THE EFFICACY OF HIGH PRESSURE PROCESSING FOR VIRUSES

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Noroviruses (NV) and hepatitis A virus (HAV) can be transmitted to humans by shellfish consumption. Depuration and relaying of oysters and clams are of limited use as an intervention strategy for shellfish-borne viruses. Our research has focused on high pressure processing (HPP) as an intervention. Different viruses display variable sensitivity to pressure but results obtained with a norovirus surrogate, feline calicivirus (FCV; strain KCD), and for HAV strain HM-175, suggest that HPP can be used as an intervention for uncooked shellfish. In DMEM tissue culture media, reduction of HAV titer was observed with treatments between 300 and 450 megapascal (MPa; approx 3,000-4,500 atm or 45,000-60,000 psi), with a room temperature 5-min treatment at 450 MPa being sufficient to completely inactivate 7-log_{10} pfu/ml. Treatment of HAV-contaminated oysters with a 1-min 400-MPa treatment at 9°C inactivated more than 3-log_{10} pfu of HAV within oyster meats. A 5-min room temperature treatment at 275 MPa is sufficient to inactivate 7-log_{10} pfu/ml of FCV. Investigation of the effects of treatment duration and temperature on FCV was determined. At room temperature, plotting log N/N_0 against treatment time at 200 MPa and 250 MPa, FCV pressure inactivation curves showed a rapid decline followed by tailing, consistent with nonlinear Weibull and log-logistic functions. Different temperature treatments of FCV at constant time and pressure indicate that inactivation above, and particularly below, room temperature result in enhanced inactivation compared to room temperature. For example, a 200-MPa 4-min treatment at -10°C and +50°C reduced the titer of FCV by 5.0 and 4.0 log_{10} respectively; while at 20°C, the same treatment only reduced the titer by 0.3 log_{10}. Recently, work with murine norovirus-1 (MNV-1), a virus closely related to human norovirus, has been undertaken. Preliminary results confirm that MNV in DMEM media is sensitive to pressure and inactivation of MNV-1 is enhanced by >3-log_{10} when treatments are performed at 5°C as compared with 20°C. Research geared toward inactivating MNV-1 within oysters, potentially the first demonstration of norovirus inactivation directly in shellfish tissues by high pressure processing, is ongoing. This work lays the groundwork for a recently-funded human volunteer study to demonstrate inactivation of non-propagable human norovirus strain 8F11b-contaminated oysters by HPP. Collectively, this work suggests optimism for the success of HPP as a virus intervention strategy for commercial shellfish.
THE COLONY OVERLAY PROCEDURE FOR PEPTIDASES TO DETECT AND ENUMERATE TOTAL VIBRIONACEAE IN MOLLUSCAN SHELLFISH AND THEIR GROWING WATERS

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Since 1925, molluscan shellfish harvesting has been regulated in the United States based on sanitary surveys of shellfish growing waters. Surveys have relied on the use of coliform standards which have effectively eliminated outbreaks of typhoid fever and other bacterial illnesses. Only the Vibrionaceae have not been controlled by the implementation of coliform or fecal coliform standards. The Vibrionaceae are indigenous to marine waters, and subsequently, can not be predicted based on the levels of fecal coliform contamination. Vibrionaceae contain members of the genera Vibrio, Aeromonas, Plesiomonas, and Photobacterium.

We developed an enzyme assay known as the colony overlay procedure for peptidases (COPP) that detects and quantifies Vibrionaceae family members in seawater and shellfish based on the presence of the enzyme phosphoglucone isomerase and its lysyl aminopeptidase activity. This procedure is relatively simple, rapid, and inexpensive compared to other Vibrio testing methods. Dilutions of oyster homogenates or seawater are spread on plates of tryptic soy agar plus 0.5% added NaCl and incubated at 37°C overnight. Countable plates are overlaid for 10 min with a cellulose acetate membrane containing a commercially available synthetic substrate, L-Lys-7-amino-4-trifluoromethylcoumarin. The membrane is then observed under UV light. Fluorescent foci corresponding with bacterial colonies indicate the presence of Vibrionaceae family members. Total Vibrionaceae counts may be determined by multiplying the total number of fluorescent foci detected by the dilution factor. High levels of total Vibrionaceae may serve as an indicator for the possible presence of pathogenic Vibrio species and signal a point when shellfish beds should be closed to harvesting or when additional, more sophisticated testing for specific pathogens is warranted.

