ISSN 0975-6299 Vol.1/Issue-3/Jul-Sep.2010

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International Journal of Pharma and Bio Sciences

MOLECULAR DIAGNOSIS OF MARINE BIRNAVIRUS IN THE ZOOPLANKTON FROM VELLAR ESTUARY (SOUTH EAST COAST OF INDIA)

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ABSTRACT

Marine birnavirus (MBVs) infect a wide range of fish and shellfish. Yet their mode of transmission is still unclear. To determine marine plankton serve as a vector for MABVs. The marine birnavirus (MABVs) is a member of Aquabirnavirus and an opportunistic pathogenic form among eukaryotic marine organisms and determine the viral presence in zooplankton as a vector, molecular diagnosis can be used. The present investigation of Zooplankton *Acartia* and *Oithona* species were collected separately. They were diagnosed for the presence of viral genome by 2 step PCR method. The result was positive in both of them and this suggests that molecular diagnosis will help us to identify viral presence in the stock larvae before they are stocked in the rearing ponds. It will be certainly helpful for the farmers whose life dependent on the healthy culturing of fishes.

KEYWORDS

Marine birnavirus (MABV), zooplankton, copepod, *Acartia*, *Oithona*.

INTRODUCTION

The viral disease in aquaculture results in direct mortalities and production loses and affect all levels in the Aquaculture. Over 30 aquatic species are currently being cultured to produce protein as a human food source. The consumption of red meat decreased that of fish reached higher. As a result the demand for fish and shelf fish continues to rise[1]. As the natural stocks have declined, aquaculture has become increasingly important as a source of fishery products, so it has become a mandatory to produce fish on fish farms.

Food from fish is rich in animal protein, omega 3– Polyunsaturated fatty acids, fat soluble vitamins (A, D and E), water soluble vitamins (B

complex) and minerals. In the world wide fish food contributes more than 25% of the total animal protein for about one billion people. Omega 3 fatty acid prevents certain diseases such as Coronary heart diseases, Stroke, Cancers of the breast, Hypertensions, etc by consuming them from sea food products. Unfortunately the losses occurred by the fish farmers related to disease is very significant. The estimates of economic loss by the viral disease in fish farm is very alarming, The trout industry reported losses during 1988 of 20.7 million fish,50% is which were lost due to disease [2].

Viruses are the most abundant members of marine ecosystem. Moreover good number of zooplankton assumes a great ecological significance in Mangrove ecosystem as they play vital role in food web of the food chain nutrient recycling and in transfer of organic matter from primary producers to secondary consumers like fishes [3]. The marine birna virus (MABV) group, a member of the birnaviridae are icosahedral undeveloped virus whose genomes comprise 2 segments of double stranded RNA designated as A and B [4].

More recent reports have indicated that marine birnavirus was examined in fish and shellfish[5]. Marine birnavirus also infects Olive Flounder in Japan and Korea[6-7]. Marine birnavirus was isolated from diseased sea squirts Halocynthia roretzi in Korea[8]. Marine birnavirus has also been detected from natural sea water [9]. Marine birnavirus genome has been detected in Zooplankton collected from the Uwa sea, Japan [10] and these viral genome also distribute widely in the ocean[11]. This virus has a broad host range in wild,cultured fish and shellfish, Most of the plankton serves as a vectors for MABV'S , because plankton are the food of most juvenile fish and shellfish. However the mode by which MABVs are transmitted remains unclear. This study aims to determine the suitability of molecular diagnosis to analysis the viral infection in the stock larvae before they are stocked in the rearing / grown out ponds. This study aims to determine if MABVs are present in plankton.

