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Molecular detection and characterization of RNA tumour Walleye dermal sarcoma virus (WDSV) phosphoprotein in Sea bass *Lates calcarifer* by using nested PCR and SDS-PAGE

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***Abstract*: The morphological evidence supports a key role for WDSV virus in the induction of skin tumours of fish. It is concluded that other biological and environmental factors mediate either expression of the tumour virus, tumour development or both. Abnormal and operculam growth of WDSV are always associated with fish tumors, which was noticed under experimental periods. In addition, mainly eyes and upper & lower jaw were recorded in middle days of experimental periods. Its clear positive indication of WDSV disorders. Irregular shape, colour, cloudiness, gas bubbles and small haemorrhagic lesions (red spots) also observed the study periods. The maximum and minimum cumulative mortality were recorded at tank A in 47th & 39th day of post inaugulation. Hence, WDSV less positive was found in 32nd day and positive bands were shown at 289bp respectively compared with 1kb DNA ladder. The moderate level was found in 39th days of experimental periods, it was shown at 479bp. Higher WDSV copies were recorded in 42nd day at 665bp, 479bp and 289bp respectively. Although the percentage of survival was noticed 100% where in 32nd day and minimum survival was observed 0 levels in ened of experimental periods (47th day). The RT-PCR detection and mortality & survival range of control tank B, were observed at 100% survival and mortality range is 0% level in experiment beginning onwards. It was shown negative house keeping gene band at 665bp, upto 47th day. The survival was noticed in minimum 0.0% and maximum was presented at 93.3%. The mortality ranges were occurred 13.3% in minimum and maximum 100% at day 32nd & 47st. The limit of detection was as severe, moderate and low infection pattern as 2000 copies/reaction (665 bp), 200 copies/reaction (479 bp) and 20 of copies/reaction (289 bp) was able to detect viral RNA from experimental animal tissues at levels ten-times lower than single tube PCR. Both higher WDSV infection and lower WDSV level infection has been amplified, and an in silico assay showed that WDSV of all genotypes can be amplified. WDSV was detected in target and non-target tissues of both diseased and asymptomatic fish. The negative control samples were showed only at 665bp, which was the product of housekeeping gene (internal control).The molecular weight marker was represented, 848 bp, 630 bp and 333 bp respectively. The lower level of infection were occurred in day 9 and severe level was found in 32 day onwards with 2000, 200 and 20 copies in under 665 bp, 479 bp and 289 bp respectively. A novel fluorescence-based staining method was developed for phosphoprotein analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Similar to the Walleye dermal sarcoma virus (WDSV) protein plays an important role in determining host range but that other viral proteins are clearly required for full phosphoprotein pathogenicity to be manifest in concern species, *Lates calcarifer* appended complex as a fluoroprobe to selectively visualize phosphorylated proteins among total proteins. The pattern of phosphorylated and non-phosphorylated proteins were presented lower to high level in on under protein marker i.e., 25kDa – 200kDa. The degree of phosphorylation were obsereved 60 minutes in under the phosphatase treatment tank. The WDSV viral proein was separated under SDS-PAGE gel, using phosphoamino acid analysis. A comparison and identification specific phosphoprotein 64kDa band intensities of 86 kDa higher and 23 kDa lower phosphorylated protein were observed in under SDS-PAGE demonsattion that the pre antibody production system can achieve detection limits of WDSV infection level and comparable those different Kda proteins resolved on a polyacrylamide gel. Since the WDSV viral protein band intensity achieved with both chain bands varies for**

different viral specific proteins. This attempt is extends the detection capability of after antibody production with associated of immuno-purification of SDS specific (64 k Da phosphoprotein) gel band, standardization of viral proteins under ELISA and preparation of antibody against WDSV, using Rabbit for developing of antibody drug against WDSV.

Keywords: **WDSV, RT-PCR, *Lates calcarifer*, experimental, mortality and SDS – PHAGE.**

I. INTRODUCTION

Viruses are the simplest forms of life yet they play a crucial role in regulating planetary processes. They are major components of the marine food web affecting bacteria, archaea and eukaryotic organisms, with consequences for nutrient and energy cycling, control of species diversity and exchange of genetic material among organisms in marine environments. It is estimated that as much as 10-20% of marine bacteria is lysed daily with 2-3% of primary production lost through viral activity. Thus the viruses play key role in energetic and as largest reservoir of genetic diversity in the marine environment [1]. A series of recent studies has shown that viruses have the ability to manipulate the life histories and evolution of their hosts in remarkable ways, challenging our understanding of the almost invisible world of viruses [2]. The Asian sea bass *Lates calcarifer* was known as barramundi, native of coastal Australia, South East and Eastern Asia. This species are farmed in cages, as well as in fresh and estuarine water. In recent years, sea bass has gained growing importance in aquaculture both as recreational and commercial fish with a high and fairly stable price. More mortality in farmed barramundi is caused by bacterial infections, but this present report described the unusual occurrence of neoplasm's in adult Asian sea bass (*Lates calcarifer*). Tumour is a common disease in fish. It caused by both viruses and non-infectious (chemicals) carcinogens. In additional, some of the reports contributing to tumour formation in fishes include viruses, bacteria, chemical or biological toxins and physical agents. More than 50% of fish tumours are related to the papilloma. The etiology of most of the tumours remains unknown. Fish tumour studies are very less in worldwide, having some reports in Indian costal water.

Walleye Epidermal Papillomatosis (WEP) or walleye demorsal Sarcolemma Virus (WDSV) occurs like outer growth of white colour tissues in many fish species, both in farmed and feral fish species. The first EP disease in fish was reported as early in 1563, when farmed carp were affected by tumor like appearance by Papillomatosis in Europe in 19th century [3]. A chemical etiology for Papillomatosis in wild fish was already alleged in the year of 1957 by Russell and Kotin. To their distinctive appearance and obvious pathological nature, tumours' of fish have been recognized by experts and laymen for centuries. Indeed, one of the first scientific documentations of a diseased fish concerned a Chaetodon species with tumour-like growths in the bones [4]. Although no present day oncologist would confirm this statement, the nature and etiology of fish tumours are still poorly understood. Morphologically, they showed light white and small bubble like structure was observed. Light microscopic observation showed over growth of tumour nodules on the skin or scales and ventral side as well of the fishes. The infected fishes showed many clusters of damaged of tissues as well as cells were obtained in the connective tissue of the epidermis at fins, gills, mouth and outer skin of the infected fish body [5].

