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White spot syndrome virus (WSSV) infection in tiger shrimp *Penaeus monodon*: A non-lethal histopathological rapid diagnostic method using paraffin and frozen sections

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Abstract. White spot syndrome virus (WSSV) infection was induced in tiger shrimp, *Penaeus monodon*, under laboratory conditions, and histopathological changes in subcuticular epithelial cells of the eye stalk and pleopod were studied sequentially at different time post-challenge. Routine histological techniques using paraffin embedded tissues, as well as frozen tissues, were used to document WSSV infection. Histological manifestations such as cellular hypertrophy in the subcuticular epithelial cells of the eyestalk and pleopod could be detected as early as 18 h post-infection (p.i.) before the manifestation of clinical signs of the disease. However, no histopathological changes could be detected before 18 h p.i.. Hypertrophy of the nuclei in the epithelial cells was pronounced after 24 h p.i. Marked necrosis, and eosinophilic intranuclear inclusions, characteristic of early stages of WSSV infection were observed between 24–36 h p.i. Clinical signs of the disease appeared at 48 h p.i. The presence of WSSV at early asymptomatic stages of p.i. has been tested in parallel samples using polymerase chain reaction, for further confirmation of WSSV. This paper discusses the potential of a non-lethal and rapid histopathological diagnostic method to document WSSV infection, using the eyestalk or pleopod, when expensive DNA based diagnostics are not available or affordable.

Introduction

During the last few years, white spot disease (WSD) has spread worldwide and caused large-scale mortalities and severe damage to shrimp culture, particularly in Asia leading to massive economic losses (Lightner 1996; Flegel 1997). The causative organism has been identified as a bacilliform virus, widely known as white spot syndrome virus (WSSV) (Lightner 1996). White spot syndrome virus is the most virulent and the largest animal virus known to affect cultured shrimp (Van Hulten et al. 2001). In the absence of an effective treatment against the WSSV, preventive measures using early and rapid diagnostics has a crucial role in the management of WSD.

DNA-based novel diagnostic tools, such as PCR, and immunological methods, such as monoclonal antibodies (MAbs), have already been developed, and are being used as health management tool (Lightner 1996). However, these diagnostic tools are expensive and require sophisticated laboratory support. A non-lethal, rapid and cost-effective method, which can be performed in an ordinary laboratory, can be useful in routine health monitoring. Hence an effort has been made to study and compare the use of the classical histopathological method and polymerase chain reaction (PCR), in the early diagnosis of WSSV.

Histopathological evidence has shown that this virus replicates in tissues of ectodermal and mesodermal origin only (Chou et al. 1995; Lightner 1996; Karunasagar et al. 1997; Mohan et al. 1997; Rajendran et al. 1999, Yoganandhan et al. 2003; Vijayan et al. 2003). Evidence of histopathological manifestations in the target tissues is one of the criteria used in the diagnosis of WSSV infection (Lightner 1996; Wang et al. 1997). During our investigation on the histopathology of the WSSV infection, we have noticed obvious histological changes characteristic of WSSV in the cuticular epidermis of eyestalk and pleopod (Rajendran et al. 1998, 1999), which is in agreement with the observation of Chang et al. 1996 and Lo et al. 1997. Epithelial cells of the eyestalk and pleopod are the preferred tissues of WSSV, and the initial sites of viral infection (Chang et al. 1996; Lo et al. 1997). This paper describes the sequential pathological changes in the eye stalk and pleopod tissue of WSSV-infected tiger shrimp, *Penaeus monodon*, and the possibility of using histological and frozen sections of these tissues as a non-lethal histological technique for an early diagnosis of the disease, when the DNA-based diagnostic (Chang et al. 1996; Durand et al. 1996; Tapay et al. 1999) facilities are not available, or affordable.

Materials and methods

Collection of experimental animals

Healthy sub-adults of *P. monodon*, (8–12 g), were collected from a farm located near Chennai, Tamil Nadu, India, without any previous history of white spot disease. These shrimp were kept in 1000-l fiberglass tanks and acclimatized for one week (salinity 20–25 ppt; ambient temperature 28–30 °C; pH 8.0–8.20). The shrimp were fed artificial pelleted feed (35% protein). The experimental animals were screened (20 random shrimps from a group of 200) for white spot virus using PCR (Takahashi et al. 1996). After screening, a total of 120 healthy animals were used in this experiment.

