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Hediste diversicolor (O.F. Müller 1776) as a Possible Model to Study White Spot Syndrome Virus Infection in Polychaetes

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Abstract

The white spot syndrome virus (WSSV) is a highly contagious shrimp pathogen world-wide for which polychaetes are among the many biological vectors. In a previous study WSSV infection was detected in the naturally infected *Dendronereis* spp. (Nereidae). To further study WSSV infection in polychaetes, a model polychaete that is easy to handle and propagate, and is free of and susceptible to WSSV infection is needed. In the present study the suitability of *Hediste diversicolor* (Nereidae) was tested as a model animal. WSSV-free *H. diversicolor* was infected by injection, feeding and immersion, and the infection was followed for 12 days post infection (dpi). In addition, polychaete survival was determined 40 dpi. *Hediste diversicolor* was able to clear the virus within 4 dpi without showing clinical signs and WSSV-associated mortality. Although a first attempt, it was concluded that *H. diversicolor* may not be an immediately suitable model animal for WSSV studies in polychaetes.

Introduction

White spot syndrome virus (WSSV) is the most damaging viral pathogen to the shrimp culture industry worldwide (Stentiford et al. 2012). White spot syndrome virus is a large double-stranded DNA virus and the only member of the genus *Whispovirus* within the family *Nimaviridae* (Lo et al. 2012).

The disease was first reported in Taiwan in 1992 and was named ‘white spot syndrome’ after the pathognomonic clinical signs in the form of small white spots that occur on the carapace of infected animals (Chou et al. 1995). The primary sites of WSSV replication in shrimp are tissues of mesodermal and ectodermal origin especially the epithelium of the foregut, the gills and the antennal

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gland of large decapod crustaceans (Escobedo-Bonilla et al. 2007). White spot syndrome virus is infectious to shrimp *per os* and horizontal transmission via water and cannibalism have been documented (Chou et al. 1998).

The virus is not only highly infectious to penaeid shrimp, but also to many other crustaceans including crabs and fresh and salt water crayfish. Hence WSSV has a very broad host range among aquatic crustaceans (Bateman et al. 2012; Escobedo-Bonilla et al. 2008; Flegel 2006; Liu et al. 2011; Marques et al. 2011; Sánchez-Paz 2010; Soowannayan and Phanthura 2011; Witteveldt 2006). The only reported non-crustacean host for WSSV is the polychaete *Dendronereis* spp. (Nereidae) (Desrina et al. 2013). Such a broad host range and tissue tropism makes WSSV a generalist virus.

Nereid polychaetes are abundantly present in soft sediments typically present in shrimp ponds. The presence of these errant polychaetes in the sediment is beneficial for improving sediment quality by bioturbation (Carvalho et al. 2007). Hence, these animals promote microbial activity, influence chemical nutrient fluxes (Brown et al. 2011; Kristensen et al. 2011) and reduce the anaerobic area thereby increasing oxidation of organic matter and pollutants. In addition, polychaetes are highly nutritious and preferred prey for shrimp (Nunes et al. 1997; Reymond and Lagardère 1990). This is why they are used as supplemental food to induce gonad maturation in broodstock (Chung et al. 2011; Nguyen et al. 2012; Poltana et al. 2007).

WSSV can be transmitted via polychaetes as evidenced from feeding experimentally infected *Marphysa* spp. (Eunicidae) (Vijayan et al. 2005) and naturally infected *Dendronereis* spp. (Haryadi et al. 2014) to naive shrimp. In contrast to what others have assumed (Escobedo-Bonilla et al. 2008; Sánchez-Paz 2010; Vijayan et al. 2005) polychaetes may potentially act as a propagative vector for WSSV. White spot syndrome virus replicates in *Dendronereis* spp. (Desrina et al. 2013) and may serve as a reservoir for this virus. As such polychaetes may play a role in the epidemiology of WSSV in pond systems.

