http://www.pjbs.org

PJBS

ISSN 1028-8880

Pakistan Journal of Biological Sciences

ANSInet
Asian Network for Scientific Information
308 Lasani Town, Sargodha Road, Faisalabad - Pakistan

Pakistan Journal of Biological Sciences 11 (24): 2660-2664, 2008
ISSN 1028-8880

Designing and Introduce a Diagnostic Kit for Detection of White Spot Syndrome Virus in Cultured *Penaeus indicus* in Iran

[1]A.M. Saberi, [2]M. Bandehpour, [3]M. Afsharnasab, [2]E. Ghayour, [2]S.A. Yousefi Namin and [2,4]B. Kazemi
[1]Research and Edjucation Organiazation, Ministry of Jihade Keshavarzi, Tehran, Iran
[2]Cellular and Molecular Biology Research Center, Shahid Beheshti University, M.C., Tehran, Iran
[3]Iranian Fisheries Research Organization, Tehran, Iran
[4]Department of Parasitology, Shahid Beheshti University, M.C., Tehran, Iran

Abstract: The aim of this study is designing a diagnostic kit for white spot syndrome virus. We designed 2 series of primers for diagnosis of viral VP24 gene and also primers as internal controls which amplify part of genome in both positive and negative samples. DNA of shrimps were extracted and PCR amplification carried out. In this research, a diagnosis kit for white spot disease of shrimps designed and tested using 32 shrimp samples which were dubious to have this disease. White spot virus were found in 23 samples and the other 9 were negative. For extra confirmation, the PCR product was sequenced and deposited to GenBank. We designed a diagnosis kit for white spot disease of shrimps and tested successfully.

Key words: PCR, white spot disease, diagnostic kit

INTRODUCTION

There is a rapid and huge development in industry of shrimp breeding since 1970, the beginning of this industry. Today, more than 50 countries export significant amount of cultured shrimps. This has made million dollars of investment and creation of hundred thousands new jobs, directly and indirectly with high income and also caused entrance of currency especially in developing countries. New viral disease in 1990s, made lots of problems for constant development and improvement of this industry in some countries especially those were located in south Asia. The first viral disease reported by Couch (1974) in Mexican Gulf which occurred for shrimp called. *Penaeus duorarum*. He named the disease *Baculovirus penaei* (BP) (Lightner, 1996). White spot disease was found in 1991. Till today it has damnified million dollars to some countries and has caused social problems such vacation of thousands of workers and employees (Takahashi *et al*., 1994). White spot disease or syndrome (WSS) involve all shrimps and has overcome all other shrimp disease. Also, it has made huge victim in shrimps culture farms in Asia (Wang *et al*., 1995; Takahashi *et al*., 1994). This disease cause spots on karapas of cultured shrimps, fast separation of cuticle from epidermis, discharge of stomach and intestine, hepatopancreas enlargement and brittleness, redden of moving organs and severe fatality which reaches to 70-100% in 2-7 days. The disease also called Bacula viral Necrosis Haematopoitic (HHNBV). In Japan, it was named Rod shaped Nuclear Virus of *penaeus japonicus* (RVPJ) and in Taiwan as Systemic Ectodermal and Mesodermal Baculovirus. (SEMBV). Lightner (1996) named the disease White Spot Syndrome (WSS). Shrimps which are members of *Penaidea* family such as *Penaeuse monodon*, *P. orientlis*, *P. indicus*, *P. merguinesis*, *P. penecilatuss*, *P. semisulactus*, all show the disease naturally or experimentally. Also, this disease has various carriers that among them *Crustacae* and specially crabs are most important one (Zhang *et al*., 2008). Considering development of shrimp culturing farms in Iran, risk of prevalence of this disease is increasing and some cases are reported too. For example, there is a report about prevalence of disease in June of 2002 in Chuibdeh of Abadan (Tokhmafshan and Tamjidi, 2004).

