Improving health controls for viruses in bivalve molluscs

David Lees

European Union Reference Laboratory for Monitoring Bacteriological and Viral Contamination of Bivalve Molluscs
Enteric viruses in shellfish

- Norovirus and hepatitis A virus principle cause of illness worldwide following consumption of faecally polluted shellfish

- Outbreaks continue to occur in most countries – impacts public health, consumer confidence, and producer interests

- Current risk assessment and management worldwide relies on faecal indicators (*E. coli* in EU) – shellfish causing outbreaks are frequently compliant with regulatory standards
Valentine’s Day oysters leave lovers feeling unromantic

Valerie Elliott, Consumer Editor

Hundres of diners fell ill this year as a result of Valentine’s Day oysters, which have been linked to The Fat Duck restaurant, in Bray, Berks.

The restaurant, adding to the list of restaurants implicated in outbreaks of food poisoning in recent years, has been closed since 15 March.

The restaurant has refused to comment, but the closure is likely to be linked to the illness and the health protection agency has launched an investigation.

The restaurant has previously been closed for a similar outbreak in 2009.

All oyster suppliers have been warned to check for contamination, which is thought to have been caused by norovirus.

Infected oysters and poor hygiene, a recipe for sickness at The Fat Duck

The restaurant has been closed since 15 March, but the outbreak is likely to be linked to the illness.

The restaurant has previously been closed for a similar outbreak in 2009.

All oyster suppliers have been warned to check for contamination, which is thought to have been caused by norovirus.
Outbreaks Associated with Molluscan Shellfish (E&W)
Data compiled by Public Health England

Shellfish species
- Oysters: 80%
- Cockles: 6%
- Mussels: 8%
- Mixed: 6%

Agent
- NoV: 55%
- HAV: 1%
- DSP: 6%
- Salmonella: 2%
- Unknown: 36%

Seasonality
Norovirus – epidemiology

- IID2 study: ~3 million cases each year in UK
Test methods for enteric viruses in shellfish

• Variety of PCR-based methods for detection of norovirus in shellfish published over last 20 years

• European food legislation anticipates the adoption of virus controls when the methods are ‘sufficiently developed and available for use’

• Cefas is the European Reference laboratory with responsibility for coordinating the application of analytical methods for shellfish testing

• Requirement for robust standardised method for detection of enteric viruses in shellfish

EURL virus proficiency testing

- World wide
- 18 distributions since 2002
- 37 countries
- 43 labs registered to participate July 2016
• Variety of methods in use – eg in 2006 23 international labs participated
  – 13 virus extraction methods
  – 29 RNA extraction methods
  – All permutations of PCR format (conventional single round, nested and semi-nested and one and two-step real-time RT-PCR)
  – 13 different sets primers/probes sets

• Performance is variable - eg in 2011
  – 27 participants including 15 EU NRLs and 8 third country laboratories
  – Clinical samples (lenticules) good performance - 24/27 labs scored 100%
  – Shellfish matrix more problematical – only 15/27 labs scored 100%
  – 6 laboratories (using in-house methods) failed to detect norovirus in an oyster sample responsible for a large international outbreak of norovirus gastroenteritis.....

• Use of more harmonised methods resulted in better performance
Standardisation - CEN/TC275/WG6/TAG4

- Technical advisory group comprised of European and International food and water virology experts, 40 members from 15 countries
- Tasked by CEN in 2004 with development of a standard method for the detection of viruses in foodstuffs
- 12 year programme of work
- CEN/ISO methods are reference methods in EU legislation
**Conceptual framework**

- **Horizontal method (all foodstuffs included)**
- **Viruses of primary focus:**
  - Norovirus
  - Hepatitis A virus
- **Matrices of primary focus:**
  - Hard surfaces
  - Salad crops
  - Soft fruits
  - Bivalve shellfish
  - Bottled water
- **Real time RT PCR with probe confirmation**
- **Quantitative and qualitative**
Framework for method

- Separate pre-processing/virus extraction methods for each food matrix
- Each tests a defined quantity of sample, e.g. 2g shellfish digestive gland, 25g soft fruit
- Viral RNA purified from defined volume of concentrate/extract by common RNA extraction method (GITC)
- Assayed by real-time (TaqMan) RT-PCR
- Extensive suite of controls
- Two parallel protocols;
  - Quantification
  - Qualitative detection
Digestive gland dissection

