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Comparison of dot blot and PCR diagnostic techniques for detection of white spot syndrome virus in different tissues of *Penaeus monodon*

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Abstract

Comparison of PCR and dot blot diagnostic techniques for detection of white spot syndrome virus (WSSV) was made on different tissues of infected *Penaeus monodon* including eye stalk, eye stalk with eye, gills, cuticle, pleopod, periopods, uropods and telson. Dot blots of crude DNA extracted from infected tissue samples showed positive reactions with all the samples; however, the sensitivity of the dot blot was reduced with the purification of DNA samples extracted from pleopod, telson and uropod. PCR was found to be more sensitive when compared to dot blot. Both crude DNA and purified DNA samples extracted from all the tissues except for eye stalk with eye showed single step nested PCR positive reaction. The amplification of all or either of the three bands of 941 bp, 525 bp and 204 bp size varied with the tissues analysed. The severity of infection assessed by PCR amplification was found to be maximum in cuticle and telson followed by gill. Other tissues such as eye stalk, pleopod, periopods and uropod were observed to have mild infection. The maximum intensity of the PCR product was for the smallest amplified product of 204 bp followed by 525 bp and the weakest intensity was observed for the 941 bp size. The limitation of PCR due to inhibiting factors present in tissues could be overcome with the use of dot blot which gave positive reaction from the DNA extracted from eye stalk including the eye but yielded no amplification by PCR.

Keywords: *P. monodon*; White spot syndrome virus; Dot blot; PCR

1. Introduction

Outbreaks of viral disease due to white spot syndrome virus (WSSV) in *Penaeus monodon* since 1994 still continues to be major problem for the shrimp aquaculture industry worldwide. In spite of extensive WSSV research, till date no therapy has been found that can contain the disease. Since large scale mortalities due to WSSV occur within 3 to 10 days in shrimp culture (Lightner, 1996), good management practice is the only option which can prevent the viral outbreaks (Flegel et al., 1997). Major emphasis is being laid for early detection of the virus by sensitive and accurate molecular diagnosis, which helps in maintaining healthy brood stock and in production of good quality shrimp larvae. Simple and rapid diagnostic tests help in taking suitable preventive measures against the disease occurrence. Several diagnostic techniques such as histopathology, PCR, immunoblot, in situ hybridization and dot blot have been developed and are widely used for

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detection of WSSV. PCR amplification as described by Takahashi et al. (1996) was one of the early works reporting successful detection of WSSV. There are numerous other reports by various workers using different sets of primers for PCR amplification of WSSV (Lo et al., 1996; Wongteerasupaya et al., 1996; Kim et al., 1998). A non-radioactively labeled digoxigenin (DIG) DNA probe has been used for dot blot analysis for various shrimp viruses such as *P. monodon* baculovirus (MBV) (Lu et al., 1993), infectious hypodermal and hematopoietic necrosis virus (IHHNV) (Nunan et al., 2000), WSSV (Edgerton, 2004; Dupuy et al., 2004), in situ hybridization for Taura syndrome virus (TSV) (Nunan et al., 1998), WSSV (Nunan and Lightner, 1997; Durand et al., 1996; Wongteerasupaya et al., 1996; Chapman et al., 2004) and Southern blot hybridization of WSSV (Wang et al., 2000).

In spite of various diagnostic tests for detection of WSSV that are available today, the choice of these tests by shrimp farmer or researcher depends on number of factors. PCR, though a very sensitive technique, can lead to false positive results if handling of samples is incorrect. In addition, it has also been reported that PCR inhibitory factors present in the tissue also have significant effect on the results (Cunningham, 2002). Whereas, reaction of shrimp endogenous peroxidase with the substrate is reported to be the cause of false positive results in immunoblot assays to detect WSSV (Zhan et al., 2003). The objective of the present study was to compare dot blot and PCR to know the sensitivity of these diagnostic techniques to detect WSSV from different infected tissues of *P. monodon*.

2. Materials and methods

2.1. Shrimp samples

White spot infected *P. monodon* shrimps were collected from disease outbreaks at shrimp farms located in the east coast regions of India. Samples were either frozen or preserved in ethanol.

2.2. Extraction of viral DNA

Infected tissues of *P. monodon* eye stalk, eye stalk with eye, gills, cuticle, pleopod, periopods, uropods and telson were homogenized separately in 300 μl of DNA extraction buffer provided in a single tube WSSV nested PCR detection kit (Bangalore Genei Ltd.). The homogenized tissue samples after heating in boiling water for 2 min were centrifuged at 11,000×*g* for 5 min at room temperature and 10 μl of supernatant (crude DNA sample) was collected both for PCR and dot blot analysis. The remaining supernatant was phenol chloroform extracted once before ethanol precipitation at −20 °C. The DNA pellet (purified sample) was washed once with 70% ethanol and dissolved in 20 μl of distilled water.

