EFSA Panel on Biological Hazards (BIOHAZ); Norovirus (NoV) in oysters: methods, limits and control options

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SCIENTIFIC OPINION

Scientific Opinion on Norovirus (NoV) in oysters: methods, limits and control options[1]

EFSA Panel on Biological Hazards (BIOHAZ)[2, 3]

European Food Safety Authority (EFSA), Parma, Italy

ABSTRACT

NoV is highly infectious, and there is no threshold infectivity limit for NoV detected by PCR. The probability of becoming infected increases with the dose but depends also on the characteristics of the organism, the food matrix and the host factors. The relationship between the number of infectious virus particles and the number of virus genome copies detected by quantitative PCR is not a constant, and it is important to realise that the infectious risk associated with low level positive oysters as determined by real-time PCR may be overestimated.

Quantitative data on viral load from areas compliant with current EU legislative requirements (*E. coli* standards) during January-March 2010 in 3 selected member states, show that a viral limit of 100, 200, 500, 1000 or 10.000 NoV PCR copies would result in 33.6-88.9%, 24.4-83.3%, 10.0-72.2%, 7.7-44.4% or 0-11.1% of non-compliant batches, respectively. Compliance with any of the above NoV limits would reduce the number of contaminated oysters placed on the market and therefore the risk for consumers to become infected. It is currently not possible to quantify the public health impact of different limits

Microbiological criteria for NoV in oysters are useful for validation and verification of HACCP-based processes and procedures, and can also be used by competent authorities as an additional control to improve risk management in production areas, during processing and retail. The Panel recommended that risk managers should consider establishing an acceptable limit for NoV in oysters to be harvested and placed on the market. NoV testing of oysters (standardized CEN method) should be used to verify compliance with the acceptable NoV limit established.

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[1] On request from the Food Safety Authority of Ireland, Question No EFSA-Q-2010-00926, adopted on 8 December 2011.

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The most effective public health measure to control human NoV infection from oyster consumption is to produce oysters from areas which are not faecally contaminated, particularly given the ineffectiveness of current depuration and relaying procedures.

KEY WORDS

Norovirus, oysters, diagnostic methods, microbiological criteria, treatment, control.

SUMMARY

The Food Safety Authority of Ireland asked the Panel on Biological Hazards to issue a scientific Opinion on: (i) The use of real-time reverse-transcription polymerase chain reaction (rRT-PCR) as a means of detection and quantification of Norovirus (NoV) in oysters; (ii) on Limits that do not pose an unacceptable risk to consumers for NoV genogroups GI and GII in oysters as determined by real-time PCR, and (iii) on Treatment regimes (post-harvest interventions) that can be relied upon to reduce NoV counts in oysters.).

On the use of PCR, the BIOHAZ Panel concluded that PCR-based detection methods exist for NoV in bivalve shellfish. Harmonization and standardization are currently ongoing for NoV in shellfish, and publication of methods is expected soon. To achieve a good sensitivity separate assays are required for NoV GI and GII detection. With the appropriate quality assurance measures, including accreditation and proficiency testing, the standardised CEN method is considered suitable for use for detection and quantification of NoV in oysters within a legislative context. The Panel also recommended that research needs to be conducted to establish the relationship between detection of NoV in oysters by PCR and human health consequences.

On the question of NoV limits, the Panel concluded that: NoV can be frequently detected by rRT-PCR in oysters during winter in European areas compliant with current legislative requirements (*E. coli* standards) for which data are available. NoV is highly infectious, and exposure of human volunteers to serial dilutions yielded a dose-dependent probability of becoming ill ranging from 0.1 (at a dose of 10^3 NoV genome copies) to 0.7 (at a dose of 10^8 virus genome copies). However, there is no threshold infectivity limit for NoV detected by rRT-PCR. From outbreak published data, it can be concluded that NoV concentrations detected in oysters linked to human cases varied greatly from less than hundred copies to more than ten thousand per gram of material analysed. The probability of becoming infected increases with the dose but depends also on the characteristics of the organism, the food matrix and the host factors. The relationship between the number of infectious virus particles and the number of virus genome copies detected by quantitative PCR is not a constant, and may vary depending on environmental conditions including time from the initial release from the host. Furthermore, the number of genome copies detected by quantitative PCR may not relate to infectious NoV particles, and as a consequence the method can only be used to provide an indirect measure of risk, and when considering what is an acceptable level of NoV in oysters, it is important to realise that the infectious risk associated with low level positive oysters as determined by rRT-PCR may be overestimated.

Despite observed differences between the ability of NoV GI and GII to cause human infection via different transmission routes, there is insufficient knowledge on the dose response for each genogroup to allow a distinction. Therefore it is appropriate to consider the total NoV load (GI+GII) when establishing microbiological criteria. Quantitative data on viral load from areas compliant with current EU legislative requirements (*E. coli* standards) during January-March 2010 in 3 member states, show that a viral limit of 100, 200, 500, 1000 or 10,000 NoV PCR genome copies would result in 33.6-88.9%, 24.4-83.3%, 10.0-72.2%, 7.7-44.4% or 0-11.1% of non-compliant batches, respectively. Compliance with any of the above NoV limits would reduce the number of contaminated oysters placed on the market and therefore the risk for consumers to become infected. The lower the limit the greater the consumer protection achieved. However, it is not currently possible to quantify the public health impact of establishment of different limits.

Microbiological criteria for NoV in oysters are useful for validation and verification of HACCP-based processes and procedures, and can be used to communicate to food business operators and other stakeholders what is an acceptable or unacceptable viral load for oysters to be placed on the market. Microbiological criteria for NoV in oysters could also be used by competent authorities as an additional control to improve risk management in production areas, during processing and retail.

The Panel recommended that on the basis of the data presented in this Opinion, risk managers should consider establishing an acceptable limit for NoV in oysters to be harvested and placed on the market. Competent authorities should consider the use NoV testing of oysters (standardized CEN method) to verify compliance with the acceptable NoV limit established. Food business operators should consider incorporating NoV testing of oysters to verify their HACCP plans to demonstrate compliance with the acceptable level. The quantitative levels of NoV within production areas and batches should be investigated further, in order to optimise sampling strategies. Furthermore, sampling schemes to comply with NoV criteria should be risk based, e.g. considering seasonality, faecal pollution levels, community outbreaks, and variability from year to year. Finally, an EU-wide baseline survey on NoV contamination in oysters should be considered, in order to estimate consumer exposure and to allow quantification of the impact on human exposure related to establishment of microbiological criteria.

On the question of post-harvest interventions to reduce NoV counts in oysters, the Panel concluded that: Current treatment regimes for products placed live on the market (depuration and relaying) as commonly practised do not effectively reduce NoV in oysters. Depuration and relaying may be improved by optimising process parameters to enhance NoV reduction (e.g. depuration times, water temperature). However, limited data is currently available. Alternative treatments such as commercial heat treatment and high pressure may be effective for NoV inactivation, but give rise to organoleptic changes which may be unacceptable to consumers. The most effective public health measure to control human NoV infection from oyster consumption is to produce oysters from areas which are not faecally contaminated, particularly given the ineffectiveness of current control regimes.

The Panel recommended that control measures for NoV in oysters should focus on avoiding contamination by either preventing human faecal contamination in mollusc production areas, or restricting commercial harvesting from faecally-contaminated areas. In addition, further studies are needed to establish and optimise the effectiveness of depuration and relaying for NoV reduction using the standardised CEN method.

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BACKGROUND AS PROVIDED BY THE FOOD SAFETY AUTHORITY OF IRELAND

Norovirus (NoV) is a major cause of epidemic and sporadic cases of acute gastroenteritis in adults and children in Ireland and around the world. In 2009 there were 1647[4] reported noroviral infections in Ireland. NoV causes outbreaks in communities and is particularly prevalent during the winter months. NoV is transmitted through consumption of food and water contaminated with faecal matter, through person-to-person contact and through contact with infected surfaces.

Bivalve molluscs are a well documented source of noroviral infection since they have the ability to accumulate and concentrate NoV particles by filtration of water contaminated with faeces. NoV is a persistent problem in coastal waters during the winter months leading to the contamination of bivalve mollusc production areas. Oysters contaminated with NoV pose a particular risk to human health since they are routinely consumed raw.

In the EU, protection of shellfish waters has been achieved by a combination of environmental surveys of the area surrounding production beds and monitoring of faecal contamination of waters by testing bivalve molluscs for levels of *E. coli* above a specified limit[5] . However, the use of indicator microorganisms of faecal pollution is not a reliable means of determining the extent of NoV contamination of shellfish. NoV detection and quantification is difficult as they are non-culturable and their biologically heterogeneity limits the applicability of immunological and serological methods for identification purposes. However, in recent years real time-PCR has enabled confirmation and quantification of NoV.

In January 2010 the FSAI was notified of noroviral food poisoning incidents linked epidemiologically to an oyster production area in Ireland[6]. The food incident eventually resulted in 31 reported food poisoning events in England and Ireland with a total of 76 people reported ill, several of whom had NoV detected in stool specimens. The oyster production area in question is a class-A growing area for shellfish, which means that shellfish may be consumed directly without further treatment. Both prior to, and during this incident the testing results for *E. coli* in the oysters were below the legal limit for a Category-A area. However, real time-PCR was able to demonstrate the presence of both genogroup one (GI) and genogroup 2 (GII) NoV. Copy numbers ranged between 200-7000 per gram for GII with less contamination by GI, which tended to be sporadically positive and rarely above the level of quantification.

The oyster production area was closed as soon as the epidemiological link was suspected. However, then as now, the producers were compliant with all EC legislative requirements including microbiological standards. This creates a problem for the competent authority as there is no clear criterion for re-opening the production area. Surveillance of oysters from the area during March and April by real time-PCR continues to show NoV present in numbers between 300 and 2500 per gram albeit reduced from levels found during February.

Historic monitoring results show that NoV is often present in oysters in low numbers during the winter months and normal depuration regimes are not effective at removing the virus. Yet no reported human illness has been attributed to consumption of these contaminated oysters. Reports in the scientific literature have suggested human resistance to NoV[7]. In addition it is suggested that PCR methods will overestimate the number of infectious NoV particles.

[4] Provisional figures reported by the Health Protection Surveillance Centre (HPSC)

[5] <230MPN/100g Regulation (EC) 2073/2005 as amended

[6] The information provided was accurate to the best knowledge of the FSAI at the time of submitting this mandate to EFSA. A recent publication from the UK has shown more accurate figures on the number of outbreaks, the number of cases and has also shown that oysters from UK suppliers were also involved along with those from the Irish suppliers (www.eurosurveillance.org/ViewArticle.aspx?ArticleId=19524)

[7] Hutson et al (2002) Norwalk virus infection and disease is associated with ABO histo-blood group type. Journal of Infectious Diseases. 185, 1335-1337 : Lindesmith et al (2003) Human susceptibility and resistance to Norwalk virus infection. Nature Medicine. 9,5,548-553

It is reasonable to conclude that there may be quantifiable level of NoV in oysters as judged by real time PCR that may not pose an unacceptable risk to consumers. If this was the case then oyster beds could be re-opened on the basis of real time-PCR detection and quantification of NoV. It is essential that there is a scientific basis for re-opening closed shellfish production areas liked to NoV outbreaks. This is an issue faced by competent authorities both in Europe and around the world and is not solely confined to Ireland.

TERMS OF REFERENCE AS PROVIDED BY THE FOOD SAFETY AUTHORITY OF IRELAND

The Food Safety Authority of Ireland requests that EFSA provides a scientific Opinion on:

1) The use of real-time PCR as a means of detection and quantification of NoV in oysters.

2) Limits that do not pose an unacceptable risk to consumers for NoV genogroups GI and GII in oysters as determined by real-time PCR (e.g. copy number per gram).

3) Treatment regimes (post-harvest interventions) that can be relied upon to reduce NoV counts in oysters.

