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Longitudinal disease studies in small-holder black tiger shrimp (*Penaeus monodon*) ponds in Andhra Pradesh, India. II. Multiple WSSV genotypes associated with disease outbreaks in ponds seeded with uninfected postlarvae

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ABSTRACT

A longitudinal study was conducted from January to August 2007 in a cluster of 61 small-holder shrimp (*Penaeus monodon*) ponds at Mallampudi (16°25′29″ N, 81°19′13 ″ E) in the Krishna District of Andhra Pradesh, India. Exhaustive PCR testing of postlarvae collected from ponds at the time of seeding detected no evidence of white spot syndrome virus (WSSV) infection. However, during grow-out, disease outbreaks occurred in 42 of the ponds (68.9%) in which the mean and median crop durations were 42.8 days and 40.5 days, respectively. Only three of the 61 ponds (4.9%) were harvested after more than 120 days of culture. Of 41 ponds sampled at harvest, 35 (85.4%) were WSSV-positive by PCR, including 27 of 28 (96.4%) disease outbreak ponds, of 17 which were graded as heavy or moderate infections. Eight of 13 (61.5%) normal harvest ponds sampled were WSSV-positive at the time of harvest, of which seven (53.8%) were graded as light or very light infections. WSSV genotype analysis was conducted on samples from 35 ponds using the ORF94 variable number tandem repeat (VNTR) marker. In total, 20 different genotypes from TRS1-TRS25 (1 to 25 repeats) were detected. Multiple TRS genotypes were detected in 27 of the 35 ponds sampled (77.1%) and 73 of the 146 individual shrimp sampled (50.0%). The most evident temporal and spatial associations of WSSV genotypes with disease outbreaks were the dominance of genotype TRS18 in seven ponds located on the eastern side and genotype TRS8 in eleven ponds in the central region of the study area. The study indicated a high risk of exposure to WSSV infection during grow-out and that multiple WSSV genotypes were circulating simultaneously in the farming area. The spatial and temporal pattern of WSSV genotype distribution suggested transmission of infection within two clusters of ponds.

1. Introduction

Small-scale holdings operated by low-income families cultivating black tiger shrimp (*Penaeus monodon*) contribute the majority of shrimp aquaculture production in India and several other Asian countries (Umesh et al., 2010). For this sector of the industry, disease is a major obstacle to reliable production. Improved health management for small-holder farmers is being addressed with some notable success through 'Better Management Practice (BMP) programs (Mohan et al., 2008; MPEDA/NACA, 2003; Padiyar, 2009; Subasinghe, 2005; Umesh et al., 2010). However, poor yields and crop losses due to disease continue to occur commonly, impacting directly on income and job security of small-holder farmers and workers in hatcheries, farms, feed mills, and processing plants, with a flow-on of impact on development in poor rural communities (Walker and Mohan, 2009).

White spot syndrome virus (WSSV) has been implicated as the major cause of disease of farmed shrimp throughout Asia (Flegel and Alday-Sanz, 1998; Lightner, 2003; Walker and Winton, 2010). WSSV has a very wide host range amongst decapod crustaceans (Corbel et al., 2001; Flegel, 2006; Hameed et al., 2003; Lo et al., 1996 and can occur as a low-level persistent infection in the absence of disease (Tsai et al., 1999). The virus can be transmitted either horizontally by

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water-borne contact or ingestion (Chou et al., 1998) or vertically during spawning (Lo et al., 1997; Lo and Kou, 1998) and white spot disease can be precipitated in persistently infected shrimp by stress or environmental influences such as changes in salinity or temperature (Joseph and Philip, 2007; Liu et al., 2006; Vidal et al., 2001). Although these fundamental biological characteristics have usefully informed disease management strategies for semi-intensive farms, little is known of the epizootiology of WSSV infection in complex and often poorly-resourced small-holder farming systems. Various farm-level risk factors have been identified (Corsin et al., 2001; MPEDA/NACA, 2003; Sahoo et al., 2010; Subasinghe, 2005; Tendencia et al., 2011) and it is well established that PCR screening of seed can significantly reduce risks of white spot disease during grow-out (Chanratchakool and Limsuwan, 1998; Peng et al., 2001; Withyachumnarnkul, 1999). However, even when PCR-screened seed is used, WSSV infection and disease commonly occur and the specific sources and dynamics of infection are poorly understood (Walker et al., 2011).

