Expert Consultation on Rapid Diagnosis of Shrimp Viral Diseases

12th – 14th June 2002, Chennai

Recommendations of the Expert Consultation

Purpose of the document

This document provides some background and the recommendations from the Expert Consultation on Rapid Diagnosis of Shrimp Viral Diseases that was held at the Central Institute of Brackishwater Aquaculture (CIBA) in Chennai, India, on 12th-14th June 2002.

The recommendations are derived from discussions at the consultation and three expert working groups and were adopted during the final plenary session of the Expert Consultation. Together they represent a powerful set of recommendations in use of rapid diagnostic tools for better health management in shrimp aquaculture. They have been put together in this summary document for rapid dissemination to potential users, researchers and other interested parties, in India and elsewhere.

A detailed report is under preparation, and will be available within 2002. For further information and comments, please contact Dr Michael Phillips, NACA (Michael.Phillips@enaca.org), Dr Peter Walker, CSIRO, Australia (Peter.Walker@csiro.au), Mr Matthew Abrahams, Director, CIBA (ciba@tn.nic.in) or Mr Vishnu Bhat, Joint Director (Aquaculture), MPEDA (vbhat@mpeda.nic.in).

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Introduction to the Expert Consultation

Background

In the past few years, use of PCR has been promoted extensively and used in India, as in other countries of Asia, to detect shrimp viruses. There is good experimental data and practical experience to indicate that PCR is a highly effective detection method. Work with white spot syndrome virus (WSSV) has shown that PCR, when properly applied for viral screening of broodstock or postlarvae and used in conjunction with good farming

practices, can significantly reduce the risk of disease and crop failure. However, PCR is a highly sensitive method requiring a high degree of technical skill for valid and reproducible implementation. Frequently, people operate PCR laboratories with minimal technical training using inadequate precaution against inadvertent contamination. The wide range of available PCR tests with varying target sites and detection sensitivities also often contributes to a lack of reliability in interpretation of test results.

Through ACIAR project FIS96/98 " Diagnostic tests and epidemiological probes for prawn viruses in Thailand and Australia", led by CSIRO, Mahidol University and the National Center for Genetic Engineering and Biotechnology (BIOTEC) in Thailand, a range of new and existing PCR methods for shrimp viruses were assessed for sensitivity and reliability and standard methods for sample preparation, storage, extraction and analysis were developed. The project also conducted training courses in PCR for a group of 24 scientists from Thailand and 6 other Asian countries. As a result of this project and follow-on activities at Mahidol University, most PCR technicians in Universities and private and government laboratories in Thailand have received uniform training and inter-calibrations of laboratory performances have been conducted. The CSIRO team has also trained PCR technicians from several Australian states in the correct use of PCR technology for detection of shrimp viruses. The final review of the ACIAR project recommended extension of the results to other Asian countries to improve their diagnostic capabilities and regional shrimp health, and the development of new research projects on the application of molecular biology to shrimp diseases in the region.

During a workshop held as part of the MPEDA/NACA technical assistance on “Shrimp Disease and Coastal Management”, experts from Mangalore University, CIBA, MPEDA and TASPARC discussed PCR and diagnostic techniques for shrimp aquaculture. The meeting expressed concern about the different PCR tests in use in India, including the use of different primers, and how to standardize the approaches being used so that some more consistent message is given to farmers.

In response, an Expert Consultation was hosted by CIBA in Chennai, on 12th-14th June 2002, to bring together some key players in India involved with PCR and other rapid diagnostic testing procedures (including some key private sector labs.) and discuss who is doing what, what the findings are and what needs to be done to move towards better standardization and offering consistent advice to farmers. Apart from the discussions on PCR and WSSV, the consultation looked into some emerging issues such as the combined infections with YHV and WSSV.

