Molecular cloning and recombinant expression of the VP28 (wsv421 gene) from Iranian white spot syndrome virus isolate

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Abstract

White spot syndrome virus (WSSV) is a highly pathogenic and prevalent virus affecting shrimp culture worldwide including Iran. In the present study, a pair of primers was designed according to the sequence of VP 28 gene of WSSV in the GenBank. VP28 gene from an Iranian WSSV isolate (IrVP28) was cloned, sequenced and expressed in *Escherichia coli* BL21(DE3) strain in order to produce VP28 protein using PET28a expression vector. The expression resulted in a protein of about 30kDa, which was purified under denaturing conditions, resulting in a highly purified final IrVP28 preparation. The obtained recombinant protein can be used in several biotechnology applications, such as production of monoclonal antibodies which could be used in development of diagnostic tools and potential oral vaccination of shrimp with vaccines consisting of VP28 proteins.

Keywords: WSSV, VP28, PET28a, Recombinant protein

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Introduction

WSSV is one of the most serious viral diseases affecting cultured shrimp worldwide (Lo *et al*., 1996; Flegel, 2007). This lethal virus causes formation of white spots in exoskeleton under carapace and can infect not only penaeid shrimps, but also other marine and freshwater crustaceans (Lo *et al*., 1996). The first outbreak of WSSV began to occur in Asia during mid-1990s and later in Americas in late 1990s, causing massive mortality rates in shrimp farms (Lightner, 2003). WSSV has been isolated from different geographic regions, such as Korea, Thailand, China and Americas (Lightner, 2003). Although the WSSV disease had been detected in Iran since 2002 (OIE, 2003), no report concerning characterization of Iranian isolates is found in the literature. Since 2002, WSSV outbreaks have been reported in southern Iran, causing losses estimated at several million dollars per year (OIE, 2003; Afsharnasab *et al*., 2007).

WSSV consists of a large enveloped virus, ovoid to baciliform in shape, containing a circular double-stranded DNA genome of 300 kb with approximately 180 putative open reading frames (Marks *et al*., 2004). Based on its unique genetic information and morphological features, WSSV represents a novel genus of DNA viruses, *Whispovirus*, belonging to the family Nimaviridae (van Hulten *et al*., 2001a). This virus contains five major structural proteins, located in the viral envelope (VP19 and VP28) and in the nucleocapsid (VP15, VP24 and VP26). VP28 (~30kDa) is the most abundant structural protein present in the envelope (Tang *et al*., 2007) and shows no similarity with other known proteins, (Robalino *et al*., 2006). This envelope protein was suggested to bind to shrimp cells, playing a crucial role in the viral penetration (Yi *et al*., 2004). Moreover, it has been shown that anti-VP28 antibodies can neutralize the virus and block the viral infection (Yi *et al*., 2004). Juvenile shrimps, *Penaeus (Metapenaeus) japonicus* were fed with commercial feeds that were mixed with recombinant VP28, a structural protein antigen of WSSV for a period of 14 days. The immune response of shrimp during oral administration of medicated feed was determined by expression analysis of fortilin, a gene that is involved in the antiviral response. There was a significant increase in the level of expression of fortilin both in gut and gills in the fed group during feeding (Caipang and Fagutao, 2013).

However, the mechanisms of virus entry into cells are still unknown but, identification of host cellular proteins interacting with WSSV will help in unraveling the repertoire of host proteins involved in WSSV infection (Biradar *et al*., 2013).

The highly conserved nature of WSSV structural proteins enables the production of recombinant proteins for different applications, such as production of poly and monoclonal antibodies that can be employed in immunodiagnostic assays, like immunochromatographic kits (Wang and Zhan, 2006). Since currently there is no effective treatment for shrimp viral diseases, and vaccines cannot be developed and used in shrimp aquaculture due to lack of adaptive immune system in crustaceans, rapid diagnosis is one of the most valid strategies to curtail WSSV infection (Liu *et al*., 2009). Moreover, structural recombinant WSSV proteins can be used in order to understand the mechanisms of WSSV infection, morphogenesis and

interaction with host molecules (Tang *et al*., 2007). With this knowledge immune responses of host cells advance to next levels, in this regard a fragment of the VP28 coding sequence from a Brazilian WSSV isolate (BrVP28) was cloned, sequenced and expressed in *E. coli* BL21(DE3) pLysS strain in order to produce the VP28 carboxyl-terminal hydrophilic region (Braunig *et al*., 2011).