Tsai *et al*. (2002) were designed a multiplex RT PCR for detection of WSSV and Taura Syndrome Virus (TSV) in penaeus shrimp in Taiwan. Vaseeharan *et al*. (2003) were detected WSSV by PCR in cultured and captured crustaceans in India. They concluded that WSSV is widespread in cultured and captured shrimp in India. Jian *et al*. (2005) compared in situ PCR with other methods for detection of WSSV in *Panaeus vannamei*. Yang *et al*. (2006) developed a multiplex PCR for detection of WSSV and other infectious virus in penaed shrimp. They believed that WSSV is important pathogen of cultured

Corresponding Author: Bahram Kazemi, Cellular and Molecular Biology Research Center, Shahid Beheshti University, M.C., Tehran, Iran Tel/Fax: +9821 22439956

shrimp that effect on cultured industry in China. Khawsak *et al.* (2008) were designed a multiplex PCR for detection of six viruses in penaeid shrimp. Their results demonstrated that WSSV was the highest prevalence at that time in Thailand. Dieu *et al.* (2004) studied molecular epidemiology of WSSV and suggested that there are different types of virus.

Diagnosis of disease is performing using transmission electron microscopy (Lei *et al.*, 2008), DNA hybridization (Dhar *et al.*, 2003), histopathology by H and E (Duangsuwan *et al.*, 2008), monoclonal antibody (Wang *et al.*, 2008) and PCR. Also, there are some commercial kits for diagnosis of disease, but it has not been made in Iran yet. The aim of this study was designing and testing a new kit in order to diagnosis of disease (white spot syndrome virus) rapidly and correctly which accessible and reliable for every laboratories near the farms.

MATERIALS AND METHODS

This study was done since April 2005 to September 2008, samples were given from shrimp farms in Khuzestan Province and transferred to Laboratory of Cellular and Molecular Biology Research Center, Tehran, Iran.

Primer designing: The whole genome sequence for WSS received from internet and 2 pairs of primers were designed (CLC Bio software) using viral VP24 gene for nested PCR. Also a pair of primers designed for host 18SrRNA as a PCR control in both positive and negative samples. Primers: Shri F 5'- GTA GGT TAA ACG CCT ACA ATG G-3', Shri R 5'- CCG GAA CTC AAA GAC TTT GGT T-3' used as PCR control for amplifying 809 nucleotides from shrimp 18SrRNA gene. Primers: WSS F1 5'- CAC CTG GGT TTG ACT ACA ATA-3', WSS R1 5'-TCT GTT TTT TTC TCT CAT GAC -3' designed to amplify 785 nucleotides (products of PCRI) from viral VP24 gene. Primers: WSS F2 5'- TCC AAA CAC AAG TGT GTT GAT C-3', WSS R2 5'-AAG ACG CCT ACC CTG TTG AAT C-3' were amplified 414 nucleotides (products of PCRII) of first PCR product.

Sampling: Referral to shrimps culture farms and investigations of exterior symptoms of dubious shrimps, 32 sample were sampled in 3 stages and transferred to laboratory inside alcohol or in freezing mode.

DNA extraction: DNA extraction was done as previously described by Bruce *et al.* (1991) with some modifications. Briefly, 50 mg of mixture of hard and soft tissue of shrimps poured into microfuge and 200 μL of lysis buffer containing 20 μg mL^{-1} proteinase K was added and put in 37°C overnight. Then, it was boiled and centrifuged. The supernatant containing DNA transferred to a new tube and extracted using phenol and chloroform. DNA precipitates dissolved in 50 μL of deionized water.

PCR reaction: PCR reaction was performed in 30 μL volume containing 1X PCR buffer, 0.2 mM dNTP mix, 1.5 mM $MgCl_2$, 40 pico mole forward and reverse primers (both host and virus), 1.25 unit Taq DNA Polymerase enzyme, 0.5 μg DNA and enough amount of water (McPherson and Moller, 2000). Then they were put in thermocycler and plan below was set: Primary denaturation of DNA 5 min in 94°C, then PCR cycles which follow these rules: for denaturation 94°C for 30 sec, annealing 56°C for 30 sec, extension 72°C for 30 sec and final extension 72°C for 5 min). PCRII was same as PCRI by just one difference, that is, the product of PCRI was used instead of DNA and also there was no need to primary denaturation.

Electrophoresis: Upon completion of the reaction, the PCR product was electrophoresed on 1% agarose gel containing ethidium bromide and DNA bands observed using 260 nm UV by UV transilluminator instrument (Boffy, 1984).

