Development of a spreadsheet model of the market chain for the live reef food fish trade

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The market for live reef fish for food (LRFF) is longstanding in Southeast Asia, with demand for live fish concentrated in Hong Kong and southern China. The total regional trade in LRFF is considered to be around 30,000 tonnes per year with an estimated 15–20,000 tonnes of this going into Hong Kong (Sadovy et al., 2004). More than 20 countries in Southeast Asia and the Pacific supply fish to this market using a variety of capture techniques and transport technologies.

A key component of this project (ADP/2002/105: Economic and market analysis of the Live Reef Food Fish Trade in Asia-Pacific) is aimed at identifying and measuring major cost and revenue components along the marketing chain using a spreadsheet model. This model will be further developed to incorporate key risk factors for the various agents along the chain from the point of capture to the point of sale; and for the case of both sea and air transport technologies.

The live reef food fish trade market chain

Market chain analysis can help to identify constraints (e.g. information flows), inequities (e.g. distribution of value) and practices (e.g. handling, quality control) along the chain that can serve to enhance benefits of trade to agents, especially those upstream agents in source countries. Within the LRFF trade however, addressing these market chain constraints and inequities can be obstructed by relationships between agents along the market chain.

The characteristics of the LRFF trade have resulted in the market chain becoming quite extended and complex. These characteristics include rudimentary storage and transport infrastructure, low technology fishing gear, the remoteness of fishing grounds from supply hubs and the considerable distances of source countries from markets. After being caught by the fisher, LRFF pass through many levels of trade before reaching restaurants in Hong Kong and China. The market chain can be shorter in some countries than in others. In Southeast Asia, the supply side of the market chain includes one or two middlemen whose role is to consolidate catches from independent fishers into sufficient quantities for movement along the chain. In Australia, the middleman role is assumed by fishing firms who employ fishers to catch fish and who sell these fish directly to wholesale exporters. In Australia, LRFF fishers receive a percentage of the ‘beach price’ paid to the fishing firm by the export wholesaler. The chain is shorter still in the Pacific with fishers being employed directly by exporters who transship this catch, almost entirely by sea.

As a high value commodity, there is a perceived potential for high economic gains along the chain. The high price of LRFF in Hong Kong has created an impression among suppliers from importing countries that the prices they receive from buyers one step further along the chain is too low (Chan, 2001). In the extended market chain for LRFF however, each agent requires an acceptable margin to continue trading. In practice these gains tend to be unevenly distributed among agents for a variety of reasons including: fishers’ lack of knowledge of final values; transport costs incurred by traders when shipping fish across large distances either by sea or air; the high risks of mortality endured by traders during transport; health
scares (e.g. ciguatera); and shocks in economic conditions (e.g. Severe Acute Respiratory Syndrome (SARS)). A hypothetical market chain, showing distribution of the final value of LRFF amongst the various agents along the chain for wild-caught LRFF, is shown in Figure 1.

While horizontal cooperation at various stages along the chain does occur, vertical cooperation, or integration, is more likely in fishery chains as a result of:

- the perishability of the product.
- variations in product quantity and quality.
- consumer awareness of product quality.
- economies of scale.

Within the LRFF trade, opportunities for vertical cooperation exist in both the wild-caught and aquaculture sectors. Vertical integration along the market chain for wild-caught LRFF usually occurs at the collection/export stage of the source country supply chain and the import/distribution stage of the import country supply chain (Figure 1). These vertical relationships will tend to obfuscate efforts to identify the distribution of final product value along the market chain. The individual agents will tend not to set prices or margins in line with their respective business operations; margins will be centrally determined for each of these ‘profit centres’.

In terms of LRFF aquaculture, there has tended to be an increased dis-aggregation at the upstream end of the market chain with increased specialisation in the production process. Hatchery, nursery and grow-out phases of LRFF aquaculture tend to be distinct components in the production stage. Vertical coordination between successive stages of the chain will occur where benefits can be demonstrated in terms of flexibility to meet variant demand conditions, access to product quality information, implementation of quality control activities and access to credit (van Anrooy, 2003).

**Schematic of the market chain model**

The current trade in LRFF is largely unregulated resulting in over-exploitation of fish stocks. It has been argued that this lack of regulation has meant small scale fishers are not receiving fair economic returns. The rationale for this has been that downstream agents bear the trade risks (i.e. fish mortality, exchange rate fluctuations and high transport costs) and these costs are passed back along the chain, leading to the relatively low prices paid to fishers.

The key objective of this project component is to measure cost and risk components of the market chain to enable options for risk reduction, improved price transparency and improved returns for small scale fishers to be examined. There are two approaches to deriving what constitutes a fair economic return:

i) A bottom-up approach of determining the costs of catching fish to derive a ‘fair’ beach price that captures this cost plus a suitable margin.

ii) A top-down approach based on the equitable distribution of the final product value (i.e. retail price) between agents based on risks and costs (e.g. transport, holding etc.).