In the present study, VP28 gene (IrVP28) from Iranian WSSV isolate was cloned in order to characterize and produce its protein. We amplified VP28 gene and cloned it in PTZ57R/T vector to aim this purpose: first, having a storehouse for gene amplification; second, checking enzymatic site on designated primers; third, just having digested the gene for ligation phase.

Materials and Methods

The WSSV-infected shrimps (*Fenneropenaeus indicus*) were obtained from shrimp farms in Abadan, southern Iran. Genomic DNA was extracted from the infected shrimp cephalothoraxes by standard phenol-chloroform extraction method (Green *et al*., 2012). Then genome purification and quantification were estimated using a spectrophotometer and also DNA concentration was assessed by agarose gel electrophoresis and NanoDrop system.

Two pairs specific primers were designed based on the VP28 sequence from an Indian WSSV isolate (GenBank: DQ681069), including 5'-ATAGGATCCAGGATGGATCTTTCTTTCACT-3' as forward and 5'-ATAGAATTCTTACTCGGTCTCAGTGCCA-3'as reverse for the amplification of 615 bp amplicon of the vp28 gene. The underlined nucleotides in the sense strand indicate the location of a BamHI site, while those in the antisense strand indicated the location of aEcoRI site. PCR amplification was performed in a final volume of 25 µl containing 1x reaction buffer, 0.2 mM of a dNTP mixture, 1.5 mM $MgCl_2$, 1 µM of each primer, 1 unit of Taq DNA Polymerase (Sinagene) and 20 ng of DNA template. PCR reaction was conducted with an initial denaturation step at 95°C for 3 min, followed by 35 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 step at 72°C for 25 min. The amplification products were resolved in 1% agarose gel, stained with gel red, visualized in a transilluminator and digitally recorded. Subsequently, PCR products were cloned into PTZ57R/T vector (Fermentas) and the recombinant plasmid (PTZ57R/T-IrVP28) was used to transform *Escherichia coli* DH5α competent cells. The transformed cells were selected on amplicillin-containing LB plates (100 µg mL^{-1}) and screened by colony PCR using the above mentioned primers. The PTZ57R/T-IrVP28 plasmid was digested (Fermentas Fast Digestion Enzyme) and the excised fragment IrVP28 was cloned into pET-28a vector (Novagen) at the BamHI and EcoRI sites, generating plasmid pET-28a-IrVP28. The fragment was inserted downstream and in-frame with a coding sequence corresponding to a histidine tag, and was further sequenced in both directions.

With the aim to produce recombinant IrVP28, the pET-28a-IrVP28 plasmid was used to transform *E. coli* BL21(DE3) strain. Single positive colonies were picked and cultured overnight at 37 °C in 10 ml of LB medium supplied with kanamycin (50µgml^{-1}). One aliquot of 5 ml of the overnight culture were inoculated into 10

ml of the same medium and were grown at 37°C until OD600nm ~0.5–0.7. Cultures were then induced with 1 mM IPTG (isopropyl-β-d-thiogalactopyranoside) and incubated at 37°C for further 5 h. All induced cells from each hour post induction as well as non induced cells were pelleted at 4000 x g for 15 min and stored at –20°C for next uses. The pellets lysed in sample buffer and the suspension was boiled for 10 min and analysed on 10% SDS- PAGE. Protein purification of the cell lysate was carried out using a Qiagen Ni-NTA column of chromatography against His-tag with a denaturation purification protocol as specified by the manufacturer (QIAGEN, USA). The purified protein was analysed on SDS-PAGE. Protein bands were visualized by staining with Coomassie Brilliant Blue.

Results

The concentration of WSSV DNA was 1500 ng/µl which was used as a template for vp28 gene amplification. The PCR product was analysed on 1% agarose gel and the size of PCR product was in accordance with part of VP28 gene size in gene bank and was 615 bp (Fig. 1). After colony PCR from 5 random selected DH5α white colonies; the PCR product was analysed on 1% agarose gel shown 2 colonies bearing the interest gene (Fig. 2). Then, the VP28 gene was cloned in PET28a vector and ligation product transformed in BL21 (DE3) which confirmed using the colony PCR (Fig. 3).