Objectives and expected outputs of the Consultation

The objectives of the consultation were:

a) To examine current PCR techniques and procedures (and other rapid diagnostic techniques) in use in shrimp culture in India.
b) To identify limitations and constraints in use of PCR and rapid diagnostic techniques as part of shrimp health management procedures in India

c) To introduce recent regional development in PCR and rapid diagnostic techniques and their application in shrimp health management elsewhere in Asia
d) To develop practical recommendations for effective use of PCR and rapid diagnostic techniques in shrimp health management procedures within India
e) To initiate a process of identifying research needs for viral disease diagnosis and shrimp health management in India

The expected outputs from the consultation were:

a) Information on current PCR methods (and rapid diagnostic techniques) in use in shrimp culture in India
b) Identified limitations and constraints in use of PCR and rapid diagnostic techniques as part of shrimp health management procedures in India.
c) Participants exposed to recent regional development in PCR and rapid diagnostic techniques and their application in shrimp health management elsewhere in Asia.
d) Consensus on a set of recommendations for effective use of PCR and rapid diagnostic techniques in shrimp health management procedures within India, such as an inter-calibration exercise, training in standard techniques etc in relation to needs.
e) Preliminary consensus on research needs for future improvement in viral disease diagnosis and shrimp health management.

Participants

The consultation brought together a unique group of 41 participants from major institutes and the private sector in India, and regional expertise from Australia and Thailand.

Expert Consultation Recommendations

Working Group 1: PCR and rapid diagnostic techniques in shrimp health management

Chair: Tim Flegel, Co-chair: M. Sudarshan Swamy. Rapporteur: CV Mohan
Members: Mr.Arun Padiyar, Dr. Michael Phillips, Konakanti Madhusudhan Reddy, Prof. R.Madhavi , Mr.Ganesh Arekere, Dr. K. Gopal Rao, Dr. T. Jawahar Abraham , Mr. Pairoj Apiruknusit, Dr Prince Jayshelan, Mr. Somanath Pai

This working group considered the issues concerning the application of PCR in shrimp health management and viral disease control.

What are the current procedures in use?
Broodstock and nauplii testing is presently followed in a small percentage of hatcheries. The constraints to testing and use of the PCR tests in health management include:

- Availability of broodstock and seasonality of broodstock supply
- Large numbers are available at any one time
- High cost of broodstock, and unwillingness to reject infected brooders
- Even if the testing is done, it is of limited use because of the mass spawning practices commonly used in hatcheries – the in built capacity of hatcheries is to handle mass spawning, therefore change may be difficult
- The maturation system has been abandoned since occurrence of white spot outbreaks (presently the industry is over dependent on gravid females)
- High (estimated around 50%) infection of broodstock common in recent years.

The working group identified the following <u>better practices</u> in broodstock and nauplii testing (emphasizing PCR for WSSV):

- Broodstock testing should follow spawning
- First step positive brooders and their progeny should be excluded
- Nested PCR positive brooders may be used but special management measures should be adopted – egg washing and nauplii testing
- Hatcheries should implement individual spawning and rearing of nauplii
- Mixing of nauplii batches should only be considered after individual spawning and PCR testing.
- MBV status should be monitored and used as a marker for hatchery practice.
- Gradual elimination of weak PLs by stressing them in PL rearing tanks.
- Better price as an incentive to produce washed, tested nauplii
- Potential carriers (crabs, clams) should not be used as feed.

<u>Recommendations</u>

- Locate wild broodstock collection areas which are less infected, and reintroduce the maturation system in hatcheries if possible – in this situation regular screening can be implemented, and infected animals can be rejected at low cost.
- Set up a "broodstock bank" programme under a society or nodal agency/statutory body
- Nauplii centers to be operated by societies.
- Long-term domestication programme should be initiated
- Certified seed should be introduced.
- Hatchery practice should be improved to increase survival of nauplii to reduce demand for wild brooders

<u>Post larvae testing</u> is presently carried out by a small percentage of PL producers (hatcheries) but a larger percentage of shrimp farmers do get the PL tested from commercial PCR labs. The testing that is done includes MBV, PCR for WSSV, vibriosis and general quality tests. The constraints include:

- PCR positive animals do not get rejected or destroyed
- Some hatchery operators influence the labs to provide incorrect results to farmers
- All PCR service providers may not achieve minimum operation standards to produce valid results

The working group identified better practice as:

- All hatcheries should have in-house testing capabilities.
- Heavily infected PL (1 step) and PL batches with high prevalence should not be used for stocking
- MBV testing should be followed as it can be an indicator of good and hygienic hatchery practice

Recommendations for implementing better practice:

- Hatcheries should have a set of minimum standards, at least covering the following:
    - Diagnosis of MBV, white spot (possible scoring system).
    - How to grade WSSV infection based on different kits used
    - How to grade moderate and severe MBV infection.
    - Methods to be used for sampling and sample size
    - Report forms from PCR labs should include details of methods used, and indicate whether 1-PCR and n-PCR used
    - 1-PCR should be rejected.
- Hatcheries receiving MPEDA subsidies for purchase of PCR should be provided with training and follow PCR testing standards.
- A standard method of assessing quality should be prepared.
- Promote farmer associations to have their own lab/diagnostic capacity.

Testing during crop: Presently few shrimp farms test shrimp during the crop. The following constraints exist:

- lack of awareness, access to facilities and labs and budget.

The working group identified better practice as:

- Regularly monitor by PCR or other farmer friendly, low cost rapid diagnostic test such as immunodot.
- Use 1-PCR or technique of similar sensitivity as an indicator of impending crash (10 numbers – to be verified) and emergency harvest
- n-PCR may be used as "red alert" but crops can continue under better management practice
- Lo (Taipei) recommends that farmers should take 10 shrimp and then check for PCR – if the result is positive, then prevalence of 20% of more. Nested PCR positive – crop may survive with good management; 1 step PCR positive – harvest immediately, otherwise it will crash.
- Monitoring of moribund shrimp should be tried as it has prognostic value.

What are the implications of PCR results?

A high level of infection and high prevalence for WSSV are high risk factors. From available evidence, low levels of infection and low prevalence are not significant risk factors.

- Positive broodstock (one step or nested PCR) prior to spawning represents a risk factor for producing infected PL.

- Reporting of PCR results usually does not allow farmers to make any distinction between levels of risk.
- PCR laboratories should have a minimum agreed set of standards with respect to sample size and reporting of test results.

There is a need for hatcheries and farmers to meet and agree on a minimum set of standards for PCR and to have a common reference for understanding the implications of PCR test results, and risks of disease occurrence from PCR results.

What are the prevalence and sampling issues that need to be considered?
The working group discussed sampling protocols and sample size. There is a range of different sampling methods being used. Examples of current practice involves:

- Varied numbers of PLs are taken. CP recommends pooled subsamples of 30-60 PL with samples collected from weaker PLs; MPEDA recommend 60-100.
- In Thailand, samples are taken from the nursery – 1000 from 5 places – and sampling of weak ones
- False negatives may be common
- In nursery samples, tissues like gills (some labs), abdomen (CP), whole samples without eyes, some whole samples are taken.
- Some laboratories recommend using the same amount of tissue weight in each test (saying that sample quantity in terms of numbers doesn't matter).

The working group identified <u>better practice</u> as:

- To detect a prevalence of 5% or above in the population with 95% confidence, the sample size should be 60.
- However, there is need for developing some minimum standard.

The working group recommended that a standard sampling procedure should be developed among key stakeholders that includes the hatchery association, farmers, labs, and technical people. The working group suggested to work within one state where there is some existing infrastructure to start with.

Regarding tests used, several indigenous and imported test kits are used. These include IQ 2000 Taiwan kit, Mangalore Biotec, Bangalore Genie. The costs: IG 2000 (60,000 for 200 reactions- Rs300/reaction); Bangalore Genie (24,000 for 100 reactions - Rs240/reaction).

The working group made the following <u>recommendations</u>:

- Many kits do not have internal controls – these should be used in PCR test kits.
- Operators should have a model or reference so that the results could be compared with that.
- In Thailand, DNA preparations were sent out to different PCR service labs to check operation efficiency in getting the result. An independent agency established by the concerned stakeholders would be useful for checking.

Who should do the testing?
There should be more emphasis on farmers organizations and local groups. Farmers should be encouraged to organize themselves into groups to do PCR testing. MPEDA should extend the same facilities and support to farmers as done in hatcheries (that were provided with PCR subsidies).

