



National Reference laboratory
for monitoring bacteriological and
viral contamination of bivalve

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UK NRL discussion paper: Detection of norovirus and hepatitis A in bivalve shellfish – current position on methodology and quality assurance

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1 Purpose and scope

The use of virus testing for bivalve molluscs to assist risk management decisions has generated requests for guidance on the appropriate methodology and quality assurance for such testing. This paper considers the current position on laboratory testing for viruses both in the EU and internationally and makes recommendations on best practice regarding laboratory methodology and quality assurance.

2 Background

Contamination of bivalve shellfish with norovirus (causing gastroenteritis) and hepatitis A virus (HAV) are recognised as the major human health risks associated with consumption of faecally contaminated shellfish. In the UK the Health Protection Agency (HPA) typically formally record 5-10 bivalve mollusc associated outbreaks in England and Wales each year with the majority being of suspect or confirmed norovirus aetiology. Historically the large majority of incidents occur in the winter and are proven to be, or highly likely to be, caused by norovirus. Examples of typical incidents are Christmas or Valentines Day banquet parties consuming oysters. High risk factors are cold weather (low water temperatures), high prevalence of norovirus gastroenteritis in the community, and high rainfall (leading to sewage system overflows). Although the majority of viral outbreaks have historically occurred during winter months a recent large outbreak in July 2007 illustrates that incidents may also occur during non-winter months. Data from the HPA infectious intestinal disease study (Wheeler *et al.*, 1999) shows that formally recognised norovirus outbreaks represent only a small part of the true burden of illness from this agent.

Risk management for bivalve molluscs aimed at control of faecal pollution risks currently relies heavily on the use of *E. coli* as an indicator of faecal (sewage) contamination and is enacted under European food regulations (Regulation 854/2004)(anon 2004a). However, although these regulations probably reduce the burden of infection, particularly for bacterial pathogens, they are not currently viewed as adequately controlling the virus risk. Particular problems are the greater robustness of viruses in the environment, and the different behaviour within bivalve molluscs, compared with bacterial faecal indicators. In the large majority of UK outbreaks shellfish are extracted from officially classified waters (generally class B), are depurated in approved plants in compliance with requirements, and are

processed in approved establishments i.e. they are produced in compliance with the EU legislation. End-product testing will generally also show products to be in compliance with the regulatory *E. coli* standard. These factors, and the importance of virus as a cause of bivalve shellfish associated human illness, is well recognised by both producers and officials.

These problems have focused attention on the need for development of methods for direct detection in bivalve molluscs of the viruses causing illness (principally norovirus and HAV) and, over the last decade, considerable progress has been made in this regard. All methods currently proposed are based on detection of virus genome using molecular techniques (- PCR). However, currently no standardised methods exist and quality assurance measures are generally not yet well developed. Because of these limitations virus testing is currently not incorporated as an element of EU legislative controls. However, the EU legislative text does foresee the adoption of virus controls when the methods are available for use and significant activity is currently focussed on this objective (see below).

However, despite the absence of a legislative framework or standardised methods, requests for virus testing arise quite frequently. Requests for testing may originate from officials, from retailers, and from producers in relation to:

- Human illness incidents of suspect or confirmed viral aetiology. Virus testing of implicated bivalve molluscs can assist investigations into the responsible food vehicle and the agent aetiology. Investigation of virus contamination in the attributed production area can contribute to risk assessment.
- Follow up investigations and checks following outbreaks. Virus surveillance in implicated production areas can help to ascribe ongoing risk and can help pinpoint sources of virus contamination.
- Responses to food alerts (eg RASFF). Official and/or producer responses to food alerts on exports (either outbreaks and/or overseas laboratory testing) may incorporate virus testing.
- Producer HACCP programmes. Following outbreaks, or other suspicion of virus contamination, virus testing may be a response to a requirement on producers to document the adequacy of measures to control virus contamination.
- Producer own-checks. Following suspect or confirmed virus outbreaks, or for due diligence purposes, producers may seek virus testing to document virus status of their products. Producers sourcing from several suppliers may also utilise virus testing to inform their stock purchasing decisions.
- Mandatory certification requirements for exports. Some overseas markets (eg Hong Kong and Singapore) have introduced product certification requirements for absence of norovirus.

These various pressures have lead to requests for virus testing services to both Cefas and other UK laboratories. The use of virus testing to assist official risk management decisions has generated requests to the NRL for guidance on the appropriate methodology and quality assurance for such testing. This paper considers the current position on laboratory testing for viruses both in the EU and internationally and makes recommendations on best practice regarding laboratory methodology and quality assurance.