RESULTS AND DISCUSSION

Thirty two samples of shrimps sampled from Khuzestan and Bushehr States and transferred to laboratory and tested using designed kit. DNA extracted from shrimp samples and after quantitative and qualitative investigation, the nested PCR was done. Viral DNA amplified in 23 samples in which 9 samples were negative. Figure 1 shows diagram for electrophoresis pattern of PCR products in both positive and negative samples.

Electrophoresis of PCR product of positive and negative sample can be shown in Fig. 2. In positive samples we can see both PCR product of white spot disease virus and host 18SrRNA. In negative samples only the host PCR product is observable.

In order to confirmation of accuracy of the kit, the PCR product (viral VP24 gene) was extracted, purified and sequenced then deposited in GenBank at Accession number DQ196431.

Different methods are using to diagnosis of white spot disease. Despite high speed of this recognition method which uses exterior symptoms like white plaque on the shrimp's body, it has some overlapping with some other shrimp disease like vibriosis or IHHNV and also the increase in water pH can not be counted as specific sign.

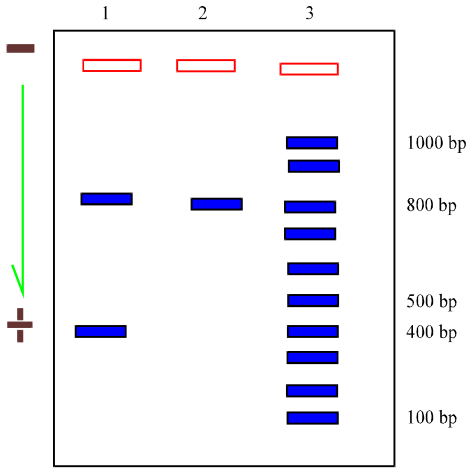

Fig. 1: Electrophoresis pattern of PCR product in positive and negative samples in compare with DNA ladder marker. In negative samples only PCR product of shrimp will observe and in positive samples both of PCR products of virus and shrimp will observe. The shrimp PCR product is a control device for PCR system

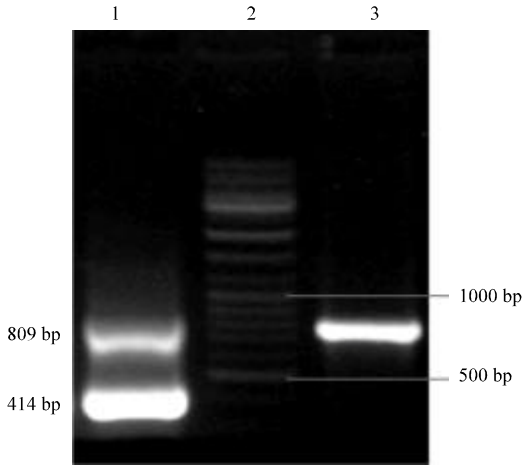

Fig. 2: Agarose gel electrophoresis of positive and negative samples. Lane 1 show PCR product of positive sample because PCR product of host and pathogen are seen and lane 3 shows negative sample because only PCR product of host is seen

Although, diagnosis of disease with pathology methods (Lightner, 1996) is an accurate method, because there are lots of similarities between this disease and some other disease like IHHNV in the results of this method, it can not be trustable alone. The most important symptoms of disease in tissue pathology is Cowdry Type A (Thakahashi *et al*., 1994; Wang *et al*., 2000), which is similar to symptoms of IHHNV (Lightner, 1996). The diagnosis of virus using electron microscope is very accurate method and lead to definite diagnosis of virus (Lightner, 1996). But despite there is a high degree of accuracy in this method to diagnosis of viral disease, it spends long time and also it costs so much. Also, this is not available method for all laboratories due to its complex equipments and it can not be used in shrimp culture farms since it is a research tool. Today, molecular methods have developed to diagnose of shrimps disease. These methods are able to diagnosis of disease in food product and shrimp culture farms. The aim of this study was to develop a home made kit for rapid diagnosis of WSSV with an internal control. The internal control will control PCR system and will amplified in negative or positive samples.

Specific properties of molecular method are their high speed and accuracy which is very important in order to prevention of disease. Among molecular methods, PCR is most remarkable due to its high speed and its accuracy. This method is able to diagnosis of 10 molecule of DNA of agent of disease in samples while the other methods need at least 100 molecules of DNA to diagnose of disease (Kou *et al*., 1998). Considering severity of fatality caused by white spot disease and this issue that this disease is in latent mode in most of the shrimps, using PCR method can diagnose the disease in lowest level of pathogens to diagnose and prevent the disease. Considering the importance of this disease, Kou *et al*. (1998) suggested using of nested PCR, because nested PCR is 1000 times more sensitive than PCR.