To study WSSV infection in polychaetes in more detail, WSSV-free polychaetes should be available. Because *Dendronereis* spp., collected in pond systems in Indonesia, was often found positive for WSSV infection (Desrina, personal communication), another nereid polychaete, *Hediste diversicolor* (Nereidae) (Müller 1776) (ragworm), was selected as model organism. This species is abundant in the Northern hemisphere where WSSV infections have not been reported. It is an euryhaline and eurythermal species and has a wide distribution in the estuaries and intertidal zone of the north Atlantic region throughout Europe (Scaps 2002). It does not have planktonic larval stages, lives in U-shaped burrows (Durou et al. 2008), and is a non-selective deposit feeder (Esselink and Zwarts 1989).

Due to the ease of handling under experimental conditions, *H. diversicolor* has been used as a model animal to study heavy metal contamination in marine sediments (Caçador et al. 2012; Durou and Mouneyrac 2007; Durou et al. 2008; Kalman et al. 2009) and to test diets and culture conditions

for commercial production (Nesto et al. 2012). We assumed that *H. diversicolor*, being a close relative to *Dendronereis* spp., could potentially be used as a more convenient model animal to study WSSV infection in polychaetes, provided WSSV replicates in this species.

The objectives of this study were to determine (i) the infectivity of WSSV in *H. diversicolor* and (ii) the time span to develop infection or WSSV persistence in this ragworm.

Materials and Methods

Hediste diversicolor

Adult *H. diversicolor* were obtained from a commercial ragworm producer (Fish-bait BV, Yerseke, the Netherlands). The ragworm can grow in captivity using sand or mud substrate (Fidalgo e Costa 1999). Upon arrival the ragworms were kept in 96-L aerated sea water aquaria with an 8 cm sand layer at the bottom. *Hediste diversicolor* naturally lives in 5-15 ºC water. The ragworms arrived in 15 ppt water at 10 ºC and were acclimatised over a 2-months period to 27 ºC and 27 ppt, the conditions conducive for WSSV to develop disease in shrimp (Chou et al. 1998; Moser et al. 2012; Rahman et al. 2006). The ragworms were fed a commercial shrimp feed twice daily at 2% of the total biomass.

A total of 360 *H. diversicolor* (average individual weight 2.1-2.3 g) were used. The ragworms were randomly distributed in groups of 15, over 24 20-L aquaria with an 8-cm sand layer and 4 L artificial sea water. Each aquarium was aerated with an air-stone laying on the bottom in the centre. During the experiment water temperature was maintained at 25-27 ºC and salinity at 27 ppt. The ragworms were fed shrimp feed at 2% of body weight per day, given in two meals per day to prevent cannibalism. Water quality was maintained by replacing 1 L per day with sterile artificial sea water. During acclimatisation, dead animals were removed and replaced so that each tank contained 15 ragworms at the start of the experiment. From the common stock population, five ragworms were tested for the presence or absence of WSSV with nested PCR prior to stocking. Since this analysis invariably gave negative results, it can be assumed that this polychaete species was free from infection at the beginning of the trial.

Inoculum preparation

Inoculum was prepared from the haemolymph of artificially WSSV- infected whiteleg shrimp *Penaeus vannamei* (Boone 1931) (*Penaeidae*). Thirty µL of purified WSSV (originated from infected *Penaeus monodon* Fabricius 1798 (*Penaeidae*) obtained from Vietnam), diluted 100x, was injected into 10 healthy specific pathogen free (SPF) *P. vannamei* (average weight 6 g· animal^{-1}). The shrimp were observed during 1 week for clinical signs. Two days post infection (dpi) shrimp became lethargic and stopped feeding. Moribund shrimp were removed, washed in cold sterile artificial sea water, dried with paper towel and stored at -20 ºC. All shrimp were tested for the