Preparation of viral inoculum

Samples of WSSV were collected from two farms at Nellore, Andhra Pradesh, India, during an epizootic in 1999. Tissue samples from the gills, stomach and

epidermal layer were prepared from the infected samples and stored at −80 °C. These samples were subsequently used to induce the disease, following the method of Takahashi et al. (1994). The infected tissues were homogenized in sterile marine phosphate buffered saline (PBS), centrifuged at 2000 rpm at 4 °C for 10 min, and the supernatant was filtered through a 0.45-μm filter fitted to a syringe. The filtrate was diluted (1:100) and injected into the second abdominal muscle of healthy shrimp (0.1% v/w). A negative control group was maintained by injecting sterile marine PBS intramuscularly into healthy shrimp.

Experimental infection and sequential pathology

Sampling was performed at 6, 12, 18, 24, 36, 48 and 72 h post-challenge. Three 500-l fiberglass tanks were filled with filtered seawater with salinity between 20 and 25 ppt, ambient temperature of 28–30 °C and pH 8.0–8.2. Each tank was stocked with 40 experimental shrimp. The replicated treatment groups were kept in tanks 1 and 2, while the third tank held the negative control. Inoculum of WSSV was administered intramuscularly using 1 ml tuberculin syringe fitted with a 22-gauge needle. At each collection time, three animals were removed from the replicate and the control groups (one each for normal histology using paraffin sections, frozen and PCR method of diagnosis).

Histology

The eyestalk (left eyestalk without compound eye) and pleopod (one of the fourth pair) were excised aseptically using standard biopsy procedures (Bell et al. 1990) and immediately fixed by submersion in Davidson's fixative. Similarly, the eyestalk and pleopod were removed for the preparation of cryosections and PCR, respectively. Histological observations were made according to the procedure outlined by Bell and Lightner (1988). Sections of 5–6 μm thickness were stained with hematoxylin and eosin (H&E).

For rapid processing of tissues, frozen sections were cut using a Leica CM 1800 Cryostat as per the following procedure. Fresh tissues excised from normal and experimentally-infected shrimp were placed in tissue embedding medium (Leica, OCT cryo embedding medium supplied by Leica, Germany), then kept in the freezing chamber at −18 to −20 °C for 35 min, and sectioned at 6–8 μm thickness. Sections were attached to warm slides and air-dried. These sections were fixed in chilled acetone and stained with Rapid H&E (Culling 1985). Photomicrographs of frozen and paraffin slides stained with H&E were taken using a WILS MPS 46 camera fitted to a Leitz Laborlux S microscope.

Polymerase chain reaction

WSSV infection at different time points was confirmed by PCR technique. Template DNA was extracted following the procedures of Vijayan et al. (1998)

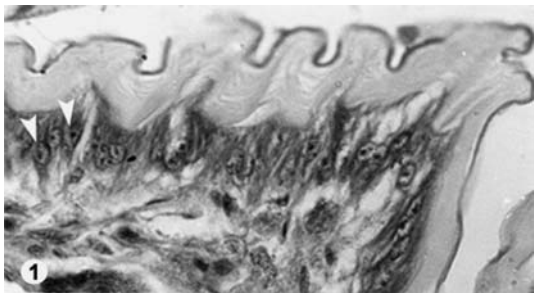

Figure 1. Cuticular epithelium of a healthy (control) *P. monodon* eyestalk. Arrow indicates the normal subcuticular epithelial cells with normal nuclei. Paraffin section – H and E, 1000×.

from the eyestalks (without compound eye) and pleopods removed from infected and control shrimp at various time intervals. The PCR was carried out according to Takahashi et al. (1996) to detect WSSV infection. PCR products were electrophoresed in 0.8% agarose gel stained with ethidium bromide, and visualized under ultraviolet transillumination.

Results

The WSSV-induced pathological changes were detected in the subcuticular epithelial cells of the eyestalk and pleopod in clinically normal shrimp, as early as 18 h post-infection (p.i.). At this time point, the nuclei of the epithelium showed discernible hypertrophy (Figures 2 and 3), while the control tissues showed normal cell architecture (Figure 1). Nuclear hypertrophy, central chromatin diminution and margination were distinct in the WSSV infected cells. No indication of a WSSV infection was detected at any earlier time point. As infection progressed, hypertrophied nuclei containing intranuclear, eosinophilic inclusions, characteristic of the early stage of WSSV infection, were noticed at 24 and 36 h p.i. Mortality and other clinical signs of the disease were detected in the experimental animals only after 48 h p.i. The appearances of white spots over the exoskeleton, pinkish-red body color, lethargy and anorexia are the common clinical signs associated with the WSSV. Shrimp that survived up to 48 h p.i. showed marked lesions in the subcuticular epithelial cells in which numerous, large, highly basophilic inclusion bodies were seen (Figures 4 and 5).

Results of the electrophoresis of the PCR products of various post-infection stages and positive and negative controls are given in Figure 6. Using PCR, WSSV infection could be diagnosed as early as 6 h p.i., demonstrated by a consistent amplification of the WSSV-DNA (643 bp) from the eyestalk and pleopod collected from shrimp at 6–72 h p.i. The negative control group was PCR-negative.

