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NOTE

White spot syndrome virus (WSSV) infectivity for *Artemia* at different developmental stages

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ABSTRACT: White spot syndrome virus (WSSV) is a major pathogen of cultivated shrimp, but its host range includes a large number of crustaceans. In this investigation, *Artemia franciscana* was tested for susceptibility to WSSV by the oral route. Both instars and adults were challenged, and the presence of WSSV was followed through to reproductive cysts and offspring using PCR. WSSV caused a much lower cumulative mortality in *Artemia* than in cultivated shrimp by 10 d post-challenge. Instars, adults and reproductive cysts were PCR positive. However, the virus was undetectable by PCR in nauplii that had hatched from PCR-positive reproductive cysts. The data indicate that WSSV or WSSV genomic DNA can be vertically transmitted from WSSV-PCR-positive instars to reproductive cysts, but this DNA is removed during hatching.

KEY WORDS: Susceptibility · *Artemia* · WSSV · Infection · PCR

INTRODUCTION

The global industry for cultivated penaeid shrimp has grown rapidly during the past 2 decades (Rosenberry 1999). White spot syndrome virus (WSSV) is a major pathogen of shrimp, and since 1993 it has caused high mortalities in the shrimp culture industry in the Asia-Pacific region (Inouye et al. 1994). WSSV has also been referred to as white spot syndrome associated baculovirus (Chang et al. 1996), or white spot bacilliform virus (Zhang et al. 2001), and its complete genomic DNA sequence was recently, in 1997, determined (van Hulten et al. 2001, Yang et al. 2001). Based on its morphology, genomic composition and on phylogenic analysis, WSSV has been assigned to the new genus *Whispovirus* within a new virus family, *Nimaviridae* (Vlak et al. 2002).

A most interesting feature of WSSV is its broad host range (Flegel 1997). It infects several species of penaeid shrimp cultivated in the Eastern/Western Hemispheres (Lu et al. 1997, Corsin et al. 2001), in addition to a wide range of other decapods and crustaceans, including crabs (Chen et al. 2000, Sahul Hameed et al. 2001) and fresh water crayfish (Huang et al. 2001, Jiravanichpaisal et al. 2001). Several studies have confirmed that many suspected decapod carriers of WSSV can transmit the virus to *Penaeus monodon* (Wongteerasupaya et al. 1996, Chang et al. 1998, Wang et al. 1998).

Artemia is widely used in hatcheries as live feed or as a feed additive for shrimp postlarvae. Chang et al. (2002) reported *Artemia* cysts that were PCR-positive for WSSV, indicating that *Artemia* might be susceptible to WSSV infection. In this investigation, *Artemia* instars and adults were challenged orally with WSSV, and PCR analysis was used to demonstrate that *Artemia* may occasionally be a passive carrier of WSSV.

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MATERIALS AND METHODS

Experimental *Artemia.* Commercial *Artemia* cysts (Sanders Company) that tested WSSV-negative by PCR (see below) were placed in a 1 l aquarium tank at 30°C at a density of 600 cysts l^{-1} (in seawater at 5 ppt). Nauplii were harvested 12 h after the start of cyst hatching to ensure a 100% homogeneous instar I population. By feeding with live microalgae, some of the nauplii were acclimatized at a density of 300 nauplii l^{-1} (in seawater at 5 ppt) prior to viral challenge, while others were transferred to another tank at a density of 300 nauplii l^{-1} and cultured from hatching instars to the adult stage, at 30°C in 100 ppt seawater. Nauplii and adult *Artemia* were collected for infectivity and vertical transmission studies to determine their susceptibility to WSSV. Seawater was sand-filtered and boiled for 10 min before use in all experiments.

Preparation of viral extract for oral challenge tests. Pond-reared *Penaeus japonicas* shrimp naturally infected with WSSV were used as the source of viral inoculum for primary laboratory infections. A stock inoculum prepared from primary infected shrimp was injected into more laboratory shrimp to provide WSSV-infected tissue for oral challenge of *Artemia*. In detail, WSSV-infected *P. japonicas* shrimp with prominent white spots were collected from culture ponds in Tong' an, Xiamen, east China, in October 1996. The infected cephalothroacic tissues (gill, stomach, midgut, etc.) were homogenized in TN buffer (20 mM Tris-HCl and 400 mM NaCl, pH 7.4) at 0.1 g ml^{-1}. After centrifugation at 2000 × *g* for 10 min, the supernatant was filtered (0.22 µm) and injected (1:100 dilution in 0.9% NaCl) intramuscularly into the lateral area of the 4th abdominal segment of healthy shrimp. Four days later, WSSV infection was verified by PCR, and abdominal muscle was collected, mixed with powdered algae in the proportion 1:5 (w:w), and 10 g of this material was homogenized in 100 ml PBS solution. This preparation was called viral feeding mixture (VFM) and stored at 4°C until used.

