RED SWAMP CRAWFISH (*PROCAMBARUS CLARKII*): AN ALTERNATIVE EXPERIMENTAL HOST IN THE STUDY OF WHITE SPOT SYNDROME VIRUS

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Received August 24, 2000; accepted November 2, 2000

Summary. – The pathogenicity of white spot syndrome virus (WSSV) for the red swamp crawfish (*Procambarus clarkii*) was investigated after infection by intramuscular (i.m.) injection and oral route. The cumulative mortality of crawfish injected i.m. with WSSV reached 100% in 5 days. After oral feeding WSSV-infected kuruma shrimp (*Penaeus japonicus)* muscle tissues to the crawfish the cumulative mortality of this host reached 100% in 11 days. On reinfection trials, all the crawfish fed WSSV-infected crawfish muscle tissues died in 9 days. All the shrimp injected with a filtrate of infected crawfish heart tissues died in 12 days with typical signs of white spot syndrome (WSS). Electron microscopy clearly demonstrated that WSSV propagated in the cells of the crawfish midgut. This study showed that the red swamp crawfish can be used as alternative experimental host in the study of WSSV.

Key words: white spot syndrome; white spot syndrome virus; kuruma shrimp; red swamp crawfish; Penaeus japonicus; Procambarus clarkii

A severe viral infection of kuruma shrimp, *Penaeus japonicus*, with high mortality was first observed in 1993. This infection was named penaeid acute viremia, caused by a penaeid rod-shaped DNA virus (PRDV) (Inouye *et al.*, 1994; Takahashi *et al.*, 1994; Inouye *et al.*, 1996). The infection inflicts extensive economic damage to the marine shrimp culture industry in Japan. Similar infections with various names have been reported in cultured black tiger shrimp, *P. monodon* and other marine shrimp in Thailand (Wongteerasupaya *et al.*, 1995), Taiwan (Chou *et al.*, 1995), China (Zhan *et al.*, 1998), Korea (Park *et al.*, 1998), India (Karunasagar *et al.*, 1998), and the USA (Lightner, 1996). The infections in these reports are thought to be the same or closely related due to typical external symptoms and histopathological changes in infected animals, and the morphology and characteristics of the virus. Lightner (1996) has grouped these infections under the term white spot syndrome (WSS), caused by WSSV.

WSS has spread rapidly in kuruma shrimp cultures in Japan. WSSV has been detected in wild population of shrimp, crabs and other decapods by polymerase chain reaction (PCR) (Lo *et al.*, 1996; Maeda *et al.,* 1998). In freshwater environment, many researchers also have made an intensive effort to investigate the host range of WSSV among crustaceans (Lo *et al.,* 1996; Chang *et al.*, 1998; Wang *et al.*, 1998; Rajendran *et al.,* 1999). Although Wang *et al.* (1998) did not find WSSV infection in wild red swamp crawfish in rivers, WSSV was detected in this experimentally infected host by PCR and *in situ* hybridization. However,

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Abbreviations: i.m. = intramuscular(ly); PCR = polymerase chain reaction; p.i. = post infection; PRDV = penaeid rod-shaped DNA virus; TEM = transmission electron microscopy; WSS = white spot syndrome; WSSV = WSS virus

these results did not provide enough evidence for the propagation of this virus in the crawfish.

The aim of the present study was to demonstrate the propagation of infectious WSSV in cells of freshwater red swamp crawfish and to discuss the possibility of utilization of this crawfish as an alternative experimental host in the study of WSSV.

Kuruma shrimp of average body weight of 10 g was purchased from a shrimp farm in Yamaguchi Prefecture where WSS has not been reported. Red swamp crawfish of average body weight of 20 g was purchased from a rearing farm in Kanagawa Prefecture. On arrival at the laboratory, the animals were randomly tested using a 2-step PCR to prove that they are WSSV-free (Maeda *et al.*, 1998). The shrimp and crawfish were kept in aquaria at 20–22°C and were fed commercial shrimp dry pellet daily until use.

