Application of PCR for improved shrimp health management in Asia

Report of PCR Training and PCR Calibration in India

Background: PCR is now widely used to screen shrimp seed prior to stocking in many countries of the region. However, disease continues to impact seriously on production due to variations in the reliability of screening, compounded by on-farm factors that may result in disease even when seed has been properly screened. Of the several possible reasons, some of the important ones include (a) low-level or low-prevalence WSSV infections that escape detection during PCR screening (b) other potential sources of WSSV infection such as naturally recruited shrimp, crustacean carriers and zooplankton, (c) other pathogenic viruses that occur commonly in shrimp and may contribute to disease, (d) lack of harmonization and inter-calibration of PCR testing capabilities of different laboratories and (e) inadequate communication to farmers of effective health management practices.

In response to such problems, the Australian Centre for International Agricultural Research (ACIAR) has developed a regional project - ***Application of PCR for improved shrimp health management in the Asian region***. The three year project was implemented in January 2005. Key partners include CSIRO in Australia; MPEDA, CIBA and College of Fisheries, Mangalore in India; Mahidol University, BIOTEC and NACA in Thailand; Ministry of Marine Affairs and Fisheries in Indonesia.

I PCR training workshop (17-21 Oct 2005, CIBA, Chennai)

The International PCR Training Workshop on **"Application of PCR for improved shrimp health management in the Asian region"** was jointly organized under the umbrella of the ACIAR project by MPEDA, CIBA-ICAR, CSIRO and NACA. Dr. P. Ravichandran, Director, CIBA-ICAR welcomed the workshop participants. The workshop was formally inaugurated by Dr. Yugraj Yadva, Member Secretary, Aquaculture Authority of India on 17th October 2005. Dr Yadava stressed the importance of such workshops in capacity building and tackling shrimp disease issues. Dr. C.V. Mohan, Aquatic Animal Health Specialist, NACA introduced the workshop concept. Dr Peter Walker, from CSIRO Australia spoke about regional cooperation in addressing shrimp health issues. Dr. J. Bojan, Director, MPEDA presided over the function and spoke about the need for promoting adoption of better management practices to reduce risks of shrimp diseases. Dr. K.K. Vijayan, CIBA, proposed the vote of thanks. The function was attended by over 120 delegates representing workshop participants, including 3 participants from Sri Lanka, Bangladesh and Myanmar,

national and international resource experts, shrimp farmers, hatchery operators, sea food processors, staff of CIBA and members from the press.

The objective of the first PCR training workshop was to provide basic training on PCR technology in order to improve the participant's understanding on the subject and to ensure quality and reliability in their results. The training workshop had lecture and practical components. The course curriculum followed a format successfully implemented by ACIAR and CSIRO in several Asian countries and further developed at Mahidol University, Thailand. The course was presented by an expert panel of instructors/demonstrators from Australia (Prof Peter Walker, Mr Nick Gudkovs, Dr Rajendran), Thailand (Prof Timothy Flegel, Dr Nusra Sittidilokratna, Dr CV Mohan) and India (Dr Vijayan, Dr Indrani Karunasagar, Dr Santiago, Dr Satlin, Ms Sanjuktha)

The Lectures covered several topics with emphasis on basic virology, molecular biology, nucleic acids, principles of PCR, PCR detection of DNA and RNA viruses, shrimp viral diseases, their diagnosis and management. The practical contents were designed to provide good understanding of PCR methodology, laboratory practices and trouble shooting in detection of both DNA and RNA viruses. To ensure effective learning and uptake, practical were conducted in 5 small batches of 6 participants and each batch was provided with one expert demonstrator. In addition, all the participants were provided with a practical course manual, detailing step-wise procedures for all the practical exercises.

Participants were selected following evaluation of their applications received in response to the advertisement issued in early August 2005. The selections were made jointly by MPEDA/CIBA/NACA based on a previously agreed criteria. From out of the 83 applications received, 25 were selected to attend the training workshop. Representation was given to all sections of the industry that are involved in PCR. The breakdown of 25 participants include; private PCR service providing laboratories (12), PCR laboratories in hatcheries (5), Government PCR service providing laboratories (6) and Research institutions (2). In addition, 3 participants, one each from Bangladesh, Sri Lanka and Myanmar attended the workshop fully supported by ACIAR. The full list of participants can be found in Annex-3. The applicants who could not be selected for the training workshop were however invited to attend the theory classes. A large number of them attended the theory classes and participated in the discussions.