Developing a bottom-up market chain model for the LRFF fishery is problematic for two related reasons. Firstly, there is a paucity of usable data, with the exception of fishing and export operations in Australia, that would enable a market chain model to be fully populated. Secondly, vertical integration between agents hampers the development of discrete sub-models for specific agents. The LRFF market chain is slightly more complex than traditional food industry value chains through the inclusion of middlemen (Sadovy et al., 2004). Traditional food industry chains consist of: producer, wholesaler, exporter, importer and retailer.

The initial spreadsheet models developed have used a hybrid top down approach. The model suite consists of two sub-models: one for fishers and fishing operations or middlemen (Figure 2); and the other for remaining market chain agents consisting of exporters, importers, distributors and retailers (Figure 3).

**Figure 1: Economic value chain model for wild-caught live reef fish for:**

a) the supply or export; and b) the demand or import sectors of the market chain. Percentage is an estimate of the final consumption value extracted at that link of the chain. The dashed boxes at export and import stages along the chain and the dashed line between these stages indicate vertical linkages between market chain agents.

(a)

(b)

The fisher/middleman or fisher/ fishing vessel sub-model

The fisher sub-model allows for costs to be derived using effort parameters and total revenues to be derived using catch parameters. Revenues can be based on either empirical beach price data or by using a margin-based approach, again
using empirical evidence. Beach prices can also be used to derive margins based on costs. Total cost and revenue information are subsequently used to develop indicators of economic returns including: net present value; annualised returns; internal rates of return and rates of return on capital (Figure 2).

**The supply chain sub-model**

In recognition of the lack of data available, a simplified model has been developed to schematically represent the supply chain (Figure 3). The current model incorporates wholesaler/exporter, importer/distributor and retailer margins based on empirical evidence. The model allows for these margins to be adjusted to explore the impacts of different margins on returns to agents and also to aid in examining the issue of ‘fair price’. The option to validate these margins based on key cost parameter will also be made available. Exchange rate movement risks have been accounted for using an expected value probability model (Figure 4).

**Distribution of value and risk**

The distribution of value of marine products has been recognised as an issue of concern in many developing country export fisheries, in terms of the percentage of final value accruing to agents along the chain and the under-pricing of resources (Jacinto, 2004). Even so, margins and value need to be considered in the context of risk borne by the respective agents along the market chain.

In the case of wild-caught fisheries, it has been suggested that fishers are usually poorly paid based on the final value of seafood products (Wood, 2001). Several factors give explanation for those receiving a relatively smaller percentage of final value. The remoteness of fishing grounds and small individual catches requires a middleman who can consolidate catches into sufficient quantities for transfer to exporters. Often these middlemen provide credit to fishers in the form of gear etc. to facilitate their fishing activities, although credit arrangements are usually not ‘mutually beneficial’.

For export fisheries, financial risks increase as the product moves along the market chain. The middlemen and exporters bear mortality risks and the costs of holding fish post-harvest. The costs of transportation to markets are borne by middlemen, wholesalers and exporters and/or importers. Shipping and freight costs can make up between 50–65% of landed price paid by importer. At the consumption end of the chain retailers incur considerable rent and wage costs (MacFadyen et al., 2003). The greater downstream risks of financial losses from mortality, prior to the product reaching consumer markets, partly explains the inequitable distribution of value.

Within the spreadsheet model, risk has been incorporated both for the fisher/fishing vessel and the supply chain sub-models. Within the fisher/fishing vessel sub-model, two types of risk have been accounted for: fish catches and fish prices. The first can account for increases or decreases in catches as a result of policy (management regulations) or environmental (overfishing) factors. The second recognises changes in demand that influence prices. Within the supply chain model, risk is associated with mortality, exchange rate fluctuations and downstream price expectations.

For each of the various risk components, an expected probability approach is used to calculate an expected value under a range of anticipated outcomes.
These expected values are used in turn to generate a cumulative probability distribution (Figure 4b). In the case of the fishing operation, risk analysis models have been incorporated for price and catch. These are reflected in the annual returns to the vessel (Figure 3). For exporters and importers, it is intended that risk analysis will be incorporated in the form of estimating expected survival rates for a consignment of live fish. The cumulative probability distribution will likely be expressed in terms of both volume (quantity) and value of a consignment and also as an annual return based on the number of monthly or annual consignments.

**Conclusions**

Despite the widespread use of market chain analysis as a means of identifying cost and revenue flows and value distribution for agents along the chain, idiosyncrasies of the LRFF trade constrain development of market chain models for use in identifying inefficiencies and distortions along the chain, in particular, inequities in value distribution.

Taking these limitations into account, this paper has outlined the initial development of an Excel spreadsheet that will be used to examine costs and revenues, and risks in the chain from the point of capture to the point of sale of LRFF.