The classification has also made for these types of viruses, able in land and vast marine ecosystem like retroviridae, heapdnaviridae, flaviviridae. Tumor causing viruses are found in DNA and RNA types but specific of DNA viruses are that they encode viral protein necessary for viral replication where RNA viruses like WDSV, cause change variant in normal host cell genes and cell receptor., which are not involved in replication. The PCR amplified product of viral nucleotides was confirmed by agarose gel electrophoresis and molecular weight of the bands were detected. The band size of WDSV is 1430bp, respectively compared with 1kb DNA ladder. the amplified PCR products of RNA based WDSV was conclude that the tumour is caused by the viruses in fishes and it may not be chemical factors and normal papillomas has been caused by chemical and physiological factors. The previous study revealed that Walleye demersal sarcoma virus from Retrovirus family were recorded that they were responsible for tumours in majority of commercial valuable fishes, detected by using RT-PCR method [6].

The RNA virus is playing a main role to causing the tumour in Indian waters as WDSV and the receptor protein potentially involved in the modulation of the virus-host interaction were identified as WDSVGAG protein receptor. In the previous study reported that the ORF gene is detected in another fish tumour virus as OMV that indicates both genes are responsible for fish tumour. Encoded Gene Sequences for the viruses (LCDV, OMV and WDSV) from Gulf of Mannar islands region were deposited to Gene Bank [7]. Protein phosphorylation is one of the most important functions in the signal transformation which is catalyzed by protein kinase and these enzume are themselfeves work as phosphoprotein

which is regulated by phosphorylation. phosphoproteins have received the most attention because of that the events of phosphorylation by kinases and dephosphorylation by phosphatases result in functional consequences, including signal transduction, apoptosis, gene expression, cell cycle progression, cytoskeletal regulation, and energy metabolism. Thus, methods developed for determining the phosphorylation status of proteins are very important. SDS-PAGE is a reliable and widely used technique for the separation, identification, and characterization of phosphoproteins. Several visualization methods have been developed for the selective detection of gel-separated phosphoproteins.

II. MATERIALS AND METHODS

Fish sampling, management and feeding

Sample collection and acclimatization phase

The fish (Sea bass *L.calcarifer*) were obtained from Gulf of Mannar coastal wares nearby Mandapam and keelakarai islands using trap and shore net with assists of fisherman. Sea bass *L.calcarifer* species was of ocean ranched origin; the size of fish is approximately 5-8 cm. and 10-12 gm, in weight. During this phase of acclimatization to the tank design, fish were fed at 1.5% of biomass per day. They were also treated with Tugon 80 against a external parasites (dose of 0.5 g m-3 during 48 hours). For fingerlings fish, portions of the kidney, spleen, pancreas and gills are combined as one sample. The tissue sample should be at least 1 g. The retina layer of the eye ball and the brain are taken for fishes exhibiting clinical signs of WDSV and combined as one sample. Tissues from a maximum of 10 fingerlings are pooled and treated as one sample. When larger fish are sampled, tissues from less than five fish are pooled. Pooled samples are of equal volume or weight.

The 47 day experiment

Fish (mean body weight 10± 4g S.D.) were starved for 24h, anaesthetized with 2-phenoxyethanol and then sorted for allocation to experimental tanks randomly. During 21 days, the fish were held in separate tanks two 50 litre capacity glass tanks in similar densities: 30 nos. The experiment was divided into two groups of 47 days each (called Tank A and Tank B) to adjust tank densities at the end of each period by biomass random removal. The physic-chemical parameters were maintained properly. 25% water exchange was made in every day. Fish were manually fed with semi floated pellet feed, once a day (in the morning). Three criteria were used to assess satiation: (1) indifference of the fish for pellets, (2) first uneaten pellets on the tank bottom and (3) first pellets collected in the particle trap of each tank. Uneaten pellets collected in the particle traps were counted after each meal. The extruded commercial feed was composed of 45% of proteins and 20% of lipids, and presented a digestible energy of 18.5 MJ per kg of feed-1.

The WDSV challenge

Viral DNA purification

Viral RNA was isolated from purified virions by treatment with proteinase K (0.2 mg/ml) and Sarkosyl (1%) at 65°C for 2 h, followed by phenol and chloroform extraction and dialysis against TE. The purity and concentration of the RNA were determined by agarose gel electrophoresis. The inoculum (viral RNA) was used to challenge WDSV-negative animals under experimental conditions. All challenged fishes displayed signs of WDSV infection thus proving the presence of infectious WDSV nodules like particles.

A challenge test was performed during 47 days on the fish from same densities: (30nos/tank), corresponding to an usual density in commercial systems. Fish were inoculated (intramuscular inoculation) with 0.2 ml of WDSV suspension SW80 (9*105 PFU per fish) [8]. Two experiments were performed using the intra muscular route. In each experimental groups of 30 shrimp (MBW = 9.40 ± 4.92 g, n = 30) were inoculated with 10, 30 or 90 ID50. In addition, control group of 10 shrimp were mock-inoculated with 50 µl PBS and used as controls. The fish were injected between above lateral line scales and below caudal peduncle area. Before and after injection, this surface was wiped with 70% ethanol. These experiments were run until all the infected fish died. Control shrimp were sacrificed at 360 h post inoculation (hpi).

Thirty fish from each density treatment were placed in triplicate tanks (30/tank). The tanks were connected to the UV filter and water temperature was maintained at 25°C. As a control, 30 fish from each density treatment were inoculated with sterile 0.5% NaCl solution. The tank used for the control group was connected to a separate line to avoid WDSV contamination. Total morbidity (i.e. mortalities and fish showing abnormal swimming behavior) was recorded daily.