What is practically known about the relationship between PCR status of seed, brood and relation to crop outcome?
There is a lot of anecdotal information available, but it is difficult to use such information to predict relations between the results of PCR tests results and crop outcome. Some information is also available from recently conducted epidemiological studies (DFID, MPEDA/NACA). The meeting emphasized the need for further epidemiological work to build understanding. In addition, the following topics need to be addressed:

- Research on potential of spermataphores for WSSV contamination during spawning
- Use of Immunodot tests for validation in the field.

What are the practical problems faced by farmers?
The group, that included private sector participants, identified the following practical problems faced by farms:

- Lack of confidence of farmers in PCR test.
- The need for farmers to have access to a reliable sample from the hatchery.

The group noted that access to samples can be better negotiated by farmers if they group together and emphasized that farmers be encouraged to form local groups for disease control. Examples of successful farmer groups at Pattukkottai, Tami Nadu, and Nagalapatnum, Tami Nadu were noted.

Additional recommendations
The working group made the following additional recommendations:

- PCR laboratories:
    - There should be a system of accreditation of laboratories, that would be useful to the hatcheries and farmers.
    - Laboratories should provide the methods used for PCR diagnosis.
    - The reports should indicate whether results are positive by first step or second step PCR.
- Crop insurance might be arranged around adoption of a Code of Practice in the farm.

For implementing the recommendations from the consultation, it was recommended that a meeting among hatchery operators and farmers be held to move towards minimum quality standards for PL quality. For PCR, there should be an agreement on a reference standard and harmonization.

Working Group 2: PCR and rapid diagnostics technologies for detection of WSSV

Chair: Dr. Richard Hodgson, Co-chair: Mr. D. Ramraj. Rap: Shri. S.V. Alavandi
Members: Miss. Allu Venkata Madhuri, Ms.Janaki, Mr. S. Pandiarajan, Mr. Phani Prakash, Mr. D. Ramraj, Dr.K.V.Rajendran , Mr.Raj kumar Singh, Dr. A. S. Sahul Hameed, Dr.K.M.Shankar, Dr T. Santiago, Ms. Subhashni

This group considered the specific issues identified as limiting the ability to provide rapid, high quality testing for WSSV and acceptance of PCR testing results.

A need for registration of PCR laboratories with MPEDA

- Registration should be mandatory for PCR laboratories. Initially would only be to provide list of laboratories available to do PCR testing
- Registration would aim to establish the laboratories that follow a code of minimum best practice standards
- Registration would require a progressive improvement in the quality standards applied to PCR testing
- Registration would require providing results from testing PCR Reference Standards

A need for inter-laboratory validation

- Identify an un-biased laboratory to hold and provide Reference Standards for PCR testing
- Positive control standards should be DNA from purified WSSV diluted into DNA from healthy (WSSV negative) shrimp.
- The positive standard set should containing a quantified range of WSSV targets from high to very low concentration WSSV
- Negative control standard should be quantified DNA from healthy shrimp

A need to simplify the reporting of PCR test results

- Unify the presentation of essential information – origin of samples tested, type of test performed, laboratory standards followed, PCR test results simplified.
- Different tests present results in different ways. Some test kit results provide +/-, others provide +/- as well as graded scale of strong, medium, mild and low infection

A need for a review of laboratory practices relating to control of contamination in PCR testing

- Laboratories should outline their separation of pre- and post-PCR processes

Better acceptance of PCR test results by producers and implementation of recommendations from PCR test results.

- Farmers have low confidence in PCR results (see working group 1 also).

Need for field level test for use by farmers (non-PCR based testing)

- Immuno-based diagnostics have potential and should be explored.