In a monthly survey of oysters and seawater in the Delaware Bay over a one-year period, we detected levels of Vibrionaceae exceeding $10^6$ per gram of oyster and $10^4$ per ml of seawater during the summer months. Vibrio vulnificus was particularly prevalent in oysters, with counts exceeding $10^5$ per gram from May – August, while Vibrio parahaemolyticus levels exceeded $10^4$ per gram in May and July. The COPP assay may find utility in identifying peak periods when Vibrionaceae are at their highest levels in East, West, and Gulf Coast oysters and growing waters and allow the correlation of total counts with the incidence of Vibrio outbreaks in those areas. The COPP assay is equally applicable in the United States and the European Union where shellfish are currently regulated based on coliform levels in seawater and shellfish meats, respectively.
Vibrio parahaemolyticus RISK MANAGEMENT IN BRITISH COLUMBIA

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In 1997 the Pacific Coast of North America experienced an unprecedented outbreak of Vibrio parahaemolyticus (Vp) illnesses attributed to the consumption of raw and lightly cooked oysters. Several hundred confirmed and clinical cases were reported, mostly following consumption of oysters from British Columbia (BC) and Washington State. In late 1997 and early 1998 regulators and stakeholders from the US and Canada worked together to develop a strategy to address Vp illnesses which eventually evolved into the Vp Interim Control Plan (ICP) section of the US Shellfish Model Ordinance.

This framework was modified and refined in BC to further minimize risk to consumers of raw oysters. The BC Vp Advisory Committee was established to address the Vp issue. The committee includes representatives from government regulators (federal, provincial and regional) and stakeholders (processors, growers and restaurant association.) The primary focus of the strategy is to minimize consumer exposure to oysters with high levels of Vp through the implementation of monitoring and stringent post-harvest time and temperature controls.

This presentation will include:

- a brief history of the 1997 Vp illness outbreak and development of the ICP;
- the 2000 project conducted to track Vp levels in intertidal oysters over a tide cycle;
- the specific components of the BC Vp risk management strategy including:
  - environmental monitoring of Vp levels in oysters by the CFIA;
  - implementation of molecular methods for detecting pathogenicity;
  - Vp monitoring at harvest sites by stakeholders;
  - post-harvest time / temperature controls;
  - record keeping under QMP / HACCP;
  - mandatory cook advisory on shucked oysters;
  - enhanced illness reporting and traceback;
  - illness statistics from 1997 to present; and
  - new developments / update for 2005, including a HACCP guidance document for BC regulators and industry.

The number of reported Vp illnesses implicating BC oysters has remained at a low level since the implementation of the modified strategy.
THE FAO/WHO RISK ASSESSMENTS ON *Vibrio* SPP. IN SEAFOODS

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*Vibrio parahaemolyticus, Vibrio vulnificus* and choleragenic *Vibrio cholerae* have been identified as the species responsible for most cases of human illness caused by *Vibrio* spp.. The FAO/WHO *Vibrio* spp. risk assessment therefore addresses all three hazards focussing on *V. parahaemolyticus* in raw oysters, finfish consumed raw and bloody clams, *V. vulnificus* in raw oysters and choleragenic *V. cholerae* O1 and O139 in warm-water shrimp in international trade. The assessments considered information on *Vibrio* spp. in seafood that was being generated and was available at regional and national levels, and this formed the substantive basis for their development. The assessments illustrate how different approaches were used to reflect the national capacity to generate data, the pathogen of concern and the commodity of concern.

The risk assessments of *V. vulnificus* in oysters and *V. cholerae* in warm water shrimp are presented as examples of the FAO/WHO risk assessments and illustrate the different purposes of risk assessment, some of the possible approaches and the impact of relevant data.