Experimental design

The experimental facility was composed of 2 rectangular tanks (50 liter) all working in continues aerators. The fish population was maintained on the basis of density treatments (30 nos for WDSV viral inoculum and 3 nos for control tank without viral inoculum) with each density treatment randomly allocated to triplicate tanks. The experimental was fed with pumped sea water and three water loops were used in order to optimise water management. The first loop generated water circulation with a pump system, the second loop produced super-oxygenated water (at 25 mg l-1) due to a cone system and the third was the treatment loop [9]. This treatment loop including the CO2 degassing column, mechanical, biological and UV filters, was designed to keep the rearing water quality below the recommended values for aquaculture [10;11].

The pH was controlled using soda addition to keep values around 7.0-7.4 [12;13]. Water temperature was maintained at the optimal level for growth at around 24°C [11] using a heat exchanger. Make up water was filtered twice (40 µm and 15 µm) before use. Tank water flows were measured and adjusted before the experiment in order to be proportional to the fish density per tank. It was necessary to double the water flow rate per km of biomass in the triplicate 10 km m^{-3} tank to ensure sufficient water circulation and avoid faeces settling in the tanks.

Measured parameters

Water quality parameters

Temperature and salinity were measured daily in all tanks, with a Checktemp1 pocket Thermometer (Hanna instruments) and an ATAGO hand refractometer S/Mill-E. The pH and oxygen were also measured every day in all tanks using an Ecoscan® pH meter and a YSI 550A oxymeter. Oxygen concentration was monitored using Oxyguard® probes connected to a computerized measuring system (Linde soft).

Fish performance parameters

Fish mortality was counted daily. At the end of each period (Tank A and B), biometrics were carried out in order to adjust the biomass in the tank according to the 2 studied experimental tanks. Before biometrics, fish were anaesthetized in their tank (2-phenoxyethanol, 220 ppm m-3). All the tank biomass was weighed and any fish in excess were removed. Fish were taken randomly and weighed (W, g) and measured at the caudal fin fork (L, mm).

Blood parameters

Blood was taken 5–10 ml from the caudal vein/aorta in the gills of moribund or weak animals (starved every 3 days) initially and after routinely necessity to take blood samples from the each tanks. Blood samples were taken within 8 min of fish capture to minimize handling stress. Serum was separated by centrifugation (15000 tpm, 5 min, 4°C) and stored at - 20°C until analysis. A pool of serum from 2–4 fish was used for the SDS-PAGE analysis for characterization of IgM antibody. Serum was stored at –20°C.

Nested PCR analysis

RNA extraction for WDSV

Extraction of RNA (the following steps shall be performed at a temperature under 4°C): For large (juvenile or adult) shrimp, take 10 mg -20 mg shrimp tissue and add 150 µl Trizol. (Crush and mix, then add more Trizol for a final volume of 500 µl). Incubate the sample from at 25°C for 5 min. Centrifuge at 12,000 g at 4° C for 10 min; pipette up the supernatant and transfer it to a new micro centrifuge tube. Add 200 µl chloroform and mix for 20 s. Incubate at 25° C for 10 min. Centrifuge at 12,000 g at 25° C for 10 min; pipette up the supernatant and transfer it to a new micro centrifuge tube. Add 670 µl isopropanol and mix. Incubate at 25° C for at least 10 min. Centrifuge at 12,000 g at 25° C for 10 min; pipette off the supernatant and discard. Rinse the pellet with 0.5 ml of 70% ethanol for at least 30 min at 25° C. Centrifuge at 12,000 g at 25° C for 10 min. Pipette off the supernatant and discard. Leave at room temperature for 20 min or until the pellet is dry. Add 25 µl diethylpyrocarbonate (DEPC)-treated water. Incubate at 56° C for 15 min. Mix gently and use for the next step to synthesize cDNA immediately or store at -70°C until ready for use.

Agarose gel preparation and electrophoresis for RNA

In a 250-ml Erlenmeyer flask take 90 ml purified water; 1 g agarose; 10 ml 10X Tris borate electrophoresis buffer (10X TBE). Cap the bottle or cover the flask but do not seal. Microwave for 2 min with high power. Swirl to mix the solution. Avoid boiling over. Microwave at high power for an additional 3 min. Use gloves to remove the bottle or flask from the

microwave and mix thoroughly by swirling (all of the agarose should be dissolved). Let the solution cool at room temperature to allow handling with bare hands. Do not over-cool, allowing the solution to solidify. Add 5 µl of 25 mM ethidium bromide (EtBr) to the cooled 1% agarose solution. Swirl to mix. Place a 20-well separation comb (1.5-mm slots) into the tray of the horizontal minigel electrophoresis system. Pour approximately 80 ml of the warm agarose solution into the tray. Allow the agarose to solidify for at least 45 min at room temperature.

Separation of RNA from Agarose Gel electrophoresis

Prepare RNA samples by mixing 1 µg of total RNA with 2 µl of 6X gelloading dye and nuclease-free water to a total volume of 12 µl. Remove comb from the solidified gel. Place the tray with gel into electrophoresis minigel box. Submerge the gel with 1X TBE (diluted from 10X TBE). Apply 8 µl of the 1 Kb plus DNA ladder (Kb ladder) to the first well by submerging the tip of the pipette into the top of the well and slowly dispensing contents. Repeat step 14 for application of samples into subsequent wells. Cover the minigel box (electrodes should be engaged). Samples will migrate towards the positive electrode. Run gels at 100 V for 1.0 hr. Stop the run, remove the gel tray, and visualize the image of RNA in the gel with the UV light box. Record the image with the camera.

Quantitation of RNA by Spectrophotometric method

- 10µl of DNA solution was diluted with 990µl of TE.
- Mixed well and absorbance at 260nm and 280nm was measured.

The absorbance at 260nm can be used to calculate the concentration of RNA as follows:

Calculations

OD_{260} of 1= 40µg/µl RNA

Dilution factor =100

$$\text{Concentration of DNA in a given solution} = \frac{40 \times OD \times \text{Dilution factor}\ \mu g/\mu l}{1 \times 1000}$$

PCR amplification

PCR Set up

A 1.0µl of Sample DNA (approximately 100 ng/µl) was added to PCR Mixture containing 100mM Tris HCl (pH 8.3), 500mM KCl (pH 8.3), 2.5µl $MgCl_2$ (25mM), 2.0µl dNTP's (2.5mM), 1.0µl Primer Forward & Reverse (each of 10pm/µl) and 1u /µl of Taq Polymerase (Bioserve Make) & the final volume made to 25 µl with nuclease free water. The primer set WDSV Fw and WDSV Rw designed in the conserved region was used for the amplification of the gene encoding Major Capsid protein gene of Lymphocystis disease virus and ORF62 region of Oncorhynchus masou Herpes virus of the test organisms and the primer sequences are presented in the Table 1.