Infectivity and vertical transmission studies. For WSSV infectivity studies, 5 replicate groups (150 ind. each) of nauplii and adult *Artemia* were fed VFM as described below. Before oral challenge, seawater was exchanged completely and the *Artemia* were starved for 12 h. Nauplii and adults were then fed twice a day with VFM at the rate of 1:1000 ml (v:v). During the challenge, aeration was sufficient to give good oxygenation and keep the VFM in suspension. Seawater was replaced totally every 24 h by filtration through micromesh gauze, on which the *Artemia* were gently but thoroughly rinsed with 3 × 100 ml sterilized seawater. The *Artemia* were then transferred to a new tank and freshly prepared VFM was added for the next 24 h challenge cycle. On Days 3 to 9, seawater was exchanged and the *Artemia* were fed with live microalgae instead of VFM to remove residual virus. Survival was determined on Day 10. After the last feeding, the challenged *Artemia* were starved for 12 h before PCR analysis. Twenty prawns were also fed with VFM every 12 h for 2 d, and then cultured for an additional 8 d with feed changed to WSSV-PCR-negative shrimp meat. Survival was determined daily until Day 10.

For vertical transmission experiments, some of the challenged instars were transferred to a new tank and cultured to sexual maturity to hatch cysts (oviparous reproduction) at 30°C in 100 ppt seawater by feeding with live microalgae. The reproductive cysts were collected and the offspring nauplii were hatched at 30°C in 5 ppt seawater. Adults, reproductive cysts, and offspring nauplii from the cysts were collected for PCR analysis.

PCR analysis. WSSV-DNA was detected using a commercial 1-step PCR detection kit (WSBV PCR Detecting Kit). Template DNA was prepared from *Artemia* according to the kit instructions. Briefly, 20 mg *Artemia* (i.e. instars, adults, reproductive cysts or hatched nauplii) was added to an Eppendorff tube containing 100 µl lysis buffer and homogenized using a sterilized toothpick. After centrifugation at 2000 × *g* for 2 min, 5 µl silica was added to the supernatant followed by gentle agitation at 4°C for 10 min. The mixture was centrifuged at 2000 × *g* for 15 s, the supernatant was discarded and the pellet was washed with 200 µl 70% ethanol and suspended in 10 µl distilled deionized water followed by incubation at 55°C for 5 min. After centrifugation at 4000 × *g* for 5 min, the supernatant was used as a template for PCR using the following cycling conditions: 94°C for 2 min followed by 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min and a final extension at 72°C for 10 min. The specific WSSV PCR amplicon (360 bp) was detected by electrophoresis in 1.2% agrose gels, ethidium bromide staining and UV transillumination.

Table 1. *Artemia.* Cumulative mortality (%) in nauplii and adult stages (5 replicates) challenged or not challenged with WSSV at Day 10 post-challenge

Replicate	Nauplii		Adult	
	Challenged	Control	Challenged	Control
1	25.3	18.7	10.0	5.3
2	26.7	16.7	19.3	8.0
3	33.3	20.0	18.7	6.7
4	25.8	15.3	13.3	6
5	32.0	20.7	18.0	8.7
Mean	28.6	18.3	15.9	6.9
t-test	9.53		6.717	
p	$p < 0.01$		$p < 0.01$	

RESULTS AND DISCUSSION

Cumulative mortality in WSSV-challenged *Artemia* on Day 10 post-challenge was significantly lower than that in marine shrimp (Fig. 1). Even though cumulative mortalities in *Artemia* were relatively low (29% for nauplii and 16% for adults), a *t*-test comparing the nauplii and adult stages showed that differences in mortality between the challenged and unchallenged *Artemia* were significant at $p < 0.01$ (Table 1). VFM was fed to a fresh batch of prawns, all of which died with prominent white spots by 10 d post-challenge (Fig. 1). In contrast to the infected prawns, WSSV-infected *Artemia* showed no obvious gross signs of infection such as lethargy and lack of appetite, and exhibited normal swimming and feeding behavior. Relatively low cumulative mortality without obvious signs of disease suggested that *Artemia* had the potential to serve as a long-lived carrier of WSSV. However, this needs to be verified in further tests in which WSSV-challenged *Artemia* are cultured for longer periods and are then used in prawn-challenge experiments.

