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Horizontal transmission of white spot syndrome virus (WSSV) between red claw crayfish (*Cherax quadricarinatus*) and the giant tiger shrimp (*Penaeus monodon*)

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ABSTRACT

Australian red claw crayfish, *Cherax quadricarinatus* was introduced into Thailand in the 1990s and is generally cultured in rice fields in many parts of the country including those where penaeid shrimp are farmed. As yet, there have been no reports of disease outbreaks in red claw caused by white spot syndrome virus (WSSV), the most serious penaeid-shrimp viral pathogen. Due to the close proximity of farms for red claw and penaeid shrimp, it is useful to know whether there is any risk of WSSV cross-infection between the two. Here we show that red claw is susceptible to WSSV infection by injection or by feeding with WSSV-infected giant tiger shrimp and that this can lead to high mortality. By contrast, co-habitation with WSSV-infected giant tiger shrimp did not lead to infection or subsequent mortality in the crayfish for up to 30 days post exposure. In contrast, WSSV could be transmitted to giant tiger shrimp within 4 days after co-habitation with WSSV-infected red claw. WSSV infections were confirmed by PCR detection, histopathology and immunohistochemistry. These results revealed that red claw is susceptible to infection and disease caused by WSSV and can transmit WSSV to the giant tiger shrimp by feeding and co-habitation. Thus, penaeid shrimp and crayfish farmers in Thailand should be aware of this possible horizontal transfer of WSSV, especially in low salinity areas where both species are cultivated or where exotic red claw have now become established.
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1. Introduction

White spot syndrome virus (WSSV) is the most serious viral pathogen of cultivated penaeid shrimp in both the eastern and western hemispheres (Alliance, 1999; Flegel and Alday-Sanz, 1998). The virus has been assigned to the new family *Nimaviridae* and genus *Whispovirus* (Vlak, et al., 2005). It was first reported to cause disease outbreaks in farmed *Penaeus japonicas* in Japan in 1993 (Inouye, et al., 1994; Nakano, et al., 1994) and it subsequently spread to other Asian countries (Chou, et al., 1995; Flegel and Alday-Sanz, 1998; Wang, et al., 1999; Wongteerasupaya, et al., 1995) and the Americas (Alliance, 1999). WSSV has been reported to infect many shrimp and other crustacean species including crayfish. The reported crayfish hosts include the American crayfish *Procambarus clarkaii* and an Australian crayfish *Cherax destructor albidus* (Shi, et al., 2005). The American crayfish in particular, has been shown to be very supportive of WSSV replication and to provide high yields of purified viral particles (Huang, et al., 2001; Xie, et al., 2005). This American crayfish was mistakenly named red claw crayfish (*Cherax quadricarinatus*) (Edgerton, 2004) in an earlier publication where it was used as a host for WSSV amplification in *vivo* (Shi, et al., 2000) and that publication has been used to mistakenly include it in lists of crustaceans reported as susceptible host of WSSV (Flegel, 2006; Stentiford, et al., 2009).

Although, there have been no reports of disease problems with red claw cultured in Thailand, studies done elsewhere on another related freshwater crayfish species *Cherax destructor albidus*, also from Australia, suggested that *C. quadricarinatus* might also be susceptible to WSSV infection by both injection and feeding on infected materials (Edgerton, 2004). Of particular concern for shrimp farmers in Thailand is the risk of disease transmission, particularly of WSSV and yellow head virus (YHV) between red claw and penaied shrimp that may be cultured in the same area. Although penaeid shrimp are considered to be marine or brackish water species, they are commonly cultivated in Thailand in freshwater areas [especially *Penaeus (Litopenaeus) vannamei*] at salinities as low as 0.5 ppt (Davis, et al., 2004; Limsuwan, 2000; Szuster and Flaherty, 2000), sometimes in co-culture with rice (Limsuwan, 2000). In addition, it is known that *C. quadricarinatus* is tolerant to salinities up to 12 ppt [(Frost, 1975; Jones, 1995) cited by (Nystrom, 2002)] and that it has escaped and become established in Thailand. In this study we describe for the first

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time, WSSV infections in the red claw crayfish *C. quadricarinatus* by means of injection and feeding on infected giant tiger shrimp (*P. monodon*) and show that the virus can be transmitted from infected red claw to giant tiger shrimp by co-habitation.