Observation of the paraffin and frozen sections in the present study showed that the cellular changes observed in both procedures are comparable in terms

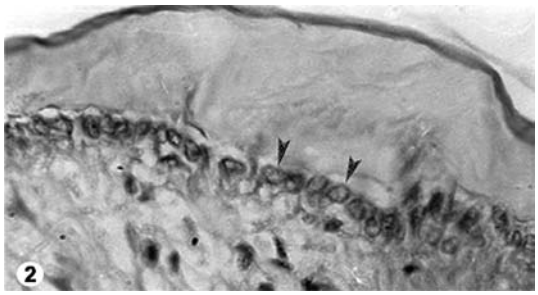

Figure 2. Cuticular epithelium of the eyestalk of *P. monodon* experimentally infected with WSSV, 18 h post-infection, arrow shows hypertrophied nuclei. Paraffin section – H and E, 1000×.

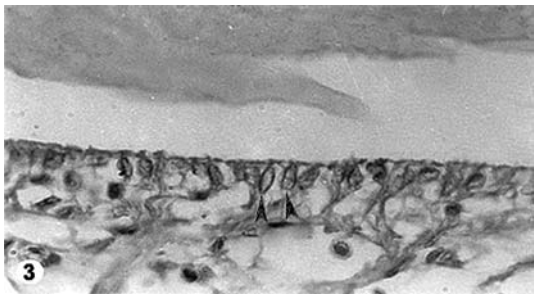

Figure 3. Cuticular epithelium of the eyestalk of experimentally infected *P. monodon* with WSSV, 18 h post-infection, arrow shows hypertrophied nuclei. Frozen section – H and E, 1000×

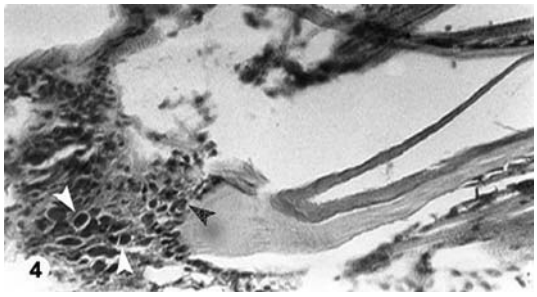

Figure 4. Cuticular epithelium of the pleopod of experimentally infected *P. monodon* with WSSV, 72 h post-infection. Arrow shows degenerated and heavily infected cuticular epithelial cells showing large basophilic inclusions characteristic of WSSV infection. Paraffin section – H and E, 400×.

of histological changes, which were subsequently used for WSSV diagnosis (Figures 2–5). By using routine paraffin sections, a conclusive diagnosis was obtained at 48–72 h post-sampling, whereas the histological procedure adopted in the present study using frozen sections and rapid H&E staining was completed in 4 h, enabling an early diagnosis. The discernible histopathological

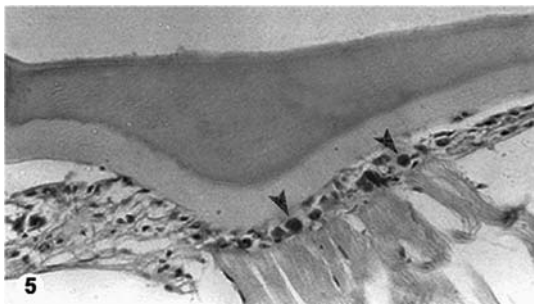

Figure 5. Cuticular epithelium of the pleopod of experimentally infected *P. monodon* with WSSV, 72 h post-infection. Arrow shows degenerated and heavily infected cuticular epithelial cells showing large basophilic inclusions characteristic of WSSV infection, Frozen section – H and E, 400×.

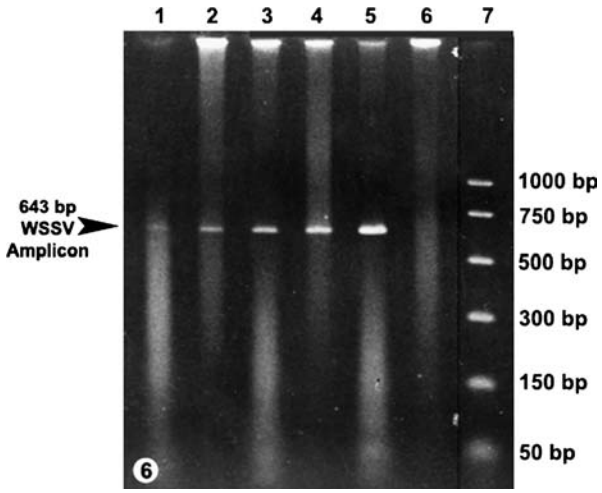

Figure 6. Electrophoretic pattern of PCR product. Pleopods collected at different time points of post-infection from *P. monodon* experimentally infected with WSSV have been used as the source of template DNA. Lanes 1-4 correspond to 6, 12, 18 and 24 h post-infection, respectively; lane 5 corresponds to positive control; lane 6 negative control and lane 7 molecular weight markers (Samples from 36, 48 and 72 h were not included).

manifestations, as early as 18 h p.i., indicated that the WSSV infection could be detected before the manifestation of clinical signs of the disease, though the diagnosis was not as specific and sensitive as PCR (Table 1).

