be performed by Reverse Transcription Polymerase Chain Reaction (RT-PCR) following a standardised method (CEN ISO/TS 15216-2:2013).

Detection of norovirus in clinical samples linked to outbreaks of gastroenteritis will be done in NHS laboratories using validated RT-PCR methods.

The method is described in detail in Appendix 8, and briefly consists of the following steps:

Upon arrival to the virology laboratory (EVU, PHE, London or Clinical Virology, RLBUHT, Liverpool), swabs will be immersed in lysis buffer with an external control (EC) added; samples can then be stored refrigerated (+4 to +8ºC) until processing (up to one week). Total nucleic acid will be extracted from the entire sample using magnetic silica beads in a semi-automated extractor system. Positive and negative controls will also be included in each run.

A fraction of the extracted nucleic acid will be used for the RT-PCR tests and the remainder will be stored immediately at -80ºC.

Each sample will be tested in three separate RT-PCR reactions for detecting RNA derived from *Norovirus* genogroup I, *Norovirus* genogroup II or the EC (*Mengovirus*). The results will be interpreted qualitatively only.

- Samples with cycle threshold (CT) values below the cut-off in either of the norovirus-specific assays will be considered positive.

- Samples with cycle threshold (CT) values above the cut-off in both of the norovirus-specific assays will be reported negative only if the EC RT-PCR results are within the expected CT range.

- Samples with cycle threshold (CT) values above the cut-off in either of the norovirus-specific assays in which EC RT-PCR results are not within the expected CT range (suggesting inhibitory samples) will be retested in a 1 in 10 dilution from the RNA extract (note that as all sample is extracted at once, it is not possible to re-extract the nucleic acid) and reported accordingly. In the event that the sample remains inhibitory in the dilution the results will be reported as invalid test.

Positive samples derived from the environmental or hand swabs or form clinical samples will be genotyped using the current reference genotyping standard methods (see Appendix 9). In brief, cDNA will be synthesised by performing a reverse transcription reaction using random hexamers. Subsequently, genotyping will be done by amplification and sequencing of a fragments of the norovirus capsid S domain, which this is sufficiently discriminatory for genotype assignment. Sequences will be 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 are of the same genotype, amplification and sequencing of the norovirus hyper-variable region (P2 domain) will be carried out,

and 100% homology between sequences from different samples indicates a common source and <100% identity indicates 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 are not obtained in the genotyping PCR, both cDNA and original PCR will be retested in the detection PCR to confirm positivity. When results are concordant, the results will be reported as "NoV RNA detected: untypable", if upon retesting the results are discordant with the original result, the sample will be result will be reported as equivocal.

4.1.5.2. Bacteriological testing

Sampling Officers can collect samples from a defined template area or from a random area at the sampling point and this must be clearly recorded on the sample request form submitted with the samples to allow testing to be directed to the right sample type.

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

- Coagulase positive Staphylococci, including Sataphylococcus aureus
- Escherichia coli
- Enterobacteriaceae

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

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

Results for swabs will be interpreted on the report as shown in Table 2.

Table 2: Interpretation for swabs collected for bacteriological testing as part of the NoVAS Study

Sample Type	Test	Interpretation		
		CONSIDERED **SATISFACTORY**	CONSIDERED **BORDERLINE**	CONSIDERED **UNSATISFACTORY**
Measured Area **(CFU per cm2)**	Aerobic colony count Escherichia coli Enterobacteriaceae	<100 <2 <2 <2	≥10^2 - <10^3 - - -	≥10^3 ≥2 ≥2 ≥2

	Coagulase positive Staphylococci			
Random Area **(CFU per swab)**	Escherichia coli Enterobacteriaceae Coagulase positive Staphylococci	<200 <200 <200	- - -	≥200 ≥200 ≥200

4.1.6. Communication

The PHE Food, Water and Environmental Microbiology Laboratories at London and Preston will meet with the Local Authorities in December, before the launch of the main study. This will constitute a training session to help standardise sample collection and also to allow any questions and issues to be resolved prior to the project start.

During sampling, Local Authorities will be able to contact the laboratory on a daily basis to ask questions, as they would do for their routine sampling. A report will be issued with the results of bacteriological samples (see interpretation of results in Table 1) in real-time and a report for norovirus testing will be reported once the testing laboratory has provided results from batch testing. Norovirus results will be reported as Norovirus RNA found (NoV PCR positive), or Norovirus RNA not found (NoV PCR negative).

Meetings will be held every four months with the Local Authorities through the main project to allow opportunities for issues to be raised and to feedback on progress to date.

A final meeting will be held with Local Authorities in each of the regions to share the final Work Package results and to allow them to feedback for the final project report.

4.1.7. Reimbursement of sampling costs

Local Authorities will be reimbursed £30 towards the cost of collecting samples for the NoVAS pilot and main project as part of Public Health England's costs for the project.

In order for PHE to pay this, Local Authorities will need to complete the PHE Payment Request Form (see Appendix 10) and return it to their local laboratory. The yellow highlighted sections of the form will need to be completed with Local Authority details. PHE will confirm the total sum each Local Authority will receive. This money will be reimbursed every three months.

5. Acknowledgments

We acknowledge the **Food Standards Agency** for the funding received for this research project (FSA Project Reference number: FS101040).

Collaborating partners on the project are (in alphabetical order):

Centre for Environment, Fisheries & Aquaculture Science (Cefas)
Food and Environment Research Agency (Fera)
Leatherhead Food Research
Liverpool Clinical Laboratories (Royal Liverpool and Broadgreen University Hospital Trust)
Public Health England
University of East Anglia
University of Liverpool

We acknowldge **Stericycle ExpertSOLUTIONS (SRCL ExpertRetrieval/QA)** for their role in performing market analysis on the study and product retrieval of samples.

6. Appendices

Appendix 1: Overall summary of Stericycle research to date (01 Jan – 17 Oct 2014)

Outlets researched to date			Outlets by food type					
Outlet type	TOTAL by outlet type	% of total	LETTUCE	% of total	RASP	% of total	OYSTERS	% of total
Restaurants	48	8%	0	0%	0	0%	48	100%
Supermarkets	309	53%	114	37%	121	39%	74	24%
Internet Sales	31	5%	0	0%	0	0%	31	100%
Fish Mongers	42	7%	0	0%	0	0%	42	100%
Wholesalers	14	2%	4	29%	5	36%	5	36%
Markets	45	8%	14	31%	31	69%	0	0%
Small Retailers	96	16%	46	48%	50	52%	0	0%
TOTAL stores researched to date	585	100%	178	30%	207	35%	200	34%

585 stores have been researched. Of these 585 stores, 309 are supermarkets (53% of the total stores assessed to date).

Lettuce was available in a total of 114 supermarkets, 37% of the 309 supermarkets visited.
Raspberries were available in a total of 121 supermarkets, 39% of the 309 supermarkets visited.
Oysters were available in a total of 74 supermarkets, 24% of the 309 supermarkets visited.

To date, the number of retail outlets researched for the availability of:

- Lettuce has been 178 (30% of overall outlets assessed)
- Raspberries has been 207 (35% of overall outlets assessed)
- Oysters has been 200 (34% of overall outlets assessed).

Areas to be sampled

Region	City
Northern Ireland	Londonderry
	Belfast
Scotland	Glasgow
	Dundee
	Aberdeen
Wales	Cardiff
	Bangor
England	Devon
	Manchester

Region	City
	Southampton
	London

Appendix 2: Summary of UK Oyster Production, Landings, Imports and Exports

Authors: Nick Taylor*, Mike Gubbins, David Ryder, Hannah Tidbury, James Lowther, David Lees.
Centre for Environment, Fisheries and Aquaculture Science (Cefas), Barrack Road, Weymouth, DT4 8UB.
*Correspondence: nick.taylor@cefas.co.uk

Background:
This report summarises available data relating to oysters produced in, exported from and imported into the UK for consumption. The aim of this study is to compile data on the origins of oysters available to UK consumers in order to help inform a structured sampling regime of oysters available at the point of sale, or provide the information required to weight estimates of *Norovirus* risk posed by oysters by their origin. In doing this we aim to highlight data gaps, assess data quality and uncertainty surrounding the available data. The study focuses on 2012 data as not all figures for 2013 were available at the time of producing this report.

Domestic Production:
All finfish and shellfish aquaculture production businesses (APB's) must be authorised. In England and Wales (E&W) this is done by the Centre for Environment, Fisheries and Aquaculture Science (Cefas), in Scotland it is done by Marine Scotland and in Northern Ireland (NI) it is done by the Department of Agriculture and Rural Development (DARD). UK is required to report aquaculture production statistics to Europe on an annual basis, though aquaculture sites are not legally required to make these figures available to each member states competent authority. Cefas compile production figures annually on behalf of the whole of the UK, with data for Scotland and NI being provided to them by Marine Scotland and DARD respectively. The annual production figures do not include data from the Channel Islands. Data is collected by Fish Health Inspectors either as part of their annual site visits or via telephone interview. Though inspectors have a good understanding of the industry and know whether the figures submitted are reasonable given the size of the production site, no formal validation is conducted. In addition to production (i.e. the laying down and on growing of stock), oysters are also harvested from beds. The majority of the beds lie in areas defined under several or regulating orders for which Cefas also collect data.

Table 1 shows there to be 451 authorised shellfish production business distributed between the countries comprising the UK in 2012. Of the 75 producers authorised in E&W only 29 were recorded as producing Oysters (Table 2), it is not possible to determine how many of the authorised shellfish sites in Scotland and NI produce oysters. **Pacific Oysters are the dominant species produced, with around 7.3 times more being produced than Native Oysters.** Though the greatest number of shellfish farms are present in Scotland, the greatest amount of Oyster production occurs in England, with very little occurring in Wales.

Table 1. UK Oyster Production (Tonnes) statistics for 2012 from authorised aquaculture production businesses.

Technical data	Scotland	Northern Ireland	Wales	England	Total
No. Authorised Shellfish Producers	335	41	7	68	451
Crassotrea gigas	251	137	6	440	834
Ostrea edulis	28	0	0	86	114
Total Production	279	137	6	526	948

In addition to this production data, oysters in E&W are also harvested from areas defined by several and regulating orders. When included in the production data the total number of Pacific and Native oysters produced in E&W is 853 tonnes and 86 tonnes respectively (Table 2). **This brings total UK production to 1333 tonnes of which 1247 tonnes are pacific oyster.** The vast majority of these oysters originate from B and long term B classified harvesting waters, with a small amount of production in C classified waters. In E&W no production occurs in class A waters (though a small proportion of Scottish Oysters are produced in class A waters). The distribution of oyster production in E&W can be seen in figure 1.

