Food Environ Virol (2010) 2:146–155
DOI 10.1007/s12560-010-9042-5

ORIGINAL PAPER

International Standardisation of a Method for Detection of Human Pathogenic Viruses in Molluscan Shellfish

David Lees · CEN WG6 TAG4

Received: 4 April 2010 / Accepted: 26 April 2010 / Published online: 16 June 2010

Abstract The viruses primarily associated with shellfish-borne illness are norovirus, causing gastroenteritis and hepatitis A virus (HAV). Recent years have seen a proliferation of publications on methods for detection of these viruses in shellfish using polymerase chain reaction (PCR). However, currently no standard harmonised procedures have been published. Standardisation is necessary before virus methods can be considered for adoption within a regulatory framework. A European standardisation working group is developing a two-part (quantitative and qualitative) standard method for virus detection in foodstuffs, including shellfish, which has the potential to be incorporated into EU legislation as a reference method. This article describes the development of the standard method and outlines the key methodology principles adopted, the controls and other quality assurance measures supporting the method and future necessary developments in the area.

Keywords Shellfish · Norovirus · Hepatitis A virus · Method · Detection · Standard

Introduction

Contamination of bivalve shellfish with norovirus (causing gastroenteritis) and hepatitis A virus (HAV) is recognised as the major human health risk associated with consumption of faecally contaminated bivalve molluscs (Lees 2000). Viruses are accumulated by bivalve molluscs during normal filter-feeding activity following faecal pollution of their production areas. The consumption of many bivalves either raw (e.g. oysters) or lightly cooked (e.g. mussels and clams), and the consumption of the whole animal including the digestive parts which contain the bulk of contaminants, accentuates the risks. Many outbreaks of illness following bivalve shellfish consumption have been reported (Lees 2000; Bosch et al. 2001; Doyle et al. 2004; Simmons et al. 2007; Le Guyader et al. 2008; Lowther et al. 2010; Westrell et al. 2010). The majority of incidents are of a gastro-enteric nature and are caused by norovirus. Outbreaks caused by HAV are reported less frequently, but the hepatitic disease caused is more severe. High risk factors are cold weather (low seawater temperatures), high prevalence of norovirus gastroenteritis or hepatitis in the community and high rainfall (leading to sewage system overflows) (Keaveney et al. 2009; Westrell et al. 2010). It is likely that the recognised outbreaks represent only a small part of the true burden of illness in most countries (Wheeler et al. 1999; Potasman et al. 2002).

In Europe faecal contamination risk management strategies for bivalve molluscs rely heavily on the use of *E. coli* as an indicator of faecal pollution in production areas (EU Regulation 854/2004, Anon 2004a). This approach generally successfully protects the shellfish consumer from infection with sewage derived bacterial pathogens (Lees 2000) but is more problematical for control of 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. Frequently in reported outbreaks shellfish are extracted from officially classified waters, are depurated (if necessary) in approved plants in compliance with requirements and are processed in approved establishments.

CEN WG6 TAG4: see Appendix for members

D. Lees (✉)
European Community Reference Laboratory, Centre for Environment, Fisheries and Aquaculture Science, The Nothe, Weymouth, Dorset DT4 8UB, UK
e-mail: david.n.lees@cefas.co.uk

End-product testing will show products to be in compliance with the regulatory *E. coli* standard (Lowther et al. 2010). These problems have focussed attention on the need for development of methods for direct detection in bivalve molluscs of the viruses causing illness. Over the last decade considerable progress has been made on detection methods for norovirus and HAV in molluscan shellfish (Lees 2000; Jothikumar et al. 2005; Loisy et al. 2005; Costafreda et al. 2006). All methods currently proposed are based on detection of virus genome using the polymerase chain reaction (PCR). However, a major factor limiting the more formal uptake of virus testing into regulatory requirements is the current absence of any standard and/or validated methods worldwide and the general absence of the accepted quality assurance framework in place for food testing. Virus testing is currently not incorporated as an element of regulatory controls within, for example, either the major EU or USA markets. However, EU Regulations do foresee the potential adoption of virus controls when the methods are available for use (Anon 2004a, 2005). This article describes the activities of a working group of expert European laboratories towards the development of a standard method for detection of human pathogenic viruses in molluscan shellfish and other food commodities. It is anticipated that should EU Regulations adopt a virus standard in the future then the CEN standard method will be specified as the reference method. Other methods could be employed but would need to be shown to give equivalent performance through comprehensive validation studies conducted in accordance with internationally accepted protocols (ISO 16140:2003).