Data limitations have dictated the form of the spreadsheet modelling approach, but in this first iteration, there are two models: for fishers and fishing vessel/middlemen; and for other agents in the chain. Adopting this approach is intended to overcome, to a degree, the lack of existing data on the operations of these downstream agents and also the need to collect the data required to populate the spreadsheet; data which will prove difficult to procure. There are a number of next steps planned for this research. These will be to modify and expand on these initial spreadsheet models to:

- explore the impacts of policies that can improve market performance and distribution of product values. (Market structures in developing fisheries with complex market chains tend to be fixed so that reducing links in the market chain to the benefit of small-scale fishers will be difficult. Governance and distributional outcomes are often skewed toward wholesalers and exporters leading to marginalisation of small-scale fishers. Opportunities for horizontal cooperation are greater in aquaculture, where supply is more able to meet variant demand and farming cooperatives, have more control over their production and supply activities).
- incorporate results from demand and supply modelling on the impacts of increased aquaculture production; most likely on beach prices for specific species or species groups.

Lastly, it is anticipated that, if there is sufficient data, spreadsheet models will be constructed not only for Australia but for at least two Southeast Asian countries and two Pacific countries. These models will aid capture fishery managers and the aquaculture sector in assessing future viability of the live reef capture and aquaculture fisheries within their countries.

**References**

Figure 4: Risk analysis for fishing vessels which: (a) uses expected probabilities of a range of catch and fish price scenarios to estimate lowest, highest and average annual returns; and (b) generates a cumulative probability distribution of expected annual returns.

(a)

Risk Analysis

**Live Catch**

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<th>Probability</th>
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<td>Minimum</td>
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<tr>
<td>Poor</td>
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</tr>
<tr>
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<tr>
<td>Good</td>
<td>0.60</td>
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<tr>
<td>Maximum</td>
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5% chance of getting between 0 and 6000 tonnes
15% chance of getting between 6000 and 9000 tonnes
40% chance of getting between 9000 and 10,000 tonnes

**Live Prices**

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<th>$/Kg</th>
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<tr>
<td>Average</td>
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<tr>
<td>Good</td>
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25% chance of getting between $25.00 and $30.00 per kilogram
35% chance of getting between $30.00 and $35.00 per kilogram
60% chance of getting between $35.00 and $40.00 per kilogram
25% chance of getting between $40.00 and $45.00 per kilogram

**FF Catch**

<table>
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<td>Minimum</td>
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<tr>
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<td>Good</td>
<td>0.60</td>
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<tr>
<td>Maximum</td>
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5% chance of getting between 0 and 2000 tonnes
15% chance of getting between 2000 and 2400 tonnes
40% chance of getting between 2400 and 2800 tonnes
40% chance of getting between 2800 and 3000 tonnes

**FF Prices**

<table>
<thead>
<tr>
<th>$/Kg</th>
<th>Probability</th>
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<tr>
<td>Poor</td>
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<td>Average</td>
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<tr>
<td>Good</td>
<td>0.75</td>
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<tr>
<td>Maximum</td>
<td>1.00</td>
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25% chance of getting between $7.50 and $8.00 per kilogram
25% chance of getting between $8.00 and $8.50 per kilogram
25% chance of getting between $8.50 and $9.00 per kilogram

(b)

Cumulative Probability Distribution

Lowest Return: $11,620
Highest Return: $12,096
Average Return: $75,818

Run Simulation
The Report of the 4th Regional Grouper Hatchery Production Training Course 2006

The 4th Regional Grouper Hatchery Training Course was another success. A total of 20 participants from 13 countries attended the training course which was hosted by the Brackishwater Aquaculture Development Centre (BADC)-Situbondo, Indonesia. The participants came from Australia, Hong Kong, Indonesia, India, Malaysia, Maldives, Myanmar, Philippines, Qatar, Saudi Arabia, Singapore, Thailand and Vietnam. The full report of the training course, which contains a lot of photographs of the activities, can be downloaded from the NACA web site.

Overall the training course met participants’ expectations. Five considered the training course was excellent and 12 said it was well organized. Sixteen participants considered the lectures organized cover all aspects of grouper hatchery production. All participants believed they have increased their knowledge and practical experience on grouper hatchery production after the course. They also agreed that they have received sufficient level of technical support throughout the training course, in spite of some language differences.

All participants were able to gather contacts for their future aquaculture activities after they returned home. The training course and the field trips provided an opportunity for the participants to obtain future contacts for supplies and marketing. Field trips were well organized based on the feedback provided by participants.

The 5th Regional Grouper Hatchery Training Course: June 11-July 1, 2007

Building on the success of the 2006 course, NACA is pleased to announce the 5th Regional Grouper Hatchery Production Training Course for the Asia-Pacific Marine Finfish Aquaculture Network is currently planned for 2007 and tentative schedule for the training course is June 11-July 1, 2007.