Discussion

We selected the eyestalk and pleopod as the target tissues for WSSV detection, for two reasons. First, pleopod and eyestalk consisted of subcuticular epithelial

Table 1. Detection of WSSV in the eyestalk and pleopod using sequential pathology and PCR, in experimentally infected *P. monodon*.

p.i.	Clinical condition	WSSV detection by paraffin sections	WSSV detection by frozen sections	WSSV detection by PCR	Mortality
0 h	Normal	Negative	Negative	Negative	Nil
6 h	Normal	Negative	Negative	Positive	Nil
12 h	Normal	Negative	Negative	Positive	Nil
18 h	Normal	Positive (+)	Positive (+)	Positive	Nil
24 h	Normal	Positive (+)	Positive (+)	Positive	Nil
36 h	Normal	Positive (++)	Positive (++)	Positive	Nil
48 h	Lethargic, Anorexia	Positive (+++)	Positive (+++)	Positive	Yes
72 h	Lethargic; Anorexia, Moribund, Pale reddish color; White spots	Positive (+++)	Positive (+++)	Positive	Yes
Control groups					
0–72 h	Normal	Negative	Negative	Negative	Nil

p.i., Post-infection time; +, Early infection; ++, Advance infection; +++, Severe infection.

cells, which are one of the target tissues of this virus. Nash and Akarajamorn (1995) and Chang et al. (1996) have reported that cuticular epithelial cells are one of the most preferred sites of the WSSV, and among the first sites the virus sets in. Second, removal of a pleopod or eyestalk is not harmful to the shrimp, hence can be used for non-lethal diagnosis in the screening of WSSV. When eyestalk removal is carried out to induce maturation, the stalk of the removed eye can be used for the diagnosis of WSSV. When eyestalks are not available, as in the case of gravid females or hatchery-reared broodstock, pleopods can be used. Removal of one of the pleopods did not hamper the maturation process or the health status of the shrimp, if the standard excision methods are followed.

The cellular pathology of WSSV infected *P. monodon* observed in the present study is in agreement with the observation of Nash and Akarajamorn (1995), Chang et al. (1996) and Yoganandhan et al. (2003). At the very early asymptomatic and subclinical stages of infection, up to 18 h p.i., only the PCR gave positive results, indicating the sensitivity and advantage of PCR over histological methods. This is the case in broodstock screening and quarantine checks, where a distinct histomorphological manifestation is absent, and hence PCR is the preferred diagnostic choice. However, from 18 h p.i., the majority of the subcuticular epidermal cells showed nuclear hypertrophy and chromatin diminution and margination, characteristic of WSSV infection. In shrimp viral infections other than WSSV, the nuclear hypertrophy, chromatin diminution and margination were not distinct and uniform among the subcuticular epidermis (Lightner 1996). The present observations on the sequential histological changes in the tissues of the eyestalk and pleopod revealed that these tissues could be used for an early diagnosis of WSSV infection. Therefore, it is suggested that in situations where the DNA-based diagnostic techniques are not

available or affordable due to high operating cost, the histological method using frozen sections could be a useful tool, as diagnosis can be made within 4 h. Further, in the epidemiological studies, where the sensitivity level of PCR is not required, the rapid histological diagnosis using frozen sections and rapid staining can be used. Histopathology on frozen sections has been a routine practice in human as well as animal diagnostics. However, in aquatic animal health, where histopathological diagnostic forms an important tool, the potential of cryosections has not been explored in any detail. The present report is the first attempt to use cryosections as a rapid histopathological diagnostic in shrimp health.

Screening of broodstock shrimp for WSSV is one of the effective ways to check the vertical transmission of disease through the hatcheries (Hsu et al. 1999). Monitoring of WSSV in the grow-out ponds has been suggested as one of the health monitoring methods in the farming of shrimp (Peng et al. 2001). The present study has indicated that histomorphological changes in the subcuticular ectoderm of eyestalk and pleopod has diagnostic potential, and can be used as a non-lethal and rapid diagnostic tool for the routine screening of shrimp broodstock and farm reared stocks.

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