ASSESSMENT

1. Introduction

Norovirus (NoV) is a genus in the family *Caliciviridae*, a group of non-enveloped, icosahedral viruses, having a single-stranded positive sense RNA genome. Bivalve molluscs are a well documented source of noroviral infection since they have the ability to accumulate and concentrate NoV particles by filtration of water contaminated with faeces. NoV is a persistent problem in coastal waters during the winter months leading to the contamination of bivalve mollusc production areas. Oysters contaminated with NoV pose a particular risk to human health since they are routinely consumed raw.

In June 2011 EFSA published a scientific Opinion updating the present knowledge on the occurrence and control of food-borne viruses[8]. In this opinion it was recommended to focus on preventive measures to avoid viral contamination rather than trying to remove/inactivate these viruses from food. In addition it was recommended to introduce microbiological criteria for viruses in bivalve molluscs unless they are labelled: "*to be cooked before consumption*", and also to introduce microbiological criteria for classification of bivalve molluscs production areas. Such criteria could be used by food business operators to validate their control options. Furthermore it was emphasised that a virus monitoring programme for compliance with these criteria should be risk based according to the findings of a sanitary survey and that using an *E. coli* standard for monitoring and classification of bivalve mollusc production areas provides general information about the background level of faecal contamination, and should be retained.

In the present mandate, EFSA is asked for a scientific opinion on the use of real-time reverse-transcription polymerase chain reaction (rRT-PCR) as a means of detection and quantification of NoV in oysters; and on limits that do not pose an unacceptable risk to consumers for NoV genogroups GI and GII in oysters as determined by real-time PCR (e.g. copy number /gram). While establishment of acceptable *vs.* unacceptable risk is a risk management task, the present opinion focuses on dose-response relationships, rRT-PCR detection methods, interpretation of NoV RT-PCR results, and on qualitative and quantitative occurrence of NoV in oysters. Also, the consequences, on compliance and thereby consumer exposure, of introducing different acceptable limits are elucidated. Ideally, the acceptable limit of a criteria should be linked through risk assessment to a realistic and appropriate or current level of protection, but data needs (availability of representative data on Nov in oysters across EU) and difficulties in knowing and recording the real number of human NoV-infections caused by oysters, makes this exercise impossible at present. In addition to rRT-PCR methods and results, the opinion also covers postharvest treatment regimes (post-harvest interventions) that can be relied upon to reduce NoV counts in oysters.

2. Dose-response relationship

The first paper reporting a volunteer study with Norwalk virus was published in 1972 and reported serial dilutions of a stool filtrate inducing disease through 3 serial passages in volunteers (Dolin et al., 1972). The frequency of disease remained high throughout these passages, with unchanged clinical spectrum but a slightly shortened incubation period (Dolin et al., 1972). Dose information was not obtained in these studies due to lack of virus detection methods; however, the agent in the Norwalk virus outbreak derived filtrates was still infectious for human volunteers after ether, acid, or heat treatment for 30 minutes at 60°C. Following this study, Teunis *et al*. showed that exposure of human volunteers to serial dilutions of the original inoculum of NoV (characterized as GI.1 Norwalk virus) yielded a dose-dependent probability of becoming ill ranging from 0.1 (at a dose of 10^3 NoV genomes) to 0.7 (at a dose of 10^8 virus genomes) (Teunis et al., 2008).

[8] EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on An update on the present knowledge on the occurrence and control of foodborne viruses. EFSA Journal 2011;9(7):2190. [96 pp.] doi:10.2903/j.efsa.2011.2190. Available online: www.efsa.europa.eu/efsajournal

After the initial outbreak of Norwalk virus in the early 1970's, this virus was hardly detected. Dose-response relations for prevalent NoV, such as GII, or any other GI NoV than Norwalk virus, have not been published to date.

The probability of becoming infected increases with the dose, as was observed in volunteer studies and during outbreaks (de Wit et al., 2007; ter Waarbeek et al., 2010; Teunis et al., 2008; Visser et al., 2010).

In addition, a correlation has been found between the number of NoV genome copies in oysters, and the amount of self reported illness in a specific study in the UK (Lowther et al., 2010) with the highest NoV RNA levels resulting in the highest rate of reported illness, suggesting a linkage between virus RNA levels and health risks.

Very few volunteer studies have been conducted with contaminated oysters. The first report described the use of volunteers to test depurated (during 7 days) oysters before marketing, with a demonstration of quite a large illness frequency (up to 37%) depending on the time of the year, and oyster sampling location (Grohmann et al., 1981). Unfortunately at this time no quantitative detection method for NoV was available. A second volunteer study was recently conducted to evaluate high pressure to inactivate NoV GI.1 Norwalk resulting in no NoV infection among subjects receiving high pressure-treated oysters as determined by RT-PCR detection of NoV RNA in subject samples (Leon et al., 2011).

In general, there is limited data on the dose response for NoVs in humans. The majority of infections recorded in the human population arise from NoV GII.4 genotypes perhaps inferring a lower infectious dose associated with this genotype than other genotypes. However greater virus survival and differences in host immunity may also play a role. By contrast, there is evidence that a disproportionate number of foodborne illnesses (including those caused by oysters) are associated with NoV GI genotype infections (Verhoef et al., 2010). This is possibly due to greater environmental survival of NoV GI genotypes, or to differential uptake and retention in oysters (Maalouf et al., 2011). Despite these apparent differences between the ability of NoV GI and GII to cause human infection via different transmission routes, there is insufficient knowledge on the dose response, illness rate or severity of symptoms for each genogroup to allow a distinction to be made between them when setting a standard in oysters.

3. Detection of NoV in oysters

As these viruses cannot be cultured, laboratory detection methods depend primarily on electron microscopy (EM) (Duizer et al., 2004), enzyme-linked immunosorbent assay (ELISA) and reverse-transcription polymerase chain reaction (RT–PCR), the choice of detection technique is depending on the sample type. Both EM and ELISA are reliable diagnostic tools for detection of NoVs in clinical samples, but they cannot be used for direct detection of NoVs in environmentally contaminated samples, such as bivalve shellfish or other foods, due to low levels of virus and matrix complexity.

Despite extensive attempts by several investigators testing different human and animal cell lines, no efficient cell culture amplification process has been found yet (Duizer et al., 2004; Guix et al., 2007; Lay et al., 2010). A cell culture system for human NoV was recently published (Straub et al., 2007). The authors demonstrated a limited growth of both GI and GII *in vitro*, using a three-dimensional model of human intestine epithelial cells under conditions of physiological fluid shear in rotating wall vessel bioreactors (Straub et al., 2007). At present, no other laboratories have been able to reproduce this result. Another cell culture model has been investigated, but results need to be confirmed (Leung et al., 2010). Although NoVs are present in several animal species such as swine, cattle and mice, the murine NoV is the only one that grows in cell culture systems. The murine NoV has been suggested as an experimental model system for the study of human NoV, specifically to improve the understanding of NoV replication, phatogenesis, virus-host interactions and host immune responses (Virgin et al., 2006). The murine NoV may be not as resistant as other NoVs (Sattar et al., 2011).

In the absence of other assays with detection levels low enough to detect viruses directly, RT–PCR is the only published method that offers the possibility of direct detection of NoVs in environmental samples such as bivalve shellfish or other foods (Lees, 2000; Mattison et al., 2010). Its high sensitivity and accuracy enables the detection of as little as 10 virus copies. rRT-PCR, performed with adequate controls and standard curves, is therefore increasingly used to quantify virus. Such high analytical sensitivity is a prerequisite for the detection of virus in food, because the number of viral particles needed to cause infection may be very low. There are various methods to assess the presence of inhibitors and determination of inhibition of target amplification due to matrix interference (Atmar et al., 1995; Le Guyader et al., 2000; Schwab et al., 1998). Adding a PCR external amplification control (EAC) to the reactions mixture is an absolute requirement to avoid any competitive effects and circumvent the loss of the quantitative properties of the assay.

In the 1990s, targets for RT-PCR assays were found in genome conserved areas, such as the polymerase gene, the ORF1-ORF2 junction, or areas in the capsid coding region. Unfortunately, no single primer pair will detect all NoVs, but many assays that detect a broad range of strains are currently available (Mattison et al., 2009; Vinje et al., 2004). In order to ensure adequate sensitivity and specificity, it is important to achieve a good quality of nucleic acid extract, and to include all controls needed to detect false negative or positive results.

3.1. General background on methods for viruses

Viruses can be present in shellfish in very low numbers and still pose a risk to consumers, making it necessary to develop highly sensitive viral extraction methods to ensure virus recovery from shellfish tissues. The observation that viruses are concentrated in digestive diverticulum tissues led to the development of a method that represented a major step in the improvement of extraction methodologies (Atmar et al., 1995; Metcalf et al., 1980). This observation was subsequently confirmed by detection of hepatitis A virus (HAV) (Romalde et al., 1994) as well as through the tissue-specific quantification of infectious enteric adenoviruses and rotaviruses in mussels previously contaminated by bioaccumulation of such viruses, and similarly of Norwalk virus in oysters and clams (Abad et al., 1997; Schwab et al., 1998). More recently the demonstration that NoV bind specifically to digestive tissues (DT) confirms this observation (Maalouf et al., 2010b). Using rRT-PCR, the concentration observed in DT, that represent usually about one tenth of the total animal weight for oysters and mussels, was at least two log higher than concentration detected in other tissues such as gills or mantle (Maalouf et al., 2011). Analysis of DT provides several advantages, including increased sensitivity, decreased processing time and decreased interference with RT-PCR by inhibitory substances (Atmar et al., 1995). With the exception of small species, such as clams or cockles, in which dissection may be technically difficult, most of recent methods are based on dissected tissues and thus will be discussed here.

Extraction of enteric viruses from shellfish is based on several steps: virus elution from shellfish tissues, recovery of viral particles, and then virus concentration. The weight analyzed generally ranges from 1.5 to 2 g of digestive tissues. Some methods propose larger weights for the first step but thereafter analyze only a fraction of the extracts (Boxman et al., 2006; Depaola et al., 2010; Kittigul et al., 2008). Viruses are eluted from shellfish digestive tissues using various buffers (i.e. chloroform-butanol or glycine) before being concentrated either by polyethylene glycol precipitation or ultracentrifugation (Atmar et al., 1995; Milne et al., 2007; Myrmel et al., 2004; Nishida et al., 2003). Direct lysis of virus particles has also been used, including methods utilizing proteinase K or Trizol to destroy shellfish tissues or Zirconia beads and a denaturing buffer for virus and/or nucleic acid elution (Greening and Hewitt, 2008; Jothikumar et al., 2005; Kittigul et al., 2008; Lodder-Verschoor et al., 2005; Lowther et al., 2008; Umesha et al., 2008).

In addition to the in-house protocols that have been used for nucleic acids extraction and purification, a number of commercial kits can also be successfully applied to accomplish this task. Advantages of the commercial kits used for nucleic acid purification include their reliability, reproducibility and ease

of use. Most of these kits are based on guanidium lysis followed by capture of nucleic acids on columns, beads or silica (Costafreda et al., 2006; de Roda Husman et al., 2007; Depaola et al., 2010; Fukuda et al., 2008; Kingsley et al., 2007; Le Guyader et al., 2009; Lodder-Verschoor et al., 2005; Milne et al., 2007; Nenonen et al., 2008; Nishida et al., 2003; Umesha et al., 2008).

One of the goals of extraction methods is to remove inhibitors of the RT and PCR reactions sufficiently to allow detection of viral nucleic acids. Polysaccharides present in shellfish tissue are at least one substance that can inhibit the PCR reaction (Atmar et al., 1993). Elimination of inhibitors is difficult to evaluate and depending on the time of the year and shellfish life, different compounds may be present (Burkhardt and Calci, 2000; Di Girolamo et al., 1970). External amplification control standards are used to detect the presence of significant sample inhibition, and the amount and frequency of sample inhibition has varied depending upon the shellfish tissue being analyzed (Atmar et al., 1995; Le Guyader et al., 2000; Schwab et al., 1998). Recent advances in food virology re-enforce the need of harmonization of methods as well as addressing quality assurance and quality control (Pinto and Bosch, 2008).