Table 2. 2012 Oyster production from aquaculture production businesses and several and regulating order areas for England and Wales based on the classification area in which they reside.

Classification	No. of Sites	Pacific Oyster production 2012 (Tonnes)	Native Oyster production 2012 (Tonnes)
Class A	0	0	0.0
Class B	2	33	2.0
Long Term Class B	25	810	83.9
Class C	2	10	0.0
Total	29	853	85.9

Oysters are also landed by fishing vessels. The Marine Management Organisation collates data submitted by fishers on all commercial species landed. These figures are not audited on submission, however records will be checked if a fishing boat is

boarded by fishery enforcement officers as part of a spot check. Fines will be levied if the records are not found to be up to date, so there is incentive for records to be kept accurately. These records show all landings into UK ports, however do not record any landings under 25kg in weight so there is the potential for substantial under reporting if small hauls of oysters are caught regularly. Table 3 shows the ports recorded as having landed oysters in 2012. The majority of these were native oysters landed on the South Coast of England (figure 1) by both UK and foreign vessels. According to the records, no oysters were landed in NI, Scotland or Wales. Compared to the production figures, the quantity of oysters landed via fishing is relatively low.

Table 3. 2012 MMO Oyster landings (tonnes) data by port. This data is collected and reported by fishing boats.

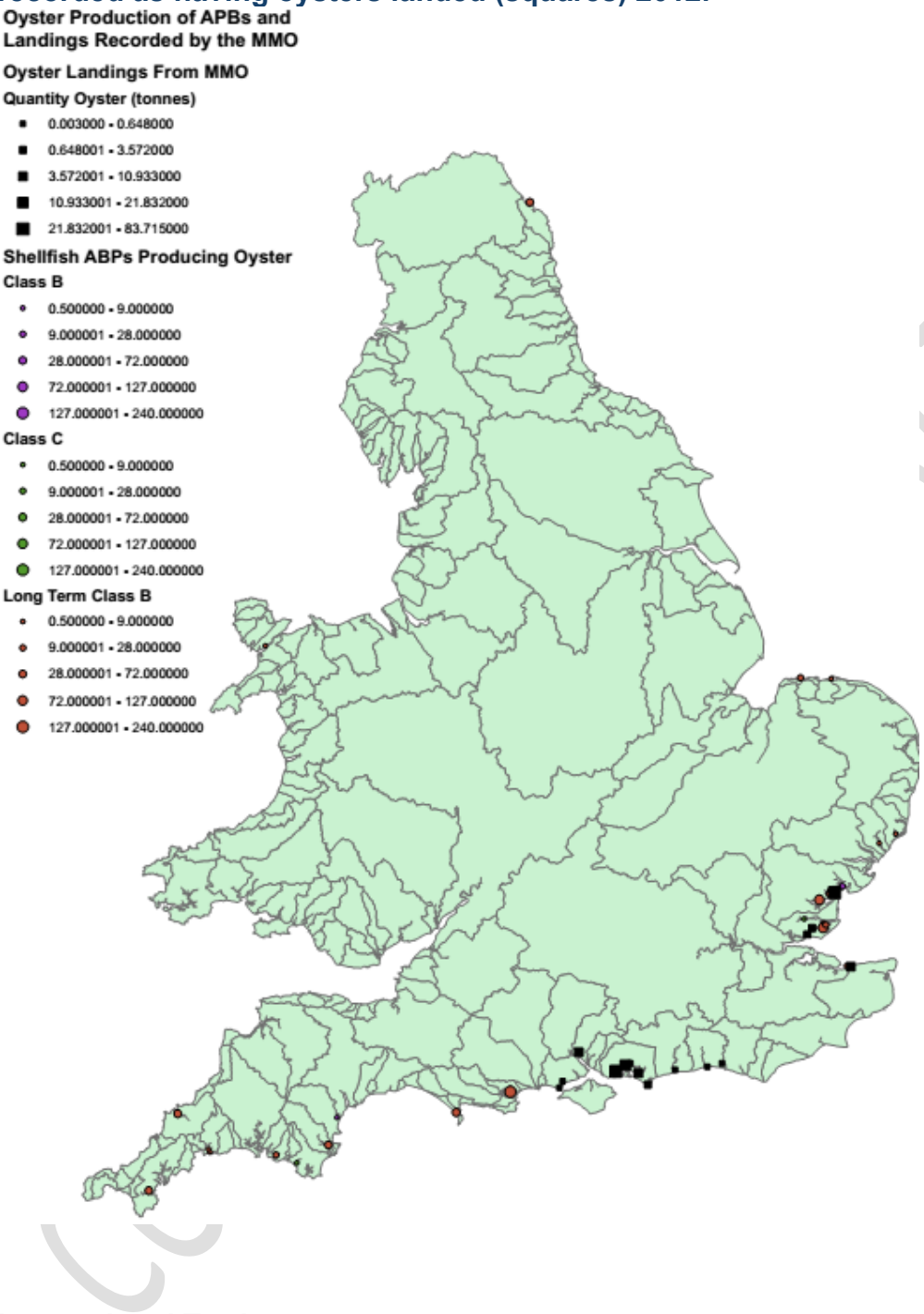
PORT NAME	NATIVE	PACIFIC	PORTUGUESE	TOTAL
BRIGHTON	0.0	0.0	0.0	0.0
EMSWORTH	0.3	0.0	0.0	0.3
ITCHENOR/EAST WITTERING	10.9	0.0	0.0	10.9
KEYHAVEN	0.6	0.0	0.0	0.6
LANGSTONE HARBOUR	21.6	0.3	0.0	21.8
LEIGH-ON-SEA	2.3	0.6	0.0	2.9
LITTLEHAMPTON	0.0	0.0	0.0	0.0
LYMINGTON	0.4	0.0	0.0	0.4
PORTSMOUTH	73.5	10.2	0.0	83.7
ROCHFORD	2.4	1.2	0.0	3.6
SELSEY	2.8	0.0	0.0	2.8
SHOREHAM	0.0	0.0	0.0	0.0
SOUTHAMPTON	4.3	4.2	0.0	8.4
SOUTHEND-ON-SEA	0.1	0.0	0.0	0.1
WEST MERSEA	53.5	0.0	0.0	53.5
WHITSTABLE	8.6	1.0	0.0	9.6
TOTAL (198.84)	181.5	17.3	0.0	198.8

Channel Islands oyster production data is collected separately to the rest of the UK, and largely relates to Jersey. **According to their official figures, Jersey produced 761 tonnes of Pacific Oysters in 2012.** Though this does not include landings or production from Guernsey (for which figures are not available), this is thought to be limited. According to figures published on the main Oyster producer in Jerseys website (http://www.jerseyoyster.com/jersey-oysters/), **around 90% of oyster produced in Jersey are exported to France**.

Adding the fishery data and that available from the Channel Islands to the other UK production figures shows that **in 2012, 2293 tonnes of oysters destined for human consumption were produced or landed in the UK and Channel Islands.**

Pacific oysters comprised 2025 tonnes of these, with only 267 tonnes of Native oysters being recorded.

Figure 1. English and Welsh oyster production sites (circles) and ports recorded as having oysters landed (squares) 2012.



International Trade

In addition to the oysters produced and landed in the UK, there is a substantial trade in oysters for the purpose of consumption. Her Majesty's Revenues and Customs (HMRC) compile figures for European and other international commodity trade in accordance with the 'General Trade System of Recording' guidelines. They have a freely available database that can be searched based on a list of available commodity codes to produce a summary of trade from different countries. UK data includes data from the Channel Islands. When data is compiled for all commodity codes containing the term 'oyster' (table 4), the results show that **in 2012 1606**

tonnes of oyster were exported from the UK, around two thirds of which went to other EU countries. A total of seven countries imported oysters to the UK in 2012, six of these were EU countries. In total **988 tonnes of oysters were imported into the UK in 2012**, 88% of which came from other EU countries with the remainder coming from South Korea. The biggest EU exporter of oysters to the UK was Germany (542 tonnes). This is unusual as very little production occurs in this country, however almost all of this product was preserved or processed and is therefore likely to have originated from other countries before redistributing after processing. **The majority of live oysters imported into the UK originate from the Irish republic (194 tonnes), followed by France (94 tonnes) and then the Netherlands (21 tonnes).** Breaking down the exported commodity into different product types shows that the vast majority of exported product is live pacific oysters (table 5). **Interestingly, almost twice the recorded UK production of Native oysters is exported from the UK,** suggesting that the available production and landings data is not representative of the total amount of UK native oyster production. In terms of imports, consistent with domestic production, **only 13% of oysters imported were Native Oysters**. Of the total 988 tonnes of oysters imported to the UK, **only 220 tonnes (22%) relates to fresh oysters (either pacific or native).** The majority of product imported to the UK has undergone some form of processing (e.g. smoking, drying, freezing) that may alter the *Norovirus* risk posed from them compared to fresh live product. It is possible that some of the oyster commodity imported to the UK actually originated in UK, but was exported to undergo processing before re-importing. It is not possible to determine this from the available data, but given that the majority of oysters imported to the UK have undergone processing, such trade could account for a substantial proportion of the total imports.

After accounting for oyster commodity exported from the UK, the figures suggest that around **685 tonnes of domestic oysters remain in the UK**. When combined with the 988 tonnes imported from overseas, the figures suggest that a total of **1673 tonnes of oysters were marketed for consumption in 2012.** Though it is known that only 220 tonnes of the 988 tonnes of oyster imported in to the UK is fresh product, it is not possible to assess the proportion of the 685 tonnes of UK produced oysters remaining in the UK that are marketed fresh.

Table 4. HMRC total oyster commodity trade data relating to countries imported to, and exported from the UK (including Channel Islands) in 2012 (Tonnes).

