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RESEARCH ARTICLE

Sandfish (*Holothuria scabra*) as potential reservoir of white spot syndrome virus (WSSV) when co-cultured with black tiger prawn (*Penaeus monodon*)

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Since the first occurrence of White Spot Disease (WSD) in 1992, it is still listed as one of the crustacean diseases by the World Organisation for Animal Health in 2022. Horizontal transmission in co-culture systems is one of the usual modes in the spread of the disease. WSD outbreak was recorded during the experimental run of the co-culture of black tiger prawn (*Penaeus monodon*) and sandfish (*Holothuria scabra*) in the grow-out phase. In this study, artificial infection through two cohabitation experiments were conducted to determine if H. scabra is a potential non-crustacean vector or reservoir of WSSV. Samples were checked using one-step and nested PCR for increased readout sensitivity of virus infection to investigate the horizontal transmission between prawn and sandfish. During the first cohabitation (5 days) where WSSV (+) prawn were cohabited with WSSV (-) sandfish, 100% of the prawn were one-step PCR positive for WSSV while 100% of the sandfish were nested PCR positive. Subsequently, WSSV (+) sandfish from the first cohabitation were transferred to another tank to be cohabited with WSSV (-) prawn. Sampling of both prawn and sandfish was done every 6 days post-infection (dpi). At 6 to 18 dpi, prawn and sandfish were nested PCR positive. At 25 dpi, there were no prawns left due to mortality and 1 of the 3 remaining sandfish was nested PCR positive. Based on the results, it elucidates the ability of sandfish to bioaccumulate the viral particles when cohabited with WSSV (+) prawn. Results suggest that WSSV is viable in the sandfish confirming its potential as a vector or reservoir due to the consistent nested PCR positive results of the prawn during the second cohabitation. Hence, it can be inferred that sandfish can be a potential non-crustacean vector or reservoir of WSSV for a limited period of time.

Introduction

One of the most intensively studied Holothurians is sandfish (*Holothuria scabra*) which has been discussed in literature since 1833. These are slow growing invertebrates that can live on sand, mud, rock and reef flats, often related with seaweeds, corals and sea grasses whilst some others live buried in the sand with their oral tentacles exposed (Hamel et al. 2001). A deposit feeder that consumes detritus, bacteria and diatoms mixed with sediments on the seabed, this is the most consumed echinoderm and has been eaten since ancient times. Sandfish are found in many countries in the Indo-Pacific, from east Africa to eastern Pacific. They are usually found between latitudes of 30°N and 30°S (Agudo 2006).

Presently, sandfish is one of the most valuable commercial species among sea cucumber and processed into dried form (trepang or *bêche-de-mer*). These delicacies are exported to China, Hong Kong Special Administrative Region (SAR), Singapore, Republic of Korea, Taiwan and Japan (Choo 2008). It has become a favorite seafood in China (Yaqing, Changqing, and Songxin 2004). Sandfish is rich in proteins and amino acids and is famous in both China (Li 2004) and in Western countries as a cure for arthritis and joint ailments (Lovatelli et al. 2004). Rapid increase in market demand in the 1990s has resulted to chronic over exploitation of natural sea cucumber population which caused extinction and collapse of stocks all over the world (Yasoda, Chi, and Ling 2006; Purcell et al. 2010). This alarming condition has led a number of institutions like the Australian Centre for International Agricultural Research (ACIAR) and World Fish Center in Malaysia to consider technologies that would allow for the culture of juvenile sandfish in hatcheries and releasing them into the wild. This will rebuild spawning biomass and hasten recovery of sandfish availability.