The addition of an external virus to a shellfish sample has been proposed as a control to evaluate the extraction efficiency of molecular virus detection methods (Costafreda et al., 2006; Depaola et al., 2010; Le Guyader et al., 2009; Lowther et al., 2008; Nishida et al., 2007). An ideal candidate would have the following properties: (i) it would be an encapsidated RNA virus with properties similar to the enteric viruses contaminating shellfish, (ii) it would normally not be present in field samples (thus RNA phages may be problematic), and (iii) it would be non-pathogenic. Based on these considerations a number of viruses such as Mengovirus or Echo type 9 (*Picornaviridae* family members) (Costafreda et al., 2006; Le Guyader et al., 2009; Nishida et al., 2007) or non-human calicivirus such as a feline calicivirus or San Miguel sea lion virus (Depaola et al., 2010; Lowther et al., 2008) have been proposed as a control for extraction efficiency.

Table 1: Overview of methods used for virus detection in shellfish.

Shellfish	Mass analysed	Virus Elution	Virus Concentration	Mass used for nucleic acid extraction	Nucleic acid extraction	% of sample used for RT-PCR	Reference
Oysters	25 g	Glycine	PEG	0.5g	QIAamp (Qiagen)	3	(Shieh et al., 1999)
	50 g	Water	PEG, precipitate	0.5g	Boiling	nc	(Chung et al., 1996)
	18 g	Glycine	Ultracentrifugation	1g	GuSCN	10	(Muniain-Mujika et al., 2003)
	50 g	Sonication	PEG	nc	GuSCN	nc	(Green et al., 1998)
	25 g	Glycine	PEG	0.4g	Tri-reagent (Sigma)	10	(Kingsley and Richards, 2001)
	1.5 g DT	Chloroform-but, CatFloc (Calgon)	PEG	1.5 g	Prot. K, CTAB	20	(Atmar et al., 1995)
	1.5 g DT	Gycine-threonine	PEG	0.12g	GuSCN+ QIAamp	16	(Beuret et al., 2003)
	1.5 g DT	Chloroform-but, Cat-floc	PEG	1.5 g	Prot. K, CTAB	20	(Schwab et al., 2001)
	1.5 g DT	Phosphate saline buffer pH7.4 Chloroform-but, Cat-floc	Ultracentrifugation	1g	QIAamp	25	(Nishida et al., 2003)
	1.5 g DT	Zirconia beads	nc	0.09g	RNEasy (Qiagen)	6	(Lodder-Verschoor et al., 2005)
	DT of 1 oyster	Stainless-steels beads	nc	nc	Silica and guanidium	17	(Ueki et al., 2005)
	2 g DT	Proteinase K	nc	0.01g	GuSCN	4*	(Jothikumar et al., 2005)
	10g DT	TRIzol (Gibco)	nc	0.08g	GuSCN	8	(Boxman et al., 2006)
	1.5 g DT	Zirconia beads	nc	nc	RNeasy	5	(Schultz et al., 2007)
	nc	Glycine, pH 10	Ultracentifugation	nc	Silica-method	10	(Hernroth and Allard, 2007)
	DT of 3 oys.	Phosphate saline buffer	PEG	1 g	QIAamp	5	(Fukuda et al., 2008)
	5 g DT	Buffer pH 8 + Prot. K	nc	0.05 g	Silica and guanidium	10	(Greening and Hewitt, 2008)

Shellfish	Mass analysed	Virus Elution	Virus Concentration	Mass used for nucleic acid extraction	Nucleic acid extraction	% of sample used for RT-PCR	Reference
	25 g flesh	Adsorption-alkaline elution	PEG (twice)	5 g flesh	RNeasy	3	(Kittigul et al., 2008)
	DT of 6 oysters	Buffer pH 8 + Prot. K	-	nc	Silica and guanidium	11	(Lowther et al., 2008)
	1.5 g DT	Glycine pH 9	PEG	1.5 g	Nuclisens (BioMerieux)	20	(Le Guyader et al., 2009)
	5 g DT	Finely chopped + Proteinase K	-	0.15g	RNeasy	4	(Gentry et al., 2009)
	25 g flesh	Adsorption elution	PEG (twice)	25 g (flesh)	RNeasy	6	(Depaola et al., 2010)
	2 g DT	Tryptose phosphate broth and chloroform butanol	PEG	nc	Silica and guanidium	nc	(Rigotto et al., 2010)
Mussels	20 g	Glycine, cat-Floc	Antigen capture	0.04g	QIAamp	21	(Lee et al., 1999)
	50 g	Glycine	PEG	nc	Guanidium, CsCl	100	(Croci et al., 2000)
	100 g	Glycine	Ultracentrifugation	1 g	GuSCN	5	(Pina et al., 1998)
	10 g	Glycine	PEG	0.8 g	RNEasy	50	(Chironna et al., 2002)
	25 g	Threonine	PEG	nc	GuSCN	2.5	(Mullendore et al., 2001)
	25 g DT	Glycine	Ultracentrifugation	1.5 g	TRIzol (Gibco)+ Boom	6	(Myrmel et al., 2004)
	2 g DT	Glycine	Ultracentrifugation	0.1 g	GuSCN	10	(Hernroth et al., 2002)
	75g total	Glycine buffer pH 9.2	PEG	8.3 g	Guanidium	100	(Croci et al., 2007)
	1.5 g DT	Glycine buffer pH 9.5	PEG	1.5	Nuclisens	5	(Vilarino et al., 2009)
	25 g DT	Glycine buffer pH 9.2	PEG twice	0.75 g	Nucleospin RNA (Macherey-Nagel)	4	(Serracca et al., 2010)
Clams	25 g	Glycine, chlorof.	Ultracentrifugation	1.25 g	Nucleospin RNA	10	(Sunen et al., 2004)
	1.5g DT	Chlorof-but, CatFloc	PEG	0.07 g	RNeasy	6	(Costafreda et al., 2006)
	1g DT	Phosphate-buffered saline	Ultracentrifugation	nc	QIAamp	1.5	(Hansman et al., 2007)

PEG: polyethylene glycol
CTAB: cetyltrimethylammonium bromide
nc: not communicated in the paper
- : no concentration step.

The key objective for developing procedures for shellfish analysis, is to end up with result in a low volume of viral concentrate, and subsequently with a highly purified nucleic acid preparation with no inhibitory effect to the PCR. The sensitivity of the RT-PCR is essentially the result of two related factors: (i) the efficiency of recovery of the extraction procedure applied to the shellfish sample, and (ii) the degree of final purity of the recovered virus. As shown in table 1, some methods begin with a large mass of sample and then extract only a portion, while other protocols are based on small weight being all extracted up to the last step. In the RT-PCR reaction the volume of sample analyzed is always very small, we can assume that all rRT-PCR assays have a limit of sensitivity above 10 viral units/ g of digestive tissues.

Even with the application of highly sensitive molecular methods such as PCR, the detection of NoV in shellfish constitutes a challenge due to: (i) the low number of virus particles needed to cause disease, (ii) the high variability of the virus genome, and (iii) the possible presence of inhibitory substances in such a complex food matrix. It must therefore be recognised that as with any method, the rRT-PCR procedure employed may underestimate the number of NoV particles present in sample. In particular the specificity of the primers employed is of critical importance. NoVs, as all RNA viruses, are prone to point mutation and recombination, which often gives rise to new variants of the viruses. It is important that PCR methods accommodate viral variation. It is therefore possible that a range of NoV genotypes may go undetected depending on the primers employed. This issue is dealt with, to some extent, in the proposed standardised method requiring the use of validated cross-reactive primers that are directed towards the ORF1-ORF2 junction region for either GI or GII NoV genotypes (Jothikumar et al., 2005; Loisy et al., 2005, Le Guyader et al. 2009). However, because of the rapid rate of evolution of NoVs rough recombination and mutation, variants of recognised genotypes and novel genotypes are regularly reported. These novel genotypes and variants may or may not be detected by currently validated primers potentially leading to false negative results in the future.

Comparing methods is a difficult challenge as no reference materials are available. However it is important to point out that many methods have never been applied to naturally contaminated shellfish.

The EU Reference laboratory for microbiological and viral contamination of bivalve molluscs (Cefas, Weymouth, UK) has, since 2003, conducted annual proficiency testing for detection of NoV in shellfish. The reports are available on the EU-RL website (www.crlcefas.org). Participants include most European National reference laboratories and specialist Government laboratories in a number of third countries. For example in the latest distribution in 2011 there were a total of 27 participants including 15 EU National Reference Laboratories (NRLs) and 8 third country laboratories including laboratories in Australia, Canada, Chile, Korea, New Zealand, Peru and Singapore. A variety of methods were used by laboratories participating in this proficiency test distribution. For virus extraction, 72% of labs providing information used proteinase K digestion of chopped digestive glands. 17% used the Atmar method (chloroform/butanol extraction in the presence of a flocculating agent on homogenised glands, followed by PEG/NaCl precipitation) (Atmar et al., 1995). Other labs used in-house methods. For RNA extraction 46% used the Biomerieux nuclisens magnetic extraction reagents, 35% used QIAGEN RNA extraction kits, other labs used other kits or in-house methods. For PCR, 85% used rRT-PCR, whilst 15% used conventional RT-PCR. Of those using real-time, 78% used one-step RT-PCR whereas 22% used two-step RT-PCR. A wide variety of PCR primer and probe combinations were used: for NoV GI, 10 combinations were used; for NoV GII, 6 combinations were used. However all sets used targeted the highly conserved ORF1/2 junction. Proficiency test samples distributed for NoV have included both stabilised virus from clinical samples (lenticules) and bivalve molluscs samples (either whole animal or dissected digestive diverticulum). Laboratory performance has, in general, been good for clinical samples but more problematical for matrix (shellfish) samples. For example in the 2011 distribution, sensitivity for lenticules was high (24/27 labs scored 100%) whereas sensitivity for shellfish samples was more variable (15/27 labs scored 100%). To illustrate the issue, 6 of the 27 laboratories participating failed to detect any NoV in an oyster sample responsible for a large international outbreak of NoV gastroenteritis, and a further 5 laboratories detected only 1 of the 2 genogroups of NoV present in the sample. It is clear from the results of proficiency testing that shellfish are a demanding matrix when compared with high titre clinical samples, and that the variety

of methods in use cannot be assumed to give equivalent results. Comparison of laboratory performance against test methodology used during both the 2011 distribution and previous rounds suggest the use of more standardised methods (as described below) generally results in better performance. For example in the 2011 distribution, indicators of good performance (defined as >80% sensitivity with shellfish samples) versus poor performance (<40% sensitivity with shellfish samples) were: the use of the standardised nuclisens approach for RNA extraction (86% of labs achieved good performance *vs.* 36% of labs using other methods), and the use of rRT-PCR (68% of labs achieved good performance *vs.* 25% of labs using conventional PCR). The proficiency testing data also clearly shows that some laboratories using in-house methods may fail to detect NoV in samples that have biologically relevant levels of virus i.e. they have caused an outbreak. This evidence underpins the importance of standardisation of methods and demonstration of their fitness for purpose through validation.