	Continent	Country	Export	Import	Dispatch	Arrival
EU	European Community	Belgium	0.0	0.0	2.8	0.0
		Cyprus	0.0	0.0	2.8	0.0
		Denmark	0.0	0.0	0.0	5.2
		France	0.0	0.0	624.5	92.8
		Germany	0.0	0.0	8.5	541.8
		Greece	0.0	0.0	0.5	0.0
		Irish Republic	0.0	0.0	60.4	205.6
		Italy	0.0	0.0	0.0	0.0
		Malta	0.0	0.0	3.6	0.0
		Netherlands	0.0	0.0	27.9	21.2
		Portugal	0.0	0.0	0.8	0.0
		Slovakia	0.0	0.0	0.8	0.0
		Spain	0.0	0.0	321.6	0.0
		Sweden	0.0	0.0	0.2	0.0
	Total		0.0	0.0	1054.5	866.6
NON EU	Asia and Oceania	China	12.3	0.0	0.0	0.0
		Hong Kong	501.4	0.0	0.0	0.0
		Malaysia	4.4	0.0	0.0	0.0
		New Zealand	0.0	0.2	0.0	0.0
		Singapore	12.2	0.0	0.0	0.0
		South Korea	0.0	121.1	0.0	0.0
	Total		530.3	121.3	0.0	0.0
	Middle East and N Africa	Lebanon	3.2	0.0	0.0	0.0
		Qatar	1.0	0.0	0.0	0.0
		Uae	16.0	0.0	0.0	0.0
	Total		20.3	0.0	0.0	0.0
	North America	Canada	0.3	0.0	0.0	0.0
		United States	0.3	0.0	0.0	0.0
	Total		0.6	0.0	0.0	0.0

	Sub-Saharan Africa	Ghana	0.1	0.0	0.0	0.0
		Kenya	0.3	0.0	0.0	0.0
		Mauritius	0.2	0.0	0.0	0.0
		Zimbabwe	0.5	0.0	0.0	0.0
	Total		1.0	0.0	0.0	0.0
	Western Europe exc EC	Norway	0.8	0.0	0.0	0.0
Total			552.9	121.3	0.0	0.0
Grand Total			552.9	121.3	1054.5	866.6

Table 5. HMRC commodity trade data relating to the form in which oysters were imported to, and exported from the UK (including Channel Islands) in 2012 (Tonnes).

	Non EU		EU	
HMRC Commodity code and descriptor	Export	Import	Dispatch	Arrival
30719900 - Oysters, even in shell, frozen, dried, salted or in brine (excl. smoked)	1.7	84.9	200.6	12.5
30719100 - Oysters, smoked, even in shell, even cooked but not otherwise prepared	0.4	0.0	2.8	0.0
30711900 - Oysters, even in shell, live, fresh or chilled (excl. live flat oysters Ostrea weighing incl. shell <= 40 g)	549.8	35.3	384.6	184.7
30711100 - Live flat oysters (Ostrea) weighing incl. shell <= 40 g	0.9	0.0	399.1	126.6
160551000 - Oysters, prepared or preserved (excl. smoked)	0.1	1.1	67.3	542.8
Total	552.9	121.3	1054.5	866.6

Inter and intra annual variability in trade and production

Only annual production data is available for the UK, it is therefore not possible to look at seasonality in production. It is however possible to compare production between 2013 and 2011 (table 6), this data suggests that annual domestic production is highly variable. The difference in production between 2013 and 2012 can be explained by the authorisation of a large new production site on the south coast of England.

Table 6. Summary of Oyster production from authorised Shellfish farms in the UK 2011 – 2013.

	2013	2012	2011
Scotland	241	279	269
Northern Ireland	137	137	261
Wales	3	6	3.5
England	936	526	735
Total	1317	948	1268.5

Imports and exports of oysters to and from the UK were fairly consistent in 2012 and 2011, they were however substantially lower in 2010 (table 7). Though this could be a result of changes in trade patterns, there have also been changes to the way different commodities are recorded and these changes may explain the disparity in figures. Limited data on trade is available with greater than annual resolution, however it has been possible to obtain monthly data for live oyster imports from the EU in 2010 (table 8). These data shows that trade varies greatly between months and species. For native oysters, imports to the UK were relatively low between April and August, but very high in December. For Pacific Oysters the greatest number of imports were observed in February and March. High trade levels correspond to the months with the greatest rainfall, known to be associated with high *Norovirus* levels in oysters. In addition to the variability in import quantities between months, there was also variation between the countries exporting to the UK, with certain countries such as Spain and Germany only supplying the UK in a single month of the year and others (e.g. France) supplying the UK every month.

Table 7. Summary of total Oyster commodity trade data based on HMRC statistics 2010 – 2012.

	2012	2011	2010
Non EU Export	553	575	275
EU Dispatch	1055	998	777
Total Exported	1608	1573	1052
Non EU Import	121	122	108
EU Arrival	867	665	184
Total imported	988	787	292

Table 8. Imports of live oysters by month and exporting country in 2010 based on HMRC data.

	Jan	Feb	Mar	Apr	May	Jun	Jul	Aug	Sep	Oct	Nov	Dec
Live Flat Oysters												
FRANCE	0	301	1620	0	0	0	0	0	3354	471	2073	681
IRISH REPUBLIC	73	61	103	61	61	61	49	49	61	7639	61	25808
ITALY	805	0	0	0	0	0	0	0	0	0	0	0
NETHERLANDS	579	768	639	512	344	509	394	509	400	414	463	297
Total	1457	1130	2362	573	405	570	443	558	3815	8524	2597	26786
Live Pacific Oysters												
DENMARK	0	0	28847	0	0	0	0	0	0	0	0	0
FRANCE	316	385	105	78	176	133	483	636	637	316	254	134
GERMANY	0	0	0	0	0	0	0	0	0	126	0	0
IRISH REPUBLIC	33	60189	12824	17	22	15	0	6959	0	0	2463	22
NETHERLANDS	1115	8443	367	984	378	446	590	317	618	3299	325	492
SPAIN	0	0	0	0	0	0	0	0	0	0	102	0
Total	1464	69017	42143	1079	576	594	1073	7912	1255	3741	3144	648

Conclusions

Based on the best data available, this report suggests that in 2012 a total of 1673 tonnes of oysters were marketed in the UK for human consumption. Around two thirds of these oysters were imported to the UK, predominately from the EU. Though UK produced oysters only constituted around a third of those marketed, it is likely that the UK production figures underestimate total production, as oysters gathered from wild beds and sold directly to purification centres, and small landings by fishing vessel of less than 25kg will not be captured in the data. This underestimate is likely to be especially relevant in terms of native oyster production, which is highlighted by the disparity between the production figures and export data. Data relating pacific oyster production and landing is likely to be more reliable, which is important as these constitute the majority of oysters marketed in the UK. Of the oysters marketed

in the UK, the majority are produced in waters classified as B or long term B, which may suggest they are exposed to a similar level of risk in terms of exposure to faecal contamination.

Though no seasonal production data was available, quantities of oysters imported into the UK are highly variable throughout the year, but are generally low in the summer and high in the winter. It will be important for the proposed study to determine whether product from different countries show differences in the *Norovirus* titres detected. If differences are detected between domestically produced and imported product, it will be necessary to obtain data on the monthly sales ratios of proportion of product originating from different countries each month in order to allow accurate estimations of risk to be made.

Three quarters of oysters imported into the UK had been processed in some way (e.g. smoked or cooked), and it is possible that some of these imported oysters originated in the UK, but were sent overseas for processing before redistribution. The impact of the various processing types on *Norovirus* titres is unknown and requires investigation if an accurate assessment of the contribution of oysters to annual *Norovirus* cases is to be derived. No data is available to determine whether the majority domestic oyster product remaining in the UK is processed prior to sale, or whether it is sold fresh. Again, this is important to understand if the risk of *Norovirus* transmission posed is to be estimated.

This report helps understand the quantities and origins of Oysters marketed in the UK annually, however, the following questions relating to consumer habits and regarding outlet types, their relative market share and sourcing practices need addressing if the risk posed by oysters in terms of contributing to annual *Norovirus* cases in the UK is to be quantified:

- What proportion of domestic product is sold fresh, frozen or processed?
- What proportion of EU imports are sold fresh, frozen or processed?
- What proportion of third country imports is sold fresh, frozen or processed?
- What proportion of fresh product is eaten raw?
- What proportion of frozen product is eaten raw?
- Types of outlet selling fresh oysters?
- What proportion of total annual oyster sales goes through each outlet type?
- What is the ratio of natives to pacific oyster sold by each outlet type?
- For each outlet type what proportion of their oysters are sourced locally, nationally or via imports?
- Is there regional variation in sourcing (e.g. London from France rest of UK via local producers)?
- For each outlet type, what proportion of the oysters are sold fresh, frozen or processed?

Sampling recommendations and justification:

Four main study options are available for the quantification of *Norovirus* risk from oysters. These depend on the amount of data available on the origins and marketing of oysters prior to the survey, and the willingness of outlets to participate in the study.

Option 1: Random sampling stratified by time, region, outlet type, product origin and type (i.e. fresh or processed). If sufficient data can be acquired prior to the survey commencing, and outlets are likely to cooperate in the study, it may be possible to design the study so that the outlets to be visited, and samples to be taken are determined in advance of visits to outlet. This study would ensure that the samples obtained are representative of what the consumer is exposed to. The limitation of this study is likely to be that given then number of samples it is possible to take each month, it will be difficult to stratify to this level an retain informative levels of statistical power. It is also unlikely that the resolution of data required to develop such a study will be available, or that outlet participation will be high enough to achieve a study of this nature.

Option 2: Random sampling stratified by time, region and outlet type. For each region, a sample population of outlets representative of the proportion of the market they constitute requires generating. Each month outlets are selected at random from each of these regions, and data on product origin and type collected at the point of sampling. If an outlet refuses to participate in the study, another can be selected at random. Post hoc adjustments to the *Norovirus* risk can be made by weighting *Norovirus* titres by data on relative contribution of different product types on market share. This study type relies on less prior information that option 1 and is more resilient to outlets failing to participate in the study. Based on data availability and confidentiality issues, this study seems the most promising option.

Option 3: Random sampling stratified by time and region. Should it not be possible to determine how much of the oyster market is supplied by different outlet types, sampling will have to be based on the best sampling frame that can be generated for each region. Sampling will have to be based on a random selection of outlets from this population, but risks particular outlet types being under or over represented. As for option 2, post hoc adjustment will be required to ensure that the risk from oysters is weighted by the relative market shares and product types.

Option 4: Cohort study of outlets willing to participate in the study. This is the least favoured option as it will produce the most limited data in terms of *Norovirus* exposure, however should willingness to participate be low, this may be a worthwhile option. By following the same sites in each region through time, the study will provide estimates of variability in *Norovirus* levels between regions and the variability within outlet types over time. The data obtained from this study type will however be limited as it will only be possible to sample a limited demographic within the population and the ability to quantify the national exposure risk will be difficult.