However, mono-culturing sandfish in hatcheries for restocking may be too expensive since juvenile sandfish require large surface areas of sand substrate to forage for detritus and bacteria (Bell et al. 2007). One of the most beneficial options to this constraint is the alternative grow-out and co-culture with compatible organisms which has been widely explored. This is due to the fact that sandfish are detritivores, and are capable of ingesting large quantities of sediment and extracting their energy from organic matter, comprised of detritus, waste feed and feces (Robinson 2013). Given their mode of nutrition, sandfish are excellent candidates for co-culture with *P. monodon* wherein the sandfish ingest the excess feeds and waste of the prawn potentially reducing nutrient loads and stratification of sediments in culture ponds (Purcell, Patrois, and Fraisse 2006). Despite the number of studies on co-culture of sea cucumbers with other organisms, such as teleost fish (Ahlgren 1998), bivalves (Slater and Carton 2007, 2009; Paltzat et al. 2008), gastropods (Kang, Kwon, and Kim 2003; Maxwell, Gardner, and Heath 2009) and shrimps (Bell et al. 2007; Watanabe et al. 2012), problems are still encountered in the co-culture of sandfish and shrimp. Pitt et al. (2004) tried co-culturing *H. scabra* with *P. monodon* which they found it favourable at most conditions; however, predation of sandfish by *P. monodon* occurred at high stocking density of the prawn and aggression was heightened when shrimp were not fed. Sandfish, together with blue shrimp juvenile *(Litopenaeus stylirostris),* appeared feasible where sandfish survived well but only grew significantly slower due to heightened levels of ammonia from the shrimp (Purcell, Patrois, and Fraisse 2006).

One of the main problems of co-culture is antagonism between species, for example, carnivorous fish can consume shrimps or shrimps attack other organisms (Martínez-Porchas et al. 2010). Other problems include acquiring of diseases such as viruses, bacteria, fungi, protista among others, and there are

at least 15 viruses known to infect cultured and wild marine penaeid shrimp which cause heavy mortalities in larval stocks within two days (Lavilla-Pitogo 1996).

One of the risks posed by the co-culture with penaeid shrimps is the horizontal transmission of WSSV, considered as one of the most serious viral pathogen of cultured shrimp and is now widespread in Asia and the Americas (de la Peña et al. 2003, 2007). Apart from shrimp, WSSV also affects a very wide host range. Studies have confirmed the following species as WSSV carriers: oysters (Vazquez-Boucard et al. 2010), polychaete worms (Vijayan et al. 2005; Supak et al. 2005), rotifers (Yan et al. 2004), insect larvae (Lo et al. 1996; Flegel, Boonyaratpalin, and Withyachumnarnkul 1997), crabs (Supamattaya et al. 1998; P.-S. Chang, Chen, and Wang 1998), lobsters (P.-S. Chang, Chen, and Wang 1998; Rajendran et al. 1999) and crayfishes (Corbel et al. 2001; Edgerton 2004). To date, there are 137 known species reported as WSSV hosts or vectors mainly consisting of penaeid shrimp, crab and a small percentage of non-crustacean (Desrina et al. 2022). A review by Sanchez-Paz (2010) implied that macro-benthic invertebrates living in the pond sediment could have the potential to acquire WSSV, due to the niche they occupy and their foraging habits in the co-culture system. Thus, it can point toward the potential of a specific macro-benthic invertebrate such as *H. scabra* as a possible WSSV vector. Studies on the horizontal transmission of WSSV usually employ one-step PCR for basic amplification of viral DNA, and further examined using nested PCR to improve the sensitivity of one-step PCR, since the PCR products of the first assay can be further amplified in the nested or second round of PCR amplification. Desrina et al. (2022), explains that the results of nested PCR enhance the detection of potential disease vectors and is useful to investigate asymptomatic carrier species.

In the experimental run of grow-out co-culture of sandfish and black tiger prawn conducted by S. Watanabe *(unpublished data)*, WSSV outbreak occurred in prawn. The sandfish was also found to be PCR-positive for WSSV. With these results, cohabitation experiments were conducted to verify that the sandfish can bioaccumulate viral particles and as a potential non-crustacean vector or reservoir of WSSV.