3.2. Standardisation of methods specifically in oysters

A major factor limiting the uptake of NoV testing into regulatory food controls for oysters is the current absence (world-wide) of any standardised and validated methods for detection of viruses in foods. In 2004 the European Committee of Standardisation (CEN) initiated the development of a standard method for detection of NoV in foodstuffs, including bivalve molluscs (e.g. oysters), based on PCR (Lees and TAG4 CW, 2010). The standard developed by a working group of expert European laboratories is now well advanced and due for publication in 2012 (www.cen.eu, WI number 00275167). Bivalve molluscs present a challenging matrix and the standard method needed to be capable of extracting low levels of contaminating virus and presenting them in a non-inhibitory extract to a sensitive PCR assay. Key aspects of the developing method were tested by inter-laboratory evaluations to ensure robust performance. Dissected digestive diverticulum (digestive gland) is used as the starting material for bivalve molluscs with further enzymatic digestion using proteinase K (Jothikumar et al., 2005). Nucleic acid is then extracted from digested extracts using guanidine thiocyanate (GITC) to denature viral coat proteins in combination with magnetic silica particles to bind released nucleic acid, purified through successive washing stages, before final elution in a small volume. Reverse transcription and PCR utilises a one-step approach using specific primers in order to simply the procedure as much as possible. However, commercial one-step kits must utilise enzymes specifically engineered for use with low abundance targets. TaqMan PCR real-time chemistries are stipulated for the amplification since: the closed tube format is less susceptible to contamination; is logistically efficient; incorporates a probe based confirmation step; can be quantitative; and is more amenable to standardisation than conventional PCR. To maximise sensitivity real-time PCR assays are run separately for NoV GI and GII. Cross-reactive real-time PCR primers and probes are directed in the ORF1-ORF2 junction region for NoV (Jothikumar et al., 2005; Loisy et al., 2005, Le Guyader et al. 2009). Exact primer/probe configuration within these regions is flexible to accommodate potential future strain variability. However the standard requires the use of peer reviewed primers/probes shown to be sufficiently sensitive and cross-reactive. The standard includes an informative annex with recommended primers/probes suitable for detection of all current strains of human NoV. The method is highly sensitive in order to detect the low levels of virus found in environmentally contaminated samples and hence also vulnerable to both cross-contamination (false positives) and potential matrix interferences (false negatives). Thus a comprehensive suite of controls was also developed to cover: positive and negative process controls; negative RNA extraction control; positive RT-PCR and RT-PCR inhibition controls; 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. During the development of the standard inter-laboratory studies by the working group successfully utilised the MC0 strain of Mengo virus (Costafreda et al., 2006) as a process control. The negative process control is a known negative sample that is taken through the entire extraction procedure and analysed. The RT-PCR inhibition control checks for potential matrix suppression by comparison of amplification of an external RNA template added to test material and a control well. Taken together the controls generate data on all aspects of the assay and are utilised to determine the

acceptability of test performance against established quality control criteria. Quantitation is based on a plasmid DNA calibration curve for each assay (NoV GI, NoV GII) with plasmid DNA concentration measured using spectrometry at 260nm. Results are reported in the standardised form of detectable virus genome copies per gram of digestive gland. Formal validation studies are planned to characterise the method according to the international requirements.

The developing CEN standard requires laboratories to report results in relation to: (i) the theoretical limit of detection (tLOD) - a level that constitutes the smallest quantity of target that can in theory be detected. This corresponds to one genome copy per volume of RNA tested in the target assay but will vary according to the test matrix and the quantity of starting material; (ii) The practical limit of detection (pLOD) - the lowest concentration of target in a test sample that can be reproducibly detected (95% confidence interval), as demonstrated by a collaborative trial or other validation; (iii) The limit of quantification (LOQ) - the lowest concentration of target in a test sample that can be quantitatively determined with acceptable level of precision and accuracy, as demonstrated by a collaborative trial or other validation.

The theoretical limit of detection (tLOD) in genome copies/g digestive tissues corresponds to the detection of a single target molecule in the total volume of RNA analysed for each sample and target. This figure is dependent on the volume of homogenate retained following treatment of the 2g portion of digestive tissues with proteinase K solution, however for a sample producing 2ml homogenate the tLOD is 20 copies/g, and for a sample producing 3ml it is 30 copies/g. pLODs and LOQs for detection of NoV in shellfish using the CEN method have been established by some laboratories but have not yet been published. The intention of the CEN working group is to establish reference LOD and LOQ values through collaborative validation trials, these trials have not yet been performed. If virus standards are adopted into EU Official Controls then it is a legal requirement under Regulation 882/2004 for the performance characteristics of test methods to be established (validated) and this will include determination of LOD and LOQ. This is also a requirement of test method accreditation under ISO 17025 which is also required of methods used for determining compliance with EU Official Controls. In considering possible legislative standards it is obviously imperative for utilised methods to have an LOQ demonstrably below the proposed legislative level. With the CEN method it is not possible to quantify numbers of viral particles below 100 genome copies per gram of digestive diverticulum. Clearly therefore legislative standards below this level are unlikely to be analytically feasible using present methodology.

3.3. Interpretation of NoV RT-PCR results

The development of a robust and reliable quantitative real-time PCR method for the detection of NoV in oysters is a major technical advance. It has the potential to provide a valuable tool for risk assessment purposes. However it must be recognised that the methodology and the current state of knowledge may offer limitations, which need to be taken into consideration.

PCR methods for detecting NoVs rely on detection of specific genomic material to establish the presence of virus in a sample. The genomic material detected by this method may or may not originate from an infectious virus particle. Therefore the number of genome copies detected by quantitative real-time PCR procedures may not relate directly to the number of infectious virus particles (Jofre and Ba, 2009). There is now clear evidence that real-time PCR methods can overestimate the number of infectious viruses (demonstrated with viruses other than NoV) present in environmental samples such as treated wastewater (Jofre and R., 2009). This will also undoubtedly apply to shellfish samples. This may be particularly true where the virus has been subjected to significant environmental pressures including wastewater disinfection before and during uptake by shellfish. This clearly leads to the potential to overestimate the infectious risk associated with NoV positive shellfish. Attempts have been made to establish a relationship between the number of infectious virus particles and the number of virus genome copies detected by quantitative PCR using a statistical (Donia et al., 2009) or methodological basis (Rodriguez et al., 2009). However these have been inconclusive or are

impractical for standardised routine use. Furthermore the situation is complicated by the fact that the ratio of infectious to non-infectious virus particles detected is likely to vary from sample to sample depending on the impact of the environmental conditions encountered (Jofre and R., 2009). In addition, the ratio between infectious and non-infectious virus particles increases over time from the initial release from the host (de Roda Husman et al., 2009). When considering what is an acceptable level of NoV in oysters it is important to realise that the infectious risk associated with low level positive oysters as determined by real-time PCR may be overestimated (See also Chapter 2).

The current test procedures being presented for standardisation employ primers used to target NoV GI and GII individually (Lees and CEN WG6 TAG4, 2010). This is because it is not possible to develop a single set of primers cross-reactive for both genogroups. The primers employed detect a comprehensive range of genotypes within each of the genogroups but do not distinguish between them without further genotyping analysis. Each primer set will detect a range of NoV GI or GII genotypes and the level determined for each genogroup is the cumulative total of all genotypes detected. Therefore the current arrangement of reporting of values for NoV GI and GII levels individually is an artefact of the testing methodology. This, aligned with the fact that illness associated with oyster consumption can cause infection with multiple NoV genotypes (Gallimore et al., 2005; Kageyama et al., 2004; Le Guyader et al., 2008; Le Guyader et al., 2006b; Nakagawa-Okamoto et al., 2009; Sugieda et al., 1996), appear to make it most appropriate to base a standard on the total level of NoVs (both NoV GI and GII) present.

A further possible important consequence of not distinguishing between genotypes is that different genotypes and associated variants may demonstrate differing infectious doses and susceptibility rates in the human population. Those genotypes with lower infectious doses and emerging GII.4 variants in particular, may present a greater risk of infection which is not specifically identified by the current PCR procedures.

3.4. Sampling strategy

Currently there is no guidance on sampling regimes for NoV monitoring in oysters or any other bivalve mollusc species. The sample size depends on the aim of the study, the anticipated prevalence and desired accuracy. For monitoring of NoV in bivalve molluscs, a relevant approach could be to determine whether contamination is present above a particular level, in order to prevent highly contaminated bivalves to reach the market with possible adverse health outcomes for the consumer. One could assume that for such monitoring, a standard method for detection of NoV in bivalve molluscs (e.g. oysters) based on PCR as described above is applied (Lees and CEN WG6 TAG4, 2010).

Table 2 shows the estimated probability that contamination will be detected when one sample is collected from a batch, and examined according to the CEN method, assuming that digestive tissues from 10 oysters are pooled, 2 g is used for nucleic acid extraction and RT-PCR is done on the concentrate and 10-fold diluted RNA, in duplicate. Results show that the success of the considered testing regime strongly depends on the NoV prevalence (batch prevalence), and the NoV concentration (genome copies per gram of digestive tissue) (Table 2).

Relating these estimates to survey data depicted in Figure 1 for the UK, and assuming for illustration purposes that this sampling is representative for a batch, gives an estimated probability of detecting contamination of at least 39% (batch prevalence ~30%, concentration of ≥100 genome copies) for November 2009. For December 2010, the batch prevalence was about 60% for a concentration of ≥500 genome copies, giving a detection probability of >93%. Examining more samples per batch would increase the probability of detecting any contamination.

Table 2: Probability of identifying a random sample from a batch positive when processed according to the CEN procedure (pool of 10), as function of the batch prevalence and concentration (genome copies of NoV per g digestive tissue).

NoV prevalence in batch	Average concentration per g digestive tissue				
	1	100	200	500	1000
1%	0.1%	4.0%	6.3%	8.9%	9.5%
2%	0.1%	7.6%	12%	17%	18%
5%	0.2%	17%	26%	37%	40%
10%	0.3%	27%	43%	61%	65%
25%	0.5%	39%	62%	88%	94%
50%	0.5%	41%	66%	93%	99%
75%	0.5%	41%	66%	93%	100%
95%	0.5%	41%	66%	93%	100%

The estimated probability that contaminated batches remain unnoticed is 1 minus the detection probability from Table 2. As indicated, the fraction that passes the screening could theoretically be large, but this depends on the prevalence of contamination within a batch. Limited data are available to support the assumption that NoV are homogeneously distributed in a batch. However, Lowther (2008) showed little between animal NoV titre variability in *C. gigas* grown in commercial oyster sacks, which supports the assumption that oysters are exposed to uniform NoV contamination levels. In that case, the NoV prevalence in batches can be expected to be large, increasing the detection probability based on a single sample screening. If more quantitative data become available on individual oysters and batches, the theoretical estimates from Table 2 could be more refined.

These estimated detection probabilities from Table 2 are dependent on the detection efficiency. The minimum number of oysters required for testing within the CEN protocol is 10 oysters. Usually oysters are tested in pools of 6 to 10 animals. Under controlled experimental conditions, analysis of pooled samples did not adversely affect NoV results (Rangdale, 2007). However, there may be an increased sensitivity of virus detection by analysing oysters individually compared to pooled samples (de Roda Husman et al., 2007). The lower the virus concentration or number of positive individuals per pool, the more false negative test results will be obtained when samples are analyzed in pools, as compared with individually analyzed oysters.

Sample variability was evaluated by Le Guyader et al. in 2009 who analyzed six naturally contaminated shellfish samples three times in separate experiments using an elution precipitation method to concentrate viral particles from digestive tissues. Nucleic acids were then extracted using the Nuclisens kit (BioMerieux, France). NoV were detected by a one step rRT-PCR assay targeting the 5' end of the ORF2 of NoV GI and GII in separate runs. Persistence of inhibitors were checked for both genogroups and are expressed as rRT-PCR efficiency percentage (Le Guyader et al., 2009).

Table 3: Reproducibility for NoV detection in six naturally contaminated samples extracted three times each.

Sample	NoV GI *r*RT-PCR efficiency [a,b]	Assay (RNA/g DT) 1	2	3	NoV GII *r*RT-PCR efficiency [a,b]	Assay (RNA/g DT) 1	2	3
1	97.0 ±1.7	330	+QL	-	100.7 ± 0.6	16	43	38
2	95.6 ± 2.5	-	-	-	100.9 ± 2.6	-	-	+QL
3	94.8 ± 4.3	-	-	-	98.2 ± 5.2	-	-	-
4	96.7 ± 1.9	-	-	250	102.4 ± 0.3	110	+DL	88
5	97.6 ± 1.7	840	-	-	99.7 ± 3.9	190	58	-
6	94.8 ± 5.0	910	-	-	100.1 ± 1.8	110	+DL	53

[a]: *r*RT-PCR efficiency was calculated based on co- amplification of GGI and GGII RNA IC with pure and one log dilution nucleic acid extract, and expressed in %.
[b]: GGI or GGII NoV concentration was calculated based on Ct values obtained for pure and one log diluted NA and corresponding standard curve.
+QL: positive sample but too close to the quantification limit.
-: below the limit of detection.