Appendix 3: Quantitative detection of *Norovirus* and hepatitis A virus in bivalve molluscan shellfish

Cefas

European Union Reference laboratory for monitoring bacteriological and viral contamination of bivalve molluscs

The Centre for Environment, Fisheries & Aquaculture Science,
Weymouth Laboratory,
Barrack Road,
The Nothe,
Weymouth,
Dorset, DT4 8UB, UK
Tel: +44 (0)1305 206600, Fax +44 (0)1305 206601

APPENDIX 3

GENERIC PROTOCOL

Quantitative detection of norovirus and hepatitis A virus in bivalve molluscan shellfish

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any information contained within. All references to Cefas must be removed if any alterations are made to this publication

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1.0 Introduction

Filter-feeding bivalve molluscan shellfish (BMS) concentrate microbial contaminants occurring in their growing waters and may present a health hazard when consumed raw or lightly cooked. Human enteric viruses including hepatitis A virus (HAV) and norovirus, occurring as a consequence of sewage contamination of growing waters, are the pathogens most frequently associated with illness following shellfish consumption.

A variety of methods for the detection of the low levels of virus found in environmentally contaminated shellfish have been published all of which are reliant upon on the polymerase chain reaction (PCR) for amplification to a detectable threshold. Detection of human enteric viruses in BMS using the PCR is complicated by the complex non-homogenous nature of the sample, by the known presence of amplification inhibitors, and by the low titre of virus present; it is therefore necessary to use a virus/RNA extraction method that produces highly clean RNA preparations that are suitable for PCR detection. Viruses are extracted from the tissues of the BMS digestive glands using treatment with a proteinase K solution. RNA is extracted using a method based on virus capsid disruption with chaotropic reagents followed by adsorption of RNA to silica particles. Real-time RT-PCR monitors amplification throughout the PCR cycle by measuring the excitation of fluorescently labelled molecules. In the 5' fluorogenic nuclease real-time RT-PCR assay the fluorescent labels are attached to a sequence-specific nucleotide probe (hydrolysis probe) that also enables simultaneous confirmation of target template. These modifications increase the sensitivity and specificity of the PCR method, and obviate the need for additional amplification product confirmation steps post PCR. Due to the complexity of the method it is necessary to include a comprehensive suite of controls. The method described in this protocol enables quantitative determination of levels of virus RNA in the test sample.

2.0 Scope

This procedure describes liberation, concentration and quantification of HAV and NoV genogroups I (GI) and II (GII), from BMS tissues. Viral RNA extraction is by lysis with guanidine thiocyanate and adsorption to silica. Extracted viral RNA is amplified and detected by real-time RT-PCR. This protocol describes a method for quantitative determination of levels of virus RNA in the test sample and includes production of control materials. The procedure is suitable for live BMS taken directly from production areas or following commercial processing and retail sale. BMS that are commercially sold frozen may also be thawed and tested using this procedure.

3.0 Principle

3.1 Virus extraction

The BMS digestive glands are removed by a simple dissection procedure, followed by chopping and protease enzyme treatment to liberate viruses. Following low speed centrifugation the virus-containing supernatant is suitable for viral nucleic acid extraction. Details of addition of a spike process control (mengo virus) to the test samples are also described.

3.2 RNA extraction

It is necessary to extract RNA using a method that yields clean RNA preparations to reduce the effect of PCR inhibitors. In this protocol the chaotropic agent guanidine thiocyanate is used to disrupt the viral capsid. RNA is then adsorbed to silica to assist purification through several washing stages. Purified viral RNA is released from the silica into a buffer prior to real-time RT-PCR.

3.3 Real-time reverse transcription polymerase chain reaction (real time RT-PCR)

This protocol uses one-step real-time RT-PCR using hydrolysis probes (TaqMan®). In one-step real-time RT-PCR, reverse transcription and PCR amplification are carried out consecutively in the same tube. TaqMan® PCR utilises a short DNA probe with a fluorescent label and a fluorescence quencher attached at opposite ends. The assay chemistry ensures that as the quantity of amplified product increases, the probe is

broken down, and the fluorescent signal from the label increases proportionately. Fluorescence may be measured at each stage throughout the cycle. The first point in the PCR cycle at which amplification can be detected for any reaction is proportional to the quantity of template, therefore analysis of the fluorescence plots enables determination of the quantity of target sequence in the sample.

4.0 Safety precautions

Standard microbiology safety precautions should be applied throughout. Laboratories should perform a full risk assessment before performing this procedure.

5.0 Equipment

- Micropipettes.
- Micropipette tips of a range of sizes, 1000µl, 200µl, 20µl and 10µl.
- Pipette filler.
- Pipettes of a range of sizes, 25ml, 10ml, 5ml.
- Vortex mixer.
- Shaking incubator operating at 37°C and 320 rpm or equivalent.
- Thermoshaker operating at 60ºC and 1400 rpm or equivalent.
- Aspirator or equivalent apparatus for removing supernatant.
- Heating block capable of operating at 95°C or equivalent.
- Waterbath capable of operating at 60°C or equivalent.
- Bench centrifuge and rotor capable of running at 3,000 x *g* with capacity for 15/50ml tubes.
- Microcentrifuge.
- Centrifuge and microcentrifuge tubes/bottles of a range of sizes, 1.5ml, 15ml, 50ml etc.
- Sterile shucking knife (for opening shellfish) or equivalent.
- Rubber block for shucking (opening) shellfish or equivalent.
- Scissors and forceps for dissecting shellfish or equivalent.
- Heavy duty safety glove.
- Sterile Petri dishes.
- Razor blades.
- NucliSens miniMAG magnetic rack. **BioMerieux.** See http://www.biomerieux.com/ for information. Cat number; 200299.
- NucliSens miniMAG instrument. **BioMerieux.** See http://www.biomerieux.com/ for information. Cat number; 200305.
- 1.5ml tubes with screw caps suitable for use with the miniMAG/easyMAG extraction systems.
- PCR machine with real-time capacity capable of supporting TaqMan® chemistry.
- Consumables for real-time PCR, e.g. optical plates and caps.

6.0 Reagents

6.1 Reagents used as purchased

- Proteinase K (30 U/mg)
- NucliSens magnetic extraction reagents. **BioMerieux**. See http://www.biomerieux.com/ for information. Cat numbers; 200293 etc.
- NucliSens lysis buffer. **BioMerieux**. See http://www.biomerieux.com/ for information. Cat numbers; 284135, 280134 etc.
- RNA Ultrasense One-step qRT-PCR system. Invitrogen.

  See http://www.invitrogen.com/content.cfm?pageid=13 for distributors. Cat number; 11732-927.
- Nuclease free water

6.2 Prepared solutions/buffers

Note: Taqman® PCR buffers must be prepared no more than 24 hours before use. Short-term storage (<24 hours) at 2-6°C is appropriate. Always prepare enough buffer for at least one reaction more than required (for larger preparations a greater number of excess reactions may be necessary). With Applied Biosystems real-time machines, Rox should be used at 1 x concentration; for the Stratagene MX3000, Rox can be either used at 0.1 x concentration, or omitted from the mastermix. For other manufacturers consult the machine instructions.

- **Proteinase K solution**

  Add 20 mg proteinase K (30U/mg) to 200ml molecular grade water. Shake to dissolve then store in working aliquots at <-15ºC for a maximum of 6 months. Once defrosted store aliquots refrigerated and use within 1 week.

- **HAV Taqman® PCR buffer**

  Add the following reagents to a 1.5ml microcentrifuge tube

5µl/reaction	RNA Ultrasense 5X Reaction Mix (from RNA Ultrasense One-step qRT-PCR system)
1.25µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5µl/reaction	ROX Reference Dye (1 x or 0.1 x; see above) (from Ultrasense system)
12.5 pmol/reaction	HAV68 (FWD) primer
22.5 pmol/reaction	HAV240 (REV) primer
6.25 pmol/reaction	HAV150 (-) probe (see Appendix 1 for sequences)

  Add nuclease free water to a total volume of 20µl/reaction and mix by vortexing.

- **Norovirus GI Taqman® PCR buffer**

  Add the following reagents to a 1.5ml microcentrifuge tube

5µl/reaction	RNA Ultrasense 5X Reaction Mix (from RNA Ultrasense One-step qRT-PCR system)
1.25µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5µl/reaction	ROX Reference Dye (1 x or 0.1 x; see above) (from Ultrasense system)
12.5 pmol/reaction	QNIF4 (FWD) primer

22.5 pmol/reaction	NV1LCR (REV) primer
6.25 pmol/reaction	TM9 probe (see Appendix 1 for sequences)

Add nuclease free water to a total volume of 20µl/reaction and mix by vortexing.

- **Norovirus GII Taqman® PCR buffer**

Add the following reagents to a 1.5ml microcentrifuge tube

5µl/reaction	RNA Ultrasense 5X Reaction Mix (from RNA Ultrasense One-step qRT-PCR system)
1.25µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5µl/reaction	ROX Reference Dye (1 x or 0.1 x; see above) (from Ultrasense system)
12.5 pmol/reaction	QNIF2 (FWD) primer
22.5 pmol/reaction	COG2R (REV) primer
6.25 pmol/reaction	QNIFS probe (see Appendix 1 for sequences)

Add nuclease free water to a total volume of 20µl/reaction and mix by vortexing.

- **Mengo virus Taqman® PCR buffer**

Add the following reagents to a 1.5ml microcentrifuge tube

5µl/reaction	RNA Ultrasense 5X Reaction Mix (from RNA Ultrasense One-step qRT-PCR system)
1.25µl/reaction	RNA Ultrasense Enzyme Mix (from Ultrasense system)
0.5µl/reaction	ROX Reference Dye (1 x or 0.1 x; see above) (from Ultrasense system)
12.5 pmol/reaction	Mengo 110 (FWD) primer
22.5 pmol/reaction	Mengo 209 (REV) primer
6.25 pmol/reaction	Mengo 147 probe (see Appendix 1 for sequences)

Add nuclease free water to a total volume of 20µl/reaction and mix by vortexing.

6.3 Control materials

- **Mengo virus process control material**

Note: for preparation of this control material laboratories will require cell culture facilities including incubator(s), preferably with controllable CO_2 levels, cell culture consumables (flasks etc.) and media.