The course structure will be similar to the previous year. For further information please download the report of the 2006 course from the NACA website. If you wish to register for the training course please contact Mr Sih Yang Sim at grouper@enaca.org. More information will be posted on the web site in mid-February 2007.
Immunological and nucleic acid based methods for detection of food borne pathogens

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The analysis of foods for the presence of both pathogenic and spoilage bacteria is a standard practice for ensuring food safety and quality. Conventional methods require several days to give results because they rely on the ability of microorganisms to multiply to visible colonies. Moreover, culture medium preparation, inoculation of plates, colony counting and biochemical characterization make these methods labour intensive. Another disadvantage of traditional methods is that cells which are viable but otherwise nonculturable cannot be detected. In the food industry there is a need for more rapid methods to provide adequate information on the possible presence of pathogens in raw materials and finished food products, for manufacturing process control and for the monitoring of cleaning and hygiene practices (Enn and Reikett, 1999). Immunological and nucleic acid based detection methods offer alternative approaches that can meet these needs.

Immunological methods

Immunological methods rely on the specific binding of an antibody to an antigen. Both polyclonal and monoclonal antibodies are used in this method. However, one of the disadvantages of using polyclonal antisera in immunoassays is the variability found in the animal’s immune response.

Homogenous and heterogenous Immunoassays: Immunoassays can be classified as homogeneous (eg: reverse passive latex agglutination (RPLA) or heterogeneous (eg: enzyme linked immunosorbent assay (ELISA). In a homogeneous assay the antigen–antibody complex formed is directly visible or measurable and there is no need to separate the bound from the unbound antibody. Incubation times are usually very short. In latex agglutination tests, latex beads are coated with antibodies that agglutinate specific antigens and form a more easily visible precipitate. These tests are available for most common pathogens. In a heterogeneous assay, the unbound antibody must be separated from the bound antibody using labeled reagents. ELISAs for pathogens have detection limits ranging from 103 to 105 colony forming units (cfu)/ml. Therefore direct detection of pathogens in foods is not possible and enrichments are required for at least 16–24 hours. Kits for the detection of bacterial toxins are based mainly on immunoassay systems, but the use of these kits does not give any information on the biological activity of the toxins (Brett, 1998).

Immunocapture based methods: These techniques include immunological binding (capture), followed by physical separation of the target organisms from a mixed enrichment culture. One application is immunomagnetic separation (IMS) in which a sample is mixed with beads coated with antibodies for the target organism. The target organisms in the sample bind to the immunomagnetic beads, which are then isolated from other sample material and microorganisms in a magnetic field. The beads are then plated on medium and incubated overnight.

Automated immunoassays: In one type of this assay, an aliquot of boiled enrichment medium is placed into a reagent strip, which is coated with antibodies. The strip contains all the ready-to-use reagents (wash solution, conjugate and substrate) required. The instrument performs all assay steps automatically. Finally, the fluorescence is measured by the optical scanner in the apparatus and analyzed automatically by the computer.

Biosensors: Biosensors consist of a microchip-based system for analyzing the formation of antigen–antibody complexes (Malmqvist, 1993). The sensor chip consists of a glass support, an overlaid gold film and a dextran matrix to which antibodies can be immobilized. The antigen is injected over the chip surface. The antibody–antigen complex changes the refraction index at the chip surface, which can be measured optically (Robison, 1997).

Nucleic acid based assays

Generally, DNA based methods have the advantage over phenotypic identification methods of not being influenced by the environmental conditions of the cells because the nucleotide sequence of the DNA is kept constant during growth. Genetic detection methods are based on the hybridization of target DNA with a specific DNA probe. Common targets for the identification of various pathogenic microorganisms are genes determining the production of toxins (Willshaw et al. 1985, Koch et al. 1993). Genes for specific enzymes, for example; thermonuclease (Liebl et al. 1987), b-galactosidase (Bej et al. 1990a), b-glucuronidase (Bej et al. 1991a) etc have also been described. Ribosomal RNA (rRNA) genes are also suitable as targets. These genes are ubiquitously distributed but show differences due to their phylogenetic divergence. A significant advantage of using rRNA as the target nucleic acid is the high copy number (>104/cell). Thus rRNA-based detection can be used for in situ and colony hybridization (Betzl et al. 1990, Salama et al. 1993) alternatively randomly chosen DNA can be used as a target region (Schmidhuber et al. 1988, Fitts et al. 1983, Scholl et al. 1990). Such random DNA-fragments may be a part of a gene of vital importance or a DNA sequence without any essential function.