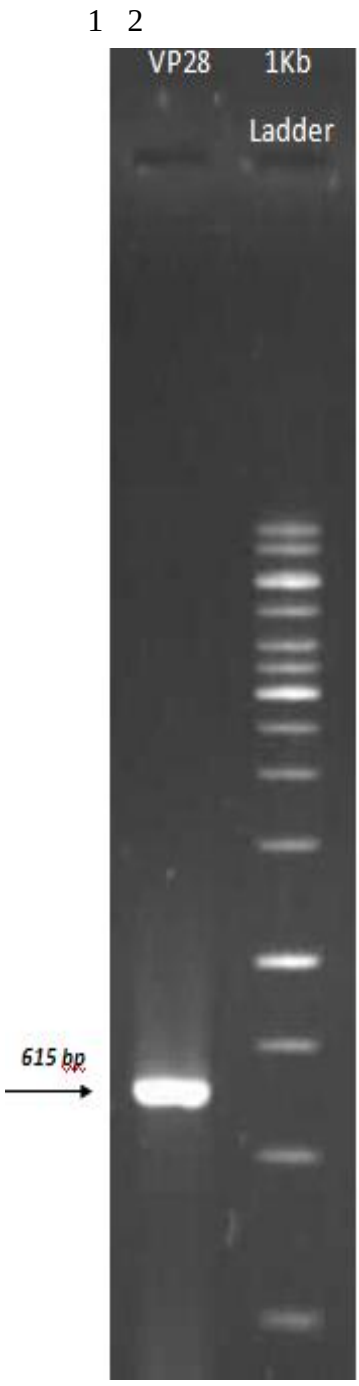

Figure 1: the part of VP28 gene size which amplified on 1% agarose gel.
Lan 1: vp28 gene, Lan 2: Fermentas 1 Kb DNA Ladder.

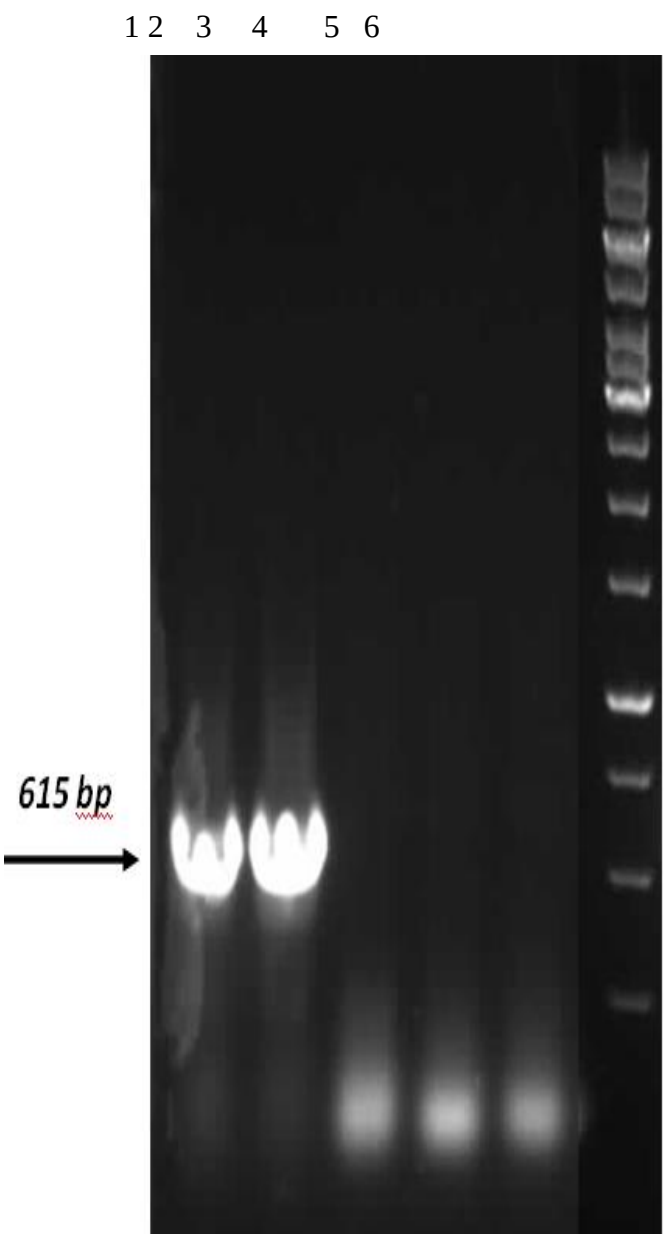

Figure 2: colony PCR from random selected DH5α white colonies on 1% agarose gel.
Lan 1 & 2: positive colonies, Lan 3, 4 &5: negative colonies, Lan 6: Fermentas 1 Kb DNA Ladder