Materials and Methods

Collection and maintenance of experimental animals

Live *P. monodon* with average body weight (ABW) of 5 g were collected from a farm in Bago City, Negros Occidental. The prawns were transported in tarpaulin bags containing oxygenated seawater at a density of five prawns/L. The tarpaulin bags were placed in styrofoam boxes with ice. Upon arrival at the Infection Building of SEAFDEC/AQD, Tigbauan, Iloilo, the prawns were acclimatized in two 500-L fiber glass tanks for five days. The prawns were provided with flow-through, aerated, and UV-sterilized seawater with temperature and salinity of 28°C and 32 ppt, respectively. The shrimp were

fed twice daily with commercial feed at 3% feed rate until the start of the experiment. *H. scabra* were provided by the SEAFDEC/AQD sandfish hatchery and were transported to Infection Building by using plastic bags filled with aerated seawater at a density of two sandfish/L. Upon arrival, sandfish were acclimatized for 1 hr in a 20 L basin supplied with aerated and UV-sterilized seawater. After acclimation, the sandfish were transferred to 500 L fiber glass tanks supplied with aerated and UV-sterilized seawater. Before any infection experiments were conducted, all experimental animals were screened for WSSV using nested PCR (Kimura et al. 1996).

Virulence enhancement of WSSV

WSSV was passed through twice in *P. monodon* to increase the virulence of the viral inoculum. For the first pass, one aquarium was filled with 10 L of UV-sterilized seawater with salinity of 30 ppt, supplied with constant aeration, and the water temperature was maintained at 28°C. Ten prawns were stocked in the aquarium. The infection was done by feeding with WSSV (+) prawn tissues twice daily for three days with a feeding rate of 3% of ABW. The tissues were one-step PCR positive for WSSV and sourced from naturally infected prawn from a grow-out pond in Negros Occidental, Philippines last 2009. Fifty percent of UV-sterilized rearing water was changed daily. Dead prawns were stored at -80°C and checked with PCR. Only one-step PCR positive tissues were used in the second pass. The feeding scheme of the first pass was followed for the second pass.

Cohabitation of WSSV (+) prawn with WSSV (-) sandfish

To prepare the WSSV (+) prawn, three aquaria were filled with 10 L of UV-sterilized seawater with salinity of 30 ppt, supplied with constant aeration, and the water temperature was maintained at 28°C. Ten WSSV (-) prawn (5 g ABW) were stocked in each aquarium. The prawn were fed with WSSV (+) prawn tissues from the second pass twice daily for three days with a feeding rate of 3%. After three days of feeding, the WSSV (+) prawns were rinsed thoroughly for three times using 3 L UV-sterilized seawater in a pail before cohabitating with WSSV (-) sandfish. Thirteen WSSV (-) sandfish (90 g ABW) and 10 WSSV (+) prawn (5 g ABW) were cohabitated using 50 L fiber glass tanks for five days in triplicate. Each tank was filled with 20 L of UV-sterilized seawater with salinity of 30 ppt, supplied with constant aeration, and water temperature of 28°C. Fifty percent of UV-sterilized rearing water was changed daily. The sandfish and prawn were separated with a screen installed in the middle of the tank. Sand was used as a substrate at the bottom of each tank where the sandfish were located. Prawn and sandfish were not fed throughout the experiment. Tanks were monitored four times a day for mortalities. Samples were collected, placed in a resealable plastic, labelled, and stored in -80 ºC for further analyses. Three sandfish and 10 prawn were processed for nested PCR after cohabitation. Four tissues of sandfish, namely: coelom, intestine, tentacle, and muscle, and gills of the prawn were aseptically dissected. Similar tissues of sandfish were pooled while prawn were individually processed.

Cohabitation of WSSV (+) sandfish with WSSV (-) prawn

Ten WSSV (+) sandfish from the first cohabitation and 10 WSSV (-) prawn were cohabited using 50 L fiber glass tanks for 25 days in triplicate. Before stocking, the experimental animals were rinsed thoroughly for three times using 3 L UV-sterilized seawater in a pail. The same experimental conditions from the first cohabitation were followed. Sampling of 2-3 sandfish and prawn per tank was done every 6 days to test for WSSV using PCR. Samples were collected, placed in a resealable plastic, labelled, and stored in -80 ºC for further analyses.