Nucleic acid hybridization

Nucleic acid hybridization is typically between a DNA or RNA molecule present in the target organism and a probe DNA that has a sequence complementary to the target sequence. Probe DNAs usually contain 15 to 30 nucleotides. The first step in these genetic methods usually is lysis of the cells and often also purification to free
the nucleic acid, so that it can hybridize with the DNA probe. Direct hybridization uses a labeled DNA probe to hybridize to nucleic acid within the sample. Radioactive and fluorescent probes allow direct detection of hybrids. Examples of direct hybridization techniques are colony hybridization and the colorimetric DNA hybridization (cDNAH) assay. The most common solid-phase formats are the southern blot and the various dot blot formats in which the target nucleic acids are immobilized on a membrane. After hybridization, positive colonies can be identified with a labeled DNA probe. The colorimetric DNA hybridization assay is based upon a sandwich hybridization assay that employs a three-component system consisting of a solid-phase bound capture probe, the target RNA and a signal generating probe. The colorimetric DNA hybridization assay is based upon a sandwich hybridization assay that employs a three-component system consisting of a solid-phase bound capture probe, the target RNA and a signal generating probe. The colorimetric DNA hybridization assay is based upon a sandwich hybridization assay that employs a three-component system consisting of a solid-phase bound capture probe, the target RNA and a signal generating probe. The colorimetric DNA hybridization assay is based upon a sandwich hybridization assay that employs a three-component system consisting of a solid-phase bound capture probe, the target RNA and a signal generating probe.

After hybridization, positive colonies can be identified with a labeled DNA probe. The colorimetric DNA hybridization assay is based upon a sandwich hybridization assay that employs a three-component system consisting of a solid-phase bound capture probe, the target RNA and a signal generating probe. The colorimetric DNA hybridization assay is based upon a sandwich hybridization assay that employs a three-component system consisting of a solid-phase bound capture probe, the target RNA and a signal generating probe. The colorimetric DNA hybridization assay is based upon a sandwich hybridization assay that employs a three-component system consisting of a solid-phase bound capture probe, the target RNA and a signal generating probe.

Sensitivity can be greatly improved through the use of the different in vitro amplification methods. The most popular method of amplification is the polymerase chain reaction (PCR) technique. In this method, first double stranded DNA is denatured into single strands and specific short DNA fragments (primers) are annealed to these DNA strands, followed by extension of the primers complementary to the single stranded DNA with a thermostable DNA polymerase. Starting from a single target DNA or RNA sequence, more than one billion product sequences can routinely be synthesized by PCR. This quantity of DNA can be visualized as a band on an ethidium bromide-stained electrophoresis gel. To increase the sensitivity and more importantly to confirm the identity of the amplification product, Southern blotting and hybridization with a specific probe should follow. Drawbacks of PCR are the inability to distinguish between live and dead cells, the presence of polymerase inhibitors in food samples leading to false negative results, and the accessibility of the target organisms. Pre-enrichment prior to PCR analysis overcomes most of these problems. For the sensitivity of PCR, the matrix of the food sample is decisive (Way et al., 1993). PCR inhibition can be prevented by separating bacteria from the food matrix prior to DNA extraction by differential centrifugation, IMS, dilution and addition of bovine serum albumin or by immunomagnetic separation of the target organism (Grant et al., 1993). PCR detection systems based on multicyclic genes (e.g. rDNA sequences, IS elements) are generally more sensitive than those based on single copy genes. The simultaneous amplification of multiple regions of a DNA template by adding more than one primer pair to the amplification reaction mixture is a process termed multiplex PCR. Multiplex assays, however, usually do not exceed a total of six primer sets because of limitations in the ability to resolve many fragments in agarose and because of the potential for generating nonspecific products that make interpretation difficult. In the field of infectious diseases, the technique has been shown to be a valuable method for identification of viruses, bacteria, fungi, and/or parasites. But the presence of more than one primer pair in the multiplex PCR increases the chance of obtaining spurious amplification products, primarily because of the formation of primer dimers. Thus, the optimization of multiplex PCR should be done to minimize or reduce such nonspecific interactions (Elnifro etal, 2000)

Real Time PCR: While conventional PCR-based methods are highly sensitive and specific, they require post-PCR detection procedures, such as gel electrophoresis. Also the results based on size discrimination may not be very accurate. Real-time PCR eliminates the need for post-PCR processing by measuring the accumulation of PCR amplicons during each cycle of PCR in real time, thus decreasing analytical time and labor. In addition, because fluorescence increases in direct proportion to the amount specific amplicons, real-time PCR can be used for quantitation (Campbell and Anita C Wright, 2003). Since fluorogenic probes target gene-specific sequences internal to the primer sites, real time PCR imparts an added degree of specificity compared to conventional PCR-based methods. Real time PCR has shown to be useful in detection of pathogenic food borne bacteria.