2. Materials and methods

2.1. Crayfish and shrimp

Healthy red claw, *Cherax quadricarinatus* (29 pieces, ~120–200 g/piece) were purchased from a crayfish farm in Srakaew province, Thailand. The animals were acclimatized and maintained in 200 l aquaria (4 per aquarium) containing water at ~5 ppt salinity and fed with commercial, penaeid-shrimp pelleted feed (at 2% of body weight) for two weeks before experiments. Average water temperature was ~28 °C during the day and ~23 °C at night.

Juvenile black tiger shrimp, *Penaeus monodon* (100 pieces, ~20 g/piece) were obtained from a specific pathogen free stock (Shrimp Genetic improvement Center, Chaiya, Surathani, Thailand). These shrimp are and have been free of many important shrimp pathogens including WSSV and yellow head virus (YHV) for more than 6 generations. The shrimp were first kept in aquaria containing ~15 ppt for two days before the salinity was gradually adjusted to 5 ppt within a 3 days period. The animals were acclimatized under these conditions for one week before experiments were begun.

During the acclimatization period, hemolymph samples were collected from all crayfish, and DNA was extracted and tested for the presence of WSSV infections using a polymerase chain reaction kit (PCR, IQ2000™ WSSV detection system, GeneReach Biotechnology Corp. Taiwan). All gave negative results for WSSV. Although some shrimp and crayfish died during transport by air (shrimp) and car (crayfish), the remainders were grossly healthy and remained so, without mortality during the acclimatization period until experiments were begun.

2.2. DNA extraction and polymerase chain reaction tests for WSSV

Hemolymph from each animal was collected in a syringe containing an equal volume of shrimp salt solution (SSS, 450 mM NaCl, 10 mM KCl, 10 mM ethylenediamine-tetra-acetic acid [EDTA], 10 mM N-[2-hydroxyethyl] piperazine-N′-2-ethanesulfonic acid [HEPES]) and DNA samples were extracted using the phenol chloroform extraction method (Sambrook, et al., 1989) with some modification as follows: one volume of hemolymph and SSS mixture (100 µl) was solubilized in 4 volumes of lysis buffer (50 mM Tris–HCl at pH 9, 100 mM EDTA, 50 mM NaCl, 2% SDS, Proteinase K 1 µg/ml) and incubated at 60 °C for 10 min in a heating block before 500 µl of phenol/chloroform/isoamyl alcohol (25:24:1) was added with thorough mixing. The mixture was then centrifuged at 15,000 g for 10 min at 4 °C before the upper aqueous phase (~200 µl) containing the DNA was collected. Absolute ethanol (500 µl) was added to the collected aqueous phase to precipitate the DNA. The tubes were left to stand at room temperature for 5 min before the precipitated DNA in the mixtures was collected by centrifugation at 15,000 g and 4 °C for 10 min. The supernatant solution was discarded and the DNA pellet was collected and washed by adding 500 µl of ice cold 70% ethanol to the sample tubes. The samples were then centrifuged again at 12,000 rpm and 4 °C for 10 min before the supernatant solution was discarded and the DNA pellet was air-dried at room temperature. The dried DNA pellet was re-suspended in 20 µl of DNase free water and kept in a freezer at −20 °C

To detect WSSV infection in shrimp and crayfish, the IQ2000™ WSSV PCR detection system (GeneReach Biotechnology Corp., Taiwan) was used. The PCR was performed according to the manufacturer's instructions except that a precise quantity of 200 ng of template DNA was used for each PCR reaction. For screening the shrimp and crayfish before the beginning of experiments, the same amount of template DNA was used from a pool of DNA samples from three to four shrimp or crayfish.