None of the samples showed significant evidence of inhibition (% of rRT-PCR efficiency varied from 89 to 99.6 for GI and 92.17 to 103.7 for GII). No GI NoVs were detected in any of the three replicates for two samples, while one or two of the replicates were positive for the other four samples. In contrast, all replicates gave the same result for four samples (one negative and three positive) in the GII NoV assay, and the other two samples had one or two replicates positive. These results suggest that either there is a heterogeneous distribution of virus within naturally contaminated samples or that the variability is due to the presence of very low concentrations of virus, as demonstrated by the detection of positive samples that were too close to the limit of detection for quantification (Le Guyader et al., 2009).

Considering these data, the same approach was used to analyse shellfish linked to an outbreak (Le Guyader et al., 2010). Following epidemiological investigations, shellfish were collected in the caterer, producer, and the harvesting area.

Table 4: NoV quantification in oysters implicated in an outbreak.

Sample	Date	Mean extraction efficiency (%)[a]	Number of Positive Replicates/Number analysed	NoV GII geometric mean concentration (range) [b]
Caterer	02/13	16.1	3/4	229 (48-2548)
Producer	02/14	13.7	2/3	144 (63- 273)
Harvesting area	02/25	12.9	4/4	301 (35- 913)
Harvesting area	03/05	12.6	4/4	776 (273-1885)

[a] Mean percentage of mengovirus RNA copy number recovered in shellfish extracts relative to the amount of mengovirus added to samples prior to extraction.
[b] Geometric mean concentration of positive samples expressed in RNA copies per g of DT (range of concentrations detected), before (uncorrected) and after (corrected) correction for the mean extraction efficiency.

Samples were extracted four times (except a sample collected from the producer which was extracted only three times due to the limited number of oysters available) for quantitative analyses. Extraction efficiencies, as measured by recovery of mengovirus seeded into the shellfish prior sample processing, ranged from 12 to 16% for all extracts (Table 4). The measured NoV concentrations for caterer and producer were quite variable, ranging from negative to thousands of RNA copies per gram of DT (Table 4). The two other samples showed a more homogenous contamination for all replicates analyzed (all four replicates positive). The geometric mean virus concentrations for all of these samples were quite similar (Le Guyader et al., 2010). There is still a paucity of data on variation of contamination of naturally-contaminated oysters, but these data showed evidence of variability in

contamination. More information is needed on variability of oyster NoV level when exposed to the same contamination event.

Under the current European control measures, shellfish harvesting areas are classified depending on the level of faecal contamination as judged by *E. coli* monitoring. To establish a sampling programme for this purpose a survey of faecal pollution inputs must be undertaken. Representative sampling points for the *E. coli* monitoring programme are then selected on the basis of this sanitary survey. European guidelines exist for conducting sanitary surveys. The information contained in the sanitary survey report should identify the source of human contamination impacting on harvesting areas. European regulations do not require that the information collected by the sanitary survey is used other than to determine the location of sampling points during routine bacteriological monitoring. However it has been proposed that this information can form the initial basis for assessing the likely extent of NoV contamination of oysters in a harvesting area (Pommepuy and Le Guyader, 2008). On the basis of the findings of a sanitary survey, combined with some background NoV monitoring, it is suggested that harvesting areas could be characterised for the relative risk of NoV contamination. This assessment combined with known seasonal and event based risk factors can be used to form a relative assessment of the likely extent of NoV contamination in harvest area. The major risk factors associated with NoV contamination of oysters are season, with winter identified as a high risk period, community outbreaks of gastroenteritis, and overflows of untreated wastewater associated with high rainfall events (Pommepuy and Le Guyader, 2008). An assessment based on these factors could be used to target NoV monitoring to at risk areas and highlight when management interventions may be required to protect public health.

4. Data on NoV in oysters

4.1. Prevalence studies

There are very limited published studies on quantification of NoV in oysters determined by real-time-PCR. Data presented on the following tables is only presence/absence data.

Table 5: Prevalence data on NoV from shellfish commercial areas (From publications later than 2000)

Country	Shellfish	No. samples	No. of positive samples	Method	Reference
USA	Oysters	45	9 (20%)	RT-PCR and hybridization	(Costantini et al., 2006)
Japan	Oysters	191	17 (9%)	rRT-PCR, typing and sequencing	(Nishida et al., 2003)
Japan	Oysters	483	33 (6,8)	rRT-PCR, typing and sequencing	(Nishida et al., 2007)
UK	Oysters	146	83 (56.8)	rRT-PCR	(Lowther et al., 2008)
Ireland	Oysters	119	37 (31%)	rRT-PCR (only NoV GII)	(Flannery et al., 2009)
France	Oysters	78	11 (14%)	rRT-PCR	Ifremer 2009[9]
Dutch	Mussels (local)	21	1 (16.6%)	Nested RT-PCR	(Boxman et al., 2006)
Spain	Clams, cockles, mussels	24	11 (45.8%)	rRT-PCR	(Vilarino et al., 2009)
Italy	Mussels, clams	120	10 (8%)	rRT-PCR	(Suffredini et al., 2008)

[9] Ifremer, Rapport annuel Laboratoire de Microbiologie- Département Environnement Microbiologie et Phycotoxine, 2009, 50 pages. www.ifremer.fr/nantes/environnement.htm

Table 6: Prevalence data on NoV from Shellfish at retail (market) (From publications later than 2000)

Country	Shellfish	No. samples	No. of positive samples	Method	Reference
Switzerland	Oysters (imported)	87	8 (9.4%)	RT-PCR capsid (GI) pol (GII)	(Beuret et al., 2003)
Hong Kong	Oysters (imported)	507	53 (10.5%)	RT-PCR and sequencing	(Cheng et al., 2005)
UK	Oysters	66	39 (59%)	rRT-PCR	(Lowther et al., 2010)
Dutch	Mussels (imported)	21	6 (28%)	Nested RT-PCR	(Boxman et al., 2006)
Italy	Clams, mussels, oysters	116	14 (12.1%)	Nested RT-PCR (NoV GII) and sequencing	(Terio et al., 2010)
USA Gulf	oysters	380	15 (3.9%)	rRT-PCR	(Depaola et al., 2010)

A few studies have been conducted on shellfish collected from the market. Prevalence data in general is difficult to compare, as methodologies used are not the same in different studies. However it is interesting to note that prevalences obtained in market product are comparable to these observed in commercial harvesting areas.

Most of the studies reported about 10% of oysters sampled being contaminated by NoV; except in the UK with prevalences above 50%. Contamination of Irish oysters was detected in about 31% , but only the presence of NoV GII was analyzed, so this prevalence may be higher for total NoV.

4.2. Quantitative data from outbreaks

Table 7: Oysters implicated in outbreaks, quantification

Date	NoV GI concn*	NoV GII concn	No Consu-mers	No. Sick people	No. stool samples	Genotype present in stool	Reference
23/03/2000	**GI.1**: 85-237	<DL	4	4		**GI.1**	(Le Guyader et al., 2003)
19-24/12/2002	**GI.4**: <QL	**GII.4**: 25-125	36 -	21 202	12 41	**GI.4, GII.4** & GII.b **GI.4**, GI.6, **GII.4** & GII.8	(Le Guyader et al., 2006a)
08/02/2006	***GI: 72-130***	<DL	2	2	2	GI.1, GI.2, GII.2 (AiV, AV)	(Le Guyader et al., 2008)
15-17/02/2006	GI.1: 5000-16000	GII.4 1600-2500 GII QL	77	37	4	GII.7, GII.b (AiV)	(Le Guyader et al., 2008)
18/02/2006	GI 2300 **G1.2**: 610-2300 GI: 260-880	GII: <QL **GII.4**: 1100 **GII.4**: LQ-79			6	GI.1, **GI.2**, GI.4, **GII.4**, GII.7, GII.17, GII.B (AiV, EV, AV, RV)	(Le Guyader et al., 2008)
05/02/2008	<DL	***GII.4: 48-2548***	34	23	5	**GII.4**, GII.2 SaV G1.2	(Le Guyader et al., 2010)
01/2007/- 05/2010	795 30 nd 35 139 22	1550 2175 8215 695 13 208		54 5 89 115 4 20		GI & GII GII GI GI & GII Unknown Unknown	(Lowther et al., 2012)
05/02/2010	<100	1736	30	11	2	GI , GII	(Baker et al. 2011)
18/02/2010	*<DL & 42*	*2040 & 2350*		>70	2	**GII.13**	Marine Institute Ireland[10]

* expressed as number of RNA copies/g of DT.
Bold and italics: left over from the consumer fridge, **bold**: same sequence detected in shellfish and stool samples
QL: quantification limit, DL: detection limit
Blank : no data available
AiV: Aichivirus, EV: enterovirus, AV: astrovirus, RV: rotavirus, SaV: sapovirus

[10]www.marine.ie/home/services/operational/ShellFish/Investigation+into+an+outbreak+of+gastroenteritis+associated+with+a+shellfish+harvesting+area.htm

Detection and quantification of viral pathogens in shellfish became more reliable with the development of molecular methods, the use of rRT-PCR, and the inclusion of quality controls has made possible sample to sample comparisons. Controls are important to be confident that concentrations expressed reflect correctly the amount of viruses present in the contaminated oysters.
Several publications report the frequent implication of NoV GI strains in oyster related outbreaks (Gallimore et al., 2005; Kageyama et al., 2004; Nakagawa-Okamoto et al., 2009). If we consider data presented in Table 7, it is evident that very low values of NoV genome copies/g may be linked to human cases. It is also apparent that in several outbreaks investigated the values of NoV genome copies/g has been above 500.

Considering these restrictions, it can be concluded that NoV concentrations detected in oysters linked to human cases varied greatly from less than hundred copies to more than ten thousand. The contamination of an oyster with multiple NoV strains complicates the investigations further. When stool samples are analyzed, variability is observed as different individuals may be infected by different strains present in the consumed oyster (Baker et al., 2011; Le Guyader et al., 2008). The genetic susceptibility of exposed consumers is an important factor, and may explain some variations in consumer illness (Le Guyader et al., 2010). There are two recognized mechanisms involved in resistance to NoV infection: genetic factors and acquired immunity. It was shown that NoV binds to histoblood group antigens (HBGAs) (Hutson et al., 2002; Marionneau et al., 2002) and the binding pattern varies between genotypes (Tan and Jiang, 2010). Thus it is likely that every person is genetically susceptible to one or more NoV genotypes (Atmar, 2010). Acquired immunity is the other resistance mechanism, even if this immunity is not long lasting (Atmar, 2010).

Considering data from outbreaks and the diverse factors that interfere with NoV infection we cannot conclude on a reliable safe limit for oyster contamination.

4.3. Quantitative data from field studies

4.3.1. United Kingdom

The UK data was generated from a Food Standards Agency funded study conducted during 2009-2011[11] . The study was organised and all analysis performed at the Cefas Weymouth laboratory using a method accredited to ISO 17025[12]. The study aimed to generate unbiased data on NoV occurrence in UK oyster production areas therefore a representative selection of sites were chosen on the basis of a risk assessment using factors including classification history, population in adjacent areas, previous association with outbreaks, etc. All sites sampled were designated and classified under EU Regulation 854/2004[13] and thus available for commercial production. Classifications ranged from class A to class C. A cross selection of 39 study sites was chosen representing low, medium and high risk according to the risk matrix criteria. Undepurated oysters from 39 study sites from around the United Kingdom were sampled monthly between May 2009 and April 2011 and despatched in temperature controlled conditions to the Cefas laboratory. Oysters were tested for the presence of GI and GII NoV RNA using a rRT-PCR method based upon the CEN TAG4 draft standard. Briefly, 2g of oyster digestive tissues were treated with proteinase K solution, RNA was then extracted from 500ul of oyster homogenate using proprietary magnetic silica reagents and each sample RNA was subjected to rRT-PCR using primers and probes as described in (Le Guyader et al., 2009), excepting the GI probe which was as described in (Hoehne and Schreier, 2006).