For PCR analysis, hearts of shrimp and crawfish were extirpated and DNA was extracted from by DNAZOL (Gibco-BRL) according to the manufacturer's instructions. The primer sets for the 1- and 2-step PCR and the reaction conditions were the same as those reported earlier (Takahashi *et al.*, 1996; Maeda *et al.*, 1998).

To infect red swamp crawfish in simple infection trials, a WSSV inoculum was prepared from moribund kuruma shrimp experimentally infected with WSSV and PCR-positive for WSSV (Maeda *et al.*, 1998). These were frozen and kept at -80°C until use. Infection of crawfish with WSSV was carried out by i.m. injection or oral administration.

For i.m. injection trials, three pairs of lymphoid organs from WSSV-infected shrimp were isolated, homogenized in 10 volumes of sterile 50% (v/v) seawater and centrifuged at 3,000 x g for 15 mins at 4°C. The supernatant was filtered through a 0.45 µm pore-size filter. A 100-fold dilution of the filtrate (0.1 ml) was injected into the muscle of the third abdominal segment of crawfish. The control group was injected with a filtrate prepared from the lymphoid organ of healthy kuruma shrimp. For oral infection trials, 10 g of WSSV-infected kuruma shrimp muscle tissue was fed to 10 crawfish everyday. The control group was fed the uninfected shrimp muscle tissue. Each group consisted of 10 crawfish that were kept in 60-liter glass aquaria at 25°C. After infection, the mortality was monitored and moribund or dead crawfish were examined by PCR.

For histopathological examination, the moribund crawfish during reinfection trial were dissected, and the stomach and midgut were isolated, fixed (Bell and Lightner, 1988), dehydrated and embedded in paraffin. Paraffin sections were made and stained with hematoxylin and eosin.

For transmission electron microscopy (TEM), the isolated organs were cut into small pieces, prefixed in 2.5% glutaraldehyde and 2.5% paraformaldehyde in 0.1 mol/l cacodylate buffer pH 7.4 for 2 hrs at 4°C, and postfixed in 2% osmium tetroxide for 1 hr at room temperature. The fixed tissues were dehydrated and embedded in Epon resin. Ultrathin sections were prepared, stained with uranyl acetate and lead citrate, and observed by a Hitachi H-7100 transmission electron microscope at 75 kV.

The crawfish, injected i.m. with the WSSV inoculum from kuruma shrimp, began to die on day 2 post infection (p.i.). The cumulative mortality of the crawfish reached

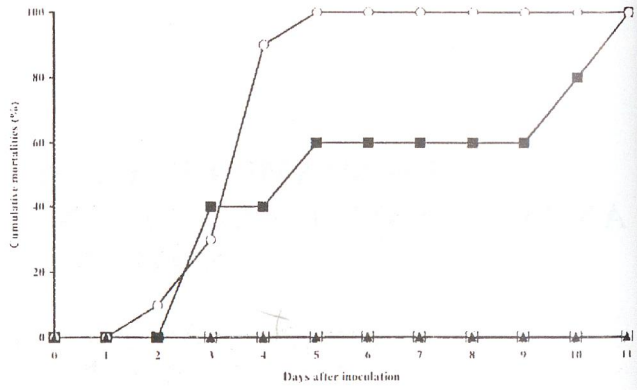

Fig. 1
Cumulative mortality of red swamp crawfish experimentally infected with WSSV derived from kuruma shrimp
Infection by i.m. injection (empty circles) and oral route (full squares); mock-infection by i.m. injection and oral route (empty squares and full triangles, respectively).

100% on day 5 p.i. No crawfish died in the control group (Fig. 1). The perorally infected crawfish started dying on day 3 p.i. The cumulative mortality reached 100% on day 11 p.i. No crawfish died in the control group. The infected crawfish showed reduction in food consumption and low movement activity. All dead crawfish were positive for WSSV in the 1-step PCR.