Mengo virus strain MC_0 (ATCC VR-1597) should be used unless proscribed by e.g. GMO regulations. In this case wild-type mengo virus (ATCC VR-1598) can be used. Mengo virus should best be grown in a 5% CO_2 atmosphere (with open vessels) or an uncontrolled atmosphere (closed vessels) on 80-90% confluent monolayers of HeLa cells (ATCC CCL-2). Recommended cell culture medium for this cell line is

Eagle's minimum essential medium with
2mM L-glutamine
Earle's BSS, adjusted to
1.5g/l sodium bicarbonate
0.1mM non-essential amino acids
1.0mM sodium pyruvate
1% streptomycin/penicillin
10% (growth) or 2% (maintenance) foetal bovine serum

Alternatively virus can be grown on FRhK-4 cells (ATCC CRL-1688). Recommended cell culture medium for this cell line is

Dulbecco's modified Eagle's medium with
4mM L-glutamine, adjusted to
1.5g/l sodium bicarbonate
4.5g/l glucose
1% streptomycin/penicillin
10% (growth) or 2% (maintenance) foetal bovine serum

To prepare mengo virus for process control, freeze and thaw a culture flask in which at least 75% cytopathic effect (CPE) has been reached, centrifuge flask contents at 3000 x g for 10min to clarify and retain supernatant. Dilute by a minimum factor of 10x in sample buffer, e.g. PBS, split into single use aliquots and store frozen at -80°C. This dilution must allow for inhibition-free detection of the process control virus genome using real-time RT-PCR but still be sufficiently concentrated to allow reproducible determination of the lowest dilution used for the process control virus RNA standard curve

- **Double-stranded DNA (dsDNA)**

Note: for preparation of these control materials laboratories will require capabilities for transformation and growth in solid and liquid media of *E.coli*, capabilities or kits for plasmid preparation, conventional PCR and purification of DNA from reaction mixes (in addition to the listed products) and a spectrophotometer capable of measuring at 260 and 280nm.

Control plasmids used by the EU-RL were developed by Prof. Albert Bosch (HAV; Costafreda et al., 2006) and Dr. Soizick LeGuyader (norovirus; Le Guyader et al., 2009). For HAV control plasmid was constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector (Promega; www.promega.com/tbs/tb086/tb086.pdf) at a *Hin*cII restriction site such that the target sequence was downstream of a promoter sequence for the SP6 RNA polymerase. For norovirus GI and GII control plasmids were separately constructed by ligating the target DNA sequence into the pGEM-3Zf(+) vector at a *Sma*I restriction site such that in each case the target sequence was downstream of a promoter sequence for the T7 RNA polymerase. The EU-RL may be able to supply these plasmids to NRLs upon request. [The EU-RL is also looking at the development of commercially available dsDNA controls].

Alternatively, separate control plasmids for each target virus can be constructed by individual labs by ligating the target DNA sequence into a suitable plasmid vector such that the target sequence is downstream of a promoter sequence for RNA polymerase.

The plasmid should be transformed and maintained in, and purified from, *E. coli* cells using standard molecular and microbiology techniques. Following purification of plasmid by e.g. commercial miniprep, **linear** DNA molecules suitable for use as quantification controls can be generated either by:-

a) linearisation of the plasmid with a single cutting enzyme which does not cut within the target insert. For the plasmids used by the EU-RL, linearise using *Eco*RI enzyme (HAV) or *Xba*I enzyme (norovirus GI and GII).

b) PCR amplification of an amplicon spanning the target region using a dilute solution of the plasmid as template. For the plasmids used by the EU-RL, the use of the pTAG 5 and pTAG 3 primers is recommended (see Appendix 1 for sequences).

Following generation of linear control DNA, a small amount should be cleaned up using e.g. a commercial PCR purification kit. The concentration of DNA can then be calculated using spectral absorption at 260 nm (spectral absorption at 280nm should also be carried out to monitor the purity of the DNA preparation, highly pure DNA should have an A260/280 ratio of around 1.8).

Multiplication of the A260 value by $5x10^{-8}$ (and by any dilution factor involved) will give the concentration of DNA in g/µl.

Divide this number by the mass in g of a single dsDNA molecule to calculate the concentration of DNA in copies/µl (the mass of an individual dsDNA molecule may be calculated by multiplying the length in bp by 607.4 (the molecular weight of an average bp) and dividing by the Avogadro constant (6.02×10^{23}) e.g. a molecule of 3000bp will have a mass of 3.02×10^{-18}g).

For the (linearised) plasmids used by the EU-RL the masses are as follows:-

HAV	$3.41x10^{-18}$g	(3383 bp)
Norovirus GI	$3.32x10^{-18}$g	(3287 bp)
Norovirus GII	$3.32x10^{-18}$g	(3292 bp)

For the pTAG PCR products amplified from the plasmids used by the EU-RL the masses are as follows:-

HAV	$3.41x10^{-19}$g	(338 bp)
Norovirus GI	$2.45x10^{-19}$g	(242 bp)
Norovirus GII	$2.50x10^{-19}$g	(247 bp)

The preparation of dsDNA should then be diluted with a suitable buffer (e.g. TE buffer with 2 ng/µl sheared salmon sperm DNA) to a concentration of approximately $1x10^{4}$ -$1x10^{5}$ copies/µl, and frozen in single use aliquots.

NOTE: do not use water only to dilute dsDNA to working concentration.

- **External control RNA (EC RNA)**

  Note: for preparation of these control materials laboratories will require capabilities for transformation and growth in solid and liquid media of *E. coli*, capabilities or kits for plasmid preparation, purification of DNA from reaction mixes (in addition to the listed products) and a spectrophotometer capable of measuring at 260nm.

  Double-stranded DNA control plasmids as described above are used for the production of EC RNA. These plasmids should be transformed and maintained in, and purified from, *E. coli* cells using standard molecular and microbiology techniques. Following purification of plasmid by e.g. commercial miniprep, a small amount should be linearised using a suitable restriction enzyme (to enable linearization of the plasmid at a point shortly downstream of the target sequence) and buffers as recommended by the manufacturer of the enzyme. For the plasmids used by the EU-RL, linearise using *Eco*RI enzyme (HAV EC RNA) or *Xba*I enzyme (norovirus GI and GII EC RNA). The reaction should then be cleaned up using e.g. a commercial PCR purification kit.

  EC RNA should be transcribed from 100-500 ng of purified linearised plasmid DNA using an in-vitro RNA transcription reaction mix prepared as recommended by the manufacturer of the relevant RNA polymerase enzyme. Following incubation, digestion of the DNA template using RNase-free DNase should be carried out according to the manufacturer's protocol.

  For the plasmids used by the EU-RL, EC RNA can be in vitro transcribed using the SP6/T7 Riboprobe combination system (**Promega**, see http://www.promega.com/catalog/country_select.asp?/default.asp&ckt=2 for information, cat no. P1460) as follows:-

  1. Add the following components at room temperature in the order listed:

5X transcription buffer	20µl
100 mM DTT	10µl

RNasin	2.5µl
rATP,rGTP,rCTP,rUTP mix (2.5mM each)	20µl
linearised template DNA (max 1µg/µl)	5µl
SP6 polymerase (for HAV EC RNA)	3µl
OR	
T7 polymerase (for norovirus GI/GII EC RNA)	3µl
Nuclease free water	39.5µl

Mix by pipetting

2. Incubate for 2 hours at 37°C.

3. Add 5µl RQ1 RNase-free DNase to the reaction.

4. Incubate for 15 mins at 37°C.

Regardless of the method used for in vitro transcription, the RNA should then be purified using RNA purification reagents (e.g. **QIAGEN** RNeasy Mini Kit [see http://www1.qiagen.com/SelectCountry.aspx for information, cat nos. 74103, 74104, 74106] using the manufacturer's RNA cleanup protocol) and eluting in 100µl RNase-free water.

The RNA preparation should be checked for freedom from significant contamination with DNA by assaying for target both with and without RT activity, for example by assaying with both TaqMan® mastermix where RT has been deactivated by heating at 95°C, and untreated mastermix. If levels of DNA contamination higher than 0.1% are found, the preparation should be subjected to further treatment(s) with DNase.

The concentration of RNA can then be calculated using spectral absorption at 260 nm.

Multiplication of the A260 value by $4x10^{-8}$ (and by any dilution factor involved) will give the concentration of RNA in g/µl.

Divide this number by the mass in g of a single EC RNA molecule molecule to calculate the concentration of DNA in copies/µl (the mass of an individual RNA molecule may be calculated by multiplying the RNA length in ribonucleotides by 320.5 (the molecular weight of an average ribonucleotide) and dividing by the Avogadro constant (6.02×10^{23}) e.g. an RNA molecule of 200 ribonucleotides will have a mass of 1.06×10^{-19} g

For the EC RNAs used by the EU-RL the masses are as follows:-

HAV	$1.33x10^{-19}$g	(250 b)
Norovirus GI	$6.73x10^{-20}$g	(126 b)
Norovirus GII	$7.00x10^{-20}$g	(131 b)

The preparation of RNA transcripts should then be diluted with a suitable buffer (e.g. TE buffer) to a concentration of approximately $1x10^{6}$ -$1x10^{8}$ transcripts/µl, and frozen in single use aliquots.

NOTE: do not use water only to dilute RNA transcripts to working concentration.

7.0 Method

7.1 Virus extraction

Immediately before any batch of samples is processed, pool together sufficient aliquots of mengo virus process control material for use with all samples (allow 10µl per sample plus 25µl excess).

Dilute a 20µl subsample of pooled material to 10^{-1} using nuclease-free water and store (for use in TaqMan®) at 4°C for a maximum of 24 hrs or in single-use aliquots at -20°C for longer periods.

Select a minimum of 10 live, or if frozen, undamaged animals (sufficient shellfish to produce 2g of digestive glands must be used). Wash off any mud from the shell.

Place the shellfish on a rubber block. Open the shells with a clean knife. Ensure the hand holding the shellfish is protected with a heavy duty safety glove.

Dissect out the digestive glands using scissors and forceps (or equivalent tools).

Transfer to a clean petri dish and chop finely with a razor blade.

Transfer a 2g portion of chopped glands into a centrifuge tube and add 10µl of mengo virus process control material.

Immediately add 2ml of proteinase K solution and mix well.

Incubate at 37°C in a shaking incubator or equivalent at 320 rpm for 60 min.

Carry out a secondary proteinase K incubation by placing the tube in a water-bath or equivalent at 60°C for 15 min.