2.3. Dot blot hybridization

The PCR product of 643 bp amplified from the WSSV infected gill tissue of *P. monodon* was used for generating probe by DIG random primed DNA labeling using DIG DNA labeling and detection kit (Roche) following manufacturer's protocol. The primers (30 pmol each) used in this study were as described by Takahashi et al. (1996): primer I: 5′GACAGAGATATGCACGCCAA 3′, primer II: 5′ACCAGTGTTTCGTCATGGAG 3′. PCR cycle consisted of initial denaturation at 95 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, followed by final extension step of 72 °C for 10 min. Quantification of the DIG labeled probe was estimated by comparing DIG labeled control provided in the kit. One microliter of serially diluted DIG labeled control DNA with final concentration of 100 pg μl^{-1}, 10 pg μl^{-1}, 1 pg μl^{-1}, 0.1 pg μl^{-1} and 0.01 pg μl^{-1} was spotted on nylon membrane. One microliter of corresponding dilutions of experimental probe was spotted in the second row on the same nylon membrane for comparison. The spotted DNA was fixed on the membrane by cross linking with UV light.

WSSV infected *P. monodon* tissues such as eye stalk, eye stalk with eye, gills, cuticle, pleopod, periopods, uropods and telson were used for dot blot analysis. Both crude DNA sample (1.0 μl) of the tissues obtained after boiling in DNA extraction buffer and the purified DNA sample (1.0 μl) obtained after phenol chloroform extraction was used for spotting on the nylon membrane. Crude DNA and purified DNA extracted from gill tissues of healthy *P. monodon* were used as negative controls. After fixing the spotted DNA on the membrane by cross linking with UV light, prehybridization, hybridization and detection with NBT/BCIP for the dot blot experiments were done as per the protocol described in the kit.

2.4. PCR

Crude DNA sample (1.5 μl) of the tissues obtained after boiling in DNA extraction buffer and the purified DNA sample obtained after phenol chloroform

extraction was used as template in the PCR reaction. The PCR reaction was carried out as per the manufacturer's protocol (Single tube WSSV nested PCR detection kit, Bangalore Genei Ltd.). PCR cycle consisted of initial denaturation at 95 °C for 2 min followed by 30 cycles of 94 °C for 1 min, 58 °C for 1 min, 72 °C for 1 min, followed by final extension step of 72 °C for 10 min.

3. Results

3.1. Dot blot

Quantification of the experimental DIG labeled probe on comparison with the DIG labeled control DNA showed positive reaction up to 0.1 pg μl^{-1} dilution. The intensity of the colour development of the experimental probe was equal to the control labeled DNA (Fig. 1A).

Dot blot of crude DNA extracted from infected tissue samples of eye stalk, eye stalk with eye, gills, cuticle, pleopod, periopods, uropods and telson showed positive reactions. In case of purified DNA, colour development was observed with the DNA extracted from eye stalk, eye stalk with eye, gills, cuticle and periopods. No colour development was observed with purified DNA extracted from pleopod, telson and uropod (Fig. 1B). The sensitivity of the dot blot was estimated by diluting the purified DNA extracted from infected gill tissue. The WSSV probe could give visible colour reaction till 1:100 dilution of the gill tissue (Fig. 1C).

3.2. PCR

Both crude DNA and purified DNA samples extracted from all the tissues except from eye stalk with eye showed PCR positive reaction. Crude DNA and purified DNA extracted from cuticle and telson showed all the three amplified PCR products of 941 bp, 525 bp and 204 bp. Two PCR products of 525 bp and 204 bp were observed with crude DNA extract and purified DNA extract of gills. Single band amplification of 204 bp PCR product was observed with crude DNA extract and purified DNA extract of eye stalk, pleopod, periopods and uropod (Fig. 2A and B).

4. Discussion

WSSV tissue specificity using DIG labeled WSSV probe by in situ hybridization has been reported. Nunan and Lightner (1997) observed positive reaction in nuclei of the cuticular epithelial cells and connective tissue cells, less frequently in antennal gland epithelium, lymphoid organ sheath cells, hematopoietic tissues and in fixed phagocytes of heart. No probe reaction was noticed in hepatopancreas and midgut mucosal epithelia as these tissues are not targeted by WSSV. In the present study, dot blot positive reaction from the crude DNA extracted from all infected tissue samples such as eye