PCR condition

PCR reaction was carried out for the amplifying gene encoding protein WDSV gag and amplification conditions are follows Initial denaturation for one cycle of 94^0C for 5min then 35cycles of

- Denaturation at 94^0C for 30s
- Annealing at 55 ^{0}C for 75s,
- Extension at 68^0C for 40s and
- Final extension at 68^0C for 7 min.
- Hold at 4^0C

Electrophoresing the Amplicons

The PCR Products (6 - 10µl) were separated by electrophoresis in 2.0 % Agarose gels containing Ethidium bromide (1µg ml^{-1}). The electropherogram obtained after electrophoresis of the PCR amplicons is shown in Fig. In the electropherogram the bands of the size ~1187bp (for sample WDSV) was observed against 100bp DNA ladder (Fermentas make). There was no overlapping of the bands in the case of test organisms and that way the bands were clear.

SDS-PAGE

SDS-PAGE diagnosis method is enables the separation of proteins and determination of their molecular weight by polyacrylamide gel electrophoresis method. The separated proteins are visualized using Coomassie staining method. In briefly, prepared 1% agarose (0.05g in 5ml of distilled water). Boiled to dissolve the agarose and poured a thin horizontal layer at the lower edge of the plates to seal the assembly. Let it solidify by allowing it to cool down for 5-10 minutes. Prepared of 12% separating gel and poured the gel in-between the plates and allow it to solidify for an hour. Immediately after the gel was poured, added distilled water to level the gel. After an hour poured off the water by inverting the casting assembly and prepared of 5% stacking gel. After addition of TEMED gently mix all the components by swirling the beaker. Poured the stacking gel on top of the separating gel and immediately placed the comb avoiding air bubbles. Allowed it to solidify for 30 minutes. Poured 1X Tris-Glycine-SDS Gel Running Buffer in the unit such that the buffer connected the two electrodes, and hence completes the flow of current. Remove the comb from the Stacking Gel carefully.

Sample Preparation

Taken 2 tubes for protein samples. Labelled them respectively. Taken 20 µl of each sample in the respective tube and added 5 µl of 5X Sample Loading Buffer to it. Boiled the tubes containing Protein Samples at 100^{o}C in a boiling water bath. Do not boil the tube containing Prestained Protein Ladder. Loaded 5 µl of Prestained Protein Ladder and 20 µl of the samples immediately after the heat treatment in the wells created by the comb in the Stacking Gel. Connected the power cord to the electrophoretic power supply according to the conventions: Red-Anode and Black- Cathode. Electrophorese at 100 volts and 10 mA until dye front reaches 0.5 cm above the sealing gel. Carefully removed the gel from in-between the plates using spatula into the plastic tray containing distilled water. Washed the gel for 1 minute. Discarded the water & proceed for staining destaining procedure.

Staining and Destaining of Gel

After removing water, added 50 ml of Staining Solution in the tray containing gel, till the bands are visible. Sometimes the gel may have to be kept overnight in the staining solution for visualization of the bands. Removed the gel from Staining Solution. The Staining Solution can be re-used 2-3 times. Washed the gel by rinsing with distilled water till a considerable amount of stain leaches out from the gel. Kept changing the distilled water for 3-4 times. Added 50 ml of Destaining Solution to the gel. Destaining should be carried out with constant moderate shaking. 5. Continued destaining till clear, distinct bands are observed. Removed the gel from Destaining Solution. The Destaining Solution can be re-used 2-3 times.

Analysis of the 64-kDa phosphoprotein

Phosphoamino acid analysis of the 64-kDa phosphoprotein, labeled in the soluble chloroplast extract and the mixed envelope fraction, was done as in. Envelope membranes and soluble proteins were labeled for 3 min under conditions described above. One half of the phosphorylation assay was analyzed by SDS-PAGE, the second half was precipitated by 200 p1 10% trichloroacetic acid. The pellet was washed three times with 5% trichloroacetic acid. Proteins were hydrolyzed in 6 M HC1 at 110°C for 2 h. Unlabeled phosphoamino acids (phosphotyrosine, phosphothreonine, phosphoserine, 20 pg each) were added to the reaction. The solvent was evaporated at 60°C under a stream of N2, resuspended in water, spotted onto a precoated Silica gel thin-layer plate (Merck, Kieselgel60) and electrophoresed under cooling for 4 h at 1000 V using glacial acetic acid/formic acid/H20 (78/25/897 by vol.) as running buffer. The phosphoamino acids were located by ninhydrin staining and radioactivity was detected by autoradiography as described above.

The 64-kDa soluble and envelope phosphoproteins were compared by partial proteolytic mapping using V8 protease. Phosphorylated proteins were separated by SDSPAGE, and dried gel slices were excised and reswollen in 125 mM Tris/HCl, pH 6.8, 1 mM EDTA, 0.1% (massjvol.) SDS (buffer A**)** for 1 h. V8 protease *(5* pl) was introduced into the wells of a second SDS/polyacrylamide gel (15% acrylamide), as a solution containing 0, *5*, 25 or 500 pg enzyme/ml in buffer A supplemented with 10% (by vol.) glycerol, and allowed to electrophorese at 20 mA for *5* min. The buffer was removed from the wells and replaced by 30 p1 buffer A containing 20% glycerol and 0.001% bromophenol blue. After 1 min the

buffer was removed and replaced by swollen gel fragments. The upper reservoir buffer was added and 5-pl aliquots of the V8 protease solutions were layered above the gel fragments. Electrophoresis was resumed, interrupted twice for 15 min, when the dye had travelled one third and two-thirds of the way through the stacking gel, and continued until the dye had reached the end of the gel.