3 Method standardisation in the EU

The European Committee on Normalisation (CEN) has an active working group addressing the development of a standard method for detection of norovirus and hepatitis A in foodstuffs (including bivalve shellfish). This working group is chaired by the European Community Reference laboratory (CRL) (Cefas, Weymouth laboratory) with active participation and practical contributions both from the UK and from a number of highly experienced European laboratories. The developing standard method has been underpinned by a number of research projects, particularly on bivalve shellfish, which have contributed methodology developments. Various stages of the developing method have been evaluated through practical inter-laboratory comparisons organised by the CRL. The current status is that a draft standard method is nearing completion and following that a full method validation exercise according to international rules is planned. The current timetable, as specified by CEN, for completion and reporting of the virus validation is 2012. It is anticipated that should EU Regulations adopt a virus standard in the future then the validated CEN method will be specified as the reference method. Other methods could be employed but would need to be shown to give equivalent performance though comprehensive validation studies conducted in accordance with international rules (ISO 16140). Given the likely future legal status of the CEN method (as a reference method), and the high level of European expertise applied to the method development and evaluation, it is recommended that, where possible, the CEN approach is adopted in the UK. Unfortunately the details of the draft CEN standard method are not yet available outside of the working group since it is still in development. However, the general principles are outlined in annex 1 and the following guidance can be given.

4 Methodology recommendations

Methods employing the polymerase chain reaction (PCR) for the detection of viruses in shellfish have been published since the mid 90's (Lees, 2000) and there are now numerous published references from laboratories world-wide. It is important to note that all published methods with demonstrable ability to detect viruses in bivalve shellfish or other foods have utilised PCR. Thus methods based on other possible approaches available for clinical samples (eg current ELISA methods) have not been demonstrated to have adequate performance (sensitivity) for foods and should not be used. Any proposals to use such methods would particularly need to demonstrate adequate sensitivity for detection of viruses in environmentally contaminated samples.

4.1 General requirements for operation of PCR laboratories and tests

PCR is an extremely sensitive technique and is well known to be susceptible to cross-contamination events within the laboratory and also to matrix interferences causing PCR inhibition. Hence the potential for both false positive and false negative results are well documented. The application of PCR to food testing requires significant investment by laboratories for both staff and equipment to ensure that analysis can be performed to a satisfactory standard. Laboratories should conduct PCR based testing in a laboratory environment consistent with internationally agreed guidance. This has significant resource implications such as the need to physically separate pre and post PCR activities to avoid cross-contamination. Laboratories not conforming with the physical separation requirements are highly likely to experience false positive test results at some point. The International Standards Organisation (ISO) have considered the laboratory and general testing requirements for analysis of food samples using PCR based methods (ISO/DIS 22118, ISO 22174:2005, ISO 20837:2006, ISO 20838:2006) and these general requirements should be carefully

evaluated and complied with. Compliance with the general ISO guidance is considered as a prerequisite in the CEN standard method.

4.2 Virus target

The principal viruses of concern in the UK and internationally are norovirus and hepatitis A virus. It is recommended that methods for bivalve shellfish specifically target these viruses. Norovirus causing human infections is classified into two genotypes norovirus genogroup I (GI) and norovirus genogroup II (GII). Assays should target both genogroups as they are both responsible for human infection and are both common contaminants of bivalve shellfish in UK waters. It should be noted that hepatitis A virus is currently a rare contaminant in UK waters.

4.3 Extraction procedure

Viruses are present in bivalve shellfish at low to very low levels compared with human clinical samples. However, unlike most enteric bacteria causing food-poisoning, enteric viruses can initiate an infection at very low levels. For example the infectious dose for norovirus is generally accepted to be around 10 infectious particles. Thus the low levels of virus generally found in bivalve shellfish have been demonstrated by several workers to pose a health risk (Bosch *et al.*, 1994; Sánchez *et al.*, 2002, Le Guyader *et al.*, 2003, 2006a). An additional complication is that the PCR is very susceptible to food matrix inhibition causing false negative reactions. This has been shown on numerous occasions to be a particular problem with bivalve shellfish. Norovirus and environmental strains of hepatitis A virus cannot be routinely grown in cell culture and thus biological amplification, a potential solution to these problems, cannot be employed. Consequently the matrix extraction procedure is critical and laboratories should be able to demonstrate that their methods are capable of recovery of low levels of contaminating virus at a purity consistent with PCR requirements (i.e. not inhibitory).