presence of WSSV with 1-step PCR and all were found positive. Four shrimps were used for inoculum preparation and the rest were used in oral route infection experiments (see the 'Infectivity of WSSV in *H. diversicolor*' section below). To prepare the inoculum, shrimps were thawed, cut into head and body portion and placed in a 50 mL- sterile- conical tube containing anticoagulant Alsever's solution (Rodriguez et al. 1995) and filled with cut yellow tips arranged compactly at the base of the tube with the fine tip end positioned toward the bottom which functioned both as channel and suction of the haemolymph. The shrimp was placed with severed parts towards the base of the tube. The tube was centrifuged at 1,500 g for 3 min at 4 ºC. Then the shrimp were removed, TNE buffer (50 mM Tris–HCl pH 7.4, 100 mM NaCl, 0.1 mM EDTA) was added and the tubes were centrifuged at 3000 x g for 8 min at 4 ºC. The clear fluid was passed through a 0.45 µm sterile membrane filter and the collected filtrate was used as inoculum and stored at -80 ºC until use.

Infection of WSSV in H. diversicolor

White spot syndrome virus was introduced into *H. diversicolor* by intra-coelomic injection (injection route), feeding (oral route) and immersion (water-borne route). Eight aquaria were randomly assigned for each route. Per route six aquaria were used in time series sampling to determine WSSV persistence in the polychaete over time. The other two aquaria were observed until 40 dpi to determine polychaete survival. Per administration route, half of the aquaria were exposed to WSSV, the other half served as the negative control.

For the injection route, individual ragworms were placed in cold sterile phosphate buffered saline (PBS, pH 7.8) until they relaxed, put onto sterile paper towel moistened with cooled PBS, injected with WSSV inoculum (diluted 100x in 330 mM NaCl) in between the antero-lateral segments (segments 10-15) with 5 µL inoculum·g^{-1} body weight while gently holding the body during 1 min to prevent contraction, which would cause loss of inoculum. The polychaetes from 'negative control' aquaria were injected with 5 µL sterile 330 mM NaCl·g^{-1} body weight. After injection, *H. diversicolor* was added to a 1-L glass beaker filled with 500 mL water from its own aquarium. Once all ragworms from one aquarium were injected, they were returned into their aquarium. The ragworms were closely observed for burrowing behaviour during 60 min. To confirm the infectivity and pathogenicity of the WSSV inoculum, five 7-g *P. vannamei* were each injected with 35 µL inoculum.

For the waterborne route, the ragworms from each tank were separately immersed for 2 h in either 0.1% inoculum solution or in sterile sea water (negative control). For the oral route, the ragworms were fed during four consecutive days minced WSSV-infected shrimp at 1% of total body weight·day^{-1} and subsequently returned to normal shrimp feed. Animals in control aquaria were fed the same ration of WSSV-free shrimp (stock shrimp and proven WSSV negative with nested PCR one day before) with the same dose. Total ammonia, pH, nitrate, nitrite and salinity were measured daily.

H. diversicolor behaviour and physical observations

The number of ragworms on the sand surface and the body condition were recorded twice daily at 7 am (after being in the dark for 12 h) and at 7 pm (after being in the light for 12 h). Afterwards, a predetermined amount of feed was placed on the bottom of each tank and feeding appetite was observed for 1 h. The number of ragworms that grabbed the food was recorded. During each time series sampling, *H. diversicolor* in each tank was observed for burrowing as follows: six ragworms from each tank were randomly taken and placed on a plastic tray (20 x 10 x 5 cm) that has been assigned for the particular tank. Colour and degree of body damage were recorded at sampling. Ragworms were left in the tray for 10 min and observed whether or not the ragworms were huddled. Next, two ragworms were taken as sample (see next section: time series sampling) and the rest were placed back in the tank. After being put back in the tank, the ragworms' digging activity was observed for 10 min.