III. RESULT

Morphological observation

The walleye dermal sarcoma virus (WDSV) is a retrovirus etiologically associated with epidermal tumor or skin tumor termed walleye dermal sarcoma (WDS) that is endemic in walleye fish throughout the world. In contrast to other tumors induced by animal retroviruses, WDS cyclically develops and regresses. Morphologically, WDSV showed light white and small bubble like structure was observed. During 32nd day of experimental periods, light microscopic observation showed over growth of tumour nodules on the skin or scales and ventral side as well of the fishes (Fig.1- a &b). The infected *L.calcarifer* fishes showed many clusters of damaged of tissues as well as cells were obtained in the connective tissue of the epidermis at fins, gills, mouth and outer skin of the infected fish body. Abnormal and operculam growth of WDSV are always associated with fish tumors, which was noticed under experimental periods of 34th day (Fig.1- c &d). In addition, mainly eyes and upper & lower jaw were recorded in the 42nd day of experimental periods (Fig. 1 – e & f). It's clear positive indication of WDSV disorders. Irregular shape, colour, cloudiness, gas bubbles and small haemorrhagic lesions (red spots) also observed the study periods.

Experimental transmission of WDSV to L.calcarifer

In generally, locally invasive tumors were observed in experimental transmission studies and their incidence was correlated with the age of fish at the time of infection. Walleye dermal sarcoma virus was experimentally transmissible to *L.calcarifer* through intramuscular injection route. The PCR detection and cumulative percent mortality of *L.calcarifer* at different time intervals after inoculum intra muscular (i.m) injection) with WDSV filtratation method. The maximum and minimum cumulative mortality were recorded at tank A in 47th & 39th day of post inaugulation. Hence, WDSV less positive was found in 32nd day and positive bands were shown at 289bp respectively compared with 1kb DNA ladder. The moderate level was found in 39th days of experimental periods, it was shown at 479bp. Higher WDSV copies were recorded in 42nd day at 665bp, 479bp and 289bp respectively (Fig..2). Although the percentage of survival was noticed 100% where in 32nd day and minimum survival was observed 0 levels in ened of experimental periods (47th day). The PCR detection and mortality & survival range of control tank B, were observed at 100% survival and mortality range is 0% level in experiment beginning onwards. It was shown negative housekeeping gene band at 665bp, upto 47th day. Molecular weight marker were representated at 848bp, 630bp and 333bp (Fig. 3). There was no mortality occurred in entire periods of experimental WDSV challenging studies (Table.3).

Nested PCR analysis

The RNA tumour WDSV is responsible for a chronic self-limiting disease that affects more than 125 teleosts. Viral isolation of WDSV is difficult, time-consuming and often ineffective; the development of a rapid and specific tool to detect and quantify WDSV is desirable for both diagnosis and pathogenic studies. In this study, nested PCR assay was using a modified IQ2000 kit based assay targeting a highly conserved region of the MCP gene. Primers were designed on a multiple alignment that included all known WDSV genotypes. The viral RNA segment was cloned within a plasmid to generate a standard curve. This system has adopted the design of Nested PCR and also inherited the reliability, sensitivity and experience from a range of IQ2000 T^{M} viral diagnostic kits for fish viral infections. The WDSV primer has an effective and reproducible detection limit of 10 viral copies while in most cases digit copy of virus can also be detected.

The limit of detection was as severe, moderate and low infection pattern as 2000 copies/reaction (665 bp), 200 copies/reaction (479 bp) and 20 of copies/reaction (289 bp) was able to detect viral RNA from experimental animal tissues at levels ten-times lower than single tube PCR. Both higher WDSV infection and lower WDSV level infection has been amplified, and an in silico assay showed that WDSV of all genotypes can be amplified. WDSV was detected in target and non-target tissues of both diseased and asymptomatic fish. The negative control samples were showed only at 910 bp, which was the product of housekeeping gene (internal control).The molecular weight marker was represented, 848 bp, 630 bp and 333 bp respectively (Fig. 2).

The lower level of infection were occurred in day 9 and severe level was found in 32 day onwards with 2000, 200 and 20 copies in under 665 bp, 479 bp and 289 bp respectively. The survival was noticed in minimum 0.0% and maximum was presented at 93.3%. The mortality ranges were occurred 13.3% in minimum and maximum 100% at day 32nd & 47st. The positive band size was varied from the level of infection in WDSV as 665, 479bp, and 289bp respectively compared with 1kb DNA ladder (Fig. 3). Amplification by the RT-PCR assay from the collected, fish infected samples followed by cell cultures supernatants of each viral template separately and of all three target templates from a mixture of three viruses were identified successfully by PCR amplification.

Detection of phosphoprotein in WDSV by SDS-PAGE

Protein phosphorylation is one of the most important processes for cellular regulation and signal transduction in eukaryotic cells. A novel fluorescence-based staining method was developed for phosphoprotein analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Similar to the Walleye dermal sarcoma virus (WDSV) protein plays an important role in determining host range but that other viral proteins are clearly required for full phosphoprotein pathogenicity to be manifest in concern species, *L.calcarifer* appended complex as a fluoroprobe to selectively visualize phosphorylated proteins among total proteins. Comparison of the sensitivity and specificity of different detection methods in SDS-PAGE with standard mixture marker proteins (A–D) and WDSV viral total proteins were analysed in different level of WDSV infection by experimental challenging studies. Marker proteins (BSA) were weighed accurately and dissolved in loading buffer containing 60mM Tris (pH 6.8), 25% (v/v) glycerol, 2% (w/v) SDS, 2% (v/v) mercaptoethanol, and 0.1% (w/v) bromophenol blue to make as standard mixture markers for 1-D electrophoresis (Xi Wang, *et al*., 2014).