The formal valedictory function was held at CIBA on 21st October 2005. Dr RaviChandran, Director of CIBA, welcomed the guests and participants. Dr Peter Walker spoke briefly about the broader aspects of the regional ACIAR project, the training workshop and the proposed PCR calibration progarmme. Mr Pedro Bueno, DG of NACA emphasized the importance of sharing regional resources and expertise to address common problems like shrimp health in the region. He also distributed the certificate of completion to all the participants. Dr Mohan Kumar, Chairman of MPEDA delivered the valedictory address and stressed the importance of providing the farmers with reliable and accurate PCR results and supported the need to organize PCR calibration programmes which could lead to some form of future accreditation schemes in the country. Representatives from amongst the participants shared their views about

the training programme and thanked the organizers and resource experts for their excellent contributions. It was widely acknowledged by the participants that the hands on training workshop was very useful for them and would help them to improve performance in their laboratories. Dr Santiago proposed the vote of thanks.

Key Recommendations: The elimination of infected seed prior to stocking is arguably the most important single factor in reducing the risks of diseases in shrimp farming. This can be achieved by proficient PCR testing of broodstock and/or seed. Considering the importance of reliable PCR screening to the profitability of the industry, the workshop made the following key recommendations:

- PCR service providers (government, private independent and hatchery-based) should consider establishing a **Professional Association of PCR Service Laboratories** and develop codes of practice for members, minimum standards of training and an email communication network;
- Resource experts involved in the project should consider developing a **better practice (BP) manual** for PCR testing in government and private laboratories and hatcheries. It should include all aspects of sampling, transport and storage, extraction, testing and analysis and reporting of data;
- Concerned organizations (MPEDA/CIBA/NACA/ACIAR) should develop and implement a transparent **PCR calibration programme** amongst interested PCR service providing laboratories and subsequently offer assistance to laboratories that need improvement;
- The project should consider developing a **way forward document - ensuring reliability of PCR testing-** for implementation by MPEDA. The document should outline implementation strategies that MPEDA/or other agencies in India could use as the basis to implement some form of quality assurance programme and possibly a mechanism of accreditation of PCR labs and a mechanism for monitoring their performance

II PCR Training Workshop (23-26 October 2006, CIBA, Chennai)

Second PCR training workshop was attended by 26 participants (23 from India and 1 each from Bangladesh, Myanmar and Sri Lanka). The same set of participants (with 2 exceptions) had attended the first PCR training workshop completed in 17-21 October 2005 at CIBA, Chennai.

The II PCR Training Workshop on was jointly organized under the umbrella of the ACIAR project by MPEDA, CIBA-ICAR, CSIRO and NACA. Dr. AG Ponniah Director, CIBA-ICAR welcomed the dignitaries, resource experts, project partners and workshop participants. The workshop was formally inaugurated by Dr. S Kannaiyan, Chairman, National Biodiversity Authority, Govt of India. Dr. C.V. Mohan, Aquatic Animal Health Specialist, NACA introduced the workshop concept. Dr Peter Walker, from CSIRO Australia spoke about regional cooperation in addressing shrimp health issues. Mr Vishnu Bhat, Director, MPEDA presided over the function and spoke about the need for promoting adoption of better management practices to reduce risks of shrimp diseases. Dr. TC Santiago, proposed the vote of thanks. The function was attended by over 60

delegates representing workshop participants, national and international resource experts, shrimp farmers, hatchery operators, sea food processors, staff of CIBA and members from the press.

The training followed a format successfully implemented by ACIAR and CSIRO in several Asian countries and further developed at Mahidol University, Thailand. The training course was presented by an expert panel of instructors/demonstrators from Australia (Prof Peter Walker and Mr Nick Gudkovs) India (Dr Rajendran, Dr Indrani Karunasagar, Dr Santiago, Mr Satlin Raj, Mr Pradeep Nair, Ms Sanjuktha) Thailand (Dr Nusra Sittidilokratna, Dr CV Mohan)

The practical contents were designed to provide good understanding of PCR methodology, laboratory practices and trouble shooting in detection of both DNA and RNA viruses. To ensure effective learning and uptake, practical were conducted in 5 small batches of 5 participants and each batch was provided with one expert demonstrator. All the participants were provided with individual operator IDs. A total of 1056 PCR reactions were run by the participants over a period of 4 days. Each participant had an opportunity to run over 40 PCR reactions with different types of samples and primers. All the participants had to enter their results against the samples provided in an excel sheet. During the daily review, this allowed everyone to check their results against the expected result. The demonstrators provided feed back to the groups and individuals in the groups. All the participants felt that the training exercise was well coordinated and completely different to running PCR tests using commercial kits. The training was rated as highly successful.