[11] www.food.gov.uk/science/research/foodborneillness/microriskresearch/p01programme/p01projlist/p01009/
[12] www.iso.org/iso/Catalogue_detail?csnumber=39883
[13] http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2004:226:0083:0127:EN:PDF

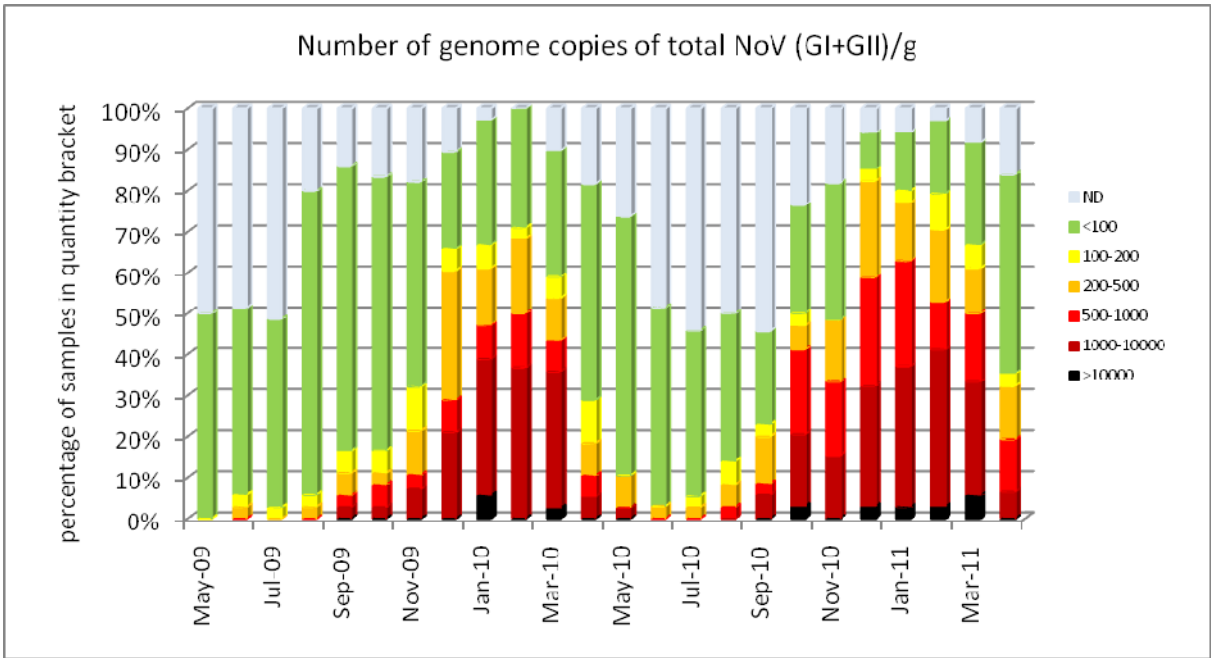

Figure 1: NoV occurrence in UK oyster samples

Of 857 samples tested, 844 (98.5%) provided valid results for NoV GI and GII. NoV was detected in 76.2% of samples, with 47.6% being positive for both NoV genogroups, 20.9% being positive for GI only and 7.7% positive for GII only. Similar NoV prevalences were found in both species of oyster tested. NoV prevalence varied from month-to-month ranging from a minimum of 45.7% samples positive in September 2010 to a maximum of 100% positive in February 2010. A strong winter seasonality was observed with 90.0% of samples taken between October and March positive compared with 62.4% of samples taken between April and September. Quantification of positive samples revealed that the majority were below the limit of quantification (100 detectable genome copies/g digestive tissues) for both genogroups. However a number of samples contained levels in excess of 10,000 copies/g – for NoV GI the maximum recorded quantity was 16,507 copies/g and for GII it was 18,024 copies/g. All 39 sites tested provided at least one NoV positive result, although prevalence varied from 21% to 100%. NoV levels varied markedly between sites with some sites scoring consistently over 1,000 copies/g during the winter while others rarely or never exceeded 100 copies/g. As with prevalence, average quantities varied markedly between seasons, with highest levels detected between December and March. The scores for NoV GI and GII in individual samples were significantly correlated with each other, however results for NoV GII tended on average to be higher than those for GI. Overall 63.5%, 85.4% and 98.9% of oyster samples were under 100, 1,000 and 10,000 genome copies per g respectively.

4.3.2. Ireland

The Irish data was generated from harvesting area monitoring conducted by the Marine Institute which is Irish National Reference Laboratory for bacteriological and virological monitoring in bivalve shellfish. Data was generated from *ad hoc* testing conducted in response to requests from producers (additional quality assurance purposes) and the competent authority in Ireland (problematic harvest areas). A total of 113 samples from 11 different harvesting areas were tested from January 2009 to January 2011. The areas tested ranged from good quality category A areas through to category B area. Two of the harvesting areas tested had been previously associated with gastroenteritis illness following consumption of oysters. Therefore it must be recognised that the data provided here is limited and may not be fully representative of levels of NoV found in oysters from Irish harvesting areas. Oysters were tested for the presence of GI and GII NoV RNA using a rRT-PCR method based

upon the CEN TAG4 draft standard. Briefly, 2g of oyster digestive tissues were treated with proteinase K solution, RNA was then extracted from 500ul of oyster homogenate using proprietary magnetic silica reagents and each sample RNA was subjected to qRT-PCR using primers and probes as described in (Le Guyader et al., 2009), excepting the GI probe which was as described in (Hoehne and Schreier, 2006). The test method was accredited to ISO 17025 for use in the Marine Institute from September 2010.

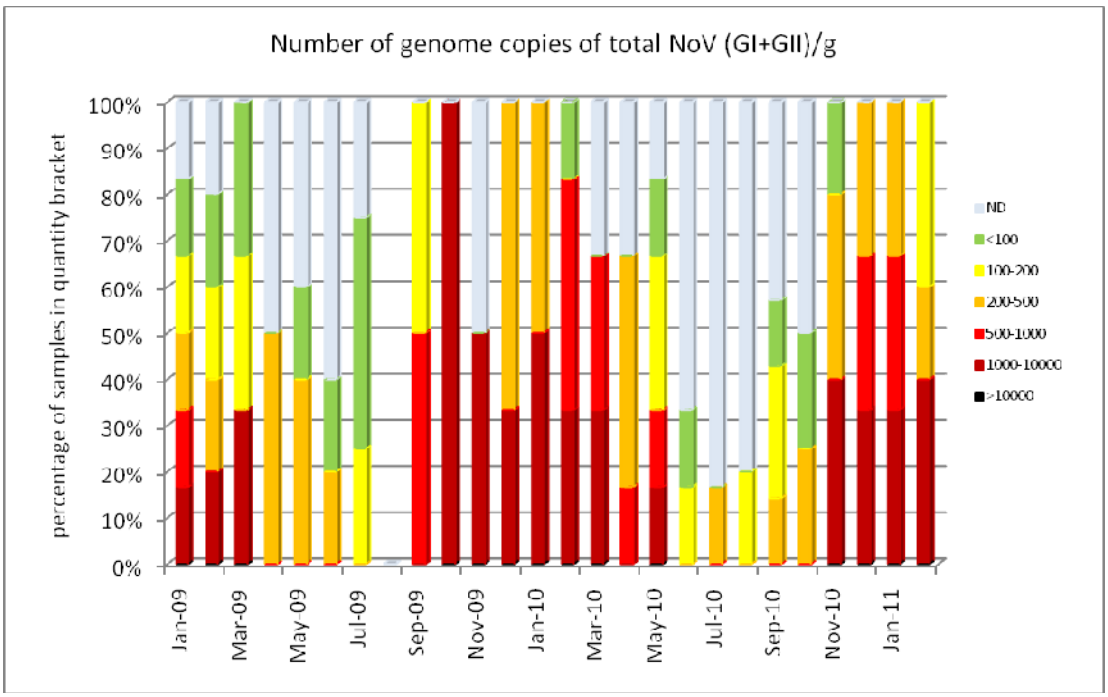

Figure 2: NoV occurrence in Irish oyster samples

The limited data set presented for Irish harvesting area is probably affected by a sampling bias and is skewed towards more problematic harvesting areas (areas associated with illness and higher contamination levels). Therefore the data probably represents a worst case scenario of levels of NoV found in Irish oyster harvest areas and conclusions from the data should be treated with caution. Both the frequency of occurrence and level of NoVs present were clearly seasonal with higher levels detected during the winter period. During the months of November through to March 88.1% of the oysters tested contained NoVs with mean level of 1328.9 genome copies per g compared 50.9% containing NoVs and mean levels of 213.4 genome copies per g in the remaining months of the year. During the winter months (Nov-March) 32.1% of oysters samples contained levels of NoV over 1,000 genome copies g (max Value 10,000) with NoVs absent in just 11.3% of samples. During the same winter period 30.2%, 52.8% and 68.0% of oyster samples were under 200, 500 and 1,000 genome copies per g respectively.

4.3.3. France

The French data was generated between January 2009 to February 2011. A total of 1,036 samples were analyzed with an average of 40 samples per month. These samples were collected through different ongoing research project in the laboratory and also as part of the French National reference laboratory activity, and therefore may not be representative of the overall situation in France. Each sample, collected from various locations along the coast of France, was constituted by at least 12 oysters. The method used, based on the CEN method, includes a concentration step by polyethylene glycol before nucleic acid extraction using the Nuclisen kit (BioMerieux). Extractions efficiencies were evaluated by using a virus control (Mengo virus) and only samples with an extraction efficiency above 10% were considered. Absence of inhibitors was checked by testing samples in duplicates and

after one log dilution. If all controls were verified then concentration was calculated using standard curves and taking into account extraction efficiency (Le Guyader et al., 2009).

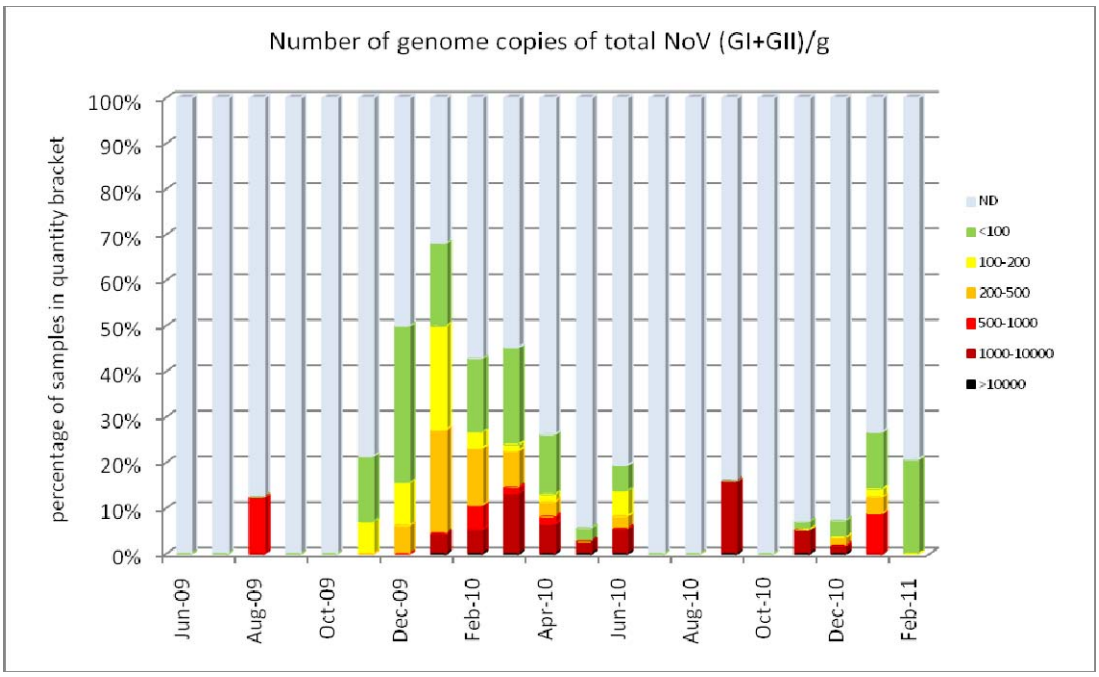

Figure 3: NoV occurrence in French oyster samples

A clear impact of season on shellfish contamination was observed, mirroring virus discharged by human population into sewage. Interestingly some contamination may be observed in August or June possibly due to local events. The seasonal impact of contamination, linked to an increase of virus in sewage correlates also with the finding that oysters are more likely to accumulate virus during winter months based on ligand analysis (Maalouf et al., 2010a). Also climatic events such as important rainfall are more likely to occur during January to April, increasing the risk of sewage input into coastal environment (Maalouf et al., 2010a).