According to the results, when mixed envelope membranes were incubated with very different centrations, we observed dramatic differences in the phosphorylation patterns (Fig. 5). Using a very low [y-32P] WDSV load concentration (33 nM), a protein, which was phosphorylated to a significant extent, had an apparent molecular mass of 64 kDa on SDS-PAGE. Only when we used much higher WDSV load (50 pM) was the previously described phosphorylation pattern observed. Under these conditions 86-kDa and 23-kDa proteins were the major phosphorylation products. It was possible to shift the phosphorylation pattern in a pulse-chase experiment. Mixed envelope membranes were first phosphorylated for 1 min using carrier-free. This resulted mainly in the phosphorylation of the 64-kDa protein for enhancing antibody against WDSV (Fig. 5). The present study, described the pattern of phosphorylated and non-phosphorylated proteins were presented lower to high level in on under protein marker i.e., 25kDa – 200kDa. The degree of phosphorylation were observed 60 minutes in under the phosphatase treatment tank. The WDSV viral proein was separated under SDS-PAGE gel, using phosphoamino acid analysis. Further we need to isolate specific phosphoprotein (64kDa) and construct to recombinant viral antibody production against RNA tumour WDSV pathogen.

IV. DISCUSSION

Abnormal and operculam growth of WDSV are always associated with fish tumors, which was noticed under experimental periods. In addition, mainly eyes and upper & lower jaw were recorded in middle days of experimental periods. Although, WDS are cutaneous mesenchymal neoplasms that are randomly distributed on the fish, arise from the superficial surface of the scales and range in size from 0.2-1.0 cm in diameter. These proliferative diseases were first reported by Walker in 1969 in walleye collected from Oneida Lake in New York State and have been reported to occur elsewhere in North America [14; 15].

In present study, WDSV was detected in target and non-target tissues of both diseased and asymptomatic fish. The negative control samples were showed only at 665 bp, which was the product of housekeeping gene (internal control).The molecular weight marker was represented, 848 bp, 630 bp and 333 bp respectively. The lower level of infection were occurred in day 9 and severe level was found in 32 day onwards with 2000, 200 and 20 copies in under 665 bp, 479 bp and 289 bp respectively. The survival was noticed in minimum 0.0% and maximum was presented at 93.3%. The mortality ranges were occurred 13.3% in minimum and maximum 100% at day 32nd & 47st. Similar studies were reported in shellfishes [15] is the two greatest challenges in the detection of enteric RNA viruses from the shellfish are to concentrate the low levels of viruses in oyster tissues to volumes suitable for RT-PCR analysis and to overcome the effects of inhibitory materials co-extracted with viral RNA by using the standard protocol of Atmar *et al*., 1993, for the extraction and detection of Norwalk viruses in shellfish tissues determined that southern hybridization increased the

occurrence of detection by 4% (2 of 46 analysis) over that of agarose gel electrophoresis [17]. The present study revealed that Walleye demersal sarcoma virus from Retrovirus family were recorded that they were responsible for tumors in sea bass fish *L.calcarifer* detected by using RT-PCR method. The negative control samples were showed only at 665 bp, which was the product of housekeeping gene (internal control).The molecular weight marker was represented, 848 bp, 630 bp and 333 bp respectively. The lower level of infection were occurred in day 9 and severe level was found in 32 day onwards with 2000, 200 and 20 copies in under 665 bp, 479 bp and 289 bp respectively. The survival was noticed in minimum 0.0% and maximum was presented at 93.3%. The mortality ranges were occurred 13.3% in minimum and maximum 100% at day 32nd & 47st. The previous study revealed that Walleye demersal sarcoma virus from Retrovirus family were recorded that they were responsible for tumours in majority of commercial valuable fishes, detected by using RT-PCR method [6].

A novel fluorescence-based staining method was developed for phosphoprotein analysis in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Similar to the Walleye dermal sarcoma virus (WDSV) protein plays an important role in determining host range but that other viral proteins are clearly required for full phosphoprotein pathogenicity to be manifest in concern species, *L.calcarifer* appended complex as a fluoroprobe to selectively visualize phosphorylated proteins among total proteins. Present study, the WDSV load concentration (33 nM), a protein, which was phosphorylated to a significant extent, had an apparent molecular mass of 64 kDa on SDS-PAGE. Only when we used much higher WDSV load (50 pM) was the previously described phosphorylation pattern observed. Under these conditions 86-kDa and 23-kDa proteins were the major phosphorylation products. It was possible to shift the phosphorylation pattern in a pulse-chase experiment. Mixed envelope membranes were first phosphorylated for 1 min using carrier-free. This resulted mainly in the phosphorylation of the 64-kDa protein for enhancing antibody against WDSV. Similar attempts to localize the 64-kDa phosphoprotein using separated inner and outer envelope membranes showed that only very little of this phosphoprotein could be detected in these fractions compared with the labeling seen in mixed envelope preparations [18] .

V. CONCLUSION

The present study revealed that, the molecular detection and characterization of RNA tumour Walleye dermal sarcoma virus (WDSV) phosphoprotein in Sea bass *Lates calcarifer* by using nested PCR and SDS-PAGE. The morphological evidence supports a key role for WDSV virus in the induction of skin tumours of fish. It is concluded that other biological and environmental factors mediate either expression of the tumour virus, tumour development or both. The pattern of phosphorylated and non-phosphorylated proteins were presented lower to high level in on under protein marker i.e., 25kDa – 200kDa. The degrees of phosphorylation were observed 60 minutes in under the phosphatase treatment tank. The radioactive 64-kDa band was localized on the gel by autoradiography, excised from each lane and treated with the V8 protease during a second round of SDS-PAGE (Cleveland, 1977). Separation of the proteolytic products revealed an identical phosphopeptide degradation pattern. Phosphoserine was the only labeled amino acid detected when the two 64-kDa phosphoproteins were hydrolyzed and analyzed by high-voltage electrophoresis on silica gel thin-layer plates (data not shown). Thus, the 64-kDa proteins in the soluble extract and in the mixed envelope fraction are indeed identical (Jurgen Soll and John Bennett, 1988). The WDSV viral proein was separated under SDS-PAGE gel, using phosphoamino acid analysis. Further we need to isolate specific phosphoprotein (64kDa) and construct to recombinant viral antibody production against RNA tumour WDSV pathogen.