Most methods in use internationally now focus on the dissected bivalve digestive diverticulum (digestive gland) as the starting material for virus extraction. This organ has been shown to be the focus of contamination within the bivalve (Metcalf *et al.*, 1980, Romalde *et al.*, 1994) and recent work suggests that this may be due to specific receptors within the digestive tissues (Le Guyader *et al.*, 2006b). Digestive tissues comprise approximately 10% of the body mass of the bivalve but contain the large majority of the contaminating virus. Thus, targeting the digestive gland avoids the need to process tissues containing little virus but many PCR inhibitors. This reduces processing time and aids both sensitivity and the quality of the extract. It is recommended that UK laboratories utilise digestive gland extractions and this is the approach taken by CEN.

There are numerous publications detailing different approaches to the treatment of bivalve digestive glands for release, concentration and purification of virus. CEN selected and evaluated two strong candidates in widespread international use through an inter-laboratory ring trial. These were the Baylor method (Atmar *et al.*, 1995) and the proteinase K digestion method (Jothikumar *et al.*, 2005) as used at Cefas. Both methods were found to perform adequately in experienced hands however they varied considerably in their robustness. A particular problem experienced was that the more complex Baylor method required higher levels of implementation commitment and, generally, inexperienced laboratories struggled to achieve satisfactory results. By contrast all laboratories achieved adequate results with the simpler proteinase K method. Consequently the CEN method has adopted

the proteinase K method of digestive gland treatment and this is recommended for UK laboratories.

4.4 Nucleic acid purification

Following initial extraction of virus from bivalve tissues most published methods require further stages of purification and concentration of either intact virus or virus nucleic acid prior to PCR. This stage is vital for both removal of PCR inhibitors and concentration of virus template to achieve sufficient sensitivity. Again many approaches to purification and concentration have been detailed in the literature. For compatibility across a wide range of food-stuffs CEN has selected the widely used Boom method (Boom *et al.*, 1990). This method utilises guanidine thiocyanate (GITC) to denature viral coat proteins in combination with silica particles to bind released nucleic acid, which is then purified through successive washing stages before final elution in a small volume. The Boom method principles are also employed by a number of commercial kits for nucleic acid extraction and clean-up. The Boom method has been found to work well in CEN inter-laboratory trials and is recommended for UK laboratories. The latest draft of the CEN method has adopted a variant of the Boom method using magnetic silica technology as this is commercially available from a number of suppliers and was reported to perform well by a number of CEN participants. Quality assured commercial reagents can improve intra-laboratory comparability through removing a source of variability. Cefas has found the magnetic silica approach to produce comparable results in field samples to the conventional Boom procedure and it offers improved method logistics and reproducibility.

4.5 Reverse transcription

Both hepatitis A virus and Norovirus are single stranded RNA viruses and therefore require reverse transcription prior to PCR (RT-PCR). A variety of formats of RT-PCR have been used for this purpose by laboratories. Main variants include the use of either random hexamers or specific primers for the RT stage, and the use of a single enzyme mix containing both reverse transcriptase and taq polymerase (one-step) or separate reaction mixes for each stage (two-step). Cefas has conventionally used a two-step approach with random hexamers for the RT stage (Jothikumar *et al.*, 2005) and this has been robust and has performed consistently well in both our and other laboratories. An advantage of random hexamers is the production of complementary DNA (cDNA) for all RNA targets present. Thus a single RT reaction can produce a cDNA archive usable for all virus (or other) targets and also useable for virus sequencing confirmation – sequencing targets different genome regions to real-time PCR. However, this approach has some disadvantages for routine analysis since it uses several additional reagents, introduces additional pipetting steps, with the potential for contamination, and complicates quantitation. CEN has therefore proposed to adopt a one-step procedure using specific primers. Inter-laboratory evaluations in the CEN group using distributed RNA (to eliminate extraction variability) showed that some laboratories experienced difficulties with the one-step procedure for some targets (in particular Norovirus GI) in comparison with in-house procedures. The main problem appeared to be poor signal strength generation during amplification at low template concentrations leading to difficulty in result interpretation and poor sensitivity. This may be a function of probe design in combination with depletion of assay reagents at very low target copy numbers i.e. towards the assay limit of detection. Recent evaluations by Cefas have proposed refinement of the assay reagents which largely overcome these problems however these have still to be discussed and approved by CEN. In the interim a two-step procedure, as described by Jothikumar *et al.* (2005), can be recommended.