Method Standardisation in Europe

In 2004 the European Committee on Standardisation (CEN) tasked a technical advisory group (TAG) chaired by this laboratory with the development of a standard method for detection of norovirus and HAV in foodstuffs (including bivalve shellfish). The TAG has been fortunate to have had active participation and collaboration from a number of highly experienced European laboratories who have all contributed to the methodology choices described. 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 within the TAG generally organised by this laboratory. Part one of the standard method (quantitative) was launched in 2009 for formal technical comment by CEN member countries with the second part (qualitative) following in 2010. This article describes the development of the standard and describes key methodological aspects. Publication of the standard by CEN is expected in 2012. It should be noted that the standard method covers a range of at risk food commodities including salad crops, food surfaces, soft fruits, bottled water and molluscan shellfish. This article focusses on molluscan shellfish; however, many procedural aspects are common to all food commodities since the standard was elaborated according to the CEN policy for horizontal methods wherever possible.

Key Method Principles

Methods employing the PCR for the detection of viruses in shellfish have been published since the mid 1990s (Lees 2000), and there are now numerous published references from laboratories worldwide (Boxman et al. 2006; Loisy et al. 2005; Nishida et al. 2003; Shieh et al. 2000). It is important to note that all published methods with demonstrable ability to detect viruses in bivalve shellfish or other foods have utilised PCR. This is because methods based on other detection systems available for clinical diagnostic samples (e.g. current ELISA methods) have not been demonstrated to have adequate performance (sensitivity) for environmentally contaminated samples such as bivalve shellfish or other foods. Hence the standard method was based on detection of viruses in foods using PCR. The European Community Reference Laboratory (CRL) organises international proficiency testing (PT) for detection of viruses in bivalve molluscs. To date all participants in this PT have employed various variants of PCR methods. However, the variable performance experienced in PT suggests that not all methods give equivalent performance (see reports on www.crlcefas.org). Bivalve molluscs present a challenging matrix and methods need to be capable of extracting low levels of contaminating virus and presenting them in a non-inhibitory extract to a sensitive PCR assay. These considerations underpinned the methodological choices described below. Important aspects were performance tested by inter-laboratory evaluations.

General Requirements for PCR

Polymerase chain reaction 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 is 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. The standard requires

laboratories to conduct PCR testing in a laboratory environment consistent with internationally agreed guidance (ISO 22174:2005). 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 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). Compliance with the general ISO guidance is considered as a prerequisite in the CEN standard method.

Virus Target

The principle viruses of concern internationally for bivalve shellfish are norovirus and HAV. The overwhelming majority of norovirus strains causing human infections are classified into two genotypes; norovirus genogroup I (GI) and norovirus genogroup II (GII). Both genogroups are targeted as they are both responsible for human infection and are both common contaminants of sewage-polluted bivalve shellfish. In non-endemic areas HAV may be a relatively rare contaminant in bivalve shellfish because of low levels in the community. However, the disease is more severe than norovirus and the consequences of an outbreak can be dramatic (Conaty et al. 2000; Bosch et al. 2001; Shieh et al. 2007). Thus, HAV is also targeted by the standard.

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; the infectious dose for norovirus is generally accepted to be around ten 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 HAV 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 methods must be able to recover 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 study 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, potentially, many PCR inhibitors. This reduces processing time and aids both sensitivity and the quality of the extract. There are numerous publications detailing different approaches to the treatment of bivalve digestive glands for release, concentration and purification of virus. Two candidates in widespread international use were selected by the TAG for evaluation 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). Both methods for processing digestive glands were found to perform adequately in experienced hands; however, they varied in their inter-laboratory 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. However, all laboratories achieved adequate results with the simpler proteinase K method. Consequently the proteinase K method of digestive gland treatment was adopted for the standard. It should, however, be noted that the digestive gland processing is only a part of the Baylor method which also incorporates further nucleic acid purification steps prior to PCR. The need to establish a horizontal standard method with common nucleic acid purification stages for all food commodities, mitigated against testing of the full Baylor procedure which focusses specifically on bivalve shellfish.

