DETECTION OF PATHOGENS IN SHELLFISH AND THE ENVIRONMENT USING MOLECULAR METHODS

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Detection of microbial pathogens in shellfish using molecular methods can provide rapid and reliable assessment for the microbiological safety of post-harvest-processed products (PHP) for the consumers. Recently the application of fluorogenic real-time PCR has progressed significantly, establishing its potential as an alternate method for the detection of *Vibrio vulnificus* and *V. parahaemolyticus* in shellfish and its surrounding waters. Detection of *V. vulnificus* in shellfish homogenate has been achieved by using SYBR Green I fluorescent dye or by dual-labeled fluorescent reporter dye on taqman probes. This detection assay targeting a 205 bp segment of the *Vibrio vulnificus* hemolysin gene includes a 5-h enrichment process, followed by the release of DNA by treatment with Instagene® matrix, and PCR amplification with appropriate reaction mixtures. The detection in seeded and natural oyster tissue homogenates or Gulf of Mexico water was achieved at an initial inoculum of <10 cfu per gram. A real-time <8h PCR assay for the detection of *V. vulnificus* in shellfish has been proposed for further validation and implementation, as an additional approach for monitoring this microorganism in shellfish.

Detection of *V. parahaemolyticus* in shellfish has been achieved by real-time PCR with Taqman probes targeting thermolabile hemolysin (tdh) for species identification, thermostable direct hemolysin (tdh) and thermostable-related hemolysin (trh) for hemolysin-producing pathogenic strains, and open-reading frame 8 (ORF8) for the detection of pandemic strains of O3:K6 serotype. A multiplexed PCR for all 4 targeted genes, using gene-specific Taqman probes and primers, was achieved after 8-16 h of enrichment of oyster homogenate and consisted of an initial level of <10 cfu/g of this microorganism. Detection for all targeted genes with an equivalent sensitivity was possible with 10^6 cfu non-*V. parahaemolyticus* background microorganisms. The application of multiplexed 4-loci Taqman PCR for the identification of total and pathogenic *V. parahaemolyticus* in shellfish distributed by the seafood industry remains promising.

In addition to the real-time PCR, the potential for comprehensive multigene-targeted natural and human-introduced pathogen detection in shellfish has been investigated by using DNA-microarray technology. The method has been tested for the presence of *V. vulnificus*, *V. parahaemolyticus*, and *V. cholerae* in seeded and natural oysters. Identification of phage-displayed peptides and the application of a biosensor technology are currently being investigated for an "intact cell"-based detection of *V. vulnificus* or other pathogenic vibrios in shellfish. Validation and implementation of molecular-based detection methods for pathogenic microorganisms in PHP oysters warrant an active partnership among industry, ISSC and academic institutions for the benefit of consumer health and the seafood industry.
DETECTION OF VIRUSES IN SHELLFISH-CHALLENGES OF METHOD STANDARDISATION, VALIDATION AND RING TRIALS

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The introduction of an internationally recognised reference method for detection and quantification of norovirus (NoV) and hepatitis A (HAV) viruses will facilitate the incorporation of viral standards into legislation and supporting monitoring programmes. The acceptance of such a method will also provide a benchmark for research and development, and comparative validation of alternative procedures for virus detection/quantification.

CEN, the European Committee for Standardization, was founded in 1961 by the national standards bodies in the European Economic Community and EFTA countries. In 2004 the working group (CEN/TC 275/WG 6/ TAG 4) on “Detection of viruses in food” was established with the aim of developing a horizontal method for NoV and HAV in foodstuffs, including shellfish. The timescale for production of a draft standard is 21 months with publication as a Technical Specification within 3 years. Thus far a consensus has been reached on a number of important aspects of method standardisation:

- use of a real time PCR (probe based) format
- extraction methods suitable for each matrix would be incorporated into the standard- for bivalve shellfish sample preparation would be targeted at the dissected digestive gland.
- acid extraction should be common to all matrices- based upon Boom silica bead/GITC method.
- use of controls - process (extraction) control (FCV, EMCV (Mengo), positive / negative RNA target control, PCR inhibition control.