4.6 PCR

Many published methods have used conventional gel based PCR giving qualitative results (Green *et al.*, 1998; Leguyader *et al.*, 2000; Formiga-Cruz, *et al.*, 2002; Atmar, *et al.*, 1995). More recently real-time or quantitative PCR has been developed as an extension of conventional PCR with significant advantages for application to food monitoring. The advantages of real-time PCR are that it is much less liable to cause cross-contamination of subsequent samples since tubes do not have to be opened after amplification; it is significantly more efficient logistically; a confirmation step is with most chemistries built into the procedure through the use of labelled probes thus avoiding the need for confirmation of positives through sequencing; it is quantitative; it is standardisable. Because of these significant advantages all recent methodological developments in this area have utilised TaqMan real-time PCR (Jothikumar *et al.*, 2005; Loisy *et al.*, 2005; Costafreda *et al.*, 2006) and this should now be regarded as the methodology of choice. Cefas has, for several years, employed TaqMan real-time PCR for all routine virus testing for bivalve molluscs. ISO have published general requirements on the use of real-time PCR in food analysis (ISO/DIS 22119) and this should be adhered to. ISO have also published guidance on performance testing of thermal cyclers (ISO/TS 20836:2005) which is given for general information.

4.7 Primers and probes

PCR primers and probes need to be cross-reactive across strains to ensure that the assay is capable of detecting the diversity of strains seen in field samples. This is a particular problem for norovirus where strain diversity is extremely high. Inappropriate choice of PCR primers or probe (for real-time assays) will render an assay over selective and prone to false negative results. Design and evaluation of virus primers and probes requires specialist knowledge and access to a wide diversity of characterised clinical samples. It is thus not generally possible for food laboratories, in isolation, to develop new PCR primers or probes. Given the technical difficulties in this area it is recommended that UK laboratories follow the procedures emerging from the CEN working group. Real-time TaqMan primers/probe for hepatitis A virus are based in the highly conserved 5' non-coding region as published by Costafreda *et al.*, (2006). Hepatitis A virus is a well conserved virus in comparison with norovirus and this recommended primer/probe combination is unlikely to require modification through the emergence of, for example, new hepatitis A virus variants. Unfortunately this is not the case for norovirus where PCR primer/probe design is an ongoing challenge and where food testing laboratories must be alert to changes in clinical prevalence of strains and thus the relevance of their chosen assay. In particular real-time primers/probes for norovirus are a significant technical challenge and are still being evaluated. It is important to note that the diversity of norovirus GI and GII strains dictates the need for separate assays for these genogroups. A breakthrough occurred with the publication by Kageyama *et al.*, (2003) showing that the ORF1-ORF2 junction region of the norovirus genome was both highly conserved and suitable for real-time TaqMan primer design. Most subsequent publications for bivalve applications (Jothikumar *et al.*, 2005; Loisy *et al.*, 2005) have targeted this region with relatively minor modifications of the original proposals, and this is the area of choice identified by CEN. However, care should be employed since recent work at Cefas suggests that some modifications, particularly the introduction of redundancies into the probe, may significantly affect sensitivity. Following extensive desk evaluations the CEN group has selected candidate sets of norovirus GI and GII TaqMan primers/probes for further practical evaluation in the validation studies, as published by da Silva *et al.*, (2007). In addition Cefas has extensive positive

experience using the norovirus GI TaqMan assay described by Jothikumar *et al.*, (2005) and the norovirus GII TaqMan assay as described by Kageyama *et al.*, (2003). However this area is still under discussion, further changes are anticipated, and this advice will need to be updated. A variety of cross-reactive conventional norovirus primers are available. Cefas has previously experienced good results with primer sets designed by the UK HPA as described by Green *et al.*, (1998).

4.8 Confirmation

The sequence diversity of noroviruses presents significant technical challenges for the design of PCR primers capable of reacting with all relevant clinical strains. An approach to this problem is the introduction of nucleotide redundancies at key positions to broaden the cross-reactivity. However, this also increases the potential for non-specific amplifications causing false positive reactions. Sequencing studies by Cefas have demonstrated that a small percentage of non-specific reactions can occur using conventional PCR and therefore that an assay confirmation stage is important for specificity. In conventional PCR this can be through incorporation of a probe hybridisation stage or through amplicon sequencing - the gold standard. An advantage of real-time PCR is the incorporation in most chemistries (including TaqMan) of additional sequence specific probes within the assay thus providing in-built confirmation. Cefas has sequenced a number (10 for norovirus GI and 14 for norovirus GII) of TaqMan real-time PCR amplicons generated in the ORF1-ORF2 junction region and has found norovirus specific sequence in all amplicons thus confirming the specificity of this approach. However, some published real-time approaches (Richards, Watson *et al.*, 2004) have suggested the use of alternative real-time PCR chemistries using non-specific intercalating dyes, such as SYBR green, rather than sequence specific probes for detection of amplification products. This approach cannot be recommended for norovirus detection in shellfish since detection of non-specific reactions (false positives) is more complicated and may not be detected. Laboratories are advised to use real-time PCR with specific labelled probes, or if not using real-time, to employ a method of PCR confirmation such as probe hybridisation or sequencing.