Time series sampling

To determine persistence of WSSV in the polychaete over time, two ragworms from each tank were taken at 2, 4, 6, 8, and 10 and 12 dpi, and pooled per sampling day: two ragworms were preserved in 70% alcohol for PCR testing, two ragworms in Davidson's solution for histopathology and two ragworms were stored at -80 ºC for RT-PCR to detect WSSV replication. However, we choose not to carry out an RT-PCR and histology analysis, when the PCR result on the viral genomic DNA was negative soon after infection and throughout the observation period.

Survival observation

Mortality, feeding activity, changes in burrowing behaviour, response to touching and changes in coloration were recorded daily until 40 dpi. At 40 dpi all ragworms were harvested and survival calculated as S (%) = ([number at T_{40} - number at T_0] / number at T_0) x 100 where T_0 and T_{40} are 0 and 40 dpi.

WSSV detection

WSSV was detected in experimental animals with 1-step and nested-PCR using primer pairs for *vp26* (Marks et al. 2005) for 1-step PCR and *vp28* and *vp28*-nested according to Desrina et al. (2013). 18s rRNA of *H. diversicolor* was used as internal control of successful DNA extraction (Desrina et al. 2013). Selected PCR products of WSSV DNA from *H. diversicolor*, *P. vannamei* along with 18s rRNA of *N. diversicolor* were sequenced (Macrogen Europe). The results were aligned to known sequences present in GenBank based on the BLAST program.

Results

Behaviour and physical observation

The ragworms crawled on the surface, dispersed and started to burrow within 30 min post infection and returned to a normal feeding pattern at 2 dpi. During the experiment, the ragworms showed normal burrowing behaviour (e.g. within 15 min ragworms were burrowing), flocked together when put together in a tray, and fed normally (the ragworm head was out of the burrow, grabbed the food and dragged it into the burrow). There was no obvious difference in body coloration, movement, feeding, and burrowing activity between the WSSV-infected *H. diversicolor* and the negative control ragworms (no virus) during 11 days of observation. Shrimp injected with WSSV inoculum became lethargic, showed reduced feeding and all died within 6 dpi.

Time series analysis

WSSV was only detected up to 4 dpi in *H. diversicolor* individuals subjected to infection by injection (Fig.1). Other infection methods (oral, immersion) gave negative results with nested-PCR. 18s rRNA of *H. diversicolor* was detected in all samples tested (Fig. 2). No WSSV was detected in the negative control animals. All whiteleg shrimps injected with the inoculum were WSSV positive with 1-step PCR (Fig.3). Alignment of sequencing results showed 100% identity with the *vp28* gene of known WSSV isolates in GenBank (Accession Numbers AF 369029, AF440570, AF 332093). The 18s rRNA of *H. diversicolor* used in this study showed 97% identity with 18s rRNA of members of genus *Nereis* (Accession number U36270, EF117897.1, AY210447.1).

Survival observation

Out of 5 *P. vannamei* used as positive control for the injection route, 3 (60%) shrimp died at 3 dpi; the other 2 died at 4 and 6 dpi, respectively. Survival of the *H. diversicolor* at 40 dpi was comparable among treatments and between each treatment and its control. The summary of survival of each treatment is presented in Table 1. Ragworms that did not survive had no particular symptoms or abnormal pathology. Apparently, no WSSV-related mortality occurred during the experiment. The experiment was considered a pilot and carried out once.