The WSSV genome displays a high level of overall sequence conservation but variable loci have been identified, including single-nucleotide polymorphisms (SNPs), insertion/deletions (INDELs) and variable numbers of tandem repeats (VNTRs) (Marks et al., 2004; van Hulten et al., 2000; van Hulten et al., 2001). Of these, VNTRs are the most useful for molecular epizootiological studies on a small spatial scale and have been used as markers to identify variations in WSSV genotypes in shrimp collected from ponds experiencing white spot disease outbreaks (Wongteerasupaya et al., 2003), distinguish WSSV genotypes infecting shrimp and wild crustaceans in the same pond (Hoa et al., 2005), identify multiple WSSV genotypes in individual shrimp (Hoa et al., 2005; Hoa et al., 2011a; Pradeep et al., 2008) and distinguish WSSV genotypes associated with disease outbreaks in different ponds (Musthaq et al., 2006; Pradeep et al., 2008; Wongteerasupaya et al., 2003). A recent longitudinal study employed VNTR markers to compare WSSV transmission routes in two black tiger shrimp farming systems in the Mekong Delta region of Vietnam (Hoa et al., 2011b) and concluded that in semi-intensive shrimp ponds WSSV transmission was mainly from neighboring ponds whereas, in improved extensive ponds, transmission was mainly due to water recycling. The study also compared the use of single and multiple VNTR markers for tracing WSSV spread in small-scale farming systems and concluded that a single marker approach based on the VNTR in WSSV ORF94 was suitable.

In this paper, we report a longitudinal study of WSSV infection and disease in 61 small-holder black tiger shrimp ponds at Mallampudi in the Krishna District of Andhra Pradesh, India. The farmers were not employing BMPs and the history of the area suggested a very high risk of disease outbreaks. The study employed conventional and quantitative PCR to determine the infection status of postlarvae at seeding and in shrimp collected at termination of the crop, and applied the ORF94 VNTR as a genetic marker to track the number and distribution of WSSV genotypes in ponds at the time of disease outbreaks and identify possible patterns of transmission.

2. Materials and methods

2.1. Longitudinal study site

The study was conducted from January to August 2007 and involved 61 ponds within a total area of approximately 1.4 km^2 on the southern side of the village of Mallampudi (16°25′29″ N, 81°19′13″ E) in the Krishna District of Andhra Pradesh, 26 km south-west of Bhimavaram (Fig. 1). The total area of study ponds was approximately 43 ha. The main water source is the Peddalanka Drain which feeds into a tributary of the Krishna River and on into the Bay of Bengal. The farmers had very low annual incomes and each operated individual or a small number of ponds. They were not organized in farmer cooperative groups and were not participating in a Better Management Practice (BMP) program, but the village had been identified as a site for future BMP implementation by the NACA/MPEDA program. Farmer cooperation and sampling opportunities were therefore somewhat restricted. However, the site had a history of unsuccessful crops and was expected to have a high probability of disease outbreaks.

2.2. Pond preparation, stocking procedure and production data collection

The ponds were stocked between 30 January and 13 March 2007 with postlarvae that had been obtained from local hatcheries. Although it was normal practice in this area for farmers to buy PCR-tested seed, the PCR screening status of postlarvae pre-stocking and the pond preparation procedures were not determined. Details of the pond area, stocking date, stocking number, duration of the crop, yield, survival rate, feed conversion ratio, total revenue and total cost of production were recorded for each pond. Dates of disease outbreaks in study ponds were also recorded. For the purpose of this study, a disease outbreak was defined empirically as: i) an abnormal reduction in feed consumption and increase in the number of shrimp with abnormal swimming behavior and/or appearance; ii) an observed increase in the number of dead or moribund shrimp; or iii) a farmer-initiated emergency harvest (Padiyar, 2009).

2.3. Sample collection

A detailed, standardized sampling protocol was prepared for the field team. However, sample numbers were sometimes fewer than specified as implementation required negotiations with individual farmers. All samples for PCR analysis were collected in 95% aqueous (v/v) analytical grade ethanol and stored at room temperature until required for testing. Two replicates of each sample were prepared to enable PCR testing at laboratories in India and Australia. Postlarvae (100 to 200) were taken from ponds at the time of stocking and each pool was divided into approximately equal numbers. Juvenile/sub-adult shrimp were collected after 4–6 weeks of culture, with further samples taken from grow out ponds at mid-crop (50–70 days of culture or before any marked change in salinity in the source water and pond water) and at the time of disease outbreaks, emergency harvests or planned harvests. The samples were either submitted whole or after longitudinal dissection of the abdominal segment and attached pleopods, and were subsequently divided into replicate sample pools. In this case of farmed shrimp, five animals from each pond were usually sampled. When available, whole wild shrimp and walking legs from crabs inside the ponds were also sampled.

2.4. WSSV testing by conventional PCR

Preliminary screening of preserved shrimp tissue to identify WSSV-positive samples was conducted using the IQ2000 WSSV PCR Kit (Farming IntelliGene Technology Corporation, Taiwan). DNA was extracted from pooled samples using the supplied DNA lysis buffer according to the manufacturer's instructions. Typically, 30 postlarvae or a pool of five pleopods from larger animals was used for DNA extraction. Because of the large number of samples, the preliminary screening was divided between the laboratories at Central Institute for Brackishwater Aquaculture (CIBA) in Chennai, and the College of Fisheries in Mangalore. WSSV-positive samples were graded as very light, light, moderate or heavy by using the banding pattern of PCR products as recommended by the manufacturer.

2.5. WSSV Taqman qPCR

Duplicates of the samples identified as WSSV-positive during preliminary screening in India were retested individually by Taqman® qPCR at the CSIRO Australian Animal Health Laboratory (AAHL) in Australia. DNA was extracted from tissue samples by homogenization