In parallel, CEFAS in its role as the EU Community Reference Laboratory for bacteriological and viral contamination of bivalve mollusces has established virus proficiency testing for NoV and HAV. The scheme is open to any laboratory with an interest in virus testing of shellfish and is now in its fourth year. In 2004/5, samples of variously high, moderate and low titre tissue culture grown HAV HM174), and faecal material containing either or genogroup I (Luton (AF439541) and II (Farmington Hills (AY502023) NoV present either singularly or in combination were distributed to up to 16 laboratories in Belgium & Luxembourg, Denmark, Finland, France, Germany, Greece, Italy, The Netherlands, New Zealand, Norway, Poland, Portugal, Slovenia, Spain, The United Kingdom and The United States. Analyses of data have enabled assessments of method specific and laboratory performance and contributed toward method standardisation. A number of themes have emerged, specifically all extraction methods do not produce equivalent results for NoV, detection of GI is more problematic than GII and laboratories experience fewer difficulties in detection of HAV than NoV. In 2004/5 the ring trial samples comprised tissue culture grown HAV, and GI and GII from faecal material. Thus whilst challenging the ability of participants’ to detect viral RNAs from mixed samples, issues associated with specificity, sensitivity and/or discrimination in a bivalve shellfish matrix were not fully addressed. The next distribution will comprise bioaccumulated bivalve Crassostrea gigas. The results of these ring trials and plans for future programmes are discussed.
DEVELOPMENT AND APPLICATION OF QUANTITATIVE MULTIPLEX RT-PCR ASSAYS FOR CALICI- AND ENTEROVIRUSES IN SHELLFISH AND WASTEWATER


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Noroviruses have been implicated as the causative agents responsible for the majority of non-bacterial gastroenteritis in shellfish consumers approximately 23 million cases of gastroenteritis in the United States annually; of these cases, 9.3 million cases are attributed to ingestion of contaminated food. These viruses are transmitted via the fecal-oral route and are found in municipal wastewater. Bivalve mollusces can become vectors of these agents if the water in which they feed is contaminated by human fecal waste or improperly treated wastewater. While certain enteroviruses can be cultured by conventional tissue culture techniques, the use of RT-PCR bases assays has increased detection sensitivity and reduced analysis time. Human noroviruses, which cause the vast majority of gastrointestinal illnesses, are placed into two genogroups, GI and GII. These genogroups are non-culturable, so their detection is based primarily upon non-quantitative RT-PCR assays. Advances in real-time quantitative RT-PCR (qRT-PCR) technology have allowed the recent development of several qRT-PCR assays for the rapid detection and enumeration of culturable Caliciviruses (San Miguel Sea Lion Virus, serotype 17), enteroviruses, and unculturable noroviruses. However, available Taqman style assay developed for norovirus requires separate simplex reactions to distinguish the GI and GII genogroups, while the SYBR Green based assay is unable to distinguish these two genogroups. This presentation describes a multiplex qRT-PCR assays developed on the Cepheid SmartCycler® system for the simultaneous the detection and enumeration of enteroviruses, norovirus genogroup I, and norovirus genogroup II as well as qRT-PCR assays for SMSV-17 and hepatitis A virus. Each of these assays has incorporated a novel quantitative internal control to prevent the reporting of false negatives due to inhibition or failure of qRT-PCR reactions. Applications of these assays include determining viral load in municipal wastewater and treatment plant efficiency, viral elimination from shellfish, shellfish-associated outbreak investigations, and determining efficiency of extracting viruses from foods.
ROTAVIRUS VIRUS LIKE PARTICLES AS SURROGATES TO EVALUATE VIRUS PERSISTENCE IN SHELLFISH

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Bivalve molluscs may accumulate viral contaminants within their tissues during feeding, and outbreaks of viral gastroenteritis have been associated with oyster consumption, and even with depurated shellfish. Little is known about viral persistence following depuration or relaying. Data on virus persistence are needed to address how long viruses may persist in contaminated oyster bed so that early reopening of beds that might pose a risk to consumer can be prevented.

Rotavirus virus-like particles (VLPs) and MS2 bacteriophages were bioaccumulated in bivalve mollusks to evaluate viral persistence in shellfish during depuration and relaying under natural conditions. Using this non-pathogenic surrogate virus, we were able to demonstrate that about one log10 of virus-like particles was depurated after one week in warm sea-water (22°C). Phages MS2 were depurated more rapidly (about two log10 in one week) than were virus-like particles, as determined using a single compartment model and linear regression analysis. After relaying in the estuary, under the influence of the tides, virus-like particles were detected in oysters for up to 82 days following seeding with high levels of VLPs (concentration range between 1010 and 109 particles per g of pancreatic tissues) and for 37 days for lower contamination levels (105 particles per g of pancreatic tissues).