4.9 Controls

Given the need for a highly sensitive PCR to detect the low levels of norovirus and hepatitis found in environmentally contaminated samples, and the susceptibility of such a sensitive PCR to cross-contamination (false positives) and also matrix interferences (false negatives), it is vitally important to incorporate alongside each test sample batch an appropriate suite of controls. The general internationally agreed requirements for PCR controls for food testing are given in ISO guidance (see references). Laboratories should carefully evaluate this guidance in the virus testing context and, where relevant, adopt it. Additional considerations exist for testing bivalve molluscs (and other food) for enteric viruses. The CEN group is considering the necessary suite of controls and this will be finalised following validation trials. In the interim the following suite of controls are recommended:

- negative PCR control
- positive PCR control
- negative extraction control
- RT-PCR control
- RT-PCR inhibition control
- process control

A negative extraction control is a known negative material (eg water, buffer, or a known negative shellfish homogenate) that is taken through the entire extraction procedure and amplified. It is thus a check of the extraction process for contamination. The positive PCR control, the negative PCR control and the extraction control should be tested for each target assay performed i.e. separately for norovirus GI, norovirus GII, and hepatitis A virus. The RT-PCR control is to check the reverse transcription stage of the RT-PCR reaction and uses an RNA template. The RT-PCR inhibition control checks for potential matrix interference or signal suppression (PCR inhibition) in the test sample. This control generally takes the form of an external RNA template added to both an aliquot of the material under test and to a well containing no test material. The difference between the reactions is used to calculate the degree of signal suppression (inhibition) caused by the test material. In the CEN method the RT-PCR control and the RT-PCR inhibition control are combined through the use of internal RNAs (IC RNAs) for each of the assays. Partners within the CEN group have developed a suite of plasmids engineered to include the specific target regions for the chosen assays (norovirus GI, norovirus GII, hepatitis A virus). Purified plasmid DNA is utilised for PCR controls; RNA run-offs from these plasmids are utilised as a combined internal RT-PCR control and inhibition control. If two-step RT-PCR is used application of RT-PCR and inhibition controls may be more complex but should be used where practically possible. The process control measures the recovery of virus during the whole extraction and test procedures using an analogous RNA virus or equivalent spiked into the test sample and assayed in parallel with the target viruses. Comparison of spiking material concentration and final extract concentration determines the recovery efficiency of the whole process. Measurement of starting and end concentrations of the process control should be determined by PCR. The principle is that the process control material should be representative of the target viruses (norovirus GI, norovirus GII, hepatitis A virus) and thus be extracted in a similar way and to the same efficiency. The target viruses themselves cannot be used as their addition to the test sample would render detection of the target virus impossible. Various process controls have been proposed including feline calicivirus, armoured RNA and FRNA bacteriophage. The CEN method proposes to use EMCV Mengo virus as described by Costafreda *et al* (2006). Mengo is a suitable analogue for hepatitis A virus and should also be suitable for norovirus thus avoiding the need for two separate process controls. This approach will be validated through the proposed inter-laboratory trials. Cefas has successfully previously utilised feline calicivirus, and is currently evaluating the use of Mengo virus. Satisfactory results have been obtained using either approach. The use of a process control gives information both about the suitability of the extraction in general (reagent batches, operator technique, etc) and also specific information about the success in extraction of the test sample. If the use of a process control is particularly problematic an alternative, but less effective approach, is to use a positive extraction control. This would comprise a positive shellfish sample containing relevant numbers of viruses, i.e. low levels, which is extracted with each test batch and shown to give test results within previously established criteria. This controls for successful extraction in general (reagents etc) but does not control for successful extraction of the particular test sample.

The laboratory should develop quality assurance acceptance criteria for each of the above controls which will determine the acceptability of each test and batch run.