- Proteinase K digestion of chopped glands
RNA extraction

• Boom technology (virus capsid disruption with chaotropic reagents, adsorption of RNA to silica particles)
RT-PCR

- One-step TaqMan (“hydrolysis probe”) RT-PCR for all targets
- Standard stipulates that primers and probes “must be published in a peer-reviewed journal and be verified for use against a broad range of strains of target virus”
- Norovirus primers must target junction of ORF1/2
- HAV primers must target 5’ NCR
Quantitation using standard curve

- Reporting in genome copies per gram of matrix tested
• Set QC criteria for: inhibition and recovery
Microbiology of food and animal feed — Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR —

Part 1: Method for quantification

Published May 2013

TAG4 chair - James Lowther
james.lowther@cefas.co.uk

EURL protocols on:
www.eurlcefas.org
Validation of ISO/TS 15216

- Maximum lifespan of technical specification 6 years; requires validation to convert to “full” standard

- European project to validate TS 15216-1 (quantification) in 7 food matrices
  - Oysters
  - Mussels
  - Raspberries
  - Lettuce
  - Spring Onions
  - Bottled Water
  - Food Surfaces (Bell Pepper)

- In two stages
  - Method characterisation in single labs (to determine LOD, LOQ, linearity, repeatability)
  - Inter-laboratory trials (10 labs per matrix; reproducibility)
Inter-laboratory trial results: reproducibility

- Quantification of norovirus GI in oysters
### Summary of method characteristics

- **Performance characteristics in Pacific oysters**

<table>
<thead>
<tr>
<th>Limit of detection</th>
<th>HAV</th>
<th>GI</th>
<th>GII</th>
</tr>
</thead>
<tbody>
<tr>
<td>Limit of detection</td>
<td>198 copies/g</td>
<td>34 copies/g</td>
<td>53 copies/g</td>
</tr>
<tr>
<td>Limit of quantification</td>
<td>198 copies/g</td>
<td>34 copies/g</td>
<td>53 copies/g</td>
</tr>
<tr>
<td>Linearity</td>
<td>316-124,000 copies/g</td>
<td>34 - 20,400 copies/g</td>
<td>53 - 9,600 copies/g</td>
</tr>
<tr>
<td>Repeatability standard deviation (logs)</td>
<td>0.19</td>
<td>0.18</td>
<td>0.22</td>
</tr>
<tr>
<td>Reproducibility standard deviation (logs)</td>
<td>0.57</td>
<td>0.53</td>
<td>0.51</td>
</tr>
</tbody>
</table>

- **Equivalent data for mussels, soft fruit, salad vegetables, food surfaces and bottled water**
ISO 15216 current status

- New draft full standard ISO DIS 15216-1 (quantification) prepared
- Includes:
  - modifications to the method following experience of validation
  - precision data (repeatability and reproducibility SDs and limits)
- Passed first vote (technical enquiry) in November 2015
- Further modifications based on comments during voting, publication anticipated by end of 2016
- ISO/TS 15216-2 (qualitative method) also due to be harmonized with 15216-1 and re-issued as a full validated standard
Application of virus methods
Two year study (May 2009 – April 2011) to establish baseline of norovirus in UK oysters (funded by FSA)

Monthly oyster samples from 39 representative sites from around UK (norovirus GI and GII)

Data on *E. coli*, water temperature and other risk factors collected in parallel

Lowther et al, AEM 2012; 78:5812
Headline results!