DNA extraction

DNAzol reagent (MRC, USA) was used for extraction of DNA according to the manufacturer's instructions. Briefly, approximately 50 mg of gill tissue of prawn and coelom, intestine, tentacle and muscle of sandfish were placed in 1.5 ml microcentrifuge tube and added with 1 ml DNAzol. The tissues were homogenized manually using a micro-pestle, followed by centrifugation for 10 min at 14,800 x *g* at 4 °C and transfer of the supernatant to a new tube. DNA was precipitated by the addition of 0.5 ml 100 % ethanol. Pelleted DNA was washed twice with 95 % ethanol by centrifugation and air-dried for a few seconds. The dried DNA pellets were suspended in 100 µl of 8 mM NaOH, incubated at 45 °C for 15 min, after which 10 µl of TE buffer was added for storage at -20 °C.

Detection of WSSV using PCR

The DNA samples were subjected to one-step and nested PCR tests using WSSV-specific primer pairs (WSSV P1, P2, P3, and P4) designed by Kimura et al. (1996). PCR reactions were carried out in a 25 µl reaction mixture which includes 10X PCR Buffer (Invitrogen, USA), 25 mM MgCl2 (Invitrogen, USA), 10 mM dNTPs (Kapa Biosystems, USA), 10 µM primers (P1-P2 and P3-P4) (Invitrogen, USA), and Taq Polymerase (Invitrogen, USA). Amplification was performed in a programmable thermal cycler (Eppendorf, Germany) with the following cycle parameters: the initial heating at 72°C for 10 min and 95°C for 6 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 57°C for 1 min and extension at 72°C for 1 min with a final extension at 72°C for 5 min before holding at 4°C until ready for electrophoresis. For the nested step, 1.0 µl of the one-step PCR product was used as the template for PCR amplification using the primer pair P3-P4 with the protocol described above. The products were separated in 1.5 % agarose gels, stained with ethidium bromide and visualized using a Gel Documentation System (UVP DigiDoc-It® 125, USA). The one-step and nested primer pairs amplified products of 982 bp and 570 bp, respectively.

Table 1. PCR results of prawn and sandfish samples from the first cohabitation of WSSV-infected *P. monodon* and WSSV-free *H. scabra* for 5 days.

Sample	Tank					
	1		2		3	
	One-step	Nested	One-step	Nested	One-step	Nested
P. monodon						
1	+	+	+	+	+	+
2	+	+	+	+	+	+
3	+	+	+	+	+	+
4	+	+	+	+	+	+
5	+	+	+	+	+	+
6	+	+	+	+	+	+
7	+	+	+	+	+	+
8	+	+	+	+	+	+
9	+	+	+	+	+	+
10	+	+	+	+	+	+
**H. scabra*						
coelom	-	+	-	+	-	+
tentacle	-	+	-	+	-	+
intestine	-	+	-	+	-	+
muscle	-	+	-	+	-	+

*Three *H. scabra* were pooled as 1 sample for each tissue per replicate

Results

Screening and virulence enhancement of WSSV in experimental animals

All screened experimental animals were nested PCR negative for WSSV. Two passes were conducted to increase the virulence of the viral inoculum. For the first pass, 10 prawns were infected by feeding WSSV (+) shrimp tissue for three days. After three days post infection (dpi), the mortality rate reached 100%. Pooled sample from the 10 infected prawns gave one-step PCR positive result. The same protocol was followed for the second pass using the previously WSSV (+) prawn as source of tissue. For the second pass, the mortality rate reached 100% after 5 dpi. All 10 prawn were individually sampled and were one-step PCR positive.