MATERIALS AND METHODS

Collection and preservation of the sample: The Zooplankton samples were collected by using horizontal twinge of plankton net (0.35 mouth diameter). The plankton net was made up of bolting silk cloth (No.10; mesh size of 158 µm).Then the plankton were washed with distilled water thrice to release in distilled water and passed through 500 µm mesh to remove fish and prawn larvae. The rinsing was made repeatedly to reduce the contamination. After rinsing, the adult copepods *Acartia* and *Oithona* species were picked from the zooplankton sample with the help of fine capillary tube and needles. Then *Acartia* and *Oithona* species were washed with double distilled water and stored in 95% ethanol under refrigerated condition for the further uses.

Extraction of viral RNA from the Acartia and Oithona species: *Acartia* and *Oithona* (Plankton) stored in refrigerated conditions were brought to the room temperature and pelletized at 6000 rpm for 5 mins. The pellet was resuspended in artificial sea water and washed thrice in order to facilitate RNA extraction. About 100 mg of dried *Acartia* and *Oithona* species were homogenized and isolation of RNA.

RNA Extraction: RNA extraction and PCR were performed[12]. Briefly, collected *Acartia* and *Oithona* species were washed with artificial sea water 3 times and dried, then 100 mg of Acarcia and Oithona species was homogenized and suspended in 45 µl TE (0.2 M TrisHCl, pH 8.3, 0.1M EDTA) buffer 5 µl proteinase K (10 mg/ml was added to the mixture and it was incubated at 55^{o}C for 2hr. Nucleic acids were extracted using the phenol; chloroform method. The extracted nucleic acid was heated at 100^{o}C for 5 mins.

Two step PCR

Amplification of Viral RNA: Diagnosis of viral infection can be possible only with PCR amplification for viral RNA present in Zooplankton, To achieve this the isolated viral RNA were mixed with specific primers like P1-P2 (P1; 5'-AGAGATCACTGACTTCACAAGTGAC-3', and P2; 5'-TGTGCACCACAGGAAAGATGACTC-3') which was procured from sanmar speciality chemical (Genie. Pvt .Ltd), Bangalore. Prior to amplification RNA was converted into cDNA with the help of reverse transcriptase.

Synthesis of cDNA: A stock of RNA containing 10-50 µg was heated at 100°C for 5 minutes. Followed by addition of p1 and p2 primers (mentioned above) with 2 µl concentration each.3µl of reverse transcriptase (Genie Pvt Ltd Bangalore) was added at 37°C and incubated for 1 hr. After the incubation over, the sample was heated up to 100°C for 5 min to inactivate the enzymes. The newly synthesized cDNA was confirmed on the agarose gel electrophoresis.

Amplification of cDNA: cDNA synthesized in the previous step was amplified in a DNA thermal cycler cDNA was amplified for 30 cycles. 5µl of RT-PCR product was used as template DNA. PCR machine was set at 95ºCfor 1 min, 48°C for 1 min and 72ºC for 1 min of 30 cycles. Prior to this template cDNA was mixed with 12 µl of each primer P3-P4.(P3; 5'-CAACACTCTTCCCCATG-3', P4; 5'-AGAACCTCCCAGTGTCT-3').After completion for 30 cycles the product was ammensed using agarose gel electrophoresis. Followed by it was analyzed in the gel documentation system. (Yercaud Biotech Pvt Ltd, Yercaud, Salem).

RESULTS

PCR amplification of viral RNA using RT–PCR shows the viral RNA in a two different fragments in both *Acartia* and *Oithona* species with the size of 900 and 960bp and which was confirmed with the help of gel documentation system (fig.1). However, the intensity of the band was found to be different with respect to the sample. This may be due to the number of viral particle present in the sample might be more. It was very obvious from our result that the PCR product from *Oithona* species found to have more amplified DNA than the *Acartia* species (fig.1).