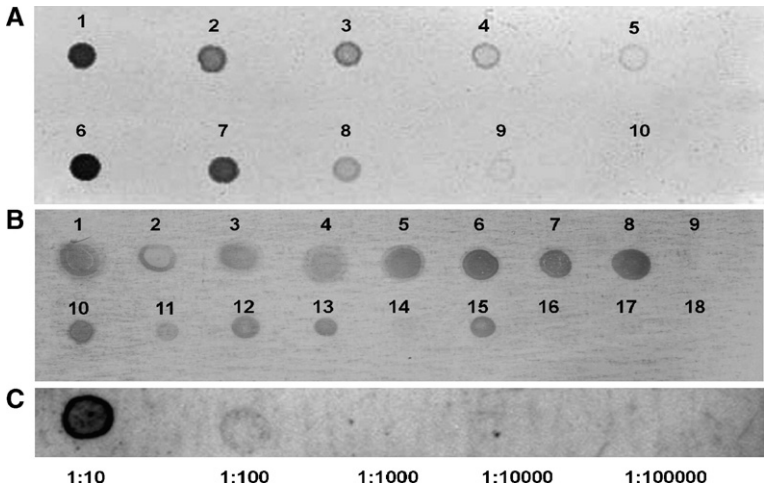

Fig. 1. Dot blot of WSSV. (A) Quantification of DIG labeled probe using 10-fold dilution of control DIG labeled probe ranging from 100 pg μl^{-1} to 0.01 pg μl^{-1} (1–5). Ten-fold dilution of experimental DIG labeled probe (6–10). (B) Dot blot hybridization of WSSV infected tissues. Crude DNA extracted from (1) eye stalk, (2) eye stalk with eye, (3) gills, (4) cuticle, (5) pleopod, (6) periopods, (7) uropods, (8) telson, (9) negative control of DNA extracted from healthy gill tissue. Purified DNA extracted from (10) eye stalk, (11) eye stalk with eye, (12) gills, (13) cuticle, (14) pleopod, (15) periopods, (16) uropods, (17) telson, (18) negative control of DNA extracted from healthy gill tissue. (C) A 10-fold dilution of purified DNA extracted from infected gill tissue.

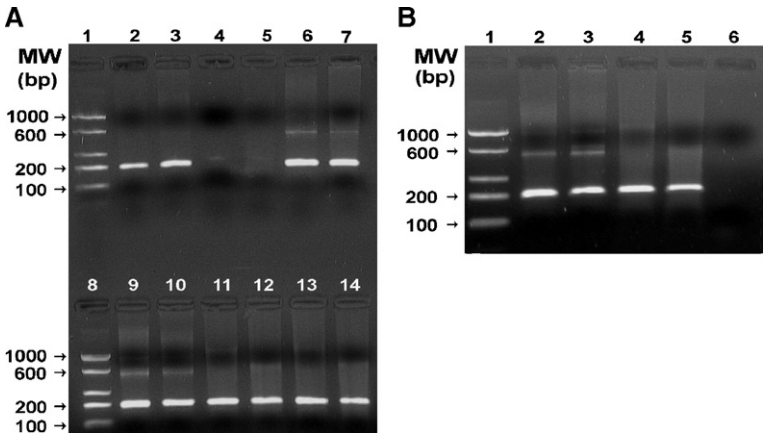

Fig. 2. PCR of crude and purified DNA extracted from different WSSV infected tissues, respectively. (A) Lane 1: 100 bp marker; lanes 2 and 3: eye stalk; lanes 4 and 5: eye stalk with eye; lanes 6 and 7: gills; lane 8: 100 bp marker; lanes 9 and 10: cuticle; lanes 11 and 12: pleopod; lanes 13 and 14: periopods. (B) Lane 1: 100 bp marker; lanes 2 and 3: telson; lanes 4 and 5: uropods; lane 6: negative control of purified DNA extracted from healthy gill tissue.

stalk, eye stalk with eye, gills, cuticle, pleopod, periopods, uropods and telson showed positive reactions; however, crude DNA extracted from eye stalk with eye showed the weakest colour development. The intensity of the colour development increased using the purified DNA extracted from eye stalk with eye when compared to the crude DNA extracted from the same tissue. The failure of colour development with purified DNA extracted from pleopod, telson and uropod may be due to the DNA purification which would have lowered the viral DNA concentration in these infected tissues as the same tissues tested positive by dot blot using the crude DNA. Similar observation has been reported in PCR results also, as DNA extraction step prior to PCR assay in case of HPV was found to be 10 times less sensitive due to loss of viral DNA than by a non-DNA extraction method (Phromjai et al., 2002).

The three PCR amplified products of 941 bp, 525 bp and 204 bp size obtained from crude DNA and purified DNA extracted from cuticle and telson varied in their band intensity. The variation in the intensity of the PCR products was observed to be dependent on the size of the amplified product. The maximum intensity was observed with the band of 204 bp followed by the amplified product of 525 bp. The weakest amplification was observed with 941 bp PCR product. The intensity of the amplified PCR products based on the size are in agreement with the observations of the other workers who have reported that, in two-step PCR, smaller fragments in PCR amplification amplify more efficiently than the larger fragments (Innis and Gefland, 1990).