The dead PCR-positive crawfish were used as the source of the virus for peroral reinfection trials in crawfish. The muscle tissue of these crawfish was fed to healthy crawfish in the same way as described above. The control group was

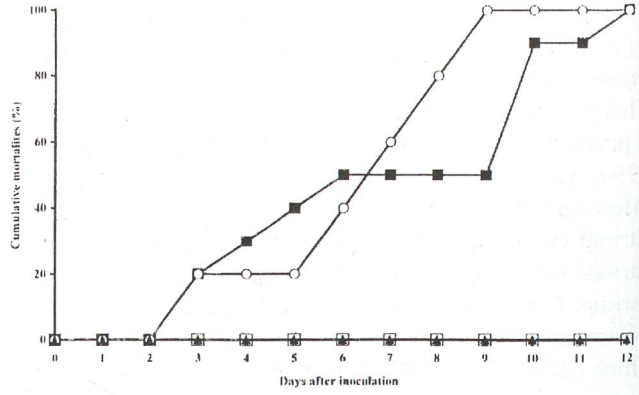

Fig. 2
Cumulative mortality of red swamp crawfish and kuruma shrimp experimentally infected with WSSV derived from red swamp crawfish
Red swamp crawfish infected with WSSV by oral route (empty circles). Kuruma shrimp infected with WSSV by i.m. injection (full squares). Red swamp crawfish and kuruma shrimp mock-infected by oral route (open squares) and i.m. injection (full triangles).

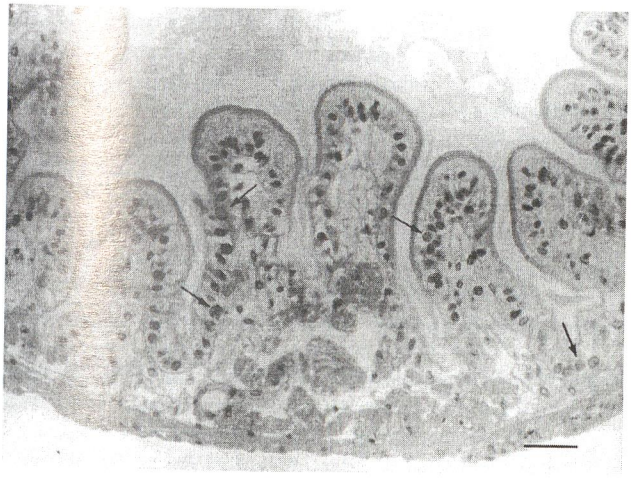

Fig. 3
Photomicrograph of cross section of midgut cells of red swamp crawfish perorally infected with WSSV derived from red swamp crawfish
Cells (arrows) show hypertrophied nuclei. Bar = 50 mm.

fed the uninfected crawfish muscle tissue. The reinfected crawfish showed a cumulative mortality of 100% in 9 days (Fig. 2). All dead crawfish were positive for WSSV in the 1-step PCR.

The dead reinfected crawfish was used in another reinfection trial (infection of kuruma shrimp with the virus from red swamp crawfish) as source of the virus. The hearts were removed from the dead crawfish and employed for preparation of a filtrate. The filtrate (0.1 ml) was injected into the muscle of the third abdominal segment of healthy kuruma shrimp (n = 10). The infected kuruma shrimp died in 12 days showing typical external symptoms with white spots on the carapace (Fig. 2).

Fig. 3 shows hypertrophied nuclei in the midgut columnar epithelial cells of crawfish from the reinfection trial. Many rod-shaped, enveloped, non-occluded virions were observed in the midgut by TEM (Fig. 4A). The size of mature virions surrounded by envelope was about 320 nm in length and about 110 nm in width, which is typical of WSSV. At higher magnification, the nucleocapsid precursors and many partially enveloped nucleocapsids were observed in nucleoplasm of crawfish from the reinfection trial (Fig. 4B).

WSSV has been reported to have a wide host range in marine crustaceans (Lo *et al.*, 1996; Momoyama *et al.*, 1997; Maeda *et al.*, 1998; Wang *et al.*, 1998). Experimental infection trials have demonstrated that some marine crustaceans are susceptible to WSSV (Kanchanaphum *et al.*, 1998; Supamattaya *et al.*, 1998). There are some reports on the occurence of WSSV infection in some crustaceans in freshwater environment (Lo *et al.*, 1996; Chang *et al.*, 1998; Wang *et al.*, 1998; Rajendran *et al.*, 1999). Although a WSSV infection of red swamp crawfish was not shown in wild freshwater in Taiwan, it was found in this host in experimental infection by PCR (Wang *et al.*, 1998) and *in situ* hybridization (Chang *et al.*, 1998). However, these authors did not provide any evidence of the propagation of infectious WSSV under these conditions.