4.3.4. Compliance scenarios

It is important to recognise that the surveillance data available from the different Northern European countries (Ireland, UK, France) may not be directly comparable. The UK study was designed to systematically sample a cross selection of commercial harvest areas and therefore can be considered to be representative of the UK situation. In comparison the data from Ireland and France was generated from studies with different objectives and may not be fully representative. Data from Ireland, for example, may over represent the extent of contamination, since at risk areas (e.g. areas associated with outbreaks) were preferentially sampled. Nevertheless it is clear that common findings emerge from these studies: (i) NoV was found in samples from all countries using the standardised CEN methods demonstrating their robustness; (ii) all the studies reveal a surprisingly large overall sample positivity rate; (iii) a strong winter seasonality for NoV contamination was observed in all the countries; (iv) the titres of NoV found in samples showed a similar range in all countries (<100 to 10,000 RNA copies per gram); (v) a standard based on virus absence (not detected by the CEN method) would have a high impact in all of the countries for which data was available.

It is clear that samples containing no detectable NoV can be considered to present the lowest level of consumer risk. However, the above surveillance studies illustrate the high impact of a standard, or microbiological criteria, based on simple absence of virus. There is now emerging evidence of a NoV RNA dose/illness relationship (dose response) both from human volunteer studies, from oyster

consumption studies, and from observation of titres found in outbreak samples (section 2). However, it is also the case that illness risk from very low levels of NoV cannot be excluded. Consequently it is not currently possible to determine a 'safe' quantitative risk threshold for NoV RNA with the available data. It is however possible to illustrate the impact (for sample failure) of different possible NoV compliance regimes. This can help to inform the risk management decision process. The available surveillance data (from the above studies) was further analysed to illustrate the impact of different possible NoV levels that could conceivably be set in a food standard or criteria (Table 8).

Table 8: Average percentage of samples that would fail during the high risk season (January to March 2010) if a maximum limit of 100, 200, 500, 1000, or 10,000 genome copies/g were set

	100 c/g	**200 c/g**	**500 c/g**	**1,000 c/g**	**10,000 c/g**
United Kingdom	65.6%	61.1%	46.9%	37.2%	2.7%
Ireland	83.3%	83.3%	72.2%	44.4%	11.1%
France	33.6%	24.4%	10.0%	7.7%	0%

It is clear that a production area NoV standard at the low end of the possible range (100 RNA copies/g) would have a high impact in all countries for which data was available (33-83% of samples non-compliant on average). It should also be remembered that this is highly seasonable in all countries, with sample failure potentially reaching near 100% during peak at risk periods (e.g. in the UK). By contrast, compliance during the summer low risk period may be much higher (e.g. >90% in France). Intermediate possible standards (e.g. 200 or 500 RNA copies/g) would have an intermediate impact. At the other extreme a comparatively high virus standard (e.g. 10,000 copies per gram) would have a relatively small impact on sample non-compliance (e.g. <2.7 % in the UK). In considering the risk management options it should be remembered that all of the areas studied in all countries were commercial production areas or monitored recreational harvest areas compliant with the microbiological standards in current EU legislation. Thus introduction of any of the possible range of virus standards illustrated in Table 8 would have a beneficial health impact over the current situation.

5. Post-harvest interventions to control NoV in oysters

5.1. The effect of depuration and relaying on NoV levels

Depuration is a commercial processing option for the treatment of oysters that is widely used worldwide. During depuration oysters are placed in tanks of seawater in an attempt to flush or purge faecal contaminants from the oysters. However, studies in both laboratory and commercial settings have shown that depuration times and conditions currently used are inadequate to remove viruses despite the rapid removal of bacterial pathogens and indicator organisms (Lees, 2000; Richards et al., 2010).

Depuration has frequently been demonstrated to be capable of rapidly eliminating *E. coli* in laboratory and commercial studies (Lees, 2000; Richards et al., 2010). However, numerous studies either directly investigating pathogenic viruses or virus indicator organisms have demonstrated minimal reductions in virus levels during depuration. Specifically, a number of studies have demonstrated the failure to eliminate NoV from oysters following depuration. NoV persistence was demonstrated in oysters responsible for outbreaks which had been depurated for several days and were compliant with *E. coli* standards (Le Guyader et al., 2008). Human pathogenic viruses including NoV were detected at the same frequency in oysters before and after commercial depuration in four European countries (Formiga-Cruz et al., 2002). Similarly, different shellfish samples (mussels, clams, oysters) collected in Italy showed no significant difference in the frequency of NoV contamination between depurated or non-depurated samples (Savini et al., 2009). Therefore NoV persistence in oysters following depuration as widely practiced in the EU has been demonstrated.

Despite the demonstrated persistence of NoVs in depurated oysters there is very limited data directly quantifying the extent of any NoV reduction during depuration. Following bioaccumulation of NoV GI.1 in oysters and subsequent depuration for 48h in a laboratory setting no significant change in virus titers were observed (Schwab et al., 1998). More recently, quantitative real-time PCR procedures have been applied to depuration studies. In a laboratory-based study using *Crassostrea gigas* that had been bioaccumulated with NoV GII.4 and then depurated for 23h hours no significant reduction in virus levels were observed (McLeod et al., 2009). Some oyster species may behave differently regarding NoV reduction during depuration. For example NoV GI.1 persisted for up to 29 days in *Crassostrea ariakensis* and 22 days in *Crassostroea virginica* (Nappier et al., 2008). Specific retention of NoV was observed by (Ueki et al., 2007) with no decline of NoV genomic copies in artificially contaminated oysters after depuration for 10 days whereas feline calicivirus could not be detected after 3 days. It has been demonstrated that some NoV genotypes bind to glycan ligands in the digestive tissue of oysters and may account for the inefficiency of depuration practices (Le Guyader et al., 2006b; Maalouf et al., 2010b). These glycan ligands are very similar to human histo-blood group antigen (HBGA) carbohydrates found in humans. A few studies have reported a limited reduction of NoV during depuration under different purification conditions. One study demonstrated almost a one log reduction in the levels of a NoV GI genotype in environmentally contaminated oysters following depuration for 3 days at 20ºC whereas no reduction was observed over the same period in oysters held at 9ºC (Henshilwood et al., 2003). During commercial depuration initial levels of 492 NoV genome copies per g were reduced by 72 % after 4 days and to <100 genome copies/g in 6 days at 17ºC (Dore et al., 2010).

It has been demonstrated that for viruses other than NoVs, factors such as the initial contamination level, depuration time, depuration system, physiological state of the shellfish, seasonal conditions, time, water temperature and salinity have an influence on the depuration dynamics of viral contaminants (De Medici et al., 2001; Dore and Lees, 1995; Kingsley and Richards, 2003; Lees, 2000). In particular depuration at elevated temperatures for extended periods has been shown to increase the extent of virus removal following depuration. However despite the limited data presented above this has not been fully demonstrated for NoVs. There is a clear requirement for further investigation to determine the full extent of NoV reduction that can be achieved under such depuration regimes.

An alternative treatment to depuration is relaying (Lees, 2000). This involves transferring sewage-contaminated oysters to pollution free marine environments and allowing them to purge sewage derived contaminants under natural environmental conditions. A limited number of studies have investigated virus removal during relaying. In studies using viruses other than NoVs, relaying has demonstrated differential reductions of viruses levels in oysters. Humphrey and Martin (1993) reported that FRNA bacteriophage were not detected after 2 to 3 weeks of relaying while somatic coliphages were still detected after 5 weeks. Rotavirus-like particles (VLPs) could be detected up to 37 days of relaying when an initial concentration of 10^5 VLPs/oyster was present (Loisy et al., 2005). As with depuration, seawater temperature appears to be an important factor in virus removal. Using FRNA bacteriophage, Dore et al., in 1998 demonstrated virus clearance after two weeks and persistence after 4 weeks in two difference relaying cycles at the same location. The authors suggest that seawater temperature was responsible for the difference between the two cycles.

Critically the usefulness of relaying as a treatment to reduce NoVs depends on the persistence of NoVs in oysters in the marine environment. Data specifically relating to NoV persistence in oysters following contamination events are still rare. After a flooding event that contaminated a production area in southern France, some oysters were implicated in cases of gastroenteritis in consumers. The Sanitary Authority closed the area, and virological analysis were conducted to evaluate NoV contamination (Table 9).

Table 9: Detection and quantification of NoV in shellfish samples.

Week	No. samples	Genogroup I		Genogroup II	
		No. pos. (%)	Mean conc*	No. pos. (%)	Mean conc*.
1	17	10 (59)	6900	12 (70)	1300
2	15	5 (33)	3100	3 (20)	120
3	13	3 (23)	120	2 (15)	DL
4	17	7 (41)	220	3 (17)	200

*: concentration expressed in RNA copies / g of digestive tissues.

During the four weeks, contamination decreased slowly both in terms of number of positive samples or mean concentration. However four weeks after the contaminating event, viruses were still present (Le Guyader et al., 2008). Similar results were observed in Ireland when contaminated oysters which had been associated with outbreaks of illness were relayed in an area of clean seawater. The NoV GII contamination was reduced from 2900 to 492 copies /g of DT after 17 days relaying. NoVs were further reduced from 492 to 136 copies/g of DT in 4 days and <100 copies/g of DT in 6 days at 17ºC for NoV GII genotypes (Dore et al., 2010). These oysters were subsequently placed on the market with no reported cases of illness.

It has been suggested that although long-term relaying may represent an effective treatment it may be impractical from a commercial standpoint because of increased production costs associated with the additional handling, it reduces product availability and also it may be problematic to find clean areas to perform relaying (Richards et al., 2010).

All EU commercial bivalve mollusc production areas must be monitored on a periodic basis for *E.coli* as an indicator of faecal pollution. This monitoring determines a classification A (cleanest), B, or C (most contaminated). It is a legal requirement for molluscs harvested from class B and C areas to be subject to post-harvest treatment prior to placing on the market for human consumption (for details see EFSA 2011[14]). Products harvested from class B areas may be treated by depuration, relaying or heat treatment. Products harvested from class C areas can only be treated by long-term relaying or heat treatment. A recent study by the EU Reference Laboratory [15] showed that about 50% of EU production areas fall into the class B category and thus require post-harvest treatment. For products placed live on the market (e.g. oysters) depuration (self-purification in tanks of clean seawater) is the predominant treatment process used. Thus depuration is very extensively used within the EU as a post-harvest treatment process for reduction of microbiological risk associated with bivalve molluscs. In EU regulations the distinction between treatments allowed for class B and class C products reflects a long standing concern over the adequacy of depuration for successful treatment of more highly contaminated products – in particular those potentially contaminated with enteric viruses. EU regulations set a microbiological standard of 230 *E. coli* (per 100g of shellfish flesh) for both class A production areas (can be harvested and directly placed on the market without further treatment) and end-products placed on the market following post-harvest treatment (Regulations (EC) No 854/2004 and 2073/2005). The regulations further require post-harvest treatment measures (such as depuration) to be performed such that products placed on the market comply with the required 230 *E. coli* standard. Critical depuration treatment parameters, such as the time period for mollusc treatment in the tanks, is not specified by legislation but rather determined by the time required to secure compliance with the *E. coli* end-product parameter. Commercial systems are not optimised for virus reduction since the compliance target is based on *E. coli* removal. Indeed, in most EU Member States previous minimum purification time standards have now been replaced by reliance on operator compliance with *E.coli* criteria – with the result that depuration times are commonly much shorter. Short depuration

[14] EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on An update on the present knowledge on the occurrence and control of foodborne viruses. EFSA Journal 2011;9(7):2190. [96 pp.] doi:10.2903/j.efsa.2011.2190. Available online: www.efsa.europa.eu/efsajournal

[15] Comparison of bivalve mollusc harvesting area classifications under EC Regulation 854/2004 across EU Member States (2009). Dated 11/4/2011. www.crlcefas.org.

times (e.g. <24 hours) are unlikely to be effective for removal of NoV. The dangers of reliance on *E. coli* criteria for regulation of key depuration parameters have been recently highlighted[16]. Now that robust and quantitative virus methods are available a much more effective strategy would be to require food business operators to validate their treatment processes (including depuration) against a NoV criteria. This would also be in conformity with the standard HACCP approach for operation of food processes. Removal of NoV to non-detectable using the standardised CEN methods would be likely to ensure a high level of consumer protection but may be difficult to achieve in practise. Alternative approaches would be to require removal to below a target level (Dore et al., 2010) suggested 200 genome copies per gram) or to require a minimum percentage removal (e.g. 95%) throughout the depuration process. Reduction of viral load during the depuration process, even if complete elimination cannot be achieved, can be considered to have a beneficial health effect since recent data suggests that risk of infection is related to viral dose consumed (See section 2). Unfortunately it is not currently possible to set a standard based on the desired health outcomes since the supporting evidence is not available – thus a risk management decision would be required.