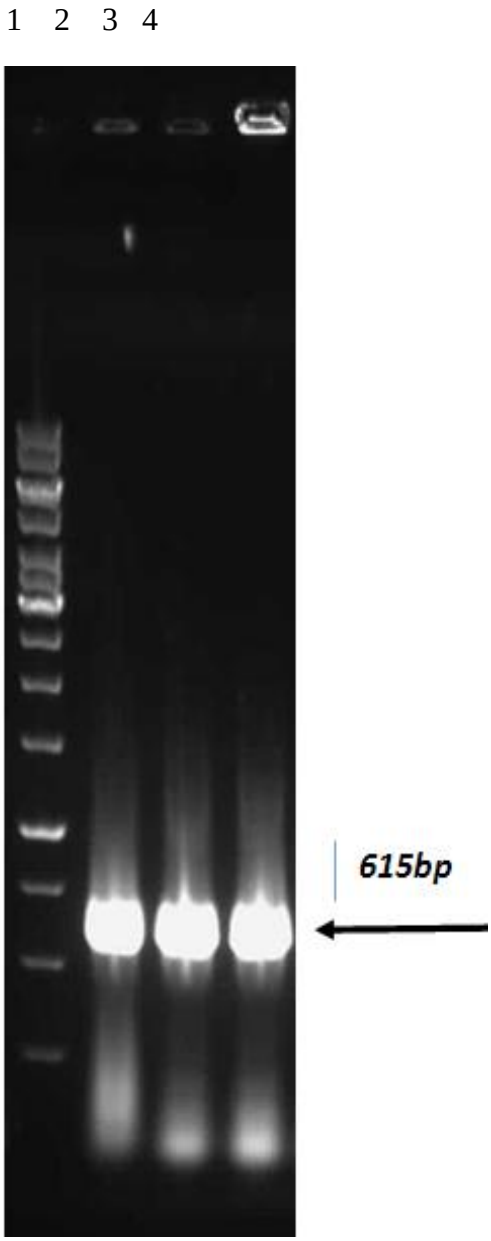

Figure 3: colony PCR from 3 random selected colonies on 1% agarose gel.
Lan 1: Fermentas 1 Kb DNA Ladder, Lan 2, 3&4: positive colonies

The sequencing result also showed that Iranian WSSV VP28 gene sequence was completely similar to other VP28 sequences published in gene bank. According to the sequencing results, it was clear that the start codon of VP28 gene was in frame with N-terminal of the His6-tag of the PET28a vector (data not shown). The VP28 was expressed as recombinant protein with His6-tag at its amino termini in *E. coli*. expected band of 30 KDa was detected on the SDS-PAGE in different duration of the induction (Fig. 4).