To conclude, the aspire of study, for the first time in the Gulf of Mannar group of islands, a molecular detection and characterization of phosphoprotein antibody against walleye dermal sarcoma virus in sea bass *Lates calcarifer* species. A comparison and identification specific phosphoprotein 64kDa band intensities of 86 kDa higher and 23 kDa lower phosphorylated protein were observed in under SDS-PAGE demonsattion that the pre antibody production system can achieve detection limits of WDSV infection level and comparable those different Kda proteins resolved on a polyacrylamide gel. Since the WDSV viral protein band intensity achieved with both chain bands varies for different viral specific proteins. This attempt is extends the detection capability of after antibody production with associated of immuno-purification of SDS specific (64 k Da phosphoprotein) gel band, standardization of viral proteins under ELISA and preparation of antibody against WDSV, using Rabbit for developing of antibody drug against WDSV. Further, require inspecting the concentration of phophoprotein antibody, which has to be developed from Rabbit and the attempt, will be made under in-vivo laboratory experimental study from the candidate species of Sea bass *L.calcarifer*. Once succeed the

drug design and control system against WDSV, then able to precede sequences of specific molecules and drug developments process with assist of pharmaceutical industries. The entire attempt will be much more helpful for researchers as well as fish farmers, those who are suffering from huge losses, due to viral disease and also we have to apply pattern, for getting authenticate in our innovative and novel research.

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APPENDICES

List of Table:

Table 1: Representing the primers of WDSV for cDNA amplification

Organisms	Forwarded primer	Reverse primer
WDSV (cDNA)	5' TGA AGC AGG AAT ACC TAC CT 3'	5' CTG TAA GTC CGT TCT CTT GT 3’

Table 2: Representing WDSV PCR components and their Composition

S.No.	Component	Quantity
1	10X PCR Buffer	2.5 µl
2	25mM MgCl2	2.0 µl
3	2.5mM dNTPs	2 µl
4	WDSV Fw Primer(10 pm/ µl)	1 µl
5	WDSV Rw (10 pm/ µl)	1 µl
6	Template DNA(100ng/ µl)	1 µl
7	Taq Polymerase(1U/ µl)	1 µl
8	Nuclease Free MilliQ water	15 µl

Table 3: PCR detection and cumulative percent mortality of *L.calcarifer* at different time intervals after inoculum intra muscular (i.m) injection) with WDSV filtrate

Time post injection required for detection	PCR detection for WDSV	Mortality & survival range of experimental tank A–intramuscular Injection (%)		PCR detection, mortality & survival range of control Tank B (%)		
Day	Tank A	Mortality	Survival	PCR	Mortality	Survival
5th day	-ve	0	100	-ve	0	100
10th day	-ve	0	100	-ve	0	100
15th day	-ve	0	100	-ve	0	100
20th day	-ve	0	100	-ve	0	100
25th day	-ve	0	100	-ve	0	100
27th day	-ve	0	100	-ve	0	100

30th day	-ve	0	100	-ve	0	100
31th day	-ve	0	100	-ve	0	100
32h day	+ve	0	100	-ve	0	100
33th day	+ve	3.3	96.7	-ve	0	100
34h day	+ve	0	96.7	-ve	0	100
35th day	+ve	0	96.7	-ve	0	100
36th day	+ve	6.7	93.3	-ve	0	100
37th day	+ve	0	93.3	-ve	0	100
38th day	+ve	0	93.3	-ve	0	100
39th day	+ve	13.3	86.7	-ve	0	100
40th day	+ve	20.0	80.0	-ve	0	100
41th day	+ve	26.7	73.3	-ve	0	100
42th day	+ve	30.0	70.0	-ve	0	100
43th day	+ve	36.7	63.3	-ve	0	100
44st day	+ve	53.3	46.7	-ve	0	100
45nd day	+ve	70.0	30.0	-ve	0	100
46rd day	+ve	83.3	16.7	-ve	0	100
47th day	+ve	100	0	-ve	0	100

List of Figure:

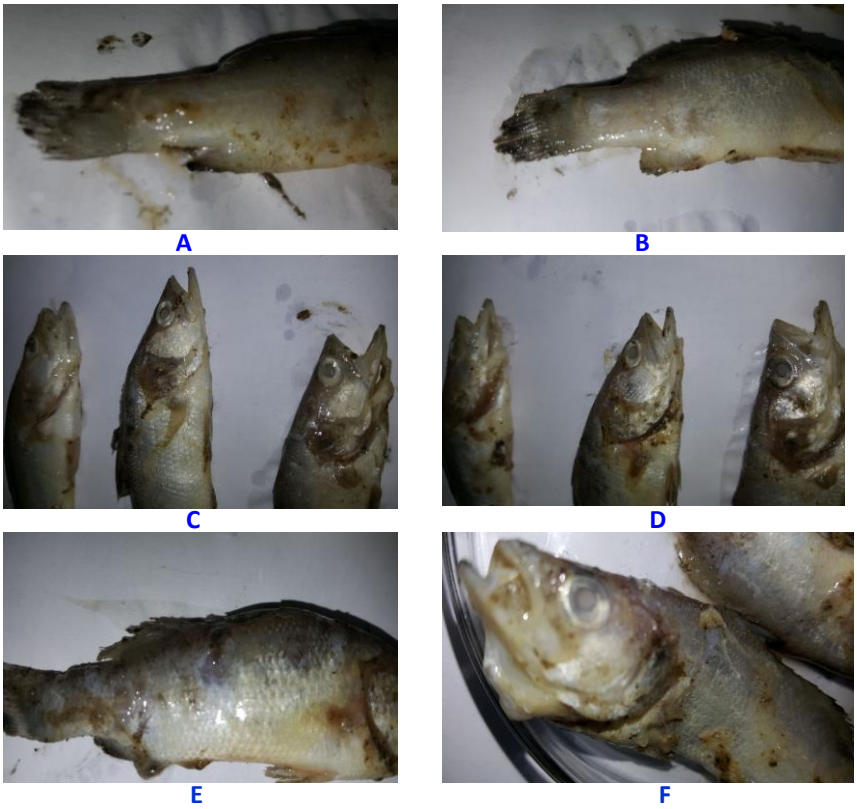

Fig. 1: Experimental transmission of WDSV infected Sea bass *L.calcarifer*, gross pathology of tumors. (A. low level infection in 32nd day at caudal peduncle region and B. moderate level of infection in 37th day at nearby dorsal fin region, C. low level infection in operculam region at 34th day, D. moderate level of infection in 36th day at snout and body region, E. severe level of infection in 47th day at dorsal and caudal region and F. severe level of infection in 47th day at individual tumor and a large mass of coalescing tumors are visible in under mouth region.