The formal valedictory function was held at CIBA on 26th October 2006. Dr CV Mohan, NACA, welcomed the guests and participants. Mr Nicholas Gudkovs provided the summary of the workshop. Two training participants gave impressions about the training programme on behalf of all the participants and thanked the organizers and resource experts for their excellent contributions. It was widely acknowledged by the participants that the hands on training workshop was very useful for them and would help them to improve performance in their laboratories. Dr Peter Walker spoke briefly about the broader aspects of the regional ACIAR project, the training workshop and the proposed PCR accreditation progarmme. Prof Sena De Silva, DG of NACA gave the valedictory address and emphasized the importance of sharing regional resources and expertise to address common problems like shrimp health in the region. He also distributed the certificate of completion. Dr C Damodaran, Former Director, Forensic Science Department, Government of Tamil Nadu gave the presidential remarks. In a lively and entertaining speech, he highlighted the value of modern molecular tools like PCR, DNA fingerprinting, etc to mankind, be it in solving crimes or screening shrimp seed for viral pathogens. Dr Santiago proposed the vote of thanks.

PCR Laboratory accreditation workshop (27th October 2006, CIBA, Chennai)

The PCR laboratory accreditation workshop was held at CIBA, Chennai on 27th morning. It was attended by all the 26 training participants, representatives from other PCR

laboratories, hatcheries, shrimp industry and all the resource experts. The workshop was facilitated by Dr Peter Walker from CSIRO and Dr CV Mohan from NACA.

PCR Laboratory accreditation: The accreditation workshop was a landmark event with general agreement from all participants to each of the proposed elements of the framework for a national accreditation program for PCR laboratories with support from MPEDA. The regional project partners will continue to develop this as a paper to MPEDA with a target date to be fully operational in late 2007. The inter-laboratory calibrations and subsequent PCR laboratory accreditation program will be a first for aquaculture globally and will surely be a model for others to follow. NACA will pursue this vigorously.

Framework for PCR Laboratory accreditation program as presented to stakeholder meeting agreed by project team

- Agreed OBJECTIVES
    - High standard of technical performance and reproducibility of PCR testing
    - High ethical standards of test reporting
    - Standardization of PCR reporting format and operating procedures
- Agreed OUTCOMES
    - Increased confidence of farmers in PCR testing
    - Reduced risk of crop failure and improved productivity of shrimp farming
- Agreed FRAMEWORK
    - MPEDA to implement with certificates issued by NABL
    - 2 full-time technical staff:
        - conduct inter-calibration, including preparation, testing, distribution of reagents
        - conduct training workshops
        - technical assistance and advice
    - 3-member technical advisory panel to oversee operations
        - selection and training f technical staff
        - implementation of process
        - review inter-calibration and other documentation
        - regular laboratory inspections
        - issue ratings and withdrawal of accreditation
    - Minimum accreditation requirements:
        - 100% score in inter-calibration
        - approved laboratory setup
        - minimum training standard for staff
        - approval of and adherence to SOPs (sample handling, sampling procedures, reporting format, etc.)
    - Star ratings published by MPEDA (*, **, ***)
        - Entry point a 1-star rating upon successful completion of all minimum requirements (including 100% successful inter-calibration result)

- Successive successful inter-calibration results to improve ratings to a maximum of 3-star
- step loss of rating until next inter-calibration if testing error is returned but accreditation would be maintained if other minimum requirements continue to be met
- laboratories loosing star ranking completely due to inter-calibration errors may remain listed as participating (non-accredited) laboratories if other minimum requirements continue to be met
- Currently accredited laboratories and star rankings to be published on MPEDA website and in local newspapers

I PCR Calibration Programme (June 2006)

Purpose: The WSSV inter-calibration exercise (ring testing) will provide an overview of the current level of performance of WSSV PCR testing within participating laboratories and assist with the identification of laboratories that may require assistance to improve their testing procedures. At the same time, individual laboratories will have an opportunity to assess their own performance compared with other laboratories undertaking PCR testing.