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 the TAG selected the widely used Boom method (Boom et al. 1990). This method utilises guanidine isothiocyanate (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 was found to work well in TAG inter-laboratory trials. The Boom method principles are employed by a number of commercial kits for nucleic acid extraction and cleanup. Quality assured commercial reagents can improve intra-laboratory comparability through removing a source of variability. However, CEN requires that no one proprietory reagent or kit should be specified in an international standard method. Therefore, throughout development commercial systems have been incorporated where possible but consideration has been given to the availability of equivalent technologies from different suppliers. For example several laboratories within the TAG have found the magnetic silica approach to produce comparable results in bivalve shellfish to the conventional Boom procedure (Green et al. 1998; Jothikumar et al. 2005) and it offers improved method logistics and reproducibility. The TAG proposes to use the commercial magnetic silica approach for nucleic acid purification in the planned validation programme for the standard.

Reverse Transcription

Both HAV and norovirus are single-stranded RNA viruses and, therefore, require reverse transcription prior to PCR. A variety of formats of reverse transcription-PCR (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 either a single reaction mix containing both reverse transcriptase and *Taq* polymerase to enable both RT and PCR stages to be carried out successively in the same tube without needing to open the tube in between (one-step), or separate reaction mixes for each stage (two-step). Some laboratories in the TAG had previously used a two-step approach with random hexamers for the RT stage (Jothikumar et al. 2005; Flannery et al. 2009), whereas other had used a one-step procedure (Costafreda et al. 2006; Loisy et al. 2005). 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 that is also useable for sequencing other regions of the virus genome for confirmation and strain identification. However, this approach has disadvantages for routine analysis since it uses several additional reagents, introduces additional pipetting steps, with the potential for contamination and complicates quantification. The TAG, therefore, adopted the one-step approach using specific primers in order to simplify the procedure as much as possible. Initial inter-laboratory evaluations 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). 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 issue was overcome by the use of one-step reverse transcription enzymes specifically engineered for use with low abundance targets. The standard requires reagents used for one-step RT-PCR to be sufficiently sensitive for the detection of the levels of virus RNA typically found in virus-contaminated foodstuffs.

PCR

Many published methods have used conventional PCR giving qualitative results (Green et al. 1998; Le Guyader 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 and it is amenable to standardisation. Owing to 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 real-time PCR is now generally regarded as the methodology of choice. A number of laboratories in the TAG have successfully employed TaqMan based real-time PCR for testing bivalve molluscs for viruses. Initial discussion in the TAG examined the practicality of this methodology for routine laboratories. However, the reducing cost of instruments, the increasing availability of platforms because of use for other types of testing and the significant technical advantages over conventional PCR were all factors in the decision to base the virus standard on detection using real-time PCR. ISO have published general requirements on the use of real-time PCR in food analysis (ISO/DIS 22119) which covers the general principles and practice of this methodology.

Primers and Probes

Polymerase chain reaction 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 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 difficult for food laboratories, in isolation, to develop new PCR primers or probes. The standard specifies the use of real-time TaqMan primers/probe for HAV based on the highly conserved 5′ non-coding region (for example, those published by Costafreda et al. 2006). The development of real-time PCR primers/probes for norovirus has been a significant, and ongoing, technical challenge. 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 (Jothikumar et al. 2005; Loisy et al. 2005; da Silva et al. 2007; Svraka et al. 2007) have targeted this region with relatively minor modifications of the original proposals, and this is the area of choice identified by the TAG for the virus standard (Le Guyader et al. 2009). However, it is recognised that food testing laboratories need to remain alert to changes in clinical prevalence of norovirus strains and thus the relevance of their assay.

PCR 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. Previous sequencing studies by TAG partners had 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. 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. To evaluate specificity of the real-time PCR assay several amplicons generated by the preferred TaqMan primers in the chosen ORF1–ORF2 junction region were sequenced. Amplicons (ten for norovirus GI and 14 for norovirus GII) were selected from a range of environmentally contaminated shellfish samples. All amplicons were found to contain norovirus specific sequence thus helping to confirm the specificity of the approach adopted by the standard.