Centrifuge at 3000 x *g* for 5 min, decant the supernatant, measure and record the volume and retain for downstream testing (RNA extraction).

7.2 RNA extraction

Note: for every set of samples a negative extraction control consisting of 500µl water should be extracted in parallel.

Add 2ml of NucliSens lysis buffer to a tube. Add 500µl of sample produced in 7.1 and mix by vortexing briefly.

Incubate for 10 min at room temperature.

Add 50µl of well-mixed magnetic silica solution to the tube and mix by vortexing briefly.

Incubate for 10 min at room temperature.

Centrifuge for 2 min at 1,500 x *g* then carefully discard supernatant by e.g. aspiration.

Add 400µl wash buffer 1 and resuspend the pellet by pipetting/vortexing.

Transfer suspension to a 1.5ml screw-cap tube. Wash for 30 sec using the automated wash steps of the miniMAG/easyMAG extraction systems or by vortexing. After washing allow silica to settle using magnet of the miniMAG/easyMAG extraction system. Discard supernatant by e.g. aspiration.

Separate tubes from magnet, then add 400µl wash buffer 1. Resuspend pellet, wash for 30 sec, allow silica to settle using magnet then discard supernatant.

Separate tubes from magnet, then add 500µl wash buffer 2. Resuspend pellet, wash for 30 sec, allow silica to settle using magnet then discard supernatant. Repeat.

Separate tubes from magnet, then add 500µl wash buffer 3. Wash for 15 sec, allow silica to settle using magnet then discard supernatant.

Note: samples should not be left in wash buffer 3 for longer than strictly necessary

Add 100µl elution buffer. Cap tubes and transfer to thermoshaker or equivalent.

Incubate for 5 min at 60ºC with shaking at 1400 rpm.

Place tubes in magnetic rack and allow silica to settle, then transfer eluate to a clean tube and retain at 4ºC for a maximum of 24 hrs or -20ºC for longer periods (up to 6 months).

7.3 TaqMan® analysis – general requirements

Note: for certain applications more or fewer TaqMan® replicates can be used for the sample or various control RNAs. In addition certain controls may be omitted or reduced in frequency e.g. where samples are normally non-inhibitory as determined by the EC RNA method, testing of 10^{-1} sample RNA may be omitted from routine analysis and only used where inhibition is detected. Where variations are made with regards to this method this approach should be subject to verification by the user laboratory.

TaqMan® analysis for all targets need not be carried out on the same plate – however the following restrictions must be observed;

Full sets of target assay control reactions (dsDNA dilution series, EC RNA and water only) should be used for every plate where sample RNA is assayed for that target.

Full sets of mengo virus assay control reactions (RNA dilution series from all relevant batches of mengo virus process control material and water only) must be included on every plate where sample RNA is assayed for mengo virus.

Prepare TaqMan® mastermixes immediately before starting procedure.

7.4 TaqMan® plate set-up - analysis of target viruses

Note: this section describes plate set-up for a single target virus.

Before starting 96 well real-time PCR plate preparation, prepare 10^{-1} dilutions of each sample RNA in nuclease free water.

Dependent on the target assays to be used, prepare 10^{-1},10^{-2},10^{-3} and 10^{-4} dilutions of target dsDNA in a suitable buffer (e.g. TE buffer).

For each sample and each target assay add 5µl of undiluted and 10^{-1} sample RNA to three wells of the plate each.

For each negative extraction control and each target assay add 5µl of undiluted RNA to one well.

For each target assay add 5µl of nuclease-free water to two wells.

For each target assay add 5µl of undiluted, 10^{-1},10^{-2},10^{-3} and 10^{-4} dsDNA to two wells each.

For each target assay add 1µl of undiluted EC RNA to one well for each undiluted sample RNA, one well for each 10^{-1} sample RNA and one well containing water only.

Add 20µl of the relevant TaqMan® mastermix to each well.

7.5 TaqMan® plate set-up - analysis of mengo virus

Defrost one aliquot of diluted (10^{-1}) mengo virus process control material for each batch used with the samples under test.

Heat at 95ºC for 5min using a heating block or equivalent to release RNA. using a heating block or equivalent.

Chill tubes rapidly, centrifuge at ≥ 3000 x *g* for 1min., then transfer the supernatant ("mengo virus RNA") to a fresh tube.

Prepare 10^{-1}, 10^{-2} and 10^{-3} dilutions of mengo virus RNA in nuclease free water for each batch of mengo virus process control.

Add 5µl of undiluted and 10^{-1} sample RNA to one well of the plate each.

For each negative extraction control add 5µl of undiluted RNA to one well.

For each batch of mengo virus process control add 5µl of undiluted, 10^{-1}, 10^{-2} and 10^{-3} mengo virus RNA to one well each.

Add 5µl of nuclease-free water to one well.

Add 20µl of the mengo virus TaqMan® mastermix to each well.

See layout on following page for example TaqMan® plate testing one sample for all four targets.

Example plate layout (single sample – all assays on one plate)

Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + HAV EC RNA	Test sample (-1) + HAV EC RNA	H2O + HAV EC RNA			
HAV dsDNA (undiluted)	HAV dsDNA (undiluted)	HAV dsDNA (-1)	HAV dsDNA (-1)	HAV dsDNA (-2)	HAV dsDNA (-2)	HAV dsDNA (-3)	HAV dsDNA (-3)	HAV dsDNA (-4)	HAV dsDNA (-4)
Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GI EC RNA	Test sample (-1) + GI EC RNA	H2O + GI EC RNA			
GI dsDNA (undiluted)	GI dsDNA (undiluted)	GI dsDNA (-1)	GI dsDNA (-1)	GI dsDNA (-2)	GI dsDNA (-2)	GI dsDNA (-3)	GI dsDNA (-3)	GI dsDNA (-4)	GI dsDNA (-4)
Test sample (undiluted)	Test sample (undiluted)	Test sample (-1)	Test sample (-1)	Test sample (undiluted) + GII EC RNA	Test sample (-1) + GII EC RNA	H2O + GII EC RNA			
GII dsDNA (undiluted)	GII dsDNA (undiluted)	GII dsDNA (-1)	GII dsDNA (-1)	GII dsDNA (-2)	GII dsDNA (-2)	GII dsDNA (-3)	GII dsDNA (-3)	GII dsDNA (-4)	GII dsDNA (-4)
-ve extraction control	H_2O	-ve extraction control	H2O	-ve extraction control	H_2O				
Test sample (undiluted)	Test sample (-1)	Process control virus RNA (undiluted)	Process control virus RNA (-1)	Process control virus RNA (-2)	Process control virus RNA (-3)	-ve extraction control	H_2O		

HAV assay
Norovirus GI assay
Norovirus GII assay
Mengo virus assay

5µl RNA (+/- 1µl EC RNA) & 20µl mastermix per well

7.6 TaqMan® assay run parameters

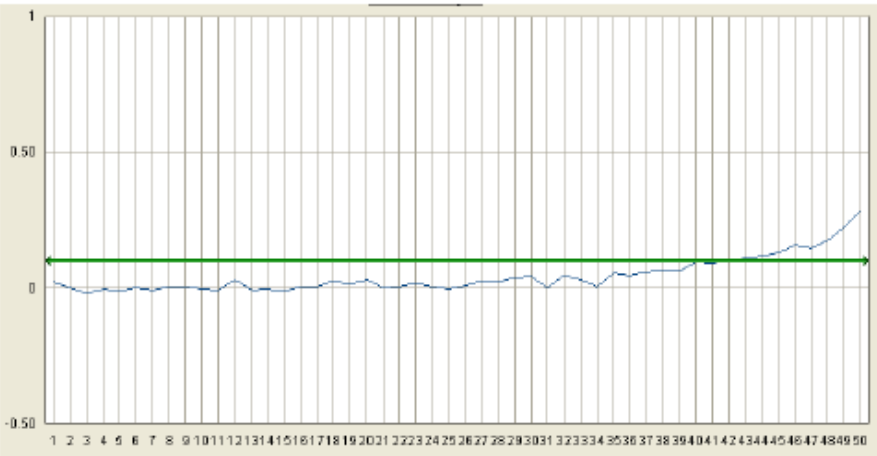
Run the TaqMan® assay with the following parameters:-

Step description		Temperature and time	Number of cycles
RT		55 °C for 1 h	1
Preheating		95 °C for 5 min	1
Amplification	Denaturation	95 °C for 15 s	45
	Annealing-extension	60 °C for 1 min 65 °C for 1 min	

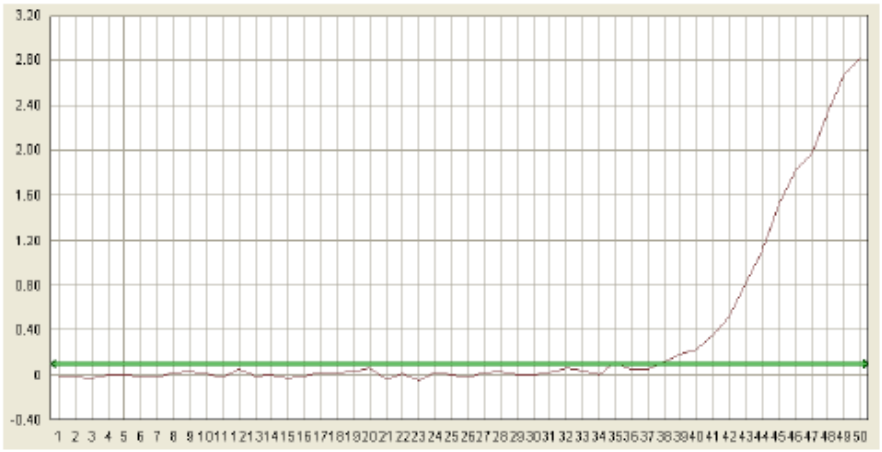
7.7 Analysis of results

Analyse the amplification plots using the approach recommended by the manufacturer of the real-time PCR machine. The threshold should ideally be set so that it crosses the area where the amplification plots (logarithmic view) are parallel (the exponential phase).

Check all amplification plots to identify false positive results caused by high or uneven background signal. Results for any wells affected in this way should be regarded as negative e.g.



Check all amplification plots to identify true positive plots where the recorded Cq value is significantly distorted by high or uneven background signal. Approximate correct Cq values should be noted (in addition to the recorded value) for any wells affected in this way. Corrected Cq values should be used for all quantity calculations.



e.g. in this case the recorded Ct value was 34.92, however it should be noted by the participating lab that the correct figure should be e.g. 38.