- DNA-hybridization based diagnostics – dot blot assays
- Tests are currently being developed

The working group made the following recommendations:

- Establish a working group to develop the code of minimum best practice standards and to promote PCR laboratory registration
    - The working group should involve members representing private testing laboratories, hatchery laboratories, research Institution laboratories, Government laboratories and producers.
- Create a set of Reference Standards for PCR testing of WSSV
    - Establish a Government reference laboratory
- Improved quality assurance standards in PCR testing
    - Continued validation of PCR testing and adoption of new technologies in Indian PCR laboratories
- National registry of PCR testing results
    - Results should be readily available to all laboratories, institutions, Government agencies and producers

Working Group 3: Researchable issues

Chair: Dr. Mathew Abraham, Co-chair: Dr. Peter Walker. Rapporteur: Dr. K. C. George.
Members: Mr.B.Vishnu Bhat, Dr.(Mrs.) Indrani Karunasagar, Mrs.A.Uma, Mr.P.C.Thakur, Dr.Toms C. Joseph, Dr.K.K.Vijayan, Mr. Mr.Viju.J.Jacob, Dr. Boonsrim Withychunnarnkul

This group identified specific research issues and identified research needs concerning PCR and rapid diagnostic tests.

PCR screening for WSSV will significantly reduce risk of disease outbreaks on farms

- Epidemiological data indicates that, even if nested-PCR is adopted, a risk of disease will remain
- The major on-farm factors contributing to the risk of disease remain unclear but may well vary in different locations and farming systems
- Events in ponds that lead to disease outbreaks must be better understood if integrated health management practices are to be applied effectively.

The working group recommended that

- Carefully designed longitudinal studies should be conducted in several locations (eg. east coast India, west coast India, Sri Lanka and Thailand) to determine the source of WSSV that causes disease in ponds.
- Recently identified genetic markers for WSSV strain variations provide powerful tools to trace the source of disease outbreaks and should be applied in the study.
- The molecular epidemiology should complement other data obtained through population-based studies
- The epidemiological study should use genotype analysis of strains in the pond environment to determine if WSSV causing individual disease outbreaks:

- Enters the pond as a low level infection in seed
- Enters the pond after seeding from an external source eg. plankton, crabs etc
- Emerges as a single dominant type from a complex mixture of genotypes in the pond
- Emerges randomly from infecting genotypes
- Emerges from the most prevalent genotype
- Emerges from the genotype with the highest viral load
- Is a common genotype or genotypes with increased tendency for disease [ie. more virulent strain(s)]
- Emerges in response to any identifiable stimulus or stress

The working group noted that pathogens other than WSSV also need to be better managed:

- In particular, the new YHV-related virus from India (+ Thailand?) should be characterized:
  - Relationship to other YH-complex viruses determined
  - PCR screening test and *in situ* hybridization tests developed
  - Prevalence of infection and host range determined
  - Source of infection and disease in ponds identified
  - Disease management strategy developed
- Screening tests for Mourilyan virus should be applied in India:
  - Determine if the virus is present and associated with disease
  - Determine relationship of Indian strain to strains detected in Australia and Malaysia
  - Develop PCR screening kit that will detect and discriminate all strains
- The effect of multiple viral infections on susceptibility to disease in ponds should be investigated

Multiple infections with WSSV, MBV, IHHNV, HPV and possibly other viruses (YH-complex, MoV) appear to occur commonly. Therefore the working group recommended that:

- The prevalence and distribution of multiple infections should be documented
- The effect of multiple viral infections on susceptibility to disease in ponds should be investigated

The other priority research issues identified by the group include:

- Application of WSSV MAbs to the development of rapid, inexpensive, pond-side tests for use by farmers.
- Initiation of work towards the development of WSSV-resistant *P. monodon*, particularly in conjunction with international efforts towards domestication and closed-cycle breeding of SPF stock.
- Understanding the basis of WSSV virulence and pathogenesis.
  - Viral virulence determinants.
  - Transition from chronic infection to acute infection and disease.
  - Identification of disease triggers

In extension and training, there is also a need for extension of currently available rapid, Level I diagnostic methods to farmers to promote improved health management practices. The working group recommended delivery of a more complete health management package

- Reliable PCR screening of broodstock and/or seed will significantly impact on shrimp health in India
- However, the risk of crop failure, particularly in small, poorly resourced farms, will remain relatively high until a more complete package of health management practices is applied.
- Development of a more complete package will be possible with a better understanding of disease caused by WSSV and other viruses that impact on production.