5 Calculation of results and reporting

A difficulty with current laboratory reports for viruses in bivalve molluscs is the difficulty in result comparison between laboratories. Reports are generally qualitative

(presence or absence), or if performed using real-time PCR, report arbitrary values such as threshold cycle (CT) values or PCR units. This arises because of the current inability to calibrate PCR assays through the usual routes of either an external reference material or an independent measurement of virus titre through culture (norovirus cannot be cultured). Thus assays may be well standardised within a laboratory, and, as demonstrated by Cefas in a recent FSA study, capable of producing consistent and comparable results. However currently it is very difficult to compare results between laboratories without extensive information about assay make-up and performance. CEN has tackled this problem by proposing a quantitative approach grounded in a DNA calibration curve for each assay (norovirus GI, norovirus GII, hepatitis A virus) with DNA concentration measured using spectrometry at 260nm. The process control and RT-PCR inhibition control provide information on the efficiency of each test assay which, for quantitative assays, might be used to calculate overall target amounts in the test sample. This approach to quantification needs further validation however. It should be noted that it is necessary to generate DNA and RNA internal control reference materials to perform these measurements. As described above partners within the CEN group have developed a suite of plasmids engineered to include the specific target regions for each of the target assays (norovirus GI, norovirus GII, hepatitis A virus). Purified plasmid DNA is utilised for the PCR calibration and RNA run-offs from these plasmids is utilised in an IC-RNA format to measure RT-PCR amplification efficiency for each test sample. Plasmid availability is currently restricted to the CEN group but they are intended to be freely available following validation. In the interim laboratories can construct their own plasmids or use an alternative approach to produce pure DNA and RNA for control purposes. Taken together these controls generate quantitative data on the performance of the assay for each test and batch. This data can then either be utilised to determine the acceptability of test performance against previously determined quality assurance criteria, or possibly utilised to report fully quantitative results (for example correcting for both virus recovery and matrix suppression). It is envisaged that normal test performance criteria would be established during the planned method validation studies. The final scheme for incorporation of control measurements to result reporting will be determined following the inter-laboratory phase of method validation and will be determined by the robustness and reproducibility of the various options. However, it is established that results will be expressed in the standardised form of virus RNA template copies per gram of material tested. Qualitative assays would report presence or absence with reference to their limit of detection.

Currently it is difficult to compare results from different laboratories due to the lack of a standardised method. A particular issue is that results are not reported in a consistent or comparable format that allows inter-laboratory comparison. It is therefore recommended that UK laboratories work towards reporting results in the standard format proposed by CEN i.e. virus RNA template copies per gram of material tested. Qualitative assays should report presence or absence with reference to the limit of detection achieved in the laboratory. Laboratories should also be able to report key method characteristics and performance data (see below) to enable inter-laboratory comparisons to be made.

6 Result interpretation (infectivity)

A major result interpretation issue is that it is not clear whether presence of virus genome, as determined by PCR, correlates with a human health risk. It is possible that, in some cases, PCR may be detecting inactivated or non-infectious virus. If virus presence were an infrequent occurrence in bivalve shellfish this would not

present a significant practical problem. Unfortunately however this does not appear to be the case - particularly for class B areas. Current data both from the UK and elsewhere shows that during winter months virus presence in shellfish is a fairly common occurrence. Various studies have shown rather high rates of viral contamination of commercially produced bivalve shellfish in a number of different countries (Costantini *et al.*, 2006; Cheng *et al.*, 2005; Chironna *et al.*, 2002; Formiga-Cruz *et al.*, 2002; Nishida *et al.*, 2003; Boxman *et al.*, 2006) illustrating the potential impact of viral monitoring. The prevalence of norovirus positives from such studies exceeds our expectation of likely disease burden based on human health incidents reported. The possibilities are either that disease reporting dramatically under estimates the actual disease burden or that PCR testing over estimates the real risk – or a combination of both factors. Further systemic studies are required to assess the incidence of viral contamination in UK production areas.

Quantitation of virus genome through real-time PCR offers the opportunity to evaluate whether genome titre correlates with health risk. This may help reconcile perceptions of disease risk with virus test results through, for example, the establishment of quantitative risk thresholds. Further work is recommended in this area to help inform the interpretation of virus positive PCR results.

In the interim it should be noted that laboratory testing cannot currently determine whether a PCR positive sample contains infectious virus.

7 Quality assurance

Given the technical complexity in this area, and the potential for both false positive and false negative results, it is vital that laboratories endeavour to comply with internationally accepted quality assurance criteria.