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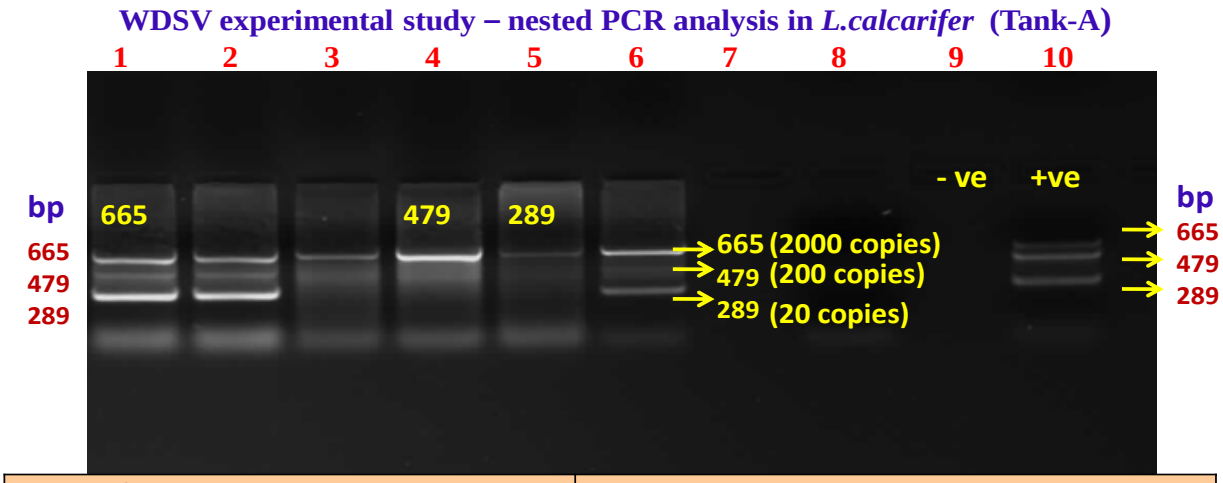
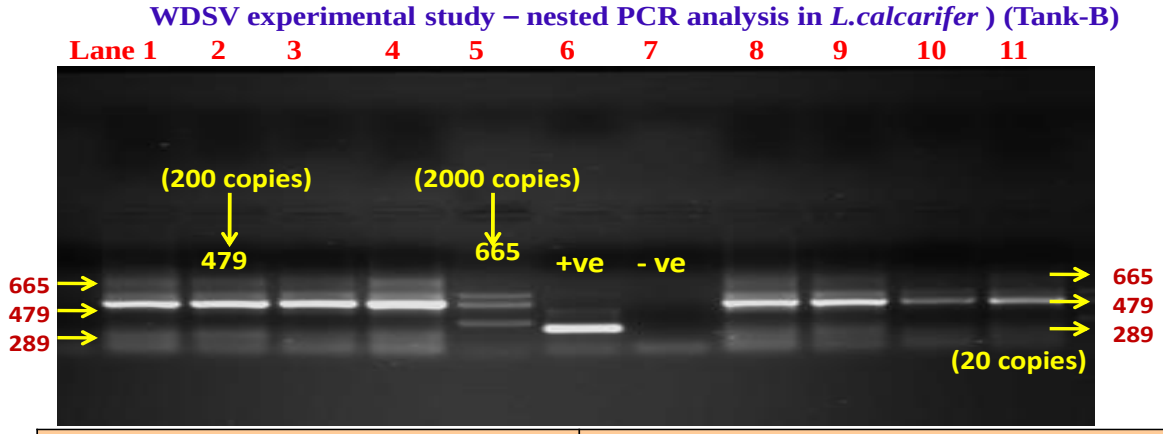


a. *L.calcarifer* Post viral inoculum			
Lane 1	Day 12, 20d WDSV low. level +ve sample (665,479,289bp) –Tank-A-sample	Lane 6	Day 55, WDSV mod. level +ve sample (479,289bp) –Tank-A-sample
Lane 2	Day 25, 35d WDSV mod. level +ve sample (479,289 bp) –Tank-A-sample	Lane 7	Negative control (yeast tRNA)
Lane 3	Day 40 D WDSV mod. level +ve sample (289bp) –Tank-A-sample	Lane 8	Day 60th d WDSV low level +ve sample (479,289bp) –Tank-A-sample
Lane 4	Day 42,45d WDSV mod. level +ve sample (289bp) –Tank-A-sample	Lane 9	Negative control (yeast tRNA)
Lane 5	Day 50 d WDSV mod. level +ve sample (289bp) –Tank-A-sample	Lane 10	Positive control (665,479,289 bp)

Fig. 2: WDSV experimental study – nested PCR analysis in *L.calcarifer* (Tank A)



A. *L.calacarifer* Post viral inoculum			
Lane 1	Day 65 WDSV low. level +ve sample (665,479,289bp) –composite sample	Lane 7	Day 82 WDSV mod. level +ve sample (479,279bp) – Tank-A-sample
Lane 2	Day 70 WDSV mod. level +ve sample (650,296bp) –composite sample	Lane 8	Day 81 WDSV sev. level +ve sample (665,479,289bp) –composite sample
Lane 3	Day 72 WDSV mod. level +ve sample (296bp) – composite sample	Lane 9	Day 80 WDSV sev. level +ve sample (665,479,289bp) –composite sample
Lane 4	Negative control (yeast tRNA)	Lane 10	Positive control (479 and 289bp - moderate)
Lane 5	Day 75 WDSV sev. level +ve sample (665,479,289bp) –composite sample	Lane 11	Positive control (665, 479 and 289bp - moderate)
Lane 6	Positive control (665,479 and 289bp)	Lane 12	Day 78 WDSV sev. level +ve sample (665,479,289bp) –Tank-A-sample

Fig. 3: WDSV experimental study – nested PCR analysis in *L.calcarifer* (Tank B)

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