- Total of 857 samples
- 76.2% positive for norovirus
- 48% positive for both genogroups
- Strong winter seasonality
- Large site by site variation
Comparison with outbreak reports

![Graph showing comparison between norovirus (copies/g) and lab reports over time from May 2009 to April 2011. The graph indicates a peak in norovirus cases in February 2010, with a corresponding peak in lab reports. There is another peak in October 2010.]
Comparison with air temperature

Spearman's rank correlation: $r^2 = 0.769$
Comparison with *E. coli*

- Little correlation between *E. coli* and norovirus on an individual sample basis

Spearman’s rank correlation; $r^2 = 0.038$
Comparison with *E. coli*

- Correlation on a site-by-site basis
- Correlation improved if results from winter only (Oct-Mar) considered

Spearman’s rank correlation; $r^2 = 0.457$
Impact of different possible virus limits
Prevalence and levels of norovirus in oyster on the retail market in the UK

• Part of wider FSA funded study: NoVAS ‘Assessing the contribution made by the food chain to the burden of UK-acquired norovirus infection’

• Market research in 21 cities/regions throughout UK identified 373 retail oyster outlets (supermarkets, fishmongers, restaurants, online, wholesale)

• Randomised sampling plan at these outlets (unweighted)

• Survey comprised 630 samples (~52/month) tested blind for NoV and E.coli during March 2015 – March 2016

• 99% Pacific oysters (C.gigas); 90% UK origin (rest EU)

University of Liverpool lead
Risk assessment: European Food Safety Authority

Food borne viruses 2011

Scientific Opinion on an update on the present knowledge on the occurrence and control of foodborne viruses

EFSA Panel on Biological Hazards (BIOHAZ)

European Food Safety Authority (EFSA), Parma, Italy

Norovirus in oysters 2012

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

EFSA Panel on Biological Hazards (BIOHAZ)

European Food Safety Authority (EFSA), Parma, Italy
Current state of play

- Virus controls under discussion at EU level
- More data required to underpin any decisions on legislative standards
- EU wide harmonised baseline survey planned
EU harmonised baseline survey of norovirus in oysters

• Objective
  – To estimate the European prevalence of norovirus-contaminated oysters at production areas and batches of oysters at dispatch centres, with a 95% level of confidence and a level of precision of 5% considering an expected prevalence of 50%

• EFSA working group developed sampling plan
  – 171 production areas sampled each 2 months
  – 197 dispatch centres sampled each 2 months
  – 2 year survey starting November 2016 (>4000 samples)
  – Norovirus analysis according to specified procedure (based on ISO 15216-1)
  – EURL providing training
  – Mandatory PT to ensure laboratory competence

• Analysis of data to estimate proportion of norovirus contaminated samples (with 95% confidence intervals) at thresholds of: <LOQ; 100; 200; 500; 1,000; 5,000; 10,000 and >10,000 copies per gram at EU level
Technical specifications for a European baseline survey of norovirus in oysters

European Food Safety Authority (EFSA)

Abstract

The European Commission requested scientific technical assistance in the preparation of a survey protocol for a European Union (EU) coordinated monitoring programme on the prevalence of norovirus (NoV) in raw oysters. The objective of the survey is to estimate the European prevalence of norovirus-contaminated oysters at production areas and batches of oysters at dispatch centres, with a 95% level of confidence and a level of precision of 5% considering an expected prevalence of 50%. The survey protocol defines the target population, the sample size for the survey, sample collection requirements, the analytical method for the quantification of NoV copy number (genotype I and genotype II), the data reporting requirements and the plan of analysis. The sample unit in production areas is a classified production area actively growing commercial oysters (whether harvesting or not is occurring) and for dispatch centres is a quantity of live oysters which are being packed and labelled (e.g. Scientific Unit, Frozen or chilled).
How does a norovirus genome titre relate to infectivity risk
Human volunteer studies show dose response
Correlation between attack rate and norovirus levels in oysters in restaurant study*
Shellfish causing outbreaks have higher levels of norovirus
EFSA conclusion: ‘infectious risk associated with low level positive oysters as determined by real-time PCR may be overestimated’

*Lowther et al., 2010. J Food Protection. 2:212
Norovirus levels in outbreak-associated batches of oysters

- Statistically significant difference between levels in outbreak and non-outbreak samples ($p<0.0001$)

Lowther et al, J Food Prot. 2012; 75:389
Health risk vs titre - conclusions

- Oyster samples where norovirus is not detected (using ISO standard methodology) unlikely to present risk of norovirus infection
- Positive samples with levels <100 copies/g have not been associated with outbreaks in our experience
- Evidence (limited) of dose response relationship as levels increase above 100 copies/g
Can we improve virus analysis for infectivity?