Reverse transcriptase polymerase chain reaction (RT-PCR): is a laboratory technique for amplifying a defined piece of a ribonucleic acid (RNA) molecule. The RNA strand is first reverse transcribed into its DNA complement or complementary DNA, followed by amplification of the resulting DNA using polymerase chain reaction. In the first step of RT-PCR complementary DNA is made from a messenger RNA template using cDNTPs and an RNA-dependent DNA polymerase, reverse transcriptase, through the process of reverse transcription. The above components are combined with a DNA primer in a reverse transcriptase buffer for an hour at 37°C. After the reverse transcriptase reaction is complete, and complementary DNA has been generated from the original single-stranded mRNA, standard polymerase chain reaction is initiated. A thermostable DNA polymerase and the upstream and downstream DNA primers are added. The reaction is heated to temperatures above 37°C to facilitate sequence specific binding of DNA primers to the cDNA. Further heating allows the thermostable DNA polymerase (‘transcriptase’) to make double-stranded DNA from the primer bound cDNA. The reaction is heated to approximately 95°C to separate the two DNA strands. The reaction is cooled enabling the primers to bind again and the cycle repeats. After approximately 30 cycles, millions of copies of the sequence of interest are generated. The original RNA template is degraded by RNase H, leaving pure cDNA (plus spare primers).

Use of reverse transcriptase PCR (RT-PCR) in detection of viable but nonculturable bacterial cells (VBNC): The viable but nonculturable (VBNC) state is a recently described survival mechanism of bacteria facing environmental stress conditions (Oliver, 2005). The VBNC state has been described for numerous gram negative bacteria, including bacteria of medical interest such as vibrios, Shigella dysenteriae, Campylobacter jejuni, Helicobacter pylori, and Escherichia coli O157:H7. When in this state, bacteria are no longer able to grow and form colonies on conventional culture media but demonstrate metabolic activity maintain their pathogenicity and, in some cases, may return to active growth when optimal conditions are restored. In this state, genetic expression may be modified, and it has been reported that DNA, RNA, and protein synthesis, as well as the concentration of ribosomal
and nucleic acids, decreased drastically in VBNC cells. Even though DNA-based techniques can detect VBNC cells, a major disadvantage of these DNA-based detection methods is that they also may amplify DNA from dead microorganisms. mRNA is a short-lived molecule (half life of few minutes) due to the presence of nucleases that digest it very rapidly. The presence of mRNA can be regarded as a valid and convincing criterion for assessing cell viability. mRNA detection by RT-PCR is one of the most appropriate methods for evaluating presence of VBNC bacteria. This has been successfully tried in the pathogens *Legionella pneumophila*, *Vibrio cholerae*, *V. vulnificus*, *Mycobacterium leprae*, *Listeria monocytogenes* and others. Yaron and Mathews (2002) have targeted the mRNA that codes for tdh1 and tdh2 genes of *Vibrio paraheamolyticus* to not only detect VBNC cells but also to allow estimation of the potential virulence of the bacteria. The retention of virulence by VBNC cells has been demonstrated in several studies (Patel et al. 1991). However, due to the short half-life of prokaryotic mRNA, it is difficult to obtain intact RNA (Belasco and Higgins 1988), and rapid lysis of the microorganisms needed for its extraction (Patel et al. 1991). Because of the much higher stability of ribosomal RNA (rRNA), the difficulties involved with handling mRNA may be circumvented by using rRNA as a target. The supposition that rRNA can be used to determine viability is supported by the observation that cell degradation is accompanied by ribosomal disappearance (Silva et al. 1987).

**Nucleic Acid Sequence Based Amplification (NASBA): An alternative to PCR is the isothermal amplification system NASBA. NASBA is specifically designed to detect RNA and employs three enzymes: a reverse transcriptase, RNaseH and T7 RNA polymerase, which act in concert to amplify sequences from an original single-stranded RNA template. Oligonucleotide primers, complementary to sequences in the target RNA, are incorporated in the reaction. One primer also contains a recognition sequence for T7 RNA polymerase. The reaction contains both dNTPs and NTPs. The first primer binds to the RNA, allowing the reverse transcriptase to form a complementary DNA strand. Then the RNase digests away the RNA and the second primer binds to the cDNA, allowing the reverse transcriptase to form a double-stranded cDNA copy of the original sequence. This double-stranded DNA then acts as a kind of mini gene, which is transcribed by the T7 RNA polymerase to produce thousands of RNA transcripts, which then cycle through the reaction. The reaction is performed at a single temperature, normally 41°C. At this temperature, the genomic DNA from the target microorganism remains double-stranded and does not become a substrate for amplification. This eliminates the necessity for DNase treatment, which is required when using RT-PCR for RNA detection (Uyttendaele, 1997) and it also offers specific detection of viable cells. The product of NASBA reaction is mainly single-stranded RNA. This may be detected on by gel electrophoresis followed ethidium bromide staining. A confirmatory step of probe hybridization can also be involved. The detection of messenger RNA has been proposed as an indicator of cell viability (Coutard etal. 2005) as defined by capability of cell division, metabolism or gene transcription. Messenger RNA can have a short half-life within viable cells, and is rapidly degraded by specific enzymes (RNases), which are themselves very stable even in environments outside the cell itself showed that NASBA can selectively amplify mRNA sequences in a background of genomic DNA, which indicated that NASBA amplification of mRNA could be used to specifically detect viable cells.