The results of our reinfection trials proved that infectious WSSV was propagated in the red swamp crawfish. Furthermore, the presence of nucleocapsid precursors and

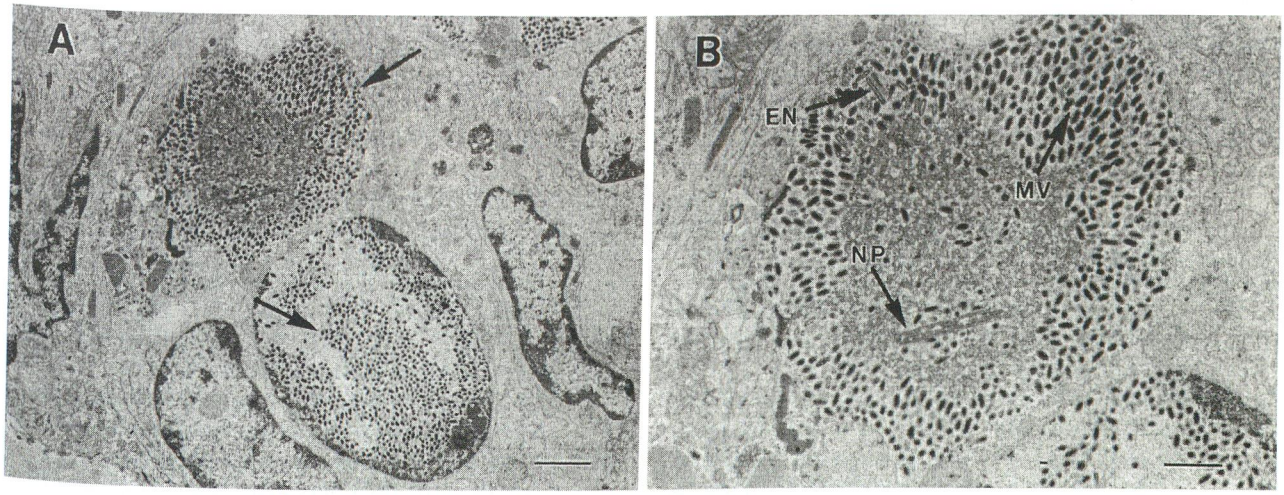

Fig. 4
TEM of virus particles found in midgut cells of red swamp crawfish perorally infected with WSSV derived from red swamp crawfish
(A) Many mature and immature virions (arrows) in nuclei with marginated chromatin. Bar = 2 mm (lower magnification). (B) Mature and immature virions in nucleoplasm. EN = empty nucleocapsid; NP = nucleocapsid precursors; MV = matured virions. Bar = 1 mm (higher magnification).

partially enveloped nucleocapsids was observed in the midgut cells of red swamp crawfish in reinfection trials, that is indicative of propagation of WSSV and similar to the findings of Durand *et al.* (1997). Our conclusion is supported by the data published earlier (Chang *et al.*, 1998; Wang *et al.*, 1998). Our results also show that the pathogenicity of WSSV for kuruma shrimp can be maintained by a passage in red swamp crawfish despite the species differences of these hosts and their rearing conditions.

Our study also showed that the susceptibility of the both compared hosts is the same by i.m. as well as oral route. It can be concluded that red swamp crawfish can be employed as alternative host of WSSV. Compared with kuruma shrimp, the advantages of red swamp crawfish are as follows: it is easier to keep them in small aquaria, their maintenance is cheaper, and they enable studies in interior, because they do not need seawater.

Note of the Editor-in-Chief. Neither WSSV nor PRDV are recognized as viruses or virus species by the presently valid virus taxonomy (Van Regenmortel *et al.*, 2000).

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