Controls

Given the need for a highly sensitive PCR to detect the low levels of norovirus and HAV 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 critically 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 (ISO 22174:2005). These are further developed in the CEN standard for viruses in food which currently incorporates the following controls: positive and negative process controls; negative RNA extraction control; positive RT-PCR and RT-PCR inhibition controls and negative and positive PCR controls. The positive process control measures the recovery of virus during the whole extraction and test procedure using a heterologous non-enveloped positive-sense ssRNA virus spiked into the test sample and assayed in parallel with the target viruses. The virus selected as the process control should provide a good morphological and physicochemical model for the targets and exhibit similar environmental persistence, and thus should be extracted in a similar way and to a comparable efficiency as the target. Comparison of spiking material concentration and final extract concentration by PCR determines the level of acceptability of the recovery efficiency of the whole process. The TAG has successfully tested the MC_0 strain of Mengo virus (a cardiovirus serologically related to encephalomyocarditis virus; Costafreda et al. 2006) in inter-laboratory studies and proposes to use this process control for further method validation practical work; however, the use of other process control viruses is permitted by the standard provided they fulfil the criteria outlined above. The use of a positive 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. The negative process control is a known negative sample that is taken through the entire extraction procedure and analysed. It is thus a check of the entire extraction process for contamination. The negative RNA extraction control on the other hand is an aliquot of target-free water subjected to RNA extraction then analysed, to control the RNA extraction portion of the process only. The positive RT-PCR control checks the entire RT-PCR reaction using 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 standard the RT-PCR control and the RT-PCR inhibition control are combined through the use of external control RNAs (EC RNAs) for each of the assays. Partners within the TAG have developed a suite of plasmids engineered to include the specific target regions for the chosen assays (norovirus GI, norovirus GII, HAV). Purified plasmid DNA is utilised as the positive PCR control (controlling only the PCR stage of the RT-PCR reaction) and for quantification purposes; RNA run-off transcripts from these plasmids are utilised as the combined positive RT-PCR control and RT-PCR inhibition control. Finally the negative PCR control is an aliquot of target-free water subjected to RT-PCR to control for contamination of the test reagents. Taken together these controls generate data on the performance of all aspects of the assay for each test and batch. The data can be utilised for either qualitative or quantitative tests to determine the acceptability of test performance. The suite of controls described covers the key elements of the methodology known, or suspected, to be problematical. The TAG has performed an inter-laboratory comparison using distributed RNA (to eliminate extraction variability) to evaluate the performance of the various reagents and control materials and all aspects have been demonstrated to perform robustly. Currently, however, there are little data available showing the value added by each control during the routine application of a well-characterised and standardised method. Further data are anticipated to be generated during the planned formal method validation studies. The CEN standard will stipulate quality assurance acceptance criteria for each of the above controls which will determine the acceptability of each test and batch run and the required frequency for running each control. These values will be finalised following formal method validation studies.

Quantitative versus Qualitative Standards

The requirement for quantitative or qualitative results depends on the particular application. For example qualitative determination of the presence of virus contamination on a food surface is sufficient to determine a risk relating to food handling. For bivalve mollucs the frequent presence of virus in samples (Lowther et al. 2008; Loisy et al. 2005; Vilarino et al. 2009) and the possible linkage of virus levels to risk (Lowther et al. 2010; Pintó et al. 2009) suggest a quantitative assay would give valuable additional risk management information although a qualitative method may suffice for example in certain outbreak situations. The TAG has developed a two-part standard covering both quantitative determination and qualitative detection for use depending on the particular application. The main differences relate to the controls and the calibration curves required for determining virus template concentrations.

Calculation of Quantitative Results and Reporting

A difficulty with current laboratory reports for viruses in bivalve molluscs is the problem of 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. This arises because of the current inability to calibrate PCR assays through the usual routes of either an external reference material (none are available) or an independent measurement of virus titre through culture (norovirus cannot be cultured). Thus, assays may be well standardised within a laboratory, and capable of producing consistent and comparable results; however, currently it is very difficult to compare results between laboratories without extensive information about assay makeup (e.g. amount of material tested) and performance (e.g. sensitivity). The CEN standard addresses this problem by developing a quantitative approach grounded in a plasmid DNA calibration curve for each assay (norovirus GI, norovirus GII, HAV) with plasmid DNA concentration measured using spectrometry at 260 nm. Results will thus be expressed in the standardised form of detectable virus genome copies per gram of material tested. Qualitative assays will report presence or absence with reference to their limit of detection. The process control and RT-PCR inhibition control provide information on the efficiency of each test assay which can be used to ensure the assay is performing within acceptable quality criteria. The CEN standard contains provisional acceptable method performance criteria for each matrix; however, it is anticipated that these will be revised as more data become available following validation studies.

Method Validation

EC Regulations expect a high level of quality assurance of methods used for official control purposes. EU Regulation 882/2004 (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 (e.g. ISO 16140:2003 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 worldwide. It should also be noted that, to our knowledge, no laboratory has yet established full method performance characteristics according to the above criteria. Following standardisation the objective of the CEN TAG4 is to formally validate the method according to the above protocols.