**Molecular subtyping methods**

A limitation in the use of nucleic acid based assays is that they indicate only the genetic potential to produce toxin or to express virulence and do not give any information on toxins already present in foods or expressed virulence. They also do not lead to the isolation of the organism and so no further characterization can be done. On the other hand, molecular based techniques may be more reliable for the detection of viable but nonculturable bacterial cells. DNA fingerprinting methods like restriction fragment length polymorphism (RFLP) and randomly amplified polymorphic DNA (RAPD) analysis are suitable for grouping of isolates in transmission studies or outbreak investigations. In the RFLP technique, DNA is cleaved by restriction enzymes and the resulting fragments are separated by gel electrophoresis. Different binding patterns (polymorphism) may be observed after transferring the DNA from the gel to hybridization filters by blotting and hybridization with labeled probes. After visualization of the label, a typical banding pattern can be observed. DNA probes used in RFLP analysis are often based on highly conserved genes coding for rRNA (in ribotyping) (Rodrigues et al., 1991; Rodtong and Tannock. 1993). RAPD is a technique based on PCR. In the RAPD assay patterns are generated by the amplification of random DNA segments with single small e.g. 10-base primers of arbitrary nucleotide sequence, and a subsequent gel electrophoresis of the amplified DNA (Williams et al., 1990). Repeated cycles of heating and cooling, generally 45 cycles per RAPD assay, lead to an exponential synthesis, and thus many copies of the amplified segments. An amplification of IV-fold copies can be expected (Kocher and Wilson, 1992). A related approach is the amplification of fragments by PCR with oligonucleotides specific for simple repetitive DNA sequences (PCR fingerprinting). Repetitive DNA sequences have been described recently in eubacteria. Based on these sequences oligonucleotides have been designed, matching repetitive extragenic palindromic (REP) elements and enterobacterial repetitive intergenic consensus (ERIC) sequences, which enabled the generation of fingerprints with PCR. Since these repetitive sequences are mainly present in enteric bacteria and some related Gram-negative bacteria, as observed by hybridization experiments with REP and ERIC probes, the applicability of this fingerprinting method is restricted to these organisms (Versalovic et al., 1991). The PCR-fingerprinting method is more robust than the RAPD method since the annealing temperature is higher, 55°C instead of 37°C, which is closer to the optimal temperature (72°C) of the Taq polymerase so the primer is extended before increase in temperature may denature the primer from the template DNA. Pulsed-field gel electrophoresis (PFGE) is considered to have both good reproducibility and sufficient resolving power for the epidemiological typing of bacterial isolates. In this technique, restriction enzymes digest the complete genome and large DNA molecules are resolved by continuous reorientation of the electric field during gel electrophoresis (Tenover et al., 1995) Bascially, the continuous reorientation of the electric field causes the DNA molecules to stretch in the direction of the field and hook when the field has changed.
DNA microarrays

Microarrays are composed of many discretely located probes on a solid substrate such as glass. Each probe is composed of a sequence that is complimentary to a pathogen-specific gene sequence. Probes are typically deposited on glass surfaces using a contact printing system such as quill pins, solid pins, or ring-and-pins. Targets may be PCR products, genomic DNA, total RNA, rRNA, cDNA, plasmid DNA, or oligonucleotides. In most cases, the targets incorporate either a fluorescent label or some other moiety such as biotin that permits subsequent detection with a secondary label. Direct or chemical labeling with Cy-3 and Cy-5 fluorescent dyes is the most common means for detecting targets on microarrays. Targets are then hybridized to the array to identify species-specific polymorphism within one or more genes. Once post-hybridization steps are completed, then arrays are imaged using a high-resolution scanner. Microarrays can also be used to “fingerprint” bacterial isolates and can be used to identify diagnostic markers suitable for developing new PCR-based detection assays.

Conclusion

The progress in recombinant DNA techniques offers opportunities for their application as analytical tools in food microbiology and food control. Improvements in the field of immunology, molecular biology, automation and computer technology continue to have a positive effect on the development of faster, more sensitive and more convenient methods in food microbiology. The possibilities of combining different rapid methods, including immunological and DNA methods, should be further explored. More research is needed on techniques for separating microorganisms from the food matrix and for concentrating them before detection by immunological or nucleic acid-based assays. As positive results of PCR tests do not indicate if the virulence or toxin gene was actually expressed, future studies should focus on the development of assays that measure biological activity. Development of the DNA chip approach is continuing at a rapid pace and for the microbiologist, the DNA chip technology will be one of the major tools for the future.

References


Quantitative evaluation of APP for the standardization of stress in naturally and artificially EUS infected Channa gachua

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In many pathological states, circulating white blood cells may show alterations in their morphology, function or concentration. Although changes in the absolute count of various types of white cell are commonly found in disease conditions and are usually non-specific, they may still be helpful in indicating the cause of a particular syndromatic condition.