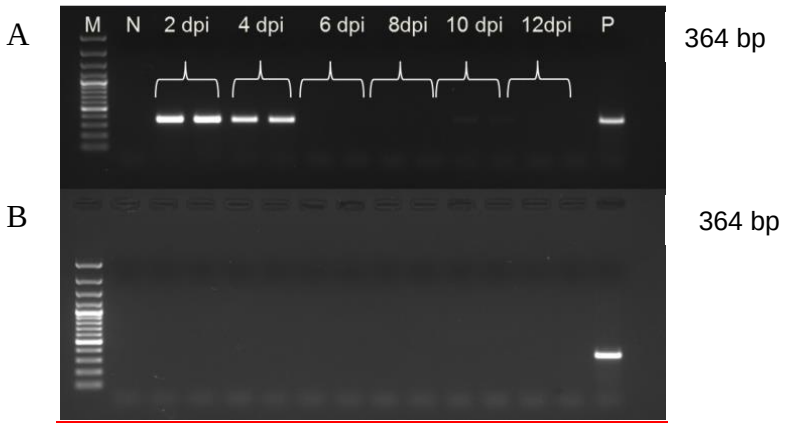

Fig. 1. WSSV was detected with nested-PCR using *vp28*-nested primer pair (amplicon 364 bp) in *H. diversicolor* upon injection up to 4 dpi (T2) (Panel A) and was not detected in negative control specimens sampled at the same time (Panel B). M = Marker (100 bp DNA ladder). N= Non-template control (NTC); P = Positive control of PCR (experimentally infected *P. vannamei*)

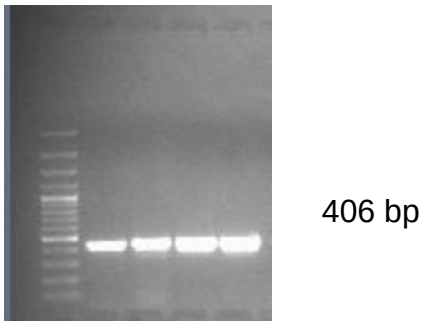

Fig. 2. 18s rRNA gene of selected *H. diversicolor* (lane 1-4) used in this experiment served as internal control for adequate DNA extraction. M= Marker (100 bp DNA ladder).

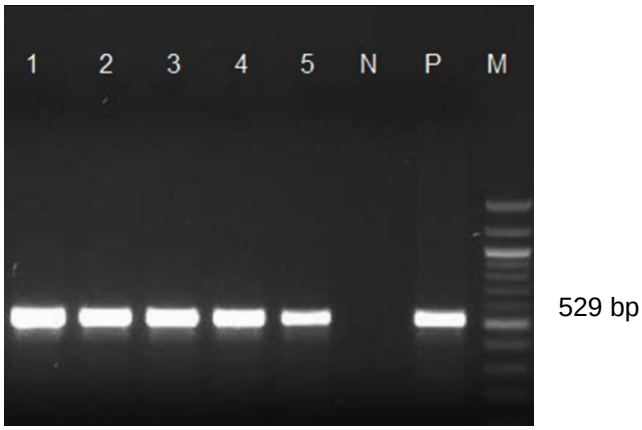

Fig. 3. WSSV DNA was detected with 1-step PCR in the *P. vannamei* injected with WSSV inoculum (positive control of infection by injection route). Lane 1= WSSV DNA obtained from *P. vannamei* at 1 dpi; Lane 2-4= WSSV DNA obtained from *P. vannamei* at 2 dpi; lane 5 = WSSV DNA obtained from *P. vannamei* at 4 dpi; N = No template control of PCR; P = positive control of PCR; M = Marker (100 bp DNA ladder).

Table 1. Survival of *H. diversicolor* infected by injection, oral and immersion routes at 40 dpi

Infection route		Survival (%)
Injection	Injection with WSSV inoculum	53
	Injection with sterile 330 mM NaCl	53
Waterborne	Immersed in 0.1% WSSV inoculum	53
	Immersed in sterile sea water	60
Oral	Fed with minced WSSV-infected shrimp	53
	Fed with minced WSSV free shrimp	66