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. These include the use of a standardised method (as described above). For laboratories performing official control testing under EU Regulation 882/2004 (Anon 2004b) accreditation of their method to the ISO 17025 standard is also required. 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 the CRL is developing stable reference materials with known reference values for norovirus and HAV which may assist laboratories towards inter-laboratory calibration. PT 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, the objective evidence provided by external PT is particularly important. The CRL runs an international PT scheme for official testing laboratories (reports are available on www.crlcefas.org).

Knowledge Gaps

Introduction of virus testing into food control legislation is a major undertaking, and there still remain several important knowledge gaps which need to be addressed prior to such movements. A crucial element in more formalised use of virus testing for official control monitoring and surveillance would be a better understanding of appropriate sampling strategies. Currently information on virus sampling strategies is very limited. Data, for example, on the distribution of contamination within bivalve molluscs (between individuals, within batches, temporally and geographically within a production area) are necessary to devise representative sampling strategies for harvesting area or consignment testing. A more systematic approach to surveillance of virus in production areas would considerably assist management and interpretation of outbreak-related incidents. A major result interpretation issue is that it is not clear whether presence of virus genome, as determined by PCR, correlates with presence of infectious virus and thus human health risk. It is a possibility 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. Various studies have shown rather high rates of viral contamination of commercially produced bivalve shellfish in a number of different counties (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, Lowther et al. 2008) 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, that presence/absence PCR testing over estimates the real risk—or a combination of both factors. A recent study provides the first evidence that the infectious risk posed by oysters may be related to the amount of norovirus RNA that they contain (Lowther et al. 2010). Further epidemiological studies are required to further explore this possibility and, if substantiated, to explore quantitative risk thresholds. The availability of the CEN standard method is critical to underpin such studies.

Acknowledgements The authors gratefully acknowledge funding by DG Sanco of the European Commission for the Community Reference Laboratory. Participants of the CEN/TC 275/WG6/TAG4 working group are funded by their respective national standardisation bodies, and this funding is gratefully acknowledged.

Appendix

The members of CEN WG6 TAG4 are David Lees (chair), European Community Reference Laboratory, Centre for Environment, Fisheries and Aquaculture Science, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom; Carl-Henrik von Bonsdorff, University of Helsinki, Finland; Leena Maunula, University of Helsinki, Finland; Anna Charlotte Schultz, National Food Institute, Søborg, Denmark; Dietrich Maede, Landesamt fuer Verbraucherschutz Sachsen-Anhalt, Halle, Germany; Reimar Johne, Federal Institute for Risk Assessment, Berlin, Germany; Barbara Becker, Life Science Technologies, Lemgo, Germany; Anja Carl, Bayerisches Landesamt für Gesundheit und Lebensmittelsicherheit, Erlangen, Germany; James Lowther, European Community Reference Laboratory, Centre for Environment, Fisheries and Aquaculture Science, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom; Rachel Rangdale, European Community Reference Laboratory, Centre for Environment, Fisheries and Aquaculture Science, The Nothe, Weymouth, Dorset, DT4 8UB, United Kingdom; Jane Sellwood, Health Protection Agency, Environmental Virology Unit, Reading, United Kingdom; Sinead Keaveney, Marine Institute, Galway, Ireland; Ingeborg Boxman, Food and Consumer Product Safety Authority, Zutphen, the Netherlands; Saskia Rutjes, National Institute for Public Health and the Environment, Bilthoven, the Netherlands; Wim van der Poel, Central Veterinary Institute, Wageningen University and Research Centre, Lelystad, the Netherlands; Elisabeth Mathijs, University of Liege, Belgium; Françoise S. Le Guyader, IFREMER, Nantes, France; Fabienne Loisy, CEERAM, Nantes, France; Sylvie Perelle, Agence Française de Sécurité Sanitaire des Aliments, Maisons-Alfort, France; Sandra Martin-Latil, Agence Française de Sécurité Sanitaire des Aliments, Maisons-Alfort, France; Christian Beuret, Spiez Laboratory, Spiez, Switzerland; Sophie Butot, Nestle Research Centre, Lausanne, Switzerland; Albert Bosch, University of Barcelona, Spain; Rosa Pinto, University of Barcelona, Spain; Rosina Girones, University of Barcelona, Spain; Gloria Sanchez, Instituto de Agroquímica y Tecnología de Alimentos, Burjassot, Spain; Luciana Croci, Istituto Superiore di Sanità, Rome, Italy; Dario DeMedici, Istituto Superiore di Sanità, Rome, Italy; Apostolos Vantarakis, University of Patras, Greece; Fouad El Tahan, Sokhna Port Laboratory, Suez, Egypt.

