Development of Rapid Diagnostic Technologies for Screening and Control of Commercially Important Shrimp Pathogens

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Introduction

Viral diseases are the major constraint to the rapidly developing shrimp industry in Malaysia. Among the important viruses are the white spot syndrome virus (WSSV), yellow head virus (YHV) and *monodon baculovirus* (MBV). White spot syndrome virus is particularly pathogenic, and has been causing massive losses to the shrimp culture since 1994. The disease causes typical white spots measuring 1 to 3 mm in diameter in the carapace. On appearance of the white spots, 100% mortality occurs within a week (Wang, 1999). Transmission of WSSV is either through water or carrier animals. In the hatcheries, postlarvae can be infected through the use of broodstocks that carry WSSV but do not show the typical signs of infection. Whereas in the growout farms, the virus can infect shrimps though water supply from the sea or crustacean such as crabs and zooplanktons that are introduced along with the water. There is no treatment for WSSV. To prevent losses to the shrimp industry, it was imperative that a sensitive method be developed to screen the postlarvae, broodstocks and the carrier animals in the water supply. In the farms, regular monitoring can also prevent or reduce losses by early detection of infection. Polymerase chain reaction (PCR) has been proven to be a powerful molecular biotechnology for early detection of WSSV infection due to its ultimate sensitivity (Wongteerasupaya *et al.* 1996).

Materials and Methods

Growout black tiger shrimp *Penaeus monodon* from a 1997 field outbreak of white spot syndrome in Johor were used for virus isolation and ultrapurification. Genomic DNA was extracted from the purified virions using DNAzol® Genomic DNA Isolation Reagent (Molecular Research Center, USA). Specific oligonucleotide primers were designed from a published conserved region of WSSV using Primer Premier version 4 (Biosoft International, USA) for the development of nested PCR assay. Primers were also designed from the conserved region of decapod as the internal control for sample DNA quality. The assay was designed to run the first and second step amplifications sequentially in the same tube after a single loading of DNA and was optimised using GeneAmp 2400 thermal cycler (Applied Biosystems, USA). The assay was tested for its capability in detecting early WSSV infection in an experimental infection (via ingestion) using growout shrimp, and was tested with other geographical WSSV isolates (Thailand, Ecuador, Indonesia, China and Sri Lanka). Autographa californica nuclear polyhedrosis virus and MBV were subjected to the assay to test the assay specificity. The assay cycling profile was tested in the thermal cyclers of other brands or model, including the DNA Thermal Cycler 480 (Applied Biosystems, USA), PTC 100 and 200 Peltier Thermal Cycler (MJ Research, USA), and Rapid Cycler (Idaho Technology, USA) for profile transferability. Different stages of shrimp (broodstocks, nauplii, postlarvae, juveniles and growouts) as well as carrier animals (crabs and wild shrimp) were tested to evaluate the detection capability in different types of samples. Gene probe was developed from the same conserved region of WSSV as an alternative detection technique of moderate sensitivity. The probe was synthesised and digoxigenin-labeled by PCR. Genomic DNA for detection of WSSV was extracted as above.

Reverse transcription PCR (RT-PCR) assay was established for detection of YHV based on the published primer sequences from Thailand. The assay was tested in experimental infection of *P. monodon* with YHV obtained from Thailand via intramuscular injection. Viral RNA for the assay was extracted from the infected shrimp using Tri Reagent® (Molecular Research Center, USA).