<table>
<thead>
<tr>
<th>Days of relaying</th>
<th>Log10 of VLPs in oyster tissues</th>
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<tbody>
<tr>
<td></td>
<td>Exp A</td>
</tr>
<tr>
<td>0</td>
<td>10.5</td>
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<td>70</td>
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</tr>
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<td>82</td>
<td>5</td>
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<tr>
<td>89</td>
<td>&lt;4</td>
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Table 1 : Reductions of VLPs in oysters under relaying condition.

After one week depuration, oysters were placed on the shore under influence of tides for a natural relaying. Samples were collected and analyzed by ELISA in duplicates. nd: analysis not done.

This is the first study that clearly demonstrates that a virus surrogate (viral-like particles) can be used in field experiments, and that they persist in shellfish for several weeks in natural environment. The data presented here also suggest that after contamination by human enteric viruses shellfish may be unsafe for human consumption for quite a long period of time.
VIRAL METHODS DEVELOPMENT AND APPLICATIONS

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Norovirus is the most important cause of outbreaks of acute gastroenteritis among people of all ages worldwide, and the principal agent of bivalve shellfish-associated gastric illness. The ability to detect norovirus (NV) in bivalve shellfish is and will in the future be of paramount importance in protecting both public health and consumer confidence related to shellfish. Until recently procedures for the detection of NV in shellfish have relied upon the use of conventional nested RT-PCR. Real-Time PCR technology offers potential improvements however, in terms of sensitivity, rapidity, quantification and ease of interpretation of test results. The high sequence diversity within and between NV strains and genogroups for some time confounded the design of broadly-reactive Real-Time PCR primers, however increases in the volume of NV sequence data available, and in particular the publication of a number of complete genome sequences has allowed the identification of regions of the genome suitable for the design of broadly-reactive primers, and a number of TaqMan™ assays for NV detection have been published in the last two years. As European Community Reference Laboratory (CRL) for monitoring bacteriological and viral contamination of bivalve molluscs, CEFAS has assessed the performance of several broadly-reactive TaqMan™ primer/probe sets in comparison to a conventional nested PCR-based method for routine NV testing. We have also used quantitative Real-Time PCR to carry out investigations into the methodology currently in use for extraction of viral RNA from shellfish and generation of cDNA template for the TaqMan™ assay, and to identify and implement improvements to the methodology. These studies have enabled us to rationally develop a complete protocol for the testing of shellfish using a Real-Time PCR format, and this data along with that generated by other European laboratories should facilitate the development of a Europe-wide standard for the detection of NV and other viruses in bivalve shellfish.
QUANTITATIVE (REAL-TIME) PCR ASSESSMENT OF POST HARVEST PROCESSING OF OYSTERS

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U.S. Gulf Coast states are currently mandated by FDA to perform post harvest processing on 25 percent of oyster shellstock in order to reduce cases of V. vulnificus disease that are related to the consumption of raw oysters. The University of Florida has partnered with the Florida Oyster Dealers Association, Florida Sea Grant, and USDA in order to establish a laboratory in Apalachicola, FL for the purpose of providing evaluation of post harvest processes. This laboratory will provide on-site certification that is necessary to validate and verify processes and can be used as a model by the seafood industry for compliance with FDA guidelines. Related research also compared and improved the methods used for evaluation of these processes, including standard microbiological analysis, DNA and PCR probe assays for confirmation species, as well as various PCR and microtiter formats for Most Probable Number (MPN) determinations. The use of Quantitative PCR (QPCR) offered a high throughput alternative to current methods for detection of Vibrio spp. in processed oysters. QPCR can be more rapid, precise, and efficient compared to standard DNA probe confirmation of MPN, while providing equivalent results. Potentially, QPCR offers greater sensitivity than current methods for determination of processes that truly reduce V. vulnificus to “non-detectable” levels.
DETECTION OF NOROVIRUSES IN SHELLFISH: DEVELOPMENT OF A METHOD FOR REGULATORY USE IN NEW ZEALAND

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Noroviruses (NV) have been detected in imported oysters and in bivalve shellfish from New Zealand shellfish growing areas. This is a problem for regulators, the shellfish industry and the general public. There is an urgent need for a sensitive assay that detects noroviruses in contaminated shellfish. Recovery of NV from shellfish is complex and there is no standard international method. Methods for NV detection in shellfish have been non-quantitative and insensitive for detection of low virus numbers, but real-time RT-PCR has allowed progress in this area. Our goal is to implement a method that is sensitive, timely, robust, reproducible and detects a wide range of noroviruses.