Cohabitation of WSSV (+) prawn with WSSV (-) sandfish

After the WSSV (-) prawn were fed with infected tissues for 3 days, WSSV (+) prawn were cohabited with WSSV (-) sandfish. For 5 days of cohabitation, the mortality rate of WSSV (+) prawns reached up to 100% while all sandfish survived. Individually sampled WSSV (+) prawn were all one-step PCR positive. On the other hand, the four different tissues (coelom, intestine, tentacle, and muscle) pooled from three sandfish per replicate were all nested PCR-positive (Table 1).

Cohabitation of WSSV (+) sandfish with WSSV (-) prawn

After the first cohabitation, WSSV (+) sandfish were cohabited with WSSV (-) prawn for 25 days. Samplings of prawn and sandfish were conducted every 6 days for the detection of WSSV. At 6 dpi, all prawn from three replicate tanks were nested PCR positive while only the sandfish from tank 1 was found to be nested PCR positive. At 12 dpi, prawn from tanks and 3 only were found to be nested PCR positive and none for the sandfish. As the experiment reached 18 dpi, the mortality rate of prawn in tank 3 reached 100%, hence, no sample was available for succeeding PCR analysis. Moreover, the remaining prawn from tanks 1 and 2 were all negative for WSSV. While the sandfish from tanks 1 and 2 were found to be negative for WSSV, however, tank 3 was found to be nested PCR positive. During the last sampling at 25 dpi, the mortality rates of prawn in all tanks reached 100%, hence, no samples were available for PCR analysis. On the other hand, only sandfish from tank 2 was found to be nested-step PCR positive (Table 2).

Discussion

Published articles suggest the sediment cleaning potential of *H. scabra* in aquaculture. In a recent study, ingestion and excretion processes of *H. scabra* has reduced the oxygen consumption rate (OCR) of the shrimp tank sediment to less than 50%. Acid volatile sulphide (AVS-S) was also reduced to less than 50% despite the low reduction rates of organic carbon and nitrogen contents. The study suggested that *H. scabra* is capable of degrading the organic matter in prawn ponds and can bioremediate pond sediment (Kodama et al. 2015). The ability of *H. scabra* to utilize organic matter in *P. monodon* ponds were also tested by Watanabe et al. (2012). In the study of Purcell et al. (2010), *H. scabra* was co-cultured with juvenile blue shrimp *Litopenaeus stylirostris* (Stimpson). Results showed high survival rate of 73-100% in both species. The above-mentioned results of the studies evidently support the beneficial role of *H. scabra* in polyculture with other economically important species such as prawn.

One of the main considerations for choosing commodities to co-culture is the potential to transmit diseases. In particular, WSSV can be transmitted horizontally through different vectors such as water, soil, polychaete, copepods, and bivalves (Y.-S. Chang et al. 2011; Vazquez-Boucard et al. 2012). To date, *H. scabra* is not known to be a potential vector or reservoir of WSSV in transmitting the disease in co-culture of prawn. However, there was a WSSV outbreak in co-cultured sandfish and prawn wherein both organisms were PCR-positive (unpublished data). Based on the results of the first cohabitation experiment with WSSV (+) prawn and WSSV (-) sandfish, pooled samples of the different tissues (coelom, intestine, tentacle, and muscle) of sandfish were all nested PCR positive. These results coincided with the cohabitation, immersion, and feeding experiments using WSSV-infected shrimp with different invertebrates, such as polychaetes, copepods, oysters, and clams (Vijayan et al. 2005; Zhang et al. 2007; Y.-S. Chang et al. 2011; Vazquez-

Table 2. PCR results of prawn and sandfish samples from second cohabitation WSSV-positive *H. scabra* cohabited with WSSV-free *P. monodon* for 25 days.