Inter-calibration not only provides a step towards accreditation but also "gives participants an opportunity to assess their own performance". Positive reactions in negative samples would suggest contamination, and failure to detect positives suggests problems with assays – both of which can be recognised and addressed as a result of the Inter-calibration.

Standard Operating Procedures: A detailed SOPs for setting up and running a PCR inter-calibration exercise was developed by CSIRO and circulated to all project partners for comments and agreement. The SOPs were finalized in December 2005 (Annex 1).

Participation: Over 70 PCR service providing labs in the country coming under the private, government, hatchery and research sectors were informed of the intended PCR inter-calibration exercise under the regional project. About 49 laboratories which applied for the first PCR training workshop in October 2005, had expressed their willingness to participate in the exercise.

At the ACIAR second project coordination meeting held on 24-25 April 2006 at Bangkok, it was decided to run the inter-calibration exercise in India in the month of June 2006. Accordingly, all the 49 laboratories (Annex 2) were informed of the process and asked to indicate their options for receiving the 10 samples from CIBA Chennai.

On 29th May 2006, the labs were provided with a three letter random identification code by NACA and were informed that the inter-calibration would begin on 5th June 2006, when the samples will be distributed. They were also provided with detailed instructions (Annex 4) to handle the 10 samples and report the results back to NACA.

Confidentiality: Participation in the inter-calibration exercise and the results of all testing is strictly confidential. Participating laboratories are identified by a code number. The laboratory identification code numbers are private and confidential. Testing laboratories will only be informed of their own identification number. At the completion of testing a summary of test results using the code numbers, will be distributed to all participants.

Inter-calibration Samples: In brief, 100 sets of 10 samples (1000 samples) were prepared and coded by scientists of CSIRO and CIBA. These included 100 sets of 5 DNA samples and 100 sets of 5 tissue samples. The preparation and coding of samples took considerable planning and very meticulous work for 2 weeks by scientists from CSIRO, Australia and CIBA, Chennai

The DNA samples were derived from WSSV experimental infection in adult *Fenneropenaeus indicus*, using infected material from a hatchery near Chennai. All DNA samples were diluted in a 1 mM Tris EDTA, pH 7.6 containing 1 ng/µl of normal shrimp DNA (estimated using Nanodrop™), to accommodate those diagnostic systems using a decapod control. Sample 1 (DNA-1) consisted of sterile DNA-free water alone. DNA-2 consisted of normal uninfected shrimp DNA at 1 ng/µl. DNA samples 3 to 5 were made by dilution of a single DNA stock (as described above). DNA-3 routinely gave a strong positive band after 1 step PCR. DNA-4 was a 1:10,000 dilution of DNA-3 and was designed to yield a moderate to light signal (faintly positive after 1 step PCR, strongly positive after 2 step). DNA-5 was a 1:4,000,000 dilution of DNA-3 and was consistently positive after 2nd step PCR. All testing was based on using a 2 µl volume of DNA template in a 25 µl reaction volume.

The tissue samples were prepared by "seeding" batches of normal tissues from uninfected PLs. Tissue-1 and Tissue-2 were duplicate samples of the uninfected tissue used to prepare the "positive" samples. A single batch of heavily infected, WSSV positive PL from the Chennai area was emulsified in 95% ethanol (sample Tissue-3), this sample was used to seed batches of emulsified uninfected normal PLs (Tissue-4 and Tissue-5). The seeding rates were determined empirically by nested (2 step) PCR testing of samples seeded at different rates (calculated as wet weight). Tissue-4 was positive after 1 step PCR and strongly positive after 2 step. Tissue-5 was consistently positive after 2nd step PCR.

Distribution of Samples: Packing of samples for distribution took lot of planning and hard work. Excellent support was provided by the CIBA Director and a team of young hard working Research Associates of CIBA under the able direction of Dr Santiago. Dr Mohan of NACA traveled to Chennai to oversee the distribution of samples on Monday the 5th June 2006. Eight laboratories collected the samples from CIBA, while for 41 laboratories, the samples packed in polystyrene insulated boxes with cool brick and dry ice were sent through couriers.