References

Anon. (2004a). Corrigendum to Regulation (EC) No 854/2004 of the European Parliament and of the Council of 29 April 2004 laying down specific rules for the organisation of official controls on products of animal origin intended for human consumption. *Official Journal of the European Union, L226*, 83–127.

Anon. (2004b). Commission Regulation (EC) No 882/2004 of the European Parliament and of the Council of 29th April 2004 on official controls performed to ensure verification of compliance with feed and food law, animal health and animal welfare rules. *Official Journal of the European Union, L191*, 1–52.

Anon. (2005). Commission Regulation (EC) No 2073/2005 of the European Parliament and of the Council of 15th November 2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union, L338*, 1–26.

Atmar, R. L., Neill, F. H., Romalde, J. L., Le Guyader, F., Woodley, C. M., Metcalf, T. G., et al. (1995). Detection of norwalk virus and hepatitis a virus in shellfish tissues with the pcr. *Applied and Environmental Microbiology, 61*, 3014–3018.

Boom, R., Sol, C. J. A., Salimans, M. M. M., Jansen, C. L., Wertheim van Dillen, P. M. E., & van der Noordaa, J. (1990). Rapid and simple method for purification of nucleic acids. *Journal of Clinical Microbiology, 28*, 495–503.

Bosch, A., Abad, F. X., Gajardo, R., & Pintó, R. M. (1994). Should shellfish be purified before public consumption? *The Lancet, 344*, 1024–1025.

Bosch, A., Sanchez, G., Le Guyader, F., Vanaclocha, H., Haugarreau, L., & Pinto, R. M. (2001a). Human enteric viruses in coquina clams associated with a large hepatitis A outbreak. *Water Science and Technology, 43*(2001), 61–65.

Bosch, A., Sánchez, G., Le Guyader, F., Vanaclocha, H., Haugarreau, L., & Pintó, R. M. (2001b). Human enteric viruses in Coquina clams associated with a large hepatitis A outbreak. *Water Science and Technology, 43*, 61–65.

Boxman, I. L. A., Tilburg, J. J. H. C., te Loeke, N. A. J. M., Vennema, H., Jonker, K., de Boer, E., et al. (2006). Detection of noroviruses in shellfish in the Netherlands. *International Journal of Food Microbiology, 108*, 391–396.

Cheng, P. K. C., Wong, D. K. K., Chung, T. W. H., & Lim, W. W. L. (2005). Norovirus contamination found in oysters worldwide. *Journal of Medical Virology, 76*, 593–597.

Chironna, M., Germinario, C., Demedici, D., Fiore, A., DiPasquale, S., Quarto, M., et al. (2002). Detection of hepatitis A virus in mussels from different sources marketed in Puglia region (South Italy). *International Journal of Food Microbiology, 75*, 11–18.

Conaty, S., Bird, P., Bell, G., Kraa, E., Grohmann, G., & McAnulty, J. M. (2000). Hepatitis A in New South Wales, Australia, from consumption of oysters: The first reported outbreak. *Epidemiology and Infection, 124*, 121–130.

Costafreda, M. I., Bosch, A., & Pintó, R. M. (2006). Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and Environmental Microbiology, 72*(6), 3846–3855.

Costantini, V., Loisy, F., Joens, L., Le Guyader, F. S., & Saif, L. J. (2006). Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. *Applied and Environmental Microbiology, 72*, 1800–1809.

da Silva, A. K., Le Saux, J. C., Parnaudeau, S., Pommepuy, M., Elimelech, M., & Le Guyader, F. S. (2007). Evaluation of removal of noroviruses during wastewater treatment, using Real-Time Reverse Transcription-PCR: Different behaviors of genogroups I and II. *Applied and Environmental Microbiology, 73*, 7891–7897.

Doyle, A., Barataud, D., Gallay, A., Thiolet, J. M., Le Guyager, S., Kohli, E., et al. (2004). Norovirus foodborne outbreaks associated with the consumption of oysters from the Etang de Thau, France, December. *Euro Surveillance, 9*, 24–26.