WSSV-challenged nauplii and adult *Artemia* yielded a clear WSSV-specific PCR amplicon (360 bp) (Fig. 2, Lanes 2 and 3), and no PCR product was amplified in the unchallenged control groups (Fig. 2, Lane 7). The prawns fed with the VFM also gave strong WSSV-PCR-positive reactions (Fig. 2, Lane 6). In the vertical transmission test, similar WSSV-positive PCR results were observed in the adults and reproductive cysts arising from challenged instars (Fig. 2, Lanes 3 and 4). However, nauplii hatched from these cysts were PCR negative (Fig. 2, Lane 5). No PCR amplicon was detected for the canned cysts used as the source of *Artemia* for all experiments (Fig. 2, Lane 1).

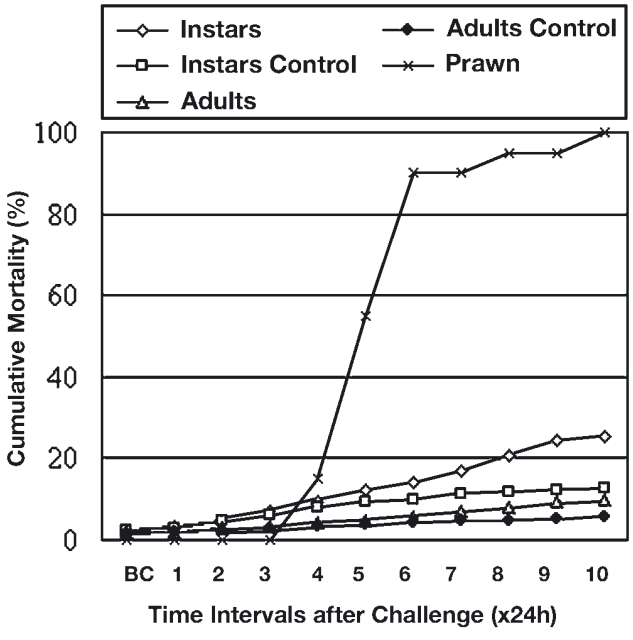

Fig. 1. *Artemia franciscana.* Cumulative mortality after oral WSSV-challenge. *Artemia* were divided into replicate challenge and control groups, each containing 150 individuals for each developmental stage (instars and adults). The positive control group comprised 20 prawns. BC: before challenge

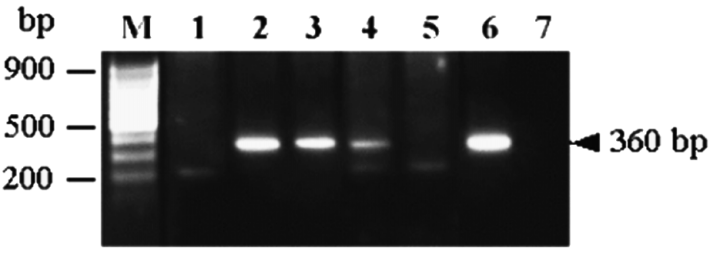

Fig. 2. PCR detection of WSSV-DNA in control and WSSV-challenged *Artemia franciscana*. M: 100 bp ladder; Lane 1: canned *Artemia* cysts; Lane 2: challenged nauplii; Lane 3: challenged adults; Lane 4: reproductive cysts produced from challenged adults; Lane 5: nauplii hatched from the reproductive cysts; Lane 6: prawn fed with viral feeding mixture (VFM) as positive control; Lane 7: unchallenged *Artemia* adults as negative control

The results we obtained from infectivity and vertical transmission studies suggest that *Artemia* may be susceptible to WSSV and may transmit the virus from hatching instars to adults and from adults to reproductive cysts. However, the finding that nauplii hatched from these cysts were WSSV-PCR negative indicates that WSSV is removed during hatching and rinsing of the nauplii. Indeed, it has been shown previously that nauplii hatched from PCR-positive cysts are non-infectious (Chang et al. 2002), suggesting that the positive PCR reaction is likely to be due to the presence of non-infectious WSSV DNA (Lan et al. 2002). However, the possibility that infectious WSSV may sometimes be present internally in reproductive cysts derived from infected *Artemia* suggests that prawn-hatchery operators should use discretion when postlarvae are fed *Artemia* biomass (larvae and adults) produced in salt works near shrimp farms.

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