One of the important tools of shrimp culture management is access to a rapid and sensitive method to diagnosis of disease, thus presence of a kit which is able to work and diagnose of disease in laboratories close to shrimp culture farms seems necessary.

Considering PCR control which is designed in this kit and is present in both positive and negative samples confirms the PCR method. Also, sequencing of PCR product related to agent of disease can confirm the reliability of the kit in disease diagnosis.

Considering amplification of viral genome in PCR and sequencing of PCR product, it can be said there is the presence of virus, agent of white spot disease in shrimp culture farm definitely and this tissue should be taken care of by the authorities. Also there are some other reports about the presence of virus in Iranian shrimp culture farms which used PCR methods and also imported kit (Afsharnasab *et al*., 2006; Afsharnasab *et al*., 2008) which all confirm the reliability of this kit.

One o f the points which should be noticed about this issue is the generic changes in viral species (Wang *et al*., 2000; Marks *et al*., 2004). The genomic differences between viral isolates is shown by Wongteerasupaya *et al*. (2003) in Thailand. They believe that different epidemics are happened by isolated viruses with different genetic structure. The advantage of this study was to working with Iranian field.

CONCLUSION

We designed a diagnostic kit based on PCR reaction with an internal control for rapid and sensitive diagnosis of WWS virus in shrimp in Iran.

ACKNOWLEDGMENTS

This study was supported by Iranian Fisheries Research Orgamization, Tehran, Islamic Republic of Iran and was done in Cellular and Molecular Biology Research Center, Shahid Beheshti University M.C. The authors wish to thank directors.

REFERENCES

Afsharnasab, M., S. Rezvani and F. Laloee, 2006. Detection of WSSD in *Penaeus indicus* using PCR. Iran. Fish. J., 14: 1-12.

Afsharnasab, M., A. Dashtiannasab and V. Yeganeh, 2008. Study on pathogenesis of white spot syndrome virus in litopenaeus vannamei. Iran. Fish. J., 16: 1-8.

Boffy, S.A., 1984. Agarose Gel Electrophoresis of DNA. In: Methods in Molecular Biology, Nucleic Acids, John Walker, M. (Ed.). Homana Press, USA., pp: 43-50.

Bruce, L.D., B.B. Trumper and D.V. Lightner, 1991. Methods for viral isolation and DNA extraction for a penaeid shrimp baculovirus. J. Virol Meth., 34: 245-254.

Couch, J.A., 1974. An enzootic nuclear polyhedrosis virus of pink shrimp: Ultrastructure, prevalence and enhancemen. J. Invertebr. Pathol., 24: 311-331.

Dhar, A.K., A. Dettori, M.M. Roux, K.R. Klimpel and B. Read, 2003. Identification of differentially expressed genes in shrimp (*Penaeus stylirostris*) infected with White spot syndrome virus by cDNA microarrays. Arch. Virol., 148: 2381-2396.

Dieu, B.T., H. Marks, J.J. Siebenga, R.W. Goldbach, D. Zuidema, T.P. Duong and J.M. Vlak, 2004. Molecular epidemiology of white spot syndrome virus within Vietnam. J. Gen. Virol., 85: 3607-3618.

Duangsuwan, P., I. Phoungpetchara, Y. Tinikul, J. Poljaroen, C. Wanichanon and P. Sobhon, 2008. Histological and three-dimensional organizations of lymphoid tubules in normal lymphoid organ of Penaeus monodon. Fish Shellfish Immunol., 24: 426-435.

Jian, X.F., L. Lu, Y.G. Chen, S.M. Chan and J.G. He, 2005. Comparison of a novel *in situ* polymerase chain reaction (ISPCR) method to other methods for white spot syndrome virus (WSSV) detection in Penaeus vannamei. Dis. Aquat. Org., 67: 171-176.

Khawsak, P., W. Deesukon, P. Chaivisuthangkura and W. Sukhumsirichart, 2008. Multiplex RT-PCR assay for simultaneous detection of six viruses of penaeid shrimp. Mol. Cell Probes, 22: 177-183.