The hematological and biochemical parameters of normal and UDN infected salmon were examined by Mulcahy (1969), Roberts (1972) and Wilkins et al, (1970) and all agreed that UDN infected fish had significantly lowered total serum protein levels. The degree of reduction varied, but since each group used a different technique for measurement, and it is probable that the degree of lesion development in the fish they examined varied also, this does not invalidate the general finding. Mulcahy (1969) has also found a *UDN specific
protein pattern*, the marked increase of a minor fraction in polyacrylamide gel electrophoreses serum from fish with early lesions. Other workers have unfortunately not described this specific feature, which could be of great value in diagnosis. Conroy (1972) studied the hematology of a small sample of UDN infected fish and found it to be very similar to that of the normal fish entering freshwater. There was a small but statistically significant increase in cells of the granulocyte series and Conroy (1972) suggested that this might be due to requirement of enzymes for autolysis of necrotized epidermal tissue. This does not seem likely, however, in view of the complete lack of invasion of lesions by granulocytes in most cases. Fleming (1958) has reported hypoproteinaemia as a feature of other fish diseases and Carbery (1970) and Mulcahy (1971) recorded it in wild trout (Salmo trutta) affected with a condition presumptively diagnosed as UDN. The marked oedema found in adult stages is also explicable simply on the basis of a marked perinuclear palisading of haemocytes. Roberts et al. (1972a) followed the growth of the disease in culture. For the purpose of present work, hematology of a small sample of UDN infected fish was collected and serum proteins.

Material and methods
For the purpose of present work, naturally infected fish was collected from local fish ponds while for experimental infection trial, snakehead, Channa gachua, was procured from a local fish market. Experimental infection trials were conducted on a total population of 170 fish ranging between 8-10 cm in total length and 25-35 g in weight. They were acclimatized in plastic pools in the laboratory for 15 days before subjecting them to experiments. During experiments, they were kept in glass aquaria of the size of 60 x 30 x 30 cm filled with underground freshwater. They were fed on pelleted feed and minced fresh goat liver @ 2-3% of their body weight. A record of main physico-chemical parameters of water such as pH temperature and dissolved oxygen (DO) was maintained.

The experimental infection trials were designed to find out the actual pathological picture of the syndrome. For this purpose, the suspected pathogens were divided into three different groups viz viruses, bacteria and fungi and four concentrations of each pathogen are used to assess the impact of concentration on the secretion of APP (Table 1). They were injected to experimental specimens intramuscularly either singly and in various possible combinations.

For the isolation of virus (SHRV), tissue cells infected with it were harvested together with the liquid medium. Viruses with harvested cell were rapidly frozen and thawed three times and centrifuged at about 400 x g for 10 minutes to remove cellular debris. This was followed by ultra centrifugation at 40,000 x g for 1 hours at 4°C. The viruses were identified by the help of neutralization test of polyclonal antiserum and isolated viron particle as per procedure followed by the authors (Tripathi and Qureshi 2006). Bacterial suspension was prepared by culturing the isolates on TCA plates at 300°C for 24 hours and harvesting them with 500 ml of 0.85 % physiological saline solution. The colony forming unit per nil (cfu/ml) of this solution was determined by plating 10-fold dilution series. For this purpose, the solution was diluted with distilled water to give the cell number 10³/ml which was also used for inoculation to fish. The fungus, Aphanomycetes invadans was cultured up to the asexual stage and allowed to ooze out sporangia before its suspension was prepared in physiological saline. The counting and staining were done by the malachite green method.

A total of 170 fish was inoculated with pathogens in pure as well as in mixed form. The fishes were kept in aquaria with temperature ranging from 14 to 20°C. The fish were divided into 17 groups, each containing one aquaria of ten fish. Feeding was stopped one day before starting the experiment. 0.01ml of suspension was inoculated intramuscularly per gram of fish body weight. The fish were first anesthetized in solution of Tricono-methonosulpho- matic (8 ml of 0.5 % solution of TMS /500 ml sterile water). The counting and inoculation of pathogens was done following the methods given by Miller (1994), Qureshi and Mastan (1998) and Robert (1994).

The hematomal and serological studies are conducted in naturally and artificially EUS infected fishes to find out the similarities between the impacts caused by the pathogens, involved naturally as well artificially, on the blood cells count and serum proteins.

Observations
In healthy Channa gachua, the total erythrocyte count (TEC) was 2.44±0.452 x 10³ /μl and total leucocytes count (TLC) was 16.20±10³ /μl. The total hemoglobin value was 11.0 gm/100 ml blood and haematocrit value was 39.4 %. In naturally infected Channa gachua, the values of TEC, TLC, hemoglobin and haematocrit value were 1.86±0.172 x 10 x 10³ /μl, 27.20 x10³/μl, 8.0 gm/100 ml and 36.8%, respectively. Among the artificially infected fish, the minimum value of TEC in set FE (1.78±0.220 x 10³ /μl) and the maximum was in set FF (2.14±0.840 x 10⁶ /μl). The minimum value of TLC