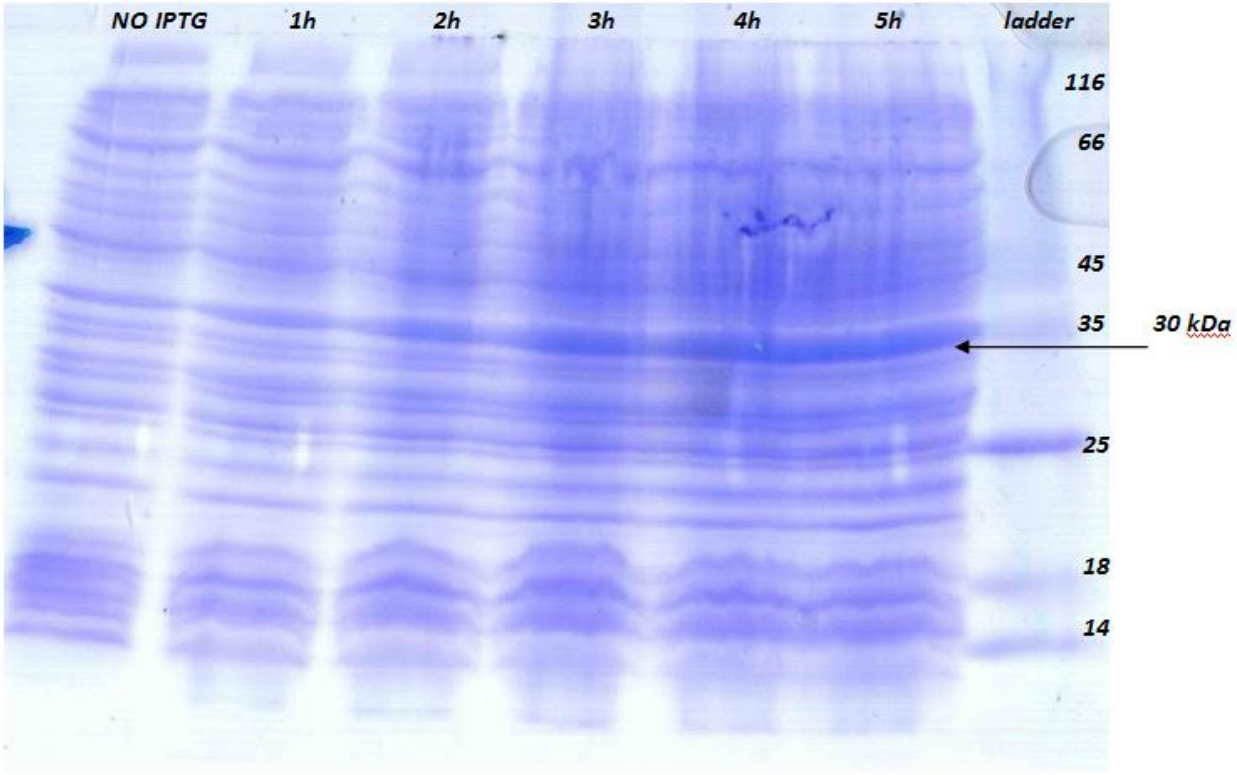

Figure 4: VP28 gene expression on SDS-PAGE.
Lan 1: transformed BL21 (DE3) with recombinant PET28a vector before adding IPTG, Lan 2: 1 hour after adding IPTG, Lan 3: 2 hour after adding IPTG, Lan 4: 3 hour after adding IPTG, Lan 5: 4 hour after adding IPTG, Lan 6: 5 hour after adding IPTG, Lan 7: Fermentas protein Ladder

As shown in Fig. 5 the expressed protein was not detected in supernatant that means it might be in insoluble form. The results showed that IPTG with concentration of 1 mM and 5 h of incubation under shaking condition after induction was optimum for expression of the protein. After expression and purification of recombinant VP28, the product was analysed on SDS-PAGE as well (Fig. 5).

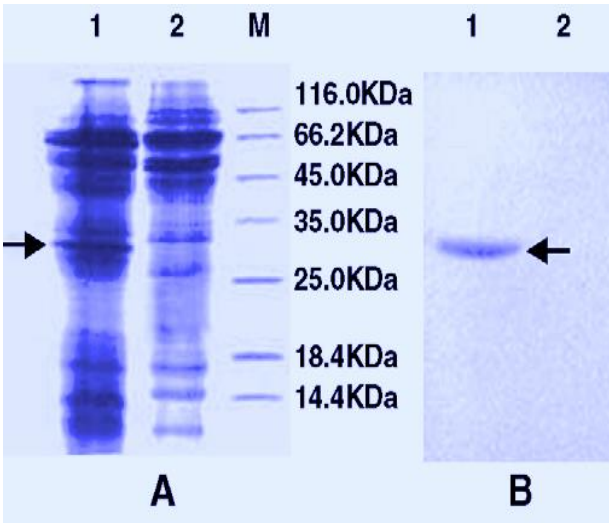

Figure 5: VP28 protein expression on SDS-PAGE.
(A) Lan 1: transformed BL21 (DE3) with recombinant PET28a after adding IPTG, Lan 2: protein expression in BL21 (DE3) with PET28a vector, Lan M: Fermentas protein Ladder, (B) Lan 1: purified protein, Lan 2: Media for soluble protein

Discussion

In this study, we have successfully cloned a novel gene (VP28) from WSSV by using a vectors library. The gene possibly encoded a structural protein. In order to locate VP28 gene, the gene was expressed in *E. coli* and the purified VP28 protein. While our studies were in progress, homology searches with the VP28 protein in GenBank using fasta and blasta showed that vp28 was homolog with Indian VP28 (GenBank: DQ681069; DQ013881; DQ013882; DQ013883).

Some studies reported purification of recombinant proteins from acrylamide gel slab (Chaivisuthangkura *et al*., 2004), but the histidine tagged protein purification protocol used in the present work was relatively simpler and provided a pure preparation without bacterial protein contaminants. Using this method of purification, a final protein yield, between 128 to 241 µg/ml, was achieved.