The variation in obtaining either or all of the three amplified products from different tissues perhaps indicates the virus load and the severity of infection in the infected tissue. For example, in the present study, as per the single tube nested PCR kit manufacturer's protocol, obtaining all the three amplified products of 941 bp, 525 bp and 204 bp indicates severe WSSV infection of above 10^5 copies. Amplification of two bands of 525 bp and 204 bp indicates moderate to high WSSV infection with 10^3 to 10^5 copies and low WSSV infection with 10 to 10^2 copies results in single band amplification of 204 bp. The amplification of all the three PCR products in cuticle and telson indicates that perhaps these tissues are the most severely infected followed by gill tissue which was moderately infected resulting in amplification of two PCR products, whereas mild infection was found in other tissues including eye stalk, pleopod, periopods and uropod which showed single band PCR product. The experimental PCR conditions also play a major role in increasing the sensitivity of PCR reaction. For example, two-step PCR reactions are more sensitive than single step reaction but in case of WSSV can also lead to false positive reactions (Chapman et al., 2004) and two-step PCR results are not good indicators for detecting WSSV field outbreaks (Lo et al., 1998).

Although PCR is reported to be a very sensitive technique, it has its own disadvantages. It has been reported that homogenates of some shrimp tissues contain PCR inhibitors (Wang et al., 1996). Nunan et al. (2000) have also reported that some crude shrimp samples which gave strong reaction in dot blot assay were PCR negative which may be due to presence of PCR inhibitors which could be removed by sample dilution. In the present study, the purification of DNA by

phenol chloroform extraction from crude samples was done to increase the DNA concentration and also to remove any polymerase inhibitors. The failure to get amplification from the crude DNA extracted from eye stalk with eye confirmed that eye itself contained some PCR inhibiting factors which prevented viral DNA amplification from the eye stalk, as DNA extracted from eye stalk without eye gave PCR positive reaction for WSSV. The purification of the DNA extracted from the same tissue was not successful in removing the PCR inhibiting factors as no amplification could be achieved using this DNA. Our earlier study showed the intensity of PCR amplification was highest for WSSV infected *P. monodon* tissue of eyestalk, followed by lymphoid organ, gills and muscle tissue. No WSSV PCR amplification could be achieved when the whole eye with stalk was processed for PCR indicating that perhaps PCR inhibiting factors present in the eye led to PCR failure (Azad et al., 2002). The selection of tissues for PCR diagnosis is therefore important to prevent false negative results due to the presence of PCR inhibiting factors. The higher concentration of SDS in lysis buffer composition is also a contributory factor for PCR inhibition. PCR failed to detect hepatopancreatic parvovirus (HPV) in *P. monodon* in the supernatant solutions of PL homogenates in lysis buffer containing 2% SDS; however, the same samples were tested PCR positive in homogenization buffer containing 0.0125% SDS (Phromjai et al., 2002).

Comparing the sensitivity of dot blot and PCR in present study, the results suggest that PCR is more sensitive as PCR bands were obtained both in crude and purified DNA samples. However, dot blot showed no reaction with the same purified samples of DNA extracted from pleopod, telson and uropod. Similar observations have been reported by other workers. For example, comparison of PCR and dot blot for sensitivity in case of IHHNV showed that PCR positive reaction could be detected in the range of 500 ng μl^{-1} to 50 fg μl^{-1} using plasmid DNA clone as DNA template. The diluted plasmid DNA was detectable in the range of 500 ng to 5 pg in the dot blot reaction (Nunan et al., 2000). Lu et al. (1993) reported detecting 0.1 pg of purified MBV DNA by dot blot using DIG labeled MBV probe. Chapman et al. (2004) found PCR to be more sensitive in detecting WSSV in shrimp tissues as compared to histological examination or in situ hybridization with estimate of sensitivity of 1.0 for PCR and 0.25 for in situ hybridization respectively. Dupuy et al. (2004) observed higher sensitivity of PCR as compared to dot blot as dot blot was unable to detect WSSV viral DNA in preparation used for injection sample which contained very small quantity of virus; however, PCR produced positive result. WSSV detection in rotifer eggs by PCR dot blot hybridization was found more sensitive when compared to PCR electrophoresis alone (Yan et al., 2004).

In summary, these experiments suggest that PCR is a sensitive diagnostic technique to detect WSSV in the infected tissues, but PCR inhibiting factors play a major role in the selection of proper tissues for DNA extraction. Dot blot has an advantage over PCR in allowing use of those tissues which prevent PCR amplification, as the crude DNA extraction was found to be suitable for getting visible positive colour development without any need for further DNA purification. Also, because of the very sensitive nature of PCR, false positive PCR reactions, which are very common phenomenon, can be avoided in diagnostic tests such as dot blot.

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