- ISO standard method detects a small section of the norovirus genome (~150bp)
Culture of norovirus?

Enteric bacteria promote human and mouse norovirus infection of B cells

Melissa K. Jones,1,3 Makiko Watanabe,1,3 Shu Zhu,1,3 Christina L. Graves,2,5,9 Lisa R. Korys,1 Katrina R. Grau,1 Marimar B. González-Hernández,2 Nicole M. Iovine,2 Cristiano R. Wobus,6 Jan Vinje,3 Scott A. Tilbeets,3 Shannon M. Waller,3,8 Stephanie M. Karst1

The cell tropism of human noroviruses and the development of an in vitro infection model remain elusive. Although susceptibility to individual human norovirus strains correlates with an individual’s histo-blood group antigen (HBGA) profile, the biological basis of this restriction is unknown. We demonstrate that human and mouse noroviruses infected B cells in vitro and likely in vivo. Human norovirus infection of B cells required the presence of HBGA-expressing enteric bacteria. Furthermore, mouse norovirus replication was reduced in vivo when the intestinal microbiota was depleted by means of oral antibiotic administration. Thus, we have identified B cells as a cellular target of noroviruses and enteric bacteria as a stimulatory factor for norovirus infection, leading to the development of an in vitro infection model for human noroviruses.

Noroviruses (NoV) are nonenveloped plus-strand RNA viruses that are the leading cause of epidemic and sporadic gastroenteritis (1–5). The cellular tropism of human NoVs (HuNoVs), and thus the development of a cultivation system for their in vitro propagation, has long eluded the NoV research community (6–10). Several pieces of data lead us to ask whether NoVs can infect B cells. First, interferon-deficient and interleukin-10-deficient mice infected with a mouse NoV (MuNoV) contained virus-positive cells in the B-cell zones of the spleen (11, 12). Second, MuNoV-infected Rag2−/− mice (which lack B and T cells) and B-cell-deficient mice had reduced virus titers compared with those of wild-type mice, suggesting the absence of a target cell (10). Last, chimpanzees infected with NoV could not be protected by passive immunization with antibodies from convalescent humans (13). The development of an in vitro infection model for human noroviruses has been thwarted by the need to infect B cells. We present a straightforward system for culturing human and mouse noroviruses in the presence of enteric bacteria. We show that human noroviruses replicate efficiently in B cells in the presence of enteric bacteria, mimicking their infection of the intestinal epithelium. We use a panel of enteric bacteria selected from our in vivo and in vitro studies to identify conditions that promote norovirus replication in B cells in vitro. We also demonstrate that noroviruses can infect B cells from any species studied with human noroviruses as well as B cells from other species of mammals.

Human norovirus culture in B cells

Melissa K Jones, Katrina R Grau, Veronica Costantini, Abimbola O Kolawole, Miranda de Graaf, Pamela Freiden, Christina L Graves, Marion Koopmans, Shanon M Wallet, Scott A Tilbetts, Stacey Schultz-Cherry, Christiane E Wobus, Jan Vinje & Stephanie M Karst

Affiliations | Contributions | Corresponding author

Published online 29 October 2015

Abstract

Human noroviruses (HuNoVs) are a leading cause of foodborne disease and severe childhood diarrhea, and they cause a majority of the gastroenteritis outbreaks worldwide. However, the development of effective and long-lasting HuNoV vaccines and therapeutics has been greatly hindered by their uncultivability. We recently demonstrated that a HuNoV replicates in human B cells, and that commensal bacteria serve as a cofactor for this infection. In this protocol, we...
Predicting norovirus impacts in shellfish production areas
• Further analysis of production area surveillance study data (Lowther et al, AEM 2012; 78:5812)
• Correlation of risk factors with norovirus levels
Site-by-site variation

- Significant difference in contamination pattern between sites
Desk study of risk factors for NoV in oysters (England and Wales)