dpi	Source	Tank					
		1		2		3	
		One-step	Nested	One-step	Nested	One-step	Nested
0	*P. monodon*	-	-	-	-	-	-
	H. scabra coelom	-	+	-	+	-	+
	tentacle	-	+	-	+	-	+
	intestine	-	+	-	+	-	+
	muscle	-	+	-	+	-	+
6	*P. monodon*	-	+	-	+	-	+
	H. scabra coelom	-	-	-	-	-	-
	tentacle	-	+	-	-	-	-
	intestine	-	-	-	-	-	-
	muscle	-	+	-	-	-	-
12	*P. monodon*	-	-	-	+	-	+
	H. scabra						
	coelom	-	-	-	-	-	-
	tentacle	-	-	-	-	-	-
	intestine	-	-	-	-	-	-
	muscle	-	-	-	-	-	-
18	*P. monodon*	-	-	-	-	No sample*	
	H. scabra						
	coelom	-	-	-	-	-	-
	tentacle	-	-	-	-	-	-
	intestine	-	-	-	-	-	-
	muscle	-	-	-	-	-	+
25	*P. monodon*	No sample*		No sample*		No sample*	
	H. scabra						
	coelom	-	-	-	-	-	-
	tentacle	-	-	-	-	-	-
	intestine	-	-	-	+	-	-
	muscle	-	-	-	+	-	-

*No sample recovered due to 100% mortalities.

Boucard et al. 2012; Desrina et al. 2013). Regarding the nested PCR positive results of the sandfish from the first cohabitation experiment, we were able to elucidate that sandfish bioaccumulated WSSV viral particles. These results were parallel to the experiment conducted by Y.-S. Chang et al. (2011) where clams (*Meretrix lusoria*) were immersed in rearing water inoculated with WSSV. Also, Vazquez-Boucard et al. (2012) were able to present and prove that oysters were able to bioaccumulate the viral particles in a WSSV infected shrimp grow-out farm. In both studies, different tissues such as gills and digestive tissues were PCR positive for WSSV.

Using the same set-up, the second cohabitation was conducted to verify if sandfish can be considered as potential vector or reservoir of WSSV. The WSSV (+) sandfish from the first cohabitation were used to potentially infect the WSSV (-) prawn. Eventually, after 6 dpi, all prawn from three replicate tanks

were nested PCR positive for WSSV while only sandfish (tentacle and muscle tissues) from tank 1 was nested PCR positive. Up until 12 dpi, there are still 2 replicate tanks that were nested PCR positive. In similar infection experiment conducted by Y.-S. Chang et al. (2011), in which WSSV-challenged clams (*Meretrix lusoria*) were fed to *Litopenaeus vannamei*, the bioassay showed the positive horizontal transmission of WSSV from the WSSV-challenged clams towards the WSSV-free shrimps. After 24-72 hours post challenged (hpc), shrimp samples were PCR positive to WSSV and mortalities were observed. In correlation with the results of Y.-S. Chang et al. (2011), bioaccumulation by bivalve mollusks and sandfish resulted in the horizontal transmission of WSSV. As suggested by Chou et al. (1995), horizontal transmission through water and feeding of infected shrimp is possible. Moreover, Flegel, Boonyaratpalin, and Withyachumnarnkul (1997) reviewed that the viability of the free virus in seawater is 3-4 days, and the virus can be spread by *P. monodon* ingestion or cohabitation. The viral load of the prawn from second cohabitation was not able to increase for our one-step PCR assay to detect since the starting viral load of sandfish was already low (Table 2). However, we can infer that the nested PCR positive sandfish was able to infect the prawn during the second cohabitation. Viability of the virus is strongly supported by the review paper of Desrina et al. (2022), in which the virus can be viable in infected animals (non-crustacean vector or reservoir), soil, and water in the absence of host species for several months. In relation to the current study, this corroborates the claim that WSSV (-) prawn can be horizontally infected when cohabited with WSSV (+) sandfish.

Based on the results of the first cohabitation, we elucidated that sandfish were able to bioaccumulate the WSSV viral particles when cohabited with WSSV (+) prawn. This is supported by the nested PCR positive results of the four target tissues (coelom, tentacle, intestine, and muscle). Relative to these findings, we considered sandfish as a potential non-crustacean vector or reservoir of WSSV since during the second cohabitation, WSSV (+) sandfish were able to infect WSSV (-) prawn.

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