Flannery, J., Keaveney, S., & Doré, W. (2009). Use of FRNA bacteriophages to indicate the risk of norovirus contamination in irish oysters. *Journal of Food Protection, 72*, 2358–2362.

Formiga-Cruz, M., Tofiño-Quesada, G., Bofill-Mas, S., Lees, D. N., Henshilwood, K., Allard, A. K., et al. (2002). Distribution of human virus contamination in shellfish from different growing areas in Greece, Spain, Sweden, and the United Kingdom. *Applied and Environmental Microbiology, 68*(12), 5990–5998.

Green, J., Henshilwood, K., Gallimore, C. I., Brown, D. W. G., & Lees, D. N. (1998). A nested reverse transcriptase pcr assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish. *Applied and Environmental Microbiology, 64*, 858–863.

ISO 16140:2003. Microbiology of food and animal feeding stuffs—protocol for the validation of alternative methods.

ISO 20837:2006. Microbiology of food and animal feeding stuffs—polymerase chain reaction (PCR) for the detection of food-borne pathogens—requirements for sample preparation for qualitative detection.

ISO 20838:2006. Microbiology of food and animal feeding stuffs—polymerase chain reaction (PCR) for the detection of food-borne pathogens—requirements for amplification and detection for qualitative methods.

ISO 22174:2005. Microbiology of food and animal feeding stuffs—polymerase chain reaction (PCR) for the detection of food-borne pathogens—general requirements and definitions.

ISO/DIS 22118. Microbiology of food and animal feeding stuffs—polymerase chain reaction (PCR) for the detection of food-borne pathogens—performance characteristics of molecular detection methods.

ISO/DIS 22119. Microbiology of food and animal feeding stuffs—real-time polymerase chain reaction (PCR) for the detection of food-borne pathogens—general requirements and definitions.

Jothikumar, N., Lowther, J. A., Henshilwood, K., Lees, D. N., Hill, V. R., & Vinjé, J. (2005). Rapid and sensitive detection of noroviruses by using TaqMan-based one-step reverse transcription-PCR assays and application to naturally contaminated shellfish samples. *Applied and Environmental Microbiology, 71*(4), 1870–1875.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., et al. (2003). Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology, 41*(4), 1548–1557.

Keaveney, S., Flannery, J., Guilfoyle, F., & Dore, W. (2009). Preliminary results from a survey of oyster production areas in Ireland for norovirus. In *Proceedings of the 6th international conference on molluscan shellfish safety, New Zealand 2007*. The Royal Society of New Zealand.

Le Guyader, F. S., Bon, F., DeMedici, D., Parnaudeau, S., Bertome, A., et al. (2006a). Detection of noroviruses in an international gastroenteritis outbreak linked to oyster consumption. *Journal of Clinical Microbiology, 44*, 3878–3882.

Le Guyader, F., Haugarreau, L., Miossec, L., Dubois, E., & Pommepuy, M. (2000). Three-year study to assess human enteric viruses in shellfish. *Applied and Environmental Microbiology, 66*(8), 3241–3248.

Le Guyader, F. S., Le Saux, J. C., Ambert-Balay, K., Krol, J., Serais, O., Parnaudeau, S., et al. (2008). Aichi virus, norovirus, astrovirus, enterovirus, and rotavirus involved in clinical cases from a French oyster-related gastroenteritis outbreak. *Journal of Clinical Microbiology, 46*, 4011–4017.

Le Guyader, F. S., Loisy, F., Atmar, R. L., Hutson, A. M., Estes, M. K., Ruvoen-Clouet, N., et al. (2006b). Norwalk virus specific binding to oyster digestive tissues. *Emerging Infectious Diseases, 12*, 931–936.

Le Guyader, F. S., Neill, F. H., Dubois, E., Bon, F., Loisy, F., Kohli, E., et al. (2003). A semi-quantitative approach to estimate Norwalk-like virus contamination of oysters implicated in an outbreak. *International Journal of Food Microbiology, 87*, 107–112.

Le Guyader, F. S., Parnaudeau, S., Schaeffer, J., Bosch, A., Loisy, F., Pommepuy, M., et al. (2009). Detection and quantification of noroviruses in shellfish. *Applied and Environmental Microbiology, 75*, 618–624.

Lees, D. N. (2000). Viruses and bivalve shellfish. *International Journal of Food Microbiology, 59*, 81–116.