Different recovery procedures, including protease digestion, acid adsorption, alkaline elution, PEG concentration, ultracentrifugation, and magnetic beads were compared. The methods were evaluated on oysters seeded with high concentrations of a GII/3 NV strain prepared from faecal specimens and titrated in conventional and real-time RT-PCR assays. Seeding was carried out by injection of titrated NV into the gut of freshly dissected oysters. A specific NV real-time RT-PCR assay was used to compare NV recovery from oysters by the different methods.

No virus concentration method was found to be significantly more efficient for virus recovery. The protease digestion method gave the greatest recovery of all methods when seeded with high levels of norovirus. Following further comparison trials with the acid adsorption and alkaline elution methods, the protease digestion method was selected for evaluation and standardisation. Studies to validate the protease digestion method, and to determine the limit of detection and limit of quantitation in shellfish contaminated with low levels of norovirus, are in progress.

Table 1. Comparison of protease digestion, acid adsorption and alkaline elution methods for NV recovery from oysters (3 replicate oyster samples per method). NV quantities recovered were determined from standard curves generated in real-time RT-PCR assays. Results show no. of replicates positive for NV.

<table>
<thead>
<tr>
<th>Method</th>
<th>2 x 10^3 NV RTPCRU</th>
<th>1 x 10^4 NV RTPCRU</th>
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<tbody>
<tr>
<td>Protease digestion</td>
<td>2/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Alkaline elution + PEG concn</td>
<td>0/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Acid adsorption + PEG concn</td>
<td>0/3</td>
<td>3/3</td>
</tr>
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</table>

The key factors required for a virus detection method are sensitivity, specificity and reproducibility. The protease digestion method, combined with a real-time generic norovirus RT-PCR assay, were chosen for further evaluation and validation as a method for New Zealand regulatory use. This combination provides efficient virus recovery, is sensitive and reproducible, of lower cost than labour-intensive methods and can be completed in 1-2 days.
THE FIRST PANDEMIC OF *Vibrio parahaemolyticus* INFECTION: EMERGENCE, SPREAD, AND EVOLUTION OF THE PANDEMIC O3:K6 CLONE

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Infection by *Vibrio parahaemolyticus* is usually sporadic and an outbreak has usually been limited to a small community scale. Starting with a collaborative study in India in 1996, we found increase in the number of *V. parahaemolyticus* infection in various Asian countries and the United States. These infections were caused by *V. parahaemolyticus* O3:K6 strains possessing the *tdh* gene but lacking the *trh* gene. In addition, these strains were shown to be clonal and distinct from O3:K6 strains isolated before 1995 by the DNA fingerprint analysis using an arbitrarily primed PCR. We thus named this new clone as a pandemic clone. This is the first emergence of the pandemic in the history of *V. parahaemolyticus* infection. We developed a PCR method to detect the strains belonging to the pandemic clone (GS-PCR). The GS-PCR detects two bases in the *toxRS* operon that are unique to the pandemic strains. This PCR allowed detection of serovariants of the pandemic strains and further spread of the pandemic strains to various parts of the world.

We carried out phylogenetic analysis of 60 strains isolated in Asia between 1983 and 2001. They belonged to the pandemic clone and related groups. Comparison of the nucleotide substitutions in eleven genes distributed in two chromosomes allowed us to confirm clonality of the pandemic strains including serovariants and to speculate evolution of the clone. A 22-kb chromosomal insert unique to the pandemic clone was identified by a subtractive hybridization method. This sequence was shown to be associated with a growth characteristic under a certain condition by a mutagenic analysis.

The pandemic strains have been isolated from molluscan shellfish in southern Thailand and Japan, from the sediments in Japan, and from the environmental water in Bangladesh. The question to be answered is how the pandemic strains were transferred across the international borders.