7.1 Method performance characterisation

EC Regulations expect a high level of quality assurance of methods used for official control purposes. EU Regulation 882/2005 (anon 2004b) details the requirements and they can be summarised as follows: methods must either be internationally recognised (for example be published as ISO or CEN standard methods) or be the subject of single laboratory validation and the method performance criteria established, in compliance with Annex III of the regulation. Annex III requires that methods of analysis must be characterised according to the following criteria: linearity and range; sensitivity; limit of detection; accuracy; recovery; precision and repeatability; limit of determination; measurement uncertainty; selectivity; reproducibility (collaborative trial); applicability (matrix and concentration range); other criteria that may be selected as required. A collaborative trial should be conducted in accordance with internationally recognised protocols (eg ISO 5725:1994 or the IUPAC International Harmonised Protocol (Thompson *et al.*, 2002) or, where performance criteria have been established, be based on compliance criteria tests. In situations where methods of analysis can only be validated within a single laboratory then they should be validated in accordance with the above international guidelines, or where performance criteria for analytical tests have been established, be based on criteria compliance tests.

It is important to note that internationally recognised methods for detection of viruses in bivalve molluscs are not yet available from any source world-wide. It should also be noted that, to our knowledge, no laboratory has yet established full method performance characteristics according to the above criteria. Thus, in the strict sense of the regulations, virus methods should not yet be used for official control analysis.

Cefas has performed aspects of the above method performance characterisation (limit of detection, linearity, limit of quantitation, repeatability) on the in-house method through a recent FSA funded study. The objective of the CEN group is to establish a standardised method and to validate it according to the above protocols.

Detection of low levels of virus template by PCR in a food matrix is known to be very demanding thus UK laboratories should be able to demonstrate to their customers satisfactory evidence of applicability i.e. that the method has adequate performance for the specified purpose. In the absence of standardised validated methods UK laboratories should ideally generate in-house method performance data according to the parameters specified in EU Regulation 882/2005 (anon 2004b) to demonstrate that their method is fit for purpose. If full performance characterisation is not feasible then at least linearity, limit of detection and limit of quantitation data should be established in the shellfish matrix being tested. In performing these studies laboratories should consider the mode of contamination of bivalve shellfish and ensure that their experimental design adequately reflects this – shellfish contaminated by the natural bioaccumulation route are the best choice of material. Once the method is set up, it may also be opportune to test the performance of the method in the field during the winter months in known polluted areas. Many research studies have shown that norovirus can commonly be detected in shellfish harvested from such areas. If a laboratory cannot replicate such a finding they should seek known positive shellfish material from another laboratory to ensure their method is functioning adequately (also see section 7.4).

7.2 Accreditation

Accreditation to the ISO 17025 standard, or working towards accreditation to this standard, is required of laboratories performing official control testing under EU Regulation 882/2005 (anon 2004b).

7.3 Reference materials

The absence of external certified or even standardised reference materials for viruses poses a major problem for laboratories in ensuring traceability of assay measurements to known reference values. Currently Cefas are developing stable reference materials with known reference values for norovirus and hepatitis A virus. It may be possible to supply these to other UK laboratories, as a first step towards inter-laboratory calibration, on a cost recovery basis.

7.4 Proficiency testing (PT)

Proficiency testing provides an external assessment of laboratory and test performance through blind analysis of samples distributed by a PT scheme organiser. Results for all participants are reported together to enable laboratories to compare their results both with other laboratories and with the reference results provided by the scheme organiser. Given the technical difficulties of testing bivalve shellfish for viruses, and the absence of other means to ensure accuracy of test results, it is considered important that laboratories endeavour to participate in external quality assurance or proficiency testing (PT). Evidence of satisfactory performance should be given to the laboratory customer. Unfortunately there are currently very few PT schemes available for virus testing in food. Cefas runs an international PT scheme for official laboratories (reports are available on www.crlcefas.org) and may be able to extend this to other UK laboratories on a cost recovery basis.

8 Sampling

A crucial element in more formalised use of virus testing for surveillance would be a better understanding of appropriate sampling strategies. This has been highlighted by the FSA. In the interim it should be noted that information on sampling strategies is currently very limited. As a minimum precaution, and to avoid bias from individual animals, it is recommended that a sample size is specified as a minimum number of individual animals. Recent data from an FSA funded study as Cefas suggests that a minimum of 10 individual animal digestive glands should be extracted for a sample. Sampling approaches for representative harvesting area or consignment testing have yet to be determined.