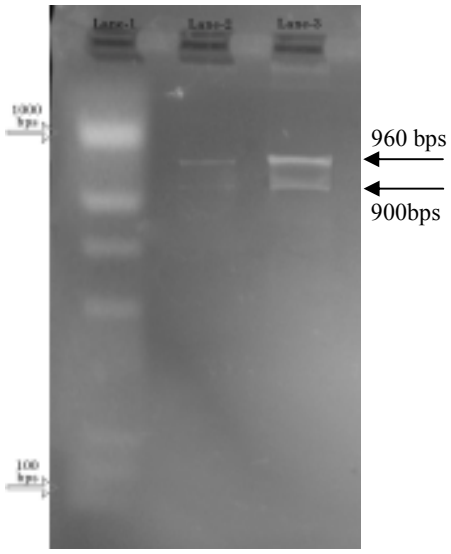

Fig. 1. *PCR amplified DNA isolated from Zooplankton*.
Lane 1. Marker DNA, Lane 2. *Acartia* species, Lane 3. Oithona species

DISCUSSION

There were many viruses reported in aquatic environments and shellfish. Many researchers have isolated RNA of birnaviruses and reported that it could have wide range of host and multiplied in number of aquatic forms [12]. Viruses infect zooplankton such as White spot syndrome virus, which is an important pathogen in shrimp and it infect copepods [13] and fish nodavirus infect zooplankton such as *Artemia* and *Rotifer*[14]. Some zooplankton such as copepods was positive for White spot syndrome virus as they were able to transmit the virus in shrimp ponds. White spot syndrome virus was detected in rotifer and rotifer resting eggs from shrimp pond sediments[15]. Birnavirus can be infected chronically and existed long term in survivors after acute infection[16,17]. PCR (polymerase chain reaction) is the opt technique to diagnosis the viral infection, It is also known for its advantages that the sensitivity of their PCR assay could be increased 10^3 to 10^4 time by using second amplification step using nested primers. It is also helpful in screening for the presence of viruses in the potential carriers and in post larvae before they are stocked in the rearing ponds[13,18]. Two steps PCR is 1000 times more sensitive than RT – PCR[19]. This was evident from our results that PCR is the best method to detect any viral infection which shows no symptoms. Recently, genetic techniques based on polymerase chain reaction (PCR) amplification of DNA have been successfully applied in qualitative studies of carnivorous insects and other organisms[20]. They have also reported that MABV genome widely distributed in the ocean. They found that MABV genome in sample of zooplankton in the Pacific Ocean and also in the Mediterranean Sea [11]. Viral infection differs with respect to the period; climatic factors and depth of the water column were found to control the infection and their growth rate. MABV genome was detected in zooplankton collected at 0m depth in September and 40 m depth in Novermber.

The PCR positive samples were 4 out of 12 (33.3%) zooplankton[10]. This confirms that MABV is concentrated in zooplankton. There are also reported that MABV genome detected in the sea water, which indicates that MABV genome originated in plankton. The specific primer used in this study was primed for the amplification of the viral RNA. It was reported to bind to specific region of the viral genome and amplify with high .It was also reported by many researchers stated that the junction regions between the genes VPS and NS is variable, but useful for the genogrouping of aqua birnavirus [21-23]. The number of zooplankton containing the MABV group increased form autumn to winter. They also reported that zooplankton may serve as a vector of MABV s in the Uwa sea of Japan. The economic loses were very significant and it was calculated that 50% of loses were only by viral disease, white spot syndrome virus (WSSV) is an important pathogen which causes severe loses without any visible symptoms. It is believed that these viruses infect zooplankton like copepods are known to be infected with WSSV and able to transmit the viruses to shrimp and other fishes. One of the researches proved that some zooplankton resting eggs deposited in the pond sediments may have been a WSSV reservoir with the potential capability of transmitting through food web to farmed shrimp[15].

CONCLUSION

Identification of these viruses will be more helpful in screening for the presence of virus in the potential carriers and in post larvae before they are stocked into rearing ponds. In the present study, we have identified the presence of (MABVs) a group of viruses from *Acartia* and *Oithona* species. These results suggest that zooplankton may serve as a vector of MABVs in these areas.

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