2.3. Preparation of viral stock inoculum

Original WSSV inoculum was obtained from hemolymph of WSSV infected shrimp from a disease outbreak that occurred in a Thai shrimp cultivation pond in 2000 and the virus was subsequently amplified by injecting this inoculum into black tiger shrimp in the laboratory. Hemolymph of WSSV-injected shrimp (3 days post injection) was withdrawn into a syringe containing an equal volume of shrimp salt solution (SSS, 450 mM NaCl, 10 mM KCl, 10 mM EDTA, 10 mM HEPES, pH 7.3). The haemocytes were removed from the mixture by centrifugation at 2000 g for 10 min. The supernatant was filtered through a 0.45 µm membrane filter and aliquots of the crude WSSV preparation were stored at −80 °C until used to infect shrimp and crayfish experimentally by tail injection.

Viral particle number in stock virus preparation (inoculums) was determined by quantitative real time polymerase chain reaction (qRT-PCR) using iQ™ SYBR® green supermix reagent (Biorad Laboratory, USA) and Applied Biosystems 7500 Real-Time PCR System (Applied Biosystem, USA). In this method, a standard curve was first created. Eight serial dilutions of WSSV Vp28 gene sequence containing plasmid (kindly provided by Dr. Saengchan Senapin of the Thai National Center for Genetic Engineering and Biotechnology) of known copy numbers (0, 10, 10^2, 10^3, 10^4, 10^5, 10^6, 10^7, 10^8 copies/µl) were prepared and used as templates for RT-PCR reactions (3 reactions or triplicates for each dilution). PCR primers used were WSSV Vp28 F: 5′tgtgaccaa-gaccatcgaaa 3′ and WSSV Vp28 R: 5′attgcggatcttgattttgc 3′. PCR conditions used are as follows: 50 °C for 5 min and 95 °C for 10 min followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Data were collected at these steps. Dissociation conditions were set as the defaults of the machine. Each RT-PCR reaction generates a cycle threshold (Ct) value, a cycle number at which the fluorescent intensity of PCR amplicons rises above that of background level. Mean Ct values for each dilution were then calculated. A standard curve was made by plotting plasmid copy numbers against their respective Ct values (mean). To quantify virus copy number in the inoculum (stock virus), DNA from the stock virus was extracted as described above and used as the template for RT-PCR (also in triplicate). Ct values (mean) obtained were then compared to those in the standard curve to extrapolate copy numbers. The stock virus was diluted with SSS before injection.

2.4. Experimental design for infection and transmission tests

In this study initial experiments were designed to determine whether red claw was susceptible to WSSV. Then experiments were carried out to determine whether WSSV could be transmitted horizontally between red claw and the giant tiger shrimp *P. monodon*. For the latter, tested modes of transmission were injection, feeding of infected *P. monodon* meat and co-habitation (sharing the same aquarium but separated by a plastic net to prevent physical contact).

For injection and cohabitation experiments with the giant tiger shrimp, 4 red claw were each injected with 100 µl of crude WSSV preparation containing 1.44×10^6 viral copies. The injected crayfish was placed in the same aquarium with 5 normal, uninjected giant tiger shrimp separated from the red claw by a plastic net to prevent physical contact. The health status of both the shrimp and red claw was monitored. Hemolymph samples from grossly diseased but living red claw (day 4 p.i.) and shrimp (2 days p.i.) were collected into a syringe containing an equal volume of shrimp salt solution (SSS) and their bodies were subsequently fixed with Davidson's fixative and processed for paraffin embedding, sectioning and hematoxylin and eosin (H&E) staining as described by Bell and Lightner (1988). The stained sections were examined using a light microscope.

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