Results and Discussion

The thermokinetics of the primers was controllable by the manipulation of assay annealing temperature, primers concentration, magnesium concentration and number of cycle. The assay was able to run the first and second step amplifications sequentially within the same tube after a single loading of sample DNA. This minimised the risk of carry-over contamination commonly encountered in the conventional two-tube nested PCR assay that involves the transfer of first step PCR products to the second step amplification. In the perspective of diagnostic laboratories, low risk of contamination means more reliable test results, and single loading means time-efficient. The assay is able to consistently detect as low as 10 copies of viral targets, thus suitable for detecting early infection in postlarvae and broodstocks. The assay was able to show semi-quantitative results indicative of heavy, moderate and light infections in shrimp. This will be helpful to the growout farm management in decision-making during outbreaks, whether to harvest (if heavy infection) or to continue culture (in light infection) with remedial measures. The decapod primers were well coordinated with the white spot primers in the kinetics of thermal cycling, and able to function as the internal control for the assay. The internal control prevents false negative assay results. In the experimental infection, viral signal was detected as early as 14 hours postin-

fection (h p.i.) in gills and integument indicating these two organs as major viral target organs. Infection only detected at 24 hours h p.i. in abdominal organ. This also suggested that gills and integument as the points of entry for WSSV in waterborne infection. The assay was able to detect other geographical WSSV isolates from Thailand, Ecuador, Indonesia, China and Sri Lanka. The assay had no amplification on autographa californica nuclear polyhedrosis virus and MBV indicating the specificity of the assay. Specificity is crucial for accurate test results. The assay cycling profile was transferable to the DNA Thermal Cycler 480, PTC 100 and 200 Peltier Thermal Cyclers, and Rapid Cycler with modification on the cycling parameters. The assay runtime, however varied from 3 h in the DNA Thermal Cycler 480, 2 h 30 min in the GeneAmp 2400, 2 h in PTC 100 and 200 Peltier Thermal Cycler, and Rapid Cycler. The variation of runtime was due to the different ramping speeds of the thermal cyclers tested. Genomic DNA suitable for the PCR assay was extracted from different stages of shrimp (broodstocks, nauplii, postlarvae, juveniles and growouts) as well as carrier animals such as crabs and wild shrimp. This suggested that the assay could be used for commercial screenings of those animals, and epidemiological studies of the disease. Apart from the PCR assay, the gene probe was also able to detect purified virus and crude lysate of infected tissue without any purification. This dot-blot technique managed to detect few hundreds copies of virus, thus not as sensitive as nested PCR. This is however applicable in farm laboratory with very minimal investment on equipments as monitoring tool for growout shrimp. Gene probe is less reliable for screening postlarvae and broodstocks in hatcheries as these animals usually have light infections. The RT-PCR assay was able to detect the RNA of YHV extracted from the experimentally infected shrimp. Although YHV is not endemic in Malaysia, the assay established can serves as a tool of management for the disease in the future.

Conclusions

Polymerase chain reaction assays and gene probe were developed and proved to be the effective molecular diagnostic methods for detection of shrimp viruses. The nested PCR assay is the first that uses single tube single loading approach to detect WSSV. This approach is completely different from the conventional nested PCR method that involves the cumbersome two tubes and transfer of PCR products. The is not only sensitive but also semi-quantitative in determining infection level in clinical samples.

Benefits from the study

The single-tube nested PCR assay has been commercialised as Fast Target™ White Spot Virus Detection Kit. This kit is the first of its kind in the world and is patent pending (PI 9904454). The innovative rapid detection system has the following advantages: 1) single tube - low risk of contamination, economical, fast and user friendly; 2) sensitive - can detect as low as 10 copies of viral DNA targets; 3) built-in false negative and positive controls - reliable results; 4) indicates infection levels (light, moderate and heavy). The kit has won the following awards: WIPO (gold), MINDS (gold), Geneva (bronze), P. Kandiah (gold), UPM Invention and Design (first). The kit is now being sold in eight countries namely Australia, Ecuador, Indonesia, Ecuador, Malaysia, Sri Lanka, Thailand and Vietnam. Technologies in gene probe and RT-PCR were also practised and improved. This improved the diagnostic capacity of the local laboratories and provided sufficient preparation in terms of advanced equipments and trained personnel for more research on the molecular biology of shrimp pathogens in the future.

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Graduate Research

None.

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