Loisy, F., Atmar, R. L., Guillon, P., LeCann, P., Pommepuy, M., & Le Guyader, F. S. (2005). Real-time RT-PCR for norovirus screening in shellfish. *Journal of Virological Methods, 123*, 1–7.

Lowther, J. A., Avant, J. M., Gizynski, K., Rangdale, R. E., & Lees, D. N. (2010). Comparison between quantitative real-time reverse transcription PCR results for norovirus in oysters and self-reported gastroenteric illness in restaurant customers. *Journal of Food Protection, 73*, 305–311.

Lowther, J. A., Henshilwood, K., & Lees, D. N. (2008). Determination of norovirus contamination in oysters from two commercial harvesting areas over an extended period, using semiquantitative real-time reverse transcription PCR. *Journal of Food Protection, 71*, 1427–1433.

Metcalf, T. G., Moulton, E., & Eckerson, D. (1980). Improved method and test strategy for recovery of enteric viruses from shellfish. *Applied and Environmental Microbiology, 39*, 141–152.

Nishida, T., Kimura, H., Saitoh, M., Shinohara, M., Kato, M., et al. (2003). Detection, quantitation, and phylogenic analysis of noroviruses in Japanese oysters. *Applied and Environmental Microbiology, 69*, 5782–5786.

Pintó, R. M., Costafreda, M. I., & Bosch, A. (2009). Risk assessment in shellfish-borne outbreaks of hepatitis A. *Applied and Environmental Microbiology, 75*, 7350–7355.

Potasman, I., Paz, A., & Odeh, M. (2002). Infectious outbreaks associated with bivalve shellfish consumption: A worldwide perspective. *Clinical Infectious Disease, 35*, 921–928.

Romalde, J. L., Estes, M. K., Szucs, G., Atmar, R. L., Woodley, C. M., & Metcalf, T. G. (1994). In situ detection of hepatitis A virus in cell cultures and shellfish tissues. *Applied and Environmental Microbiology, 60*, 1921–1926.

Sánchez, G., Pintó, R. M., Vanaclocha, H., & Bosch, A. (2002). Molecular characterization of hepatitis A virus isolates from a transcontinental shellfish-borne outbreak. *Journal of Clinical Microbiology, 40*, 4148–4155.

Shieh, Y. C., Khudyakov, Y. E., Xia, G., Ganova-Raeva, L. M., Khambaty, F. M., Woods, J. W., et al. (2007). Molecular confirmation of oysters as the vector for hepatitis A in a 2005 multistate outbreak. *Journal of Food Protection, 70*, 145–150.

Shieh, Y. C., Monroe, S., Fankhauser, R. L., Langlois, G. W., Burkhardt, W., & Baric, R. S. (2000). Detection of Norwalk-like virus in shellfish implicated in illness. *The Journal of Infectious Diseases, 181*(5), 1523–1874.

Simmons, G., Garbutt, C., Hewitt, J., & Greening, G. (2007). A New Zealand outbreak of norovirus gastroenteritis linked to the consumption of imported raw Korean oysters. *New Zealand Medical Journal, 120*, U2773.

Svraka, S., Duizer, E., Vennema, H., de Bruin, E., van der Veer, B., Dorresteijn, B., et al. (2007). Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. *Journal of Clinical Microbiology, 45*, 1389–1394.

Thompson, M., Ellison, S. L. R., & Wood, R. (2002). Harmonized guidelines for single laboratory validation of methods of analysis (IUPAC technical report). *Pure and Applied Chemistry, 74*(5), 835–855.

Vilarino, M. L., Le Guyader, F. S., Polo, D., Schaeffer, J., Krol, J., & Romalde, J. L. (2009). Assessment of human enteric viruses in cultured and wild bivalve molluscs. *International Microbiology, 12*, 145–151.

Westrell, T., Dusch, V., Ethelberg, S., Harris, J., Hjertqvist, M., Jourdan-da Silva, N., Koller, A., Lenglet, A., Lisby, M., & Vold, L. (2010). Norovirus outbreaks linked to oyster consumption in the United Kingdom, Norway, France, Sweden and Denmark, 2010. *Euro Surveill, 15*, pii:19524.

Wheeler, J. G., Sethi, D., Cowden, J. M., Wall, P. G., Rodrigues, L. C., Tompkins, D. S., et al. (1999). Study of infectious intestinal disease in England: Rates in the community, presenting to general practice, and reported to national surveillance. *BMJ, 318*, 1046–1050.