Development of diagnostic clones against an emerging marine fish virus disease: A methodological improvisation in the field of Red Sea Bream Iridovirus disease surveillance

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Red sea bream iridovirus (RSIV) is an OIE-listed viral disease that causes extensive mortality in marine fishes. The first outbreak of this economically significant disease in India was reported during 2018, followed by the second report from ICAR-CMFRI during 2020. Both of these outbreaks occurred in Asian Seabass (*Lates calcarifer*) cultured in open estuarine cages along the west coast of India and caused around 50 to 90% mortality rate in this economically important food fish species. RSIV is now considered an emerging disease in Indian aquaculture systems, and proper control strategies are necessary to prevent the spread of disease and consequent economic loss to the fish farmers. Further, as RSIV has a broad host range and has been isolated from fishes in open water cages, the risk of spreading to wild fish stock is very high. Thus, it becomes pertinent to do both active and passive surveillance of this disease using PCR techniques among diverse marine animals. The rapid and sensitive PCR-based assay can detect RSIV infection at a phase sufficiently early to be an aid in fish disease management. Thus, PCR-based viral disease diagnosis is potentially a fast and reliable method; however, it has certain drawbacks like the possibility of false-negative or -positive results. Standardized positive and negative controls are necessary for PCR-based diagnostic assays to overcome this problem, PCR false-negative results usually occur due to PCR inhibitors and suboptimal reaction conditions. Accordingly, the establishment of recombinant bacterial clones containing antigenic portions having the diagnostic significance of the important pathogens is necessary for diagnostic labs. Keeping the fact, recombinant bacterial clones containing antigenic portions of RSIV was established in the marine biotechnology division of ICAR-CMFRI, as a methodological improvisation in the field of RSIV disease diagnosis. The protocols followed had the approval from Institute Biosafety Committee of ICAR-CMFRI (No.2/IBSC/CMFRI dated 18/11/2021).

As the first step, total DNA was extracted from the brain tissue of the infected cage farmed Asian Seabass (collected and preserved during 2020 disease outbreak), through the phenol-chloroform method, so that the total DNA of 675.7 ng/µl concentration was obtained, having OD 260/280 nm ratio of 1.94. Then PCR was performed using the total DNA and the specific primer sets that can amplify a fragment 568 bp in length from RSIV (RSIV-For: 5'- CGGGGGCAATGACGACTACA-3', RSIV Rev: 5'-CCGCCTGTGCCTTTTCTGGA- 3'), so that an amplicon

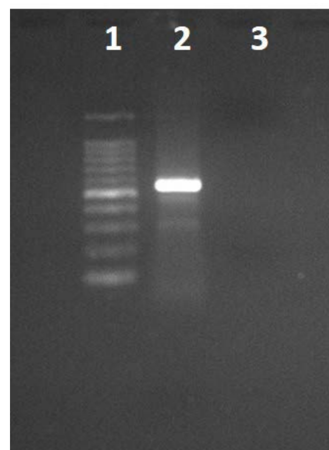

Fig. 1. PCR amplification the diagnostic fragment of RSIV

Lane 1: 100bp DNA ladder (Takara), Lane 2: PCR product, Lane 2: Negative control

of 568 bp was obtained (Fig. 1). The PCR product was then sequenced to confirm the specific amplification, and the obtained sequence was submitted in NCBI GenBank under the accession number MZ224281. The confirmed PCR product was then purified and ligated into pMD20-T vector plasmid using the Mighty TA cloning kit. Finally, the ligated plasmid was transformed into competent *E. coli DH5* cells, the *E. coli* strain which lacks virulence properties. After transformation, the cells were allowed to grow in the media containing ampicillin, X-gal (colorless analog of lactose), and IPTG (isopropyl -D-1-thiogalactopyranoside), so that the white recombinant colonies could be observed after overnight incubation(Fig. 2). The white colonies were picked and screened by colony PCR using the gene-specific primers (RSIV for and RSIV rev). After confirmation by colony PCR, the positive clones were inoculated to LB Broth containing ampicillin. The glycerol stocks were made from overnight incubated cultures, and stored at -80°C. The prepared diagnostic clones were confirmed through the isolation of plasmid DNA followed by PCR using the target gene-specific primers, and further DNA sequencing.

Fig. 2. Plate after transformation showing blue and white colonies

In conclusion, the establishment of recombinant bacterial clones containing antigenic portions of an emerging fish viral pathogen namely, RSIV was done through the present study, as a methodological improvisation in the field of RSIV disease surveillance. The clones can be used as the positive control in RSIV diagnostic and molecular surveillance protocols of the national fish disease surveillance program, to ensure 100% accurate results in the PCR protocol. Now, future investigations on RSIV circulating in the country through extensive active and passive surveillance programs in both healthy and diseased fish are warranted, to have specific stringent control measures against this emerging viral pathogen in the marine aquaculture industry.