Check Cq values of all standard curves for any points that do not fall close to the line of best fit. These Cq values should not be incorporated into standard curve calculations. Points from a minimum of 3 (mengo virus RNA) or 4 dilutions (dsDNA) must be included. Curves with r^2 values of <0.98 should not be used for calculations.

To determine the amplification efficiency for each sample and each target calculate the average Cq values for the undiluted sample RNA + EC RNA wells. Use this value to estimate amplification efficiency by reference to the Cq value of the water + EC RNA well and the slope of the dsDNA standard curve. A sample (+ EC RNA) producing the same Cq values as undiluted EC RNA will have an amplification efficiency of 100%.

If the amplification efficiency is >25% results for the undiluted RNA should be used for that sample and target. If the amplification efficiency is <25% repeat calculation with the 10^{-1} sample RNA + EC RNA wells for the same target. If the amplification efficiency using the 10^{-1} RNA is >25% results for the 10^{-1} RNA should be used for that sample and target. If amplification efficiencies for both undiluted and 10^{-1} sample RNA are <25% results are not valid and the sample should be retested.

Use the Cq value for the mengo virus assay from the test sample RNA well (undiluted or 10^{-1} dependent on the amplification efficiency results; see above). to estimate mengo virus recovery by reference to the mengo virus RNA standard curve (if 10^{-1} sample RNA results are used multiply by 10 to correct for the dilution factor). A sample producing the same Cq value as undiluted mengo virus RNA will have a mengo virus recovery of 100%. To determine the extraction efficiency divide the mengo virus recovery by 0.5 and multiply by the total measured homogenate volume. Where the extraction efficiency is <1% sample results are not valid and the sample should be retested.

For each sample and target virus, take the Cq values for the sample RNA only wells (undiluted or 10^{-1} dependent on the amplification efficiency results; see above) and use these to calculate target concentrations (in detectable virus genome copies/µl RNA) ***for each replicate*** by reference to the relevant dsDNA standard curve. Negative replicates should be ascribed zero quantities. For each sample calculate the average of the concentrations for both replicates.

Multiply this value by 100 (undiluted RNA) or 1000 (10^{-1} RNA) to calculate the quantity of target in 100µl RNA/ 500µl extracted virus. Calculate the detectable quantity in the entire sample by dividing the above value by 0.5 and multiplying by the total homogenate volume.

To obtain the estimated quantity of target virus in detectable virus genome copies per g divide the number of genome copies in the entire sample by the starting weight (normally 2g) of the sample.

8.0 Uncertainty of test results

Uncertainty inherent in any test method, i.e. instruments, media, analyst performance etc. can be assessed by the repeatability and reproducibility of test results. These should be monitored through control tests analysed alongside sample tests, in-house comparability testing between analysts and external intercomparison exercises, which would highlight any uncertainties within the test methods.

9.0 References

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10.0 Appendix 1: Primer and probe sequences

HAV

HAV68 (FW):	TCA CCG CCG TTT GCC TAG	[Costafreda et al., 2006]
HAV240 (REV):	GGA GAG CCC TGG AAG AAA G	[Costafreda et al., 2006]
HAV150(-) (PROBE):	CCT GAA CCT GCA GGA ATT AA	[Costafreda et al., 2006]

Probe labelled 5' 6-carboxyfluorescein (FAM), 3' MGBNFQ (minor groove binder/non-fluorescent quencher)

Norovirus GI

QNIF4 (FW):	CGC TGG ATG CGN TTC CAT	[da Silva et al., 2007]
NV1LCR (REV):	CCT TAG ACG CCA TCA TCA TTT AC	[Svraka et al., 2007]
TM9 (PROBE):	TGG ACA GGA GAT CGC	[Hoehne & Schreier, 2006]

Probe labelled 5' FAM, 3' MGBNFQ

Norovirus GII

QNIF2 (FW):	ATG TTC AGR TGG ATG AGR TTC TCW GA	[Loisy et al., 2005]
COG2R (REV):	TCG ACG CCA TCT TCA TTC ACA	[Kageyama et al., 2003]
QNIFS (PROBE):	AGC ACG TGG GAG GGC GAT CG	[Loisy et al., 2005]

Probe labelled 5' FAM, 3' 6-carboxy-tetramethylrhodamine (TAMRA)

Mengo virus

Mengo 110 (FW):	GCG GGT CCT GCC GAA AGT	[Pinto et al., 2009]
Mengo 209 (REV):	GAA GTA ACA TAT AGA CAG ACG CAC AC	[Pinto et al., 2009]
Mengo 147 (PROBE):	ATC ACA TTA CTG GCC GAA GC	[Pinto et al., 2009]

Probe labelled 5' FAM, 3' MGBNFQ

pTAG (for pGEM series plasmids)

pTAG 5:	GCT ATG ACC ATG ATT ACG CCA A	[Maguire et al., 1999]
pTAG 3:	TGT AAA ACG ACG GCC AGT GAA	[Maguire et al., 1999]

Appendix 4: Environmental Sampling for Norovirus in food premises

Environmental sampling for norovirus in food premises.

This protocol is for environmental sampling of surfaces and equipment for detecting the presence of noroviruses in food premises.

Swabs will be used to sample surfaces of the kitchen environment and the toilets used by staff and should include:

- the refrigerator door handle
- a food preparation surface (e.g. chopping board)
- another frequently touched surfaces such as sink taps
- the inside door handle of the toilet used by staff members
- the toilet flush of the toilet used by staff members
- in addition to the above any surfaces known or suspected to have been contaminated, such as the floor, areas or objects in frequently used and touched (e.g. sink, taps, door handles, telephone, etc.) will also be swabbed in premises involved in a confirmed or suspected outbreaks of norovirus.

In addition swabs will also be taken from the hands of up to five members of the staff that handle food at the premises: the right hand if the individual is right-handed or the left hand if the individual is left-handed.

Swabbing technique:

1. Use a sterile cotton swab (provided by the PHE Food, Water and Environmental Microbiology Laboratory for use in this study) per surface to be samples, moisten with the sterile saline or phosphate buffered saline provided.
2. Swab the area, object or palm of the hand including the fingers and finger tips, applying a small amount of pressure. For surfaces and objects record the approximate size and shape of the area swabbed, and for flat surfaces choose a maximum area of 10 x 10 cm (100 cm^2).
3. Place the swab back into its original sheath (**without** adding any additional transport medium or buffer)
4. Label each swab with a unique reference and use the enclosed form to record the number of swabs taken, details of the areas swabbed Hand swabs should be anonymised and should not include any details that would allow identification of the staff sampled.
5. Pack swabs and completed form together and transfer to the Food Water and Environmental laboratory for testing using the dedicated courier system.

Samples should be sent to:

In London:
Public Health England
Food, Water and Environmental Microbiology Laboratory, London
PHE Colindale, 61 Colindale Avenue, London NW9 5EQ
Email: FWEM@phe.gov.uk
Telephone: 0208 327 6548/6550/6551
Local Project Coordinator: Nicola Elviss

In the North West of England:
Public Health England
Food, water and Environmental Microbiology Laboratory, Preston
Royal Preston Hospital, Sharoe Green Lane North, Preston PR2 9HT
Email: LabFwePreston@phe.gov.uk
Telephone: 01772522759
Local Project Coordinator: Andrew Fox

Environmental sampling for norovirus in food premises: Record sheet

Name of establishment			Post code		
Local Authority					
EHO responsible for sampling					
Pre-visit Food Hygiene Rating Score	Confidence in management systems		Practices	Structures	
0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐					
New Food Hygiene Rating Score	Confidence in management systems		Practices	Structures	
0 ☐ 1 ☐ 2 ☐ 3 ☐ 4 ☐ 5 ☐					
Premises type	Hotel ☐	Pub ☐	Cafe ☐	Take away ☐	Other ☐ *Please state*
Number of Kitchen Staff		Number of covers			
Number of sinks in the kitchen:		Number of toilets Dedicated to staff:		Type of hand drying: Paper towels ☐ Air (above) ☐ Air (blade) ☐	
Number of washbasins in the kitchen:					
Surface cleaning product in use					
Does cleaning product comply to British Standard?	Yes ☐ No ☐				
Date of Sampling		Surveillance ☐	Outbreak investigation ☐		
Total number of Swabs taken[1]					

FOR LABORATORY USE ONLY:			
Date received		Time Received	
Received By		Received From	
Cool box reference		Datalogger reference	
Temperature on receipt		Sample receipt:	Satisfactory ☐ Unsatisfactory ☐

[1] For routine surveillance maximum number of swabs to be taken is 10. During outbreak investigation up to 15 swabs in total can be taken ad referred for investigation.

Environmental Samples: Viral (Cotton-tipped, pre-moistened Swab ONLY) – up to five for routine sampling visit or up to 10 in an incident investigation

UKFSS code / Sample reference	Swab number	Surface sampled	Laboratory Number
	EV1		
	EV2		
	EV3		
	EV4		
	EV5		
	EV6		
	EV7		
	EV8		
	EV9		
	EV10		

Hand Swabs: Viral Only (Cotton-tipped, pre-moistened Swab ONLY) – up to five

UKFSS code / Sample reference	Swab number	Hand swab identifier	Attended Food Hygiene Training?	Laboratory Number
	HV1		☐	
	HV2		☐	
	HV3		☐	
	HV4		☐	
	HV5		☐	

Environmental Samples: Bacteriological (SpongeSicle™ Swab ONLY) – up to five for routine sampling visit or up to 10 in an incident investigation

UKFSS code / Sample reference	Swab number	Surface sampled	Laboratory Number
	EB1		
	EB2		
	EB3		
	EB4		
	EB5		
	EB6		
	EB7		
	EB8		
	EB9		
	EB10		

Additional Notes:

Appendix 5: Number of premises and swabs taken by LA, premises and food hygiene rating during the pilot study

North West region

Local Authority	Number Premises sampled	Premise type: Cafe	Hotel	Other (not stated)	Pub	Restaurant	Take away	Environmental swabs	Number of hand swabs
Allerdale BC	**3**				3			16	9
Salford City Council	**4**				1	2	1	20	8
Sefton Council	**3**		2	1				15	6
Wyre Borough Council	**3**	1	1				1	16	5
Grand Total	**13**	1	3	1	4	2	2	67	28

	Number of premises by Food Hygiene rating					
Local Authority	**0**	**1**	**2**	**3**	**4**	**5**
Allerdale BC			1	1		1
Salford City Council	1			1	2	
Sefton Council				1	1	1
Wyre Borough Council		1			1	5
Grand Total						