- Linear correlation (Pearson’s r) analyses
- Linear regression

Levels of NoV and *E. coli* in oysters from 31 sampling points

- Catchment resident population
- Catchment population density
- Catchment urbanised area
- Water temperature
- Fluvial distance from sampling point to discharge
- Tidal range
- Mean high water springs
- Rainfall (day of sampling/cumulative 7 days prior to sampling)
- River flows (day of sampling/cumulative 7 days prior to sampling)
- Base flow index
- Number of discharges (continuous/intermittent) to shellfish water
- Number of discharges (continuous/intermittent) in the catchment
- Size (volume) of continuous discharges
- Number of trade discharges in the catchment
- Frequency/duration of sewage spills

Desk study of risk factors for NoV in oysters (England and Wales)
Norovirus levels in oysters vs potential risk factors

Elevated NoV concentrations at a site correlated with:

- Water temperature
- Sewage discharges (number and volume)
- River flows
- Frequency of storm overflows
- No correlation with demographic parameters

Relationship between NoV in oysters and number of CSO spills:

Field studies investigating the quantitative relationship between sewage discharges and norovirus contamination in shellfish

- Shallow linear estuary
- Coastal embayment

- Sewage discharge
- Shellfish sampling cage
Field studies

- Estuary 7 stations (2012 – 2016)
- STW - 170 sewage samples from 2 principal works and 2 possible contributors
- Shellfish - 257 shellfish samples from 30 sampling runs
- E. coli and norovirus analysis in both sewage and shellfish
- Environmental data collected
Characterisation of norovirus and *E. Coli* in sewage effluents

### Total removal ($\log_{10}$) of *E. coli* and norovirus:

<table>
<thead>
<tr>
<th>Process</th>
<th>E. coli</th>
<th>Norovirus (genogroup II)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Min.</td>
<td>Max.</td>
</tr>
<tr>
<td>Activated sludge+humus tanks+UV disinfection</td>
<td>3.25</td>
<td>6.41</td>
</tr>
<tr>
<td>Trickling filters+sand filters+humus tanks+</td>
<td>3.86</td>
<td>5.77</td>
</tr>
<tr>
<td>UV disinfection</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Trickling filters+humus tanks</td>
<td>0.37</td>
<td>3.41</td>
</tr>
<tr>
<td>Biofilters+humus tanks</td>
<td>0.94</td>
<td>3.45</td>
</tr>
</tbody>
</table>
E. coli and norovirus in oysters
**E. coli** and norovirus in oysters

Linear models:

- **E. coli** $R^2$ 26.6%
- Norovirus $R^2$ 32.2%

Estimating effluent dilution using dye

160l of Rhodamine WT dye mixture injected for 12.4h

Dr Greg Goblick
Dilution of dye-tagged effluent evaluated using:

- Fluorometer towed from boat to identify the extent of spatial distribution of dye plume at the surface.
- Fluorometers attached to oyster sampling cages.
Norovirus in oysters vs effluent dilution

Predicted mean concentrations of norovirus at four dilution ratios:

<table>
<thead>
<tr>
<th>Dilution ratio</th>
<th>Mean concentration of norovirus (copies/g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>300:1</td>
<td>1,200</td>
</tr>
<tr>
<td>1,000:1</td>
<td>600</td>
</tr>
<tr>
<td>5,000:1</td>
<td>200</td>
</tr>
<tr>
<td>10,000:1</td>
<td>100</td>
</tr>
</tbody>
</table>

Site A – coastal embayment
Site B – estuary

Minimum dilution in the receiving water

Norovirus (GI+GII) (copies/g)

$R^2$ (adj)=75.4%
Data needed to predict virus impact (buffer zones)

- Input levels of norovirus from local community
  - and/or
- Output levels from STWs and storm overflows etc
- Effluent dilution in the environment (hydrographical models or dye tracing)
- Seasonal variation in shellfish virus equilibrium (seawater temperature)
Summary

- Standardised methods for detection of viruses in foods now available
- Unexpectedly high levels of norovirus RNA in UK (and EU) oyster production areas
- Virus standards for shellfish under consideration, informed by EU baseline survey
- Quantitative predication of virus risk developing – for buffer zones?
- But... determination of virus viability remains a challenge
Acknowledgments

UK Food Standards Agency
DG Santé of the EU Commission
James Lowther, Lisa Cross, Carlos Campos - Cefas
CEN TAG4 participants
Jane Richardson, - EFSA
Greg Goblick - US FDA