Discussion

The goal of this study was to evaluate *H. diversicolor* as a potential model animal to further investigate WSSV infection in polychaetes. This polychaete did not show basic indicators of WSSV infection in penaeid shrimp, such as clinical signs, body coloration, and/or behavioral changes. The ragworms fed and burrowed shortly after infection and during the experiment and appeared to be healthy, hence, the presence of the virus did not affect this ragworm. Burrowing activity of *H. diversicolor* is an indicator of the ragworm's well-being (Esselink and Zwarts 1989) and has been used to study effects of heavy metal contamination in this polychaete (Bonnard et al. 2009; Kalman et al. 2010; Kalman et al. 2009; Mouneyrac et al. 2010). We adapted the parameters to our study because generally, reduced feeding and abnormal movement were the first behavioural change attributed to disease or adverse environmental conditions in fish or shrimp. Therefore, burrowing activity is an applicable proxy to assess *H. diversicolor*'s health condition. Furthermore, there was no difference in body coloration between infected and mock-infected ragworms, even at 40 dpi.

Injection, immersion and feeding are three commonly used methods to test infectivity of WSSV in shrimps and crabs (Bateman et al. 2012; Chen et al. 2000; Liu et al. 2011). We used all three types of exposure to overcome the limitation of each method. Published studies on WSSV infectivity in polychaetes gave variable results. *Marphysa* spp. developed light infection within 7 days post exposure to WSSV by immersion in sediment-contaminated WSSV, as evidenced by nested PCR (Vijayan et al. 2005). Laoaroon et al. (2005) were able to induce light infection in the Nereidae, *Perinereis nuntia* (Savigny in Lamarck 1818) fed with WSSV infected *P. monodon* and by immersion methods as evidenced by nested PCR. In the latter report, 40-90% of the tested polychaetes were found infected within 2 weeks pi. However, these results should be regarded with caution because the experiment did not start with WSSV-free animals. In contrast, we very likely failed to induce infection in *H. diversicolor* by both feeding and immersion.

It is possible that in our case the dose used was too low to initiate infection in this polychaete, although the same dose was readily able to induce infection in the control positive shrimp.

There may be a huge difference in susceptibility between nereids and penaeids, and among nereids. Another explanation may be that the virus was diluted quickly in the coelomic cavity.

The WSSV inoculum mixed with 1 µL of patent Blue V colour was spread and diluted in the coelomic cavity within 30 min (data not shown) indicating that this may have been the case. In addition, the polychaete species and type of sediment used in our experiment as compared to the literature (Laoaroon et al. 2005; Vijayan et al. 2005), may have attributed to the different outcome. The behaviours and lack of external symptoms in WSSV infected *H. diversicolor* were in accordance with the PCR result, showing that *H. diversicolor* was able to clear the virus within 4 days.

WSSV occurrence in wild *Marphysa* spp. (Vijayan et al. 2005) and *P. nuntia* (Laoaroon et al. 2005) was reported. The authors hypothesised that WSSV was naturally acquired along with ingested sediment and that the virus accumulated in the polychaete to make it only a passive vector. Later, Desrina et al. (2013) showed that WSSV replicated in *Dendronereis* spp., which makes this polychaete an active WSSV carrier. Likewise, *H. diversicolor* can be a potential carrier of WSSV once the virus establishes itself in the environment where this polychaete lives. On the basis of this first attempt though, it seems that *H. diversicolor* is not an immediately suitable proxy model animal for WSSV studies in polychaetes. No clinical or typical signs were noted and no WSSV-associated mortality occurred, not even in ragworms infected by injection. It is possible that (i) WSSV in polychaetes is attenuated because of the phylogenetic distance between polychaete and penaeid shrimp as natural host of WSSV, including the associated immune response or (ii) there is specific coadaptation between WSSV and polychaete that enable them to adapt to each other.

Conclusions

Studies on WSSV infection in polychaetes and roles played by polychaetes in transmission of WSSV in shrimp ponds are still at early stage. To further study WSSV infection in polychaetes, a model animal is needed. In the present study, WSSV infection could not be induced in *H. diversicolor* through oral, injection and immersion routes. Therefore, other routes of infection such as indirect transmission through virions attached to sediment particles or benthic algae should be explored. If the latter would be not successful, then the use of other nereids polychaetes as model species for WSSV infection studies in polychaetes should be explored.

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