London region

Local Authority	Number of Premises Sampled	Premises Type						Environmental swabs	Hand swabs
		Cafe	Hotel	Other (not stated)	Pub	Restaurant	Take Away		
Royal Borough of Greenwich	4	1			1	1	1	16	3
London Borough of Southwark	4				1	2	1	39	9
London Borough of City of London	3			1		2		38	5
London Borough of Ealing	1					1		5	5
London Borough of Tower Hamlets	1			1					
Grand Total									

Local Authority	Number of premises by Food Hygiene Rating Scheme score					
	0	1	2	3	4	5
Royal Borough of Greenwich			1	1	1	1
London Borough of Southwark	1			1	1	1
London Borough of City of London		1			1	1
London Borough of Ealing	1					
London Borough of Tower Hamlets						
Grand Total						

Appendix 6: Example of sampling plan by month and LA

Month	Local Authority 1	Local Authority 2	Local Authority 3	Local Authority 4	Local Authority 5
1	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4
2	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3
3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5
4	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4
5	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3
6	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5
7	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4
8	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3
9	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5
10	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4
11	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3
12	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5	Two Premises: 1 x FHRS 0, 1 x FHRS 3	Two Premises: 1 x FHRS 1, 1 x FHRS 4	Two Premises: 1 x FHRS 2, 1 x FHRS 5

Appendix 7: Premises type and FHRS by local authority

		RatingValue							
LA Name	BusinessType	0	1	2	3	4	5	AwaitingInspection	Exempt	Grand Total
Southwark	Distributors/Transporters				3	5	7		1	16
	Farmers/growers					1	2			3
	Hospitals/Childcare/Caring Premises			3	7	37	98		4	149
	Hotel/bed & breakfast/guest house				2	4	16		2	24
	Importers/Exporters					2	1			3
	Manufacturers/packers		1		7	9	9		2	28
	Mobile caterer			3	9	5	20			37
	Other catering premises	1	9	6	29	61	70		4	180
	Pub/bar/nightclub		9	26	40	78	77	1	4	235
	Restaurant/Cafe/Canteen	6	59	56	137	164	277	2	4	705
	Retailers - other	3	74	33	136	229	202	1	103	781
	Retailers - supermarkets/hypermarkets		3	1	6	13	37			60
	School/college/university		1	1	6	20	85			113
	Takeaway/sandwich shop		32	31	65	53	53			234
Total		10	188	160	447	681	954	4	124	2568

		RatingValue								
LA Name	BusinessType	0	1	2	3	4	5	AwaitingInspection	Exempt	Grand Total
Greenwich	Distributors/Transporters	1			3	1	1	1	2	9
	Hospitals/Childcare/Caring Premises			2	10	14	68	30	7	131
	Hotel/bed & breakfast/guest house				1	3	6	8		18
	Manufacturers/packers		1		5	8	4	4	1	23
	Mobile caterer		4	1	11	20	15	12		63
	Other catering premises		3	1	20	30	48	44	10	156
	Pub/bar/nightclub		9	2	21	20	31	45		128
	Restaurant/Cafe/Canteen	3	24	11	80	80	124	51		373
	Retailers - other	3	33	9	52	35	39	121	90	382
	Retailers - supermarkets/hypermarkets		2		4	7	9	21		43
	School/college/university		3		15	33	52	27	3	133
	Takeaway/sandwich shop	7	29	10	54	44	40	13	3	200
Total		14	108	36	276	295	437	377	116	1659

		RatingValue							
LA Name	BusinessType	0	1	2	3	4	5	Awaiting Inspection	Exempt	Grand Total
Ealing	Distributors/Transporters		1		1		1		4	7
	Farmers/growers						1			1
	Hospitals/Childcare/Caring Premises		5	2	24	44	107	5	7	194
	Hotel/bed & breakfast/guest house			1	7	2	6			16
	Importers/Exporters		1			1				2
	Manufacturers/packers	2	7	1	17	17	37	13	6	100
	Mobile caterer		3	1	10	8	13	11		46
	Other catering premises	2	3	14	35	30	60	27	49	220
	Pub/bar/nightclub		16	10	45	32	58	9		170
	Restaurant/Cafe/Canteen	18	71	44	151	109	161	24	1	579
	Retailers - other	11	113	53	164	82	100	17	159	699
	Retailers - supermarkets/hypermarkets		4	4	6	8	32	1		55
	School/college/university		1	6	8	15	72	3		105
	Takeaway/sandwich shop	10	25	26	39	36	47	9		192
Ealing Total		43	250	162	507	384	695	119	226	2386

		RatingValue							
LA Name	BusinessType	0	1	2	3	4	5	Awaiting Inspection	Exempt	Grand Total
Tower Hamlets	Distributors/Transporters		6	1	16	25	22	2		72
	Hospitals/Childcare/Caring Premises		1			8	28			37
	Hotel/bed & breakfast/guest house						2			2
	Manufacturers/packers		6		8	14	33	28		89
	Mobile caterer				2	2	6	3		13
	Other catering premises		3	2	6	8	24	6		49
	Pub/bar/nightclub		7	3	47	53	67	8		185
	Restaurant/Cafe/Canteen	10	87	22	147	218	429	68		981
	Retailers - other	3	61	8	98	168	125	33	1	497
	Retailers - supermarkets/hypermarkets			2	1	7	33	2		45
	School/college/university		1	1	3	18	77			100
	Takeaway/sandwich shop	5	52	7	50	72	98	20		304
Total		18	224	46	378	593	944	170	1	2374

		RatingValue								
LA Name	BusinessType	0	1	2	3	4	5	Awaiting Inspection	Exempt	Grand Total
City of London Corporation	Distributors/Transporters						4			4
	Hospitals/Childcare/Caring Premises					1	5			6
	Hotel/bed & breakfast/guest house		1		1	2	5			9
	Importers/Exporters					2				2
	Mobile caterer		1		1		5	1		8
	Other catering premises		1	3	5	32	118	2	19	180
	Pub/bar/nightclub	3	11	25	30	89	91	2		251
	Restaurant/Cafe/Canteen	6	37	42	79	148	397	18	8	735
	Retailers - other		4	2	8	22	81	1	45	163
	Retailers - supermarkets/hypermarkets			1		4	23			28
	School/college/university		1			5	2			8
	Takeaway/sandwich shop	10	15	21	45	78	171	5		345
City of London Corporation Total		19	71	94	169	383	902	29	72	1739

LA Name	BusinessType	RatingValue 0	1	2	3	4	5	Awaiting Inspection	Exempt	Grand Total
Allerdale, Cumbria	Distributors/Transporters					2			2	4
	Farmers/growers								2	2
	Hospitals/Childcare/Caring Premises			1	5	9	23	1	7	46
	Hotel/bed & breakfast/guest house			3	14	65	157		1	240
	Manufacturers/packers			2	3	5	9		6	25
	Mobile caterer			3	2	8	10			23
	Other catering premises	1		2		7	25	1	26	62
	Pub/bar/nightclub	1	1	4	19	28	33	1	31	118
	Restaurant/Cafe/Canteen		5	6	19	65	113	1	9	218
	Retailers - other			9	12	37	64	1	39	162
	Retailers - supermarkets/hypermarkets				1	10	10		1	22
	School/college/university				1	9	64		7	81
	Takeaway/sandwich shop	1		10	14	24	31			80
Allerdale Total		3	6	40	90	269	539	5	131	1083

LA Name	BusinessType	RatingValue 0	1	2	3	4	5	Awaiting Inspection	Exempt	Grand Total
Fylde, Lancashire	Distributors/Transporters						1		1	2
	Farmers/growers								1	1
	Hospitals/Childcare/Caring Premises		2	1	6	23	41		27	100
	Hotel/bed & breakfast/guest house		2	1	3	11	18	1		36
	Manufacturers/packers				2	3	2		1	8
	Mobile caterer		1	3	3	10	13	1	1	32
	Other catering premises			2	4	3	19	4	20	52
	Pub/bar/nightclub		4	6	11	20	33	3	1	78
	Restaurant/Cafe/Canteen	1	10	11	18	36	59	3	8	146
	Retailers - other		2	2	7	22	45	1	58	137
	Retailers - supermarkets/hypermarkets			2		9	11			22
	School/college/university			1	1	2	32		1	37
	Takeaway/sandwich shop	1	4	11	14	21	31	3		85
Fylde Total		2	25	40	69	160	305	16	119	736

LA Name	BusinessType	RatingValue 0	1	2	3	4	5	Exempt	Grand Total
Salford, G Manchester	Hospitals/Childcare/Caring Premises		3	4	12	27	81	19	146
	Hotel/bed & breakfast/guest house		4		2	3	13		22
	Mobile caterer		3	2	5	26	44	1	81
	Other catering premises		3	1	8	13	95	12	132
	Pub/bar/nightclub		12	4	16	44	120	1	197
	Restaurant/Cafe/Canteen	2	23	15	65	65	168	25	363
	Retailers - other	5	89	11	52	82	195	32	466
	Retailers - supermarkets/hypermarkets		1	1	3	2	30		37
	School/college/university			1	3	16	75	1	96
	Takeaway/sandwich shop	5	58	23	57	63	75		281
Salford Total		12	196	62	223	341	896	91	1821

LA Name	BusinessType	RatingValue 0	1	2	3	4	5	Awaiting Inspection	Grand Total
Sefton, Cheshire and Merseyside	Farmers/growers				1	1			2
	Hospitals/Childcare/Caring Premises		2		9	31	191		233
	Hotel/bed & breakfast/guest house		3		1	18	42		64
	Mobile caterer				4	6	34		44
	Other catering premises		2		3	27	123		155
	Pub/bar/nightclub		7	2	25	60	153	2	249
	Restaurant/Cafe/Canteen		21	7	43	63	268		402
	Retailers - other	2	24	6	37	72	165	3	309
	Retailers - supermarkets/hypermarkets		4	1	2	5	47		59
	School/college/university				3	10	106		119
	Takeaway/sandwich shop	6	19	10	50	45	101	2	233
Sefton Total		8	82	26	178	338	1230	7	1869

Appendix 8: Bench protocols

bench protocols (SOPs) v9 PDF (3).pd

Appendix 9: Standard methods for norovirus genotyping

V-6897 Genotyping and Characterisation

Appendix 10: PHE Payment Request Form

PHEPayment-request -form.xlsx