9 Status of virus testing services in the UK

There are currently limited options for virus testing of bivalve molluscs in the UK.

9.1 Official Control laboratories

As virus testing is not currently a testing requirement within EU legislation there is, formally, no Official Control testing requirements and hence no designated Official Control laboratories in the UK. Other public sector laboratories in the UK undertaking Official Control testing have expressed an interest in virus testing for bivalve molluscs but, to our knowledge; do not yet offer a virus testing service.

9.2 Cefas

Cefas has developed and standardised an in-house method for testing for norovirus and hepatitis A in oysters and mussels. The method has been in use for many years, is well documented, and incorporates a comprehensive suite of method controls. We have contributed key aspects, such as the shellfish extraction procedure, to the development of the CEN method which is therefore similar, but not identical, to our method. Many of the principles of the CEN approach, such as the necessary method controls, are already incorporated into the Cefas method. We are currently in the process of adopting additional aspects of the CEN method which is expected to be finalised by summer 2008. Cefas, as the UK NRL, participates in the CRL virus ring testing (using the in-house method) and has performed well in comparison with other international participants. As the UK NRL we will also participate in the planned CEN method validation studies. Cefas is, subject to agreement of funding, able to offer virus analysis in the context of human health or pollution incidents, and other official control activities as requested by FSA or local authorities, such as harvesting area surveillance or sanitary survey investigations.

9.3 Laboratories providing services to the private sector

There are a number of Labs, in the UK and in other EU Member States, offering a commercial testing service for norovirus in shellfish. Growing demand for virus testing from the producer and retail sector, and the introduction of virus testing in several export markets, suggests that requests for testing in this sector will increase.

10 Best practice recommendations

Considering the above factors the UK NRL offers the following recommendations on best practice for laboratory methodology, and quality assurance, in relation to testing bivalve molluscs for viruses:

10.1 Method. The only methods currently demonstrated to work at the required sensitivity are based on PCR. Hence methods based on other possible approaches, eg ELISA, are not appropriate. The European CEN working group is developing a standard method using a proteinase K digestion of molluscs

digestive glands followed by nucleic acid clean-up and one-step reverse transcription probe based real-time PCR and this is the general approach recommended.

10.2 Laboratory environment. PCR is very susceptible to cross-contamination events within the laboratory and to matrix interferences. Hence the potential for both false negative and false positive results are well documented. Laboratories should conduct PCR based testing in a laboratory environment consistent with internationally agreed guidance such as that published by ISO (see references). This has significant resource implications such as the need to physically separate pre and post PCR activities. Laboratories not conforming with the physical separation requirements are highly likely to experience false positive test results.

10.3 Controls. Given the sensitivity of PCR and its susceptibility to matrix interferences it is critical to incorporate within each test batch an appropriate suite of controls. The general requirements for PCR controls are given in ISO guidance (see reference list). Our advice is that controls for testing of viruses in shellfish should include a:

- negative PCR control
- positive PCR control
- negative extraction control
- RT-PCR control
- RT-PCR inhibition control
- process control

The RT-PCR controls may be combined through the use of internal RNA controls. If two-step RT-PCR is used application of RT-PCR and inhibition controls may be more complex but should be used where practically possible. If a process control is particularly problematical then a positive extraction control (a well characterised previously positive shellfish sample) could be an alternative but less effective approach. If the PCR test employed is claimed to be quantitative then numerical performance criteria should be determined for the above controls and used to assess batch run acceptability. Otherwise qualitative criteria should be established.

10.4 Results reporting. Laboratories should work towards reporting results in the standard format proposed by CEN (virus RNA template copies per gram of material tested) since this will facilitate comparability between laboratories.

- 10.5 Test performance evidence. Detection of low levels of virus template by PCR in a food matrix is known to be very demanding and laboratories should be able to demonstrate to their customer's satisfactory evidence of test performance. In the absence of characterised and validated methods laboratories should be able to provide single laboratory test performance data on method linearity, limit of detection, and limit of quantitation (if quantitative). Such studies should be performed on shellfish matrix material contaminated with virus by a route representative of that occurring in the field (eg natural bioaccumulation). In addition the laboratory should be able to demonstrate applicability through documenting the presence of norovirus in field samples from known polluted areas during winter months (many studies have shown this to be a common occurrence).
- 10.6 Proficiency testing. The laboratory should endeavour to participate in external quality assurance or proficiency testing (PT) for virus testing in order to compare its performance with that of other laboratories. Evidence of satisfactory performance should be given to the laboratory customer.

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