VP28 protein was reported to play a key role in the initial steps of systemic infection of WSSV in shrimp (van Hulten *et al*., 2001a, b; Leu *et al*., 2013). The recombinant VP28 protein expressed in *E. coli* has been successfully utilized to 'vaccinate' shrimp (Namikoshi *et al*., 2004). Although it is effective using *E. coli* to produce recombinant proteins, the expressed proteins are usually in form of inclusion bodies that must be solubilized and renatured before they can become biologically active. Therefore, the use of rVP28 to produce monoclonal and polyclonal antibodies targeting rVP28 for diagnosis was suggested (Anil *et al*., 2002; Chaivisuthangkura *et al*., 2004; Makesh *et al*., 2006) and recently new projects are advanced (Fu *et al*., 2010; Du *et al*., 2013).

In vivo neutralization experiments with neutralizing antibodies have been used for many viruses and have led to passive immunization strategies for WSSV (van Hulten *et al*., 2001b; Kim *et al*., 2004; Wu *et al*., 2005; Musthaq *et al*., 2006; Robalino *et al*., 2006; Natividad *et al*., 2007). Recombinant VP28 is wildly used to control WSS although the protection mechanism is unclear and its efficacy varies. One possible mechanism of rVP28 is that it binds to shrimp VP28 binding protein, preventing the binding of WSSV to cells (Xu *et al*., 2006).

Another possibility suggested by many studies is that rVP28 stimulates shrimp immunity or acts as a vaccine (Bright Singh *et al*., 2005; Du *et al*., 2006; Vaseeharan *et al*., 2006; Witteveldt *et al*., 2006; Jha *et al*., 2007; Rout *et al*., 2007; Mavichak *et al*., 2009). Although, the evidence that rVP28 increases the shrimp survival rate against WSSV, the protection ability of rVP28 varies and seems to rely on the amount of proteins received (Jha *et al*., 2006).

Cloning of the vp28 gene in the pET-28a expression system led to the expression of a

protein with approximately 30 kDa MW. In this system, an additional bp from the vector corresponding to the His-6tag was also coexpressed with the complete VP28 protein. Expression was standardized with different concentrations of IPTG and different time durations after induction. Purification was achieved under denaturation conditions (Fig. 5). Therefore, in this study we attempt to produce IrVP28 isolated WSSV from Iran to survey its antigenicity to use in serological diagnosis and oral vaccines in future. In this case a new generation of oral vaccine is suggested (Kulkarni *et al*., 2013)

Recombinant VP28 protein production from Khozestan Province isolated from WSSV by PET28a vector is possible. According to the importance of this protein and for future studies, the possibility of this protein production is available. Our results revealed that isolated VP28 protein of Iran is insoluble.

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References

Afsharnasab, M., Dashtyannasab, A., Yeganeh, V. and Soltani, M., 2007. Incidence of white spot disease (WSD) in *Penaeus indicus* farms in Bushehr Province, Iran. *Iranian Journal of Fisheries Sciences*, 7, 15-26.

Anil, T.M., Shankar, K.M. and Mohan, C.V., 2002. Monoclonal antibodies developed for sensitive detection and comparison of white spot syndrome virus isolates in India. *Diseases of Aquatic Organisms*, 51, 67-75.

Biradar, V., Narwade, S., Paingankar, M. and Deobagkar, D., 2013. White spot syndrome virus infection in *Penaeus monodon* is facilitated by housekeeping molecules. *Journal of Bioscience*, 38(**5**), 917–924.

Braunig, P., Diego da Rosa, R., Seibert, C.H., Borsa, M., Stoco, P.H., Grisard, E.C. and Pinto A.R., 2011. Molecular cloning and recombinant expression of the VP28 carboxyl-terminal hydrophilic region from a Brazilian white spot syndrome virus isolate. *Brazilian Archives of Biology and technology*, 54(**2**), 399-404.

Bright Singh, I.S., Manjusha, M., Pai, S.S. and Philip, R., 2005. *Fenneropenaeus indicus* is protected from white spot disease by oral administration of inactivated white spot syndrome virus. *Diseases of Aquatic Organisms*, 66, 265-270.

Caipang, C.M.A. and Fagutao, F.F., 2013. Transcriptional upregulation of fortilin in shrimp, *Penaeus (Metapenaeus) japonicus* fed diets containing recombinant VP28, an antigenic protein of white spot syndrome virus (WSSV). *International Journal of the Bioflux Society*, 6(**4**), 345-351.