The risk assessment of *V. vulnificus* in raw oysters successfully investigated how an established *V. parahaemolyticus* model (FDA, 2001) could be adapted to a different pathogen. Despite the fact that data were only available from the USA, this risk assessment was undertaken because *V. vulnificus* illness has one of the highest mortality rates of any foodborne disease and has emerged as an important issue in a number of countries and regions including the USA, New Zealand, Japan, Republic of Korea, and Europe. The work clearly demonstrated the utility of adapting the framework and parameters for the *V. parahaemolyticus* risk assessment to another *Vibrio* spp.. Additional retail study data served to validate the exposure assessment predictions. The dose-response relationship was established using exposure predictions together with the reported frequency of illness. The risk assessment also evaluated the establishment of target levels (3/g, 30/g and 300/g) as an example of a management intervention and estimated that substantial reductions in risks were associated with target levels of 3/g and 30/g.

The *V. cholera* risk assessment was undertaken to estimate the likelihood of contracting cholera from the consumption of warm-water shrimp (cooked and uncooked) in international trade. No previous risk assessments had been carried out on this pathogen-product combination. A range of approaches were employed, including qualitative, semi-quantitative and quantitative techniques, to undertake a risk assessment using the available data. In all cases, based on the available data, the risk of acquiring cholera from imported warm-water shrimp was estimated to be very low.

These two examples illustrate the flexibility of risk assessment as a food safety management tool and the range of approaches that can be applied therein. They provide a good basis from which countries can develop their own risk assessments, an overview of the currently available data and the data gaps and identify some of the issues to be addressed when undertaking a risk assessment.
VIBRIOS, VIRUSES, VERDICTS AND VIRTUOSOS

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It is now 11 years since we held the first International Molluscan Shellfish Safety Conference in Sydney, Australia. Public health issues of major concern at the time were Vibrios, particularly Vibrio vulnificus, and viruses.

In 2005, as scientists, health professionals and industry how have we measured up? The statistics may tell us some of the story:

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<tr>
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<th>1994</th>
<th>2004</th>
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<tr>
<td>Vv cases USA</td>
<td>11</td>
<td>19</td>
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<td>Virus outbreaks associated with seafood</td>
<td>7</td>
<td>3</td>
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<td>Restricted harvest areas in NZ</td>
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Epidemiologists and virologists can no more stop seafood illness or environmental degradation than economists can stop inflation. However, the power of science is surely its capacity to change things, and to promote a better environment in which to live. Environmental science without decision making or policy context is a waste of time.

HACCP and Risk Assessment were supposed to be the new weapons against illness. Risk assessment tools quickly make us realize that Vibrios and viruses will take two entirely different management approaches. Vibrios, being natural pathogens are going to force society to focus on how much disease is acceptable to a population.

Viruses, however take us back to the root of shellfish sanitation programmes. Our shellfish sanitation forbears were able to prevent typhoid, why can we not prevent viral illnesses associated with shellfish consumption? We find ourselves once again dealing with environmental issues – specifically sewage contamination. To combat typhoid four groups were necessary to ensure public health reforms; politicians, professional groups, lobby groups and the press.

This knowledge must reach decision makers (whoever they are) and in a timely fashion, in a form they understand, and in a manner which encourages a positive response.

Our challenge therefore is not only to collect the science and data, but to provide clear information and explicit requirements to the policy analysts who act as brokers with the political decision makers, the press and the public. This conference has a wealth of professional skills which could be used to produce guidelines for the policy makers e.g. providing clear information on the appropriate sewage treatment systems. Let us hope 2114, this conference will have made an impact on the statistics.
DEVELOPMENT OF IMPROVED RISK ASSESSMENT AND MANAGEMENT TOOLS FOR Vibrio vulnificus: WHAT MAKES THEM PATHOGENIC FOR HUMANS?