Validation and Quality Control: New packets of certified RNase/DNase free tubes were closed and labeled. All WSSV *negative* samples were aliquoted into the tubes and

stored frozen at -80C. WSSV positive samples were then prepared and frozen, using a single batch of stock for each sample.

After all the samples were dispensed and stored, 5 batches of each sample were examined by CSIRO, Australia using a TaqMan Real-Time assay and 3 different nested (2 step) PCR systems (including the OIE method) and 1 commercial 2-step WSSV PCR kit. On the day that samples were dispatched from CIBA, 3 sets of samples were removed from the freezer and maintained at 4 ^{o}C, another 3 sets of samples were stored at room temperature. These 6 sets of samples were extracted and tested over a time course of 24, 48 and 72 hours to verify the stability of samples over time. ALL samples tested consistently throughout.

Summary of Results and Feedback to Participating Labs: In the instructions issued, the labs were asked to report the results to NACA on or before 14th June 2006. Of the 49 labs which received the samples, 37 laboratories returned the results. The results were collated and a summary table of results was prepared. The summary results along with a covering letter were sent to all the participating laboratories on 25th June 2006 individually, to ensure confidentiality of the identity of the participating labs. The summary table allows the participating labs to review their individual results and at the same time compare the performance with other labs.

Summary of Performance: Of 37 participating laboratories in private, hatchery, government and research categories, 17 correctly identified all positive and negative samples. A further 3 laboratories failed to detect only one low positive sample. Of the remaining 17 laboratories:

- 9 laboratories reported positive results for negative samples. This is indicative of problems with test contamination.
- 6 laboratories reported negative results for positive samples. This is indicative of a problem with test sensitivity.
- 2 laboratories reported incorrect results for both positive and negative samples. This is indicative of a general failure of PCR testing capability or errors in sample handling.

Second PCR calibration programme (February 2007)

Participation: At the second PCR training workshop held in CIBA in October 2006, it was decided to run the second PCR calibration in Jan-Feb 2007. Over 80 PCR service providing labs in the country coming under the private, government, hatchery and research sectors were informed of the intended second PCR inter-calibration exercise under the regional project. About 51 laboratories expressed their willingness to participate in the exercise. These labs were provided with a two letter random identification code by NACA and were informed that the inter-calibration would begin on 2nd February 2007, when the samples will be distributed. They were also provided with detailed instructions to handle the 10 samples and report the results back to NACA.

Summary of Results and Feedback to Participating Labs: In the instructions issued, the labs were asked to report the results to NACA on or before 28th February 2007. Of the 51 labs which received the samples, 33 laboratories returned the results. The results were collated and a summary table of results was prepared. The summary results along with a covering letter were sent to all the participating laboratories on 3rd April 2007 individually, to ensure confidentiality of the identity of the participating labs. The summary table allows the participating labs to review their individual results and at the same time compare the performance with other labs.

Summary of Performance: Of 33 participating laboratories in private, hatchery, government and research categories, 6 correctly identified all positive and negative samples. A further 13 laboratories failed to detect only one low positive sample. Of the remaining laboratories:

- 2 laboratories reported positive results for negative samples. This is indicative of problems with test contamination.
- 6 laboratories reported negative results for positive samples. This is indicative of a problem with test sensitivity.
- 6 laboratories reported incorrect results for both positive and negative samples. This is indicative of a general failure of PCR testing capability or errors in sample handling.

Financial and Technical Support for the programme:

Financial support for the activities in India was provided from MPEDA. This covered all expenses connected with the conduct of 2 PCR training workshops and 2 PCR calibration programmes. Technical support for the progarmme was provided from other ACIAR project partners, namely CSIRO, CIBA, NACA, Mahidol Biotech and College of Fisheries, Mangalore. The cost of travel of project partners and DSA for the time spent in Chennai was covered by the ACIAR project funds, as was the salary of the principal trainer Mr Nick Gudkovs. CSIRO contributed the salary of ACIAR project leader Dr Peter Walker. In addition, CIBA, CSIRO and NACA contributed significantly in kind for the preparation and implementation of the training workshop and the proposed PCR calibration programme.

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