Chaivisuthangkura, P., Tangkhabuanbutra, J., Longyant, S., Sithigorngul, W., Rukpratanporn, S., Menasveta, P. and Sithigorngul, P., 2004. Monoclonal antibodies against a truncated viral envelope protein (VP28) can detect white spot syndrome virus (WSSV) infections in shrimp. *Sci. Asia*, 30, 359-363.

Du, H.H., Hou, C.L., Wu, X.G., Xie, R.H. and Wang, Y.Z., 2013. Antigenic and immunogenic properties of truncated VP28 protein of white spot syndrome virus in *Procambarus clarkii*. *Fish and Shellfish Immunology*, 34, 332-338.

Du, H., Xu, Z., Wu, X., Li, W. and Dai, W., 2006. Increased resistance to white spot syndrome virus in *Procambarus clarkii* by injection of envelope protein VP28 expressed using recombinant baculovirus. *Aquaculture*, 260, 39-43.

Flegel, T.W., 2007. Update on viral accommodation, a model for host-viral interaction in shrimp and other arthropods. *Developmental and Comparative Immunology*, 31, 217-231.

Fu, L.L., Shuai, J.B., Xu, Z.R., Li, J.R. and Li, W.F., 2010. Immune responses of *Fenneropenaeus chinensis* against white spot syndrome virus after oral delivery of VP28 using *Bacillus subtilis* as vehicles. *Fish and Shellfish Immunology*, 28, 49-55.

Green, M.R., Sambrook, J. and McCallum, P., 2012. Molecular cloning: A Laboratory Manual, fourth edition. Cold Spring Harbor Laboratory Press, NY, USA.

Jha, R.K., Xu, Z.R., Shen, J., Bai, S.J., Sun, J.Y. and Li, W.F., 2006. The efficacy of recombinant vaccines against white spot syndrome virus in *Procambarus clarkia*. *Immunology Letters*, 105: 68-76.

Jha, R.K., Xu, Z.R., Bai, S.J., Sun, J.Y., Li, W.F. and Shen, J., 2007. Protection of *Procambarus clarkii* against white spot syndrome virus using recombinant oral vaccine expressed in *Pichia pastoris*. *Fish and Shellfish Immunology*, 224, 295-307.

Kim, D.K., Jang, I.K., Seo, H.C., Shim, S. O., Yang, S.Y. and Kim J.W., 2004. Shrimp protected from WSSV disease by treatment with egg yolk antibodies (IgY) against a truncated fusion protein derived from WSSV. *Aquaculture*, 237, 21-30.

Kulkarni, A., Rombout, J.H.W.M., Bright Singh, I.S., Sudheer, N.S., Vlak, J.M., Caipang, C.M.A., Brinchmann, M.F. and Kiron, V., 2013. Truncated VP28 as oral vaccine candidate against WSSV infection in shrimp: an uptake and processing study in the midgut of *Penaeus monodon*. *Fish and Shellfish Immunology*, 34,159-166.

Leu, J.H., Lin, S.H., Huang, J.Y., Chen, T.C. and Lo, C.F., 2013. A model for apoptotic interaction between white spot syndrome virus and shrimp *Fish and Shellfish Immunology*, 34, 1011-1017.

Lightner, D.V., 2003. The penaeid shrimp viral pandemics due to IHHNV, WSSV, TSV and YHV. History in the Americas and current status. 32nd Aquaculture Panel Meeting, California, USA, 120P.

Liu, H., Söderhäll, K. and Jiravanichpaisal, P., 2009. Antiviral immunity in crustaceans. *Fish and Shellfish Immunology*, 27, 79-88.

Lo, C.F., Ho, C.H., Peng, S.E., Chen, C.H., Hsu, H.C., Chiu, Y.L., Chang, C.F., Liu, K.F., Su, M.S., Wang C.H. and Kou, G.H., 1996. White spot syndrome baculovirus (WSBV) detected in cultured and captured shrimp, crabs and other arthropods. *Diseases of Aquatic Organisms*, 27, 215-225.

Makesh, M., Koteeswaran, A., Chandran, N.D.J., Manohar, B.M. and Ramasamy, V., 2006.

Development of monoclonal antibodies against VP28 of WSSV and its application to detect WSSV using immunocomb. *Aquaculture*, 261, 64-71.