Kou, G.H., S.E. Peng, Y.L. Chin and C.F. LO, 1998. Tissue Distribution of White Spot Syndrome Virus (WSSD) in Shrimp and Crabs. In: Advance in Shrimp Biotechnology, Flegel, T.W. (Ed.). National Center for Genetic Engineering and Biotechnology, Bangkok.

Lei, Y., H. Chen, H. Dai, Z. Zeng, Y. Lin, F. Zhou and D. Pang, 2008. Electroless-plated gold films for sensitive surface plasmon resonance detection of white spot syndrome virus. Biosens. Bioelect., 23: 1200-1207.

Lightner, D.V., 1996. A Handbook of Shrimp Pathology and Diagnostic Procedures for Diseases Of Cultured Penaeid Shrimp. World Aquaculture Society, Baton Rouge, Louisiana.

Marks, H., R.W. Goldbach, J.M. Vlak and M.C. van Hulten, 2004. Genetic variation among isolates of White spot syndrome virus. Arch. Virol., 149: 673-697.

McPherson, M.J., S.G. Moller, R. Beynon and C. Howe, 2000. PCR The Basics from Background to Bench. Bios Scientific Publishers, Understanding PCR, USA., pp: 9-21.

Takahashi, Y., T. Itami, M. Kondo, M. Maeda, R. Fujii, S. Tomonaga, K. Supamattya and S. Boonyaratpalin, 1994. Electron microscopic evidence of bacilliform virus infection in kuruma shrimp (*Penaeus japonicus*). Fish Pathol., 29: 121-125.

Tokhmafshan, M. and B. Tamjidi, 2004. Histopathology and clinical signs of WSSD in *Penaeus indicus*. Iran. Fish. J., 12: 15-18.

Tsai, J.M., L.J. Shiau, H.H. Lee, P.W. Chan and C.Y. Lin, 2002. Simultaneous detection of White Spot Syndrome Virus (WSSV) and Taura Syndrome Virus (TSV) by multiplex Reverse Transcription-Polymerase Chain Reaction (RT-PCR) in pacific white shrimp *Penaeus vannamei*. Dis. Aquat. Org., 50: 9-12.

Vaseeharan, B., R. Jayakumar and P. Ramasamy, 2003. PCR-based detection of white spot syndrome virus in cultured and captured crustaceans in India. Lett. Applied Microbiol., 37: 443-447.

Wang, C.H., C.H. Lo, J.H. Leu, C.M. Chou, P.Y.H.Y. Yeh, M.C. Tung, C.F. Chang, M.S. Su and G.H. Kou, 1995. Purification and genomic analysis of baculovirus associated with white spot syndrome (WSBV) of *Penaeus monodon*. Dis. Aquat. Org., 23: 239-242.

Wang, Q., L.M. Nunan and D.V. Lightner, 2000. Identification of genomic variations among geographic isolates of white spot syndrome virus using restriction analysis and Southern blot hybridization. Dis. Aquat. Org., 43: 175-181.

Wang, Y., X. Zhang, L. Yuan, T. Xu, Y. Rao, J. Li and H. Dai, 2008. Generation of recombinant monoclonal antibodies to study structure-function of envelope protein VP28 of white spot syndrome virus from shrimp. Biochem. Biophys. Res. Commun., 372: 902-906.

Wongteerasupaya, C., P. Pungchai, B. Withyachumnarnkul, V. Boonsaeng, S. Panyim, T.W. Flegel and P.J. Walker, 2003. High variation in repetitive DNA fragment length for White Spot Syndrome Virus (WSSV) isolates in Thailand. Dis. Aquat. Org., 54: 253-257.

Yang, B., X.L. Song, J. Huang, C.Y. Shi, Q.H. Liu and L. Liu, 2006. A single step multiplex PCR for simultaneous detection of white spot syndrome virus and infectious hypodermal and haematopoietic necrosis virus in penaeid shrimp. J. Fish Dis., 29: 301-305.

Zhang, J.S., S.L. Dong, Y.M. Dong, X.L. Tian and C.Q. Hou, 2008. Bioassay evidence for the transmission of WSSV by the harpacticoid copepod *Nitocra* sp. J. Invertebr. Pathol., 97: 33-39.