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Vibrio vulnificus, a Gram-negative halophilic bacterium common to estuarine and marine waters, colonizes shellfish and the intestinal contents of fish. Food borne infections caused by this opportunistic pathogen through ingestion of raw or undercooked shellfish are life threatening, with a high mortality rate. In addition to the human health costs, outbreaks of V. vulnificus infections cause a significant economic impact on the shellfish industry. In spite of considerable efforts to modify harvesting, transportation and storage practices during the warmer summer months, to develop post harvest treatment methods to eliminate the pathogen, and to educate the at-risk population about the risks of raw oyster consumption, the annual number of clinical cases and mortality has remained largely unchanged. It has long been recognized that there is a wide range of virulence, as measured in various animal models. Moreover, human clinical infections with this pathogen usually arise from a single strain, even though an individual oyster can harbor hundreds to thousands of strains as determined by clamped homogeneous electric field gel electrophoresis. Clearly, management of the shellfishery would be aided by a method to assess risk that could identify potentially virulent strains from other environmental strains that are less likely to cause human disease. However until recently, efforts to develop genotypic and/or phenotypic methods to differentiate strains have been largely unsuccessful.

We and others have shown that V. vulnificus can be divided into two or more groups, type A and type B, based on a 17 nucleotide polymorphism between two alleles of the 16S rRNA gene, of which there are nine copies in the V. vulnificus genome. We have demonstrated a high correlation between human clinical isolates and the type B genotype. We have recently extended the original T-RFLP assay used to differentiate the 16S rRNA type to a rapid assay using real-time PCR. This test has the potential to be used in monitoring programs that would allow fishery or public health managers to better gauge risk of shellfish harboring this organism. Type A and B strain differentiation can also be used to focus basic molecular pathogenesis research towards the identification of V. vulnificus virulence determinants important for human infection. Taken together, research based on this data should replace assumptions about the virulence of V. vulnificus with empirical data.

In our laboratory, research on V. vulnificus and V. parahaemolyticus is largely supported by the NOAA West Coast Center for Oceans and Human Health. The NOAA Oceans and Human Health Initiative is poised to leverage resources that can be used to address critical gaps in risk assessment and risk management for marine pathogens and shellfish safety.
BRIEF HISTORY OF MICROBIAL INDICATORS IN THE NATIONAL SHELLFISH SANITATION PROGRAM, USA

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Microbial indicators of fecal contamination have been studied and employed to assess the sanitation of waters and foods since 1880. The National Shellfish Sanitation Program (NSSP), initiated in 1925, places high importance on water quality as the first critical control point in the production of safe molluscan shellfish, because shellfish can accumulate pathogens, toxins, and chemicals from their surrounding waters. The NSSP is a voluntary safety program implemented by individual states, with federal oversight, and enforced collectively through the Interstate Shellfish Sanitation Conference (ISSC). State authorities classify shellfish growing areas, control the harvest of molluscan shellfish, and certify dealers. Classification of shellfish growing areas requires periodic shoreline surveys to identify and assess all actual and potential sources of contamination and periodic water quality testing to verify classifications.

The NSSP requires state authorities to determine indicator bacteria levels in shellfish waters. Beginning in 1925 the NSSP indicators were coliform bacteria determined by fermentation testing. However, uniform water standards for classifying areas were not formally prescribed until after 1941. Even so, from the inception of the NSSP most states applied water standards that approximated a Most Probable Number (MPN) of 70 coliform bacteria per 100 ml. Following a U.S. Public Health Service report in 1941, the NSSP formally prescribed a median or geometric mean water MPN of 70 coliform bacteria per 100 ml for approved areas in 1946. In 1958, the NSSP prescribed a water standard for restricted areas, and in 1959 90th percentile values for approved and restricted area classifications were added. The 90th percentile values allow for variability inherent to the test method, and establish a two-part standard for the classification of shellfish areas. In 1961 fecal coliforms were proposed as an alternative to coliforms, and based on numerous studies, the NSSP formally added fecal coliforms as an indicator in 1974. A median or geometric mean MPN values of 14 fecal coliform bacteria per 100 ml was prescribed for approved areas, with 90th percentile values per 100 ml for also included. In 1986, values for restrict areas were prescribed.

It has long been recognized that bacterial indicators do not readily index the potential occurrences of viruses sometimes present in fecal contamination. Nonetheless, the requirements and restrictions established by the NSSP, based in part on coliform and fecal coliform bacterial water quality standards, have proven to be substantially effective for producing commercial shellfish that are safe for consumers. In 2005, for the first time, a proposal has been made to introduce a viral indicator in the NSSP as a means to re-open areas following atypical sewage contamination events. The proposal will be deliberated at the biennial meeting of the ISSC in August 2005.