Marks, H., Goldbach, R.W., Vlak, J.M. and van Hulten, M.C.W., 2004. Genetic variation among isolates of white spot syndrome virus. *Archives of Virology*, 149, 673–697.

Mavichak, R., Kondo, H., Hirono, I., Aoki, T., Kiyono, H. and Yuki, Y., 2009. Protection of pacific white shrimp, *Litopenaeus vannamei* against white spot virus following administration of N-terminus truncated recombinant VP28 protein expressed in Gram positive bacteria, *Brevibacillus choshinensis*. *Aquaculture Science*, 57, 83-90.

Musthaq, S., Yoganandhan, K., Sudhakaran, R., Kumar, S. and Hameed, A.S., 2006. Neutralization of white spot syndrome virus of shrimp by antiserum raised against recombinant VP28. *Aquaculture*, 253, 98-104.

Namikoshi, A., Wu, J.L., Yamashita, T., Nishizawa, T., Nishioka, T., Armoto, M. and Muroga, K., 2004. Vaccination trials with *Penaeus japonicus* to induce resistance to white spot syndrome virus. *Aquaculture*, 229, 25–35.

Natividad, K.D.T., Higio, M., Tanaka, M., Nomura, N. and Matsumura M., 2007. White spot syndrome virus (WSSV) inactivation in *Penaeus japonicas* using purified monoclonal antibody targeting viral envelope protein. *Aquaculture*, 269, 54-62.

OIE, 2003. Diagnostic manual for aquatic animal diseases, 4th Edition. Office International des Epizooties (OIE). Paris, France. 358P.

Robalino, J., Payne, C., Parnell, P., Shepard, E., Grimes, A.C., Metz, A., Prior, S., Witteveldt, J., Vlak, J.M., Gross, P.S., Warr, G. and Browdy, C.L., 2006. Inactivation of white spot syndrome virus (WSSV) by normal rabbit serum: implications for the role of the envelope protein VP28 in WSSV infection of shrimp. Virus Research, 118, 55-61.

Rout, N., Kumar, S., Jaganmohan, S. and Murugan, V., 2007. DNA vaccines encoding viral envelope proteins confer protective immunity against WSSV in black tiger shrimp. *Vaccine*, 25, 2778-2786.

Tang, X., Wu, J., Sivaraman, J. and Hew, C.L., 2007. Crystal structures of major envelope proteins VP26 and VP28 from white spot syndrome virus shed light on their evolutionary relationship. Journal of Virology, 81, 6709-6717.

Van Hulten, M.C.W., Witteveldt, J., Peters, S., Kloosterboer, N., Tarchini, R., Fiers, M., Sandbrink, H., Lankhorst, R.K. and Vlak, J.M., 2001a. The white spot syndrome virus DNA genome sequence. *Virology*, 286, 7-22.

Van Hulten, M.C.W., Witteveldt, J., Snippe, M. and Vlak, J.M., 2001b. White spot syndrome virus envelop protein VP28 is involved in the systemic infection of shrimp. *Virology*, 285, 228–233.

Vaseeharan, B., PremAnand, T., Murugan, T. and Chen, J.C., 2006. Shrimp vaccination trials with the VP292 protein of white spot syndrome virus. *Letters in Applied Microbiology*, 43, 137-142.

Wang, X. and Zhan, W., 2006. Development of an

immunochromatographic test to detect white spot syndrome virus of shrimp. *Aquaculture*, 225, 196-200.

Witteveldt, J., Vlak, J.M. and van Hulten, M.C., 2006. Increased tolerance of *Litopenaeus vannamei* to white spot syndrome virus (WSSV) infection after oral application of the viral envelope protein VP28. *Diseases of Aquatic Organisms*, 70, 167-170.

Wu, W., Wang, L. and Zhang, X., 2005. Identification of white spot syndrome virus (WSSV) envelope proteins involved in shrimp infection. *Virology*, 332, 578-583.

Xu, Z., Du, H., Xu, Y., Sun, J. and Shen, J., 2006. Crayfish *Procambarus clarkii* protected against white spot syndrome virus by oral administration of viral proteins expressed in silkworms. *Aquaculture*, 253, 179-183.

Yi, G., Wang, Z., Qi, Y., Yao, L., Qian, J. and Hu, L., 2004. VP28 of shrimp white spot syndrome virus is involved in attachment and penetration into shrimp cells. *Journal of Biochemistry and Molecular Biology*, 37, 726-734.