In summary it is clear that commercial depuration as currently practiced cannot be relied upon as a control measure to effectively remove NoVs from oysters. The limited quantitative data available demonstrates that depuration at elevated temperatures for extended periods can reduce NoV levels in oysters by between 0.8 and 1 $\log_{10}$ (Dore et al., 2010; Henshilwood et al., 2003). Relaying combined with depuration at elevated temperatures has been demonstrated to achieve a reduction of greater than 1.46 $\log_{10}$ (Dore et al., 2010). However genotype specific binding patterns may mean that meaningful reductions of NoVs during relaying and/or depuration may not be feasible for all NoV genotypes. There remains a clear need for further investigations to establish elimination patterns of NoVs from oysters during depuration and relaying regimes.

5.2. Effects of other post-harvest treatments used in food processing on viruses

NoV are quite persistent and are difficult to eliminate when in shellfish body. Usually to reduce viruses from any food product, cooking is the most efficient method. However, in shellfish, thorough cooking may change organoleptic characteristics, and home or restaurant cooking is generally inadequate for the elimination of NoV (Alfano-Sobsey et al., 2011; Richards et al., 2010).

Studies to evaluate the thermal resistance of NoV is hampered by the lack of infectivity assay and different testing methods, different virus strains or surrogates, and different shellfish species may impact results. Total inactivation (5 $\log_{10}$) of MNV-1 was achieved in PBS after 3 min at 72°C (Wolf et al., 2009), but less thermal inactivation would be expected in viruses protected within shellfish tissues as for example boiling for 3 min gave an internal temperature of 92°C, and steaming for the same period gave an internal temperature of only 63°C (Richards et al., 2010). Canned oysters are likely to be safe from a virus standpoint, since the canning process provides sufficient heat to essentially sterilize the product (Richards et al., 2010). Slomka and Appleton (1998) investigated the inactivation of feline calicivirus (FCV) by immersion of cockles in boiling water for 0.5 min and found 1.7 $\log_{10}$ reduction of FCV. At that time, the internal temperature of the cockles reached approximately 60°C. After 1 min, the internal temperature reached 78°C and FCV (initially 4.5 $\log_{10}$ TCDI50/g present) could not be detected anymore.

High pressure processing (HPP) has emerged as a potential treatment to inactivate viruses in shellfish (based on observation done for vibrios) and to facilitate oyster shucking. Commercial processors use around 275−300 MPa of pressure for about 3 min, keeping taste and texture of raw product, that remain plump and juicy, and have a slightly cooked appearance from partial denaturation of oyster proteins by the pressure treatment. In 2002, Kingsley and colleagues were the first to demonstrate that pressure of 250 MPa for 5 min was sufficient to inactivate 7 $\log_{10}$ of FCV in culture media containing

[16] EFSA Panel on Biological Hazards (BIOHAZ); Scientific Opinion on An update on the present knowledge on the occurrence and control of foodborne viruses. EFSA Journal 2011;9(7):2190. [96 pp.] doi:10.2903/j.efsa.2011.2190. Available online: www.efsa.europa.eu/efsajournal

10% foetal bovine serum (Kingsley et al., 2002). However it was later demonstrated that food matrices, salt concentrations, pH, and temperature affect virus inactivation rates (Kingsley and Chen, 2008). The advent of a quantitative assay for MNV-1, a closer relative of human NoV than FCV, led to a study showing a 4 $\log_{10}$ decrease in virus infectivity after pressure treatment of oysters for 5 min at 400 MPa (Kingsley et al., 2007), therefore, it was uncertain which surrogate was more representative of human NoV inactivation under high pressure. A human challenge study was conducted to assess the effectiveness of HPP to inactivate human NoV in oysters. Over 50 volunteers were challenged either with oysters that were inoculated into their stomach cavity with NoV GI.1 and pressure-treated or with oysters that were similarly inoculated with virus but were not pressure treated. Only oysters treated with 600 MP for 5 minutes at 6°C, were effective at inactivating NoV (no sick volunteer), but induced a mildly cooked whitish appearance, presumably not acceptable to consumers (Leon et al., 2011). Such study clearly demonstrate that human NoV in oysters is more resistant to HPP than either FCV or MNV-1, which again stresses the limited usefulness of studies involving surrogate viruses. It may also be possible that resistance may vary among the different genetic clusters.

Enteric virus inactivation studies have included work on the effects of ultraviolet light and ionization radiation on virus levels. Ultraviolet irradiation is effective in reducing NoV surrogates on the surface of product, but does not have penetrating power to inactivate viruses deep within the shellfish. Similarly, gamma irradiation at 0.5, 0.3, and 0.1 kGy produced 3 $\log_{10}$ decreases in FCV, canine NoV, and MS2 titers, respectively, in low protein solutions, but high amounts of protein appreciably reduced the effectiveness of ionizing irradiation (de Roda Husman et al., 2004).

Freezing of shellfish is a potential processing method of limited value. Raw shellfish meats are often frozen to await subsequent processing, such as breading or cooking either at the restaurant or at home. Although freezing tends to preserve viruses, there can be an initial loss in virus titer with each freeze-thaw cycle. That loss in titer may reduce virus levels in minimally contaminated product to enhance safety. Freezing by itself seems inadequate to protect the consumer from even lightly contaminated shellfish, but when freezing is combined with cooking or HPP, the additive effect of both processes would further enhance shellfish safety (Richards et al., 2010).

Smoking is another commercial method of processing shellfish. There are various, non-standardized methods for smoking shellfish and the amount of heat applied in this process is likely to vary considerably from one facility to another and perhaps from one batch of shellfish to another. It is uncertain if smoking alone is effective in reducing virus levels in shellfish, but many smoked products are also canned, which provides sufficient heat to inactivate viruses (Richards et al., 2010).

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

Answer to ToR 1: The use of real-time PCR as a means of detection and quantification of NoV in oysters.

- PCR-based detection methods exist for NoV in bivalve shellfish. Harmonization and standardization are currently ongoing for NoV in shellfish under the auspices of the European Committee of Standardisation (CEN), and publication of methods is expected soon.

- To achieve a good sensitivity separate assays are required for NoV GI and GII detection.

- With the appropriate quality assurance measures, including accreditation and proficiency testing, the standardised CEN method is considered suitable for use for detection and quantification of NoV in oysters within a legislative context.

Answer to ToR 2: Limits that do not pose an unacceptable risk to consumers for NoV genogroups GI and GII in oysters as determined by real-time PCR.

- NoV can be frequently detected by rRT-PCR in oysters during winter in European areas compliant with current legislative requirements (*E. coli* standards) for which data are available.

- NoV is highly infectious. Exposure of human volunteers to serial dilutions yielded a dose-dependent probability of becoming ill ranging from 0.1 (at a dose of 10^3 NoV genome copies) to 0.7 (at a dose of 10^8 virus genome copies). However, there is no threshold infectivity limit for NoV detected by rRT-PCR. From outbreak published data, it can be concluded that NoV concentrations detected in oysters linked to human cases varied greatly from less than hundred copies to more than ten thousand per gram of material analysed.

- The probability of becoming infected increases with the dose but depends also on the characteristics of the organism, the food matrix and the host factors.

- The relationship between the number of infectious virus particles and the number of virus genome copies detected by quantitative PCR is not a constant, and may vary depending on environmental conditions including time from the initial release from the host.

- The number of genome copies detected by quantitative PCR may not relate to infectious NoV particles, and as a consequence the method can only be used to provide an indirect measure of risk.

- When considering what is an acceptable level of NoV in oysters it is important to realise that the infectious risk associated with low level positive oysters as determined by rRT-PCR may be overestimated.

- Despite observed differences between the ability of NoV GI and GII to cause human infection via different transmission routes there is insufficient knowledge on the dose response for each genogroup to allow a distinction. Therefore it is appropriate to consider the total NoV load (GI+GII) when establishing microbiological criteria.

- Quantitative data on viral load from areas compliant with current EU legislative requirements (*E. coli* standards) during January-March 2010 in 3 member states, show that a viral limit of 100, 200, 500, 1000 or 10,000 NoV PCR genome copies would result in 33.6-88.9%, 24.4-83.3%, 10.0-72.2%, 7.7-44.4% or 0-11.1% of non-compliant batches, respectively.

- Compliance with any of the above NoV limits would reduce the number of contaminated oysters placed on the market and therefore the risk for consumers to become infected. The lower the limit the greater the consumer protection achieved. However, it is not currently possible to quantify the public health impact of establishment of different limits.

- Microbiological criteria for NoV in oysters are useful for validation and verification of HACCP-based processes and procedures, and can be used to communicate to food business operators and other stakeholders what is an acceptable or unacceptable viral load for oysters to be placed on the market.

- Microbiological criteria for NoV in oysters could also be used by competent authorities as an additional control to improve risk management in production areas, during processing and retail.

Answer to ToR 3: Treatment regimes (post-harvest interventions) that can be relied upon to reduce NoV counts in oysters

- Current treatment regimes for products placed live on the market (depuration and relaying) as commonly practised do not effectively reduce NoV in oysters.
- Depuration and relaying may be improved by optimising process parameters to enhance NoV reduction (e.g. depuration times, water temperature). However, limited data is currently available.
- Alternative treatments such as commercial heat treatment and high pressure may be effective for NoV inactivation, but give rise to organoleptic changes which may be unacceptable to consumers.
- The most effective public health measure to control human NoV infection from oyster consumption is to produce oysters from areas which are not faecally contaminated, particularly given the ineffectiveness of current control regimes.

RECOMMENDATIONS

- Control measures for NoV in oysters should focus on avoiding contamination by either preventing human faecal contamination in mollusc production areas, or restricting commercial harvesting from faecally contaminated areas.
- On the basis of the data presented in this Opinion, risk managers should consider establishing an acceptable limit for NoV in oysters to be harvested and placed on the market.
- Competent authorities should consider the use NoV testing of oysters (standardized CEN method) to verify compliance with the acceptable NoV limit established.
- Food business operators should consider incorporating NoV testing of oysters (standardized CEN method) to verify their HACCP plans to demonstrate compliance with the acceptable level.
- The quantitative levels of NoV within production areas and batches should be investigated further, in order to optimise sampling strategies.
- Sampling schemes to comply with NoV criteria should be risk based, e.g. considering seasonality, faecal pollution levels, community outbreaks, and variability from year to year.
- An EU-wide baseline survey on NoV contamination in oysters should be considered, in order to estimate consumer exposure and to allow quantification of the impact on human exposure related to establishment of microbiological criteria.
- Research needs to be conducted to establish the relationship between detection of NoV in oysters by PCR and human health consequences.
- Further studies are needed to establish and optimise the effectiveness of depuration and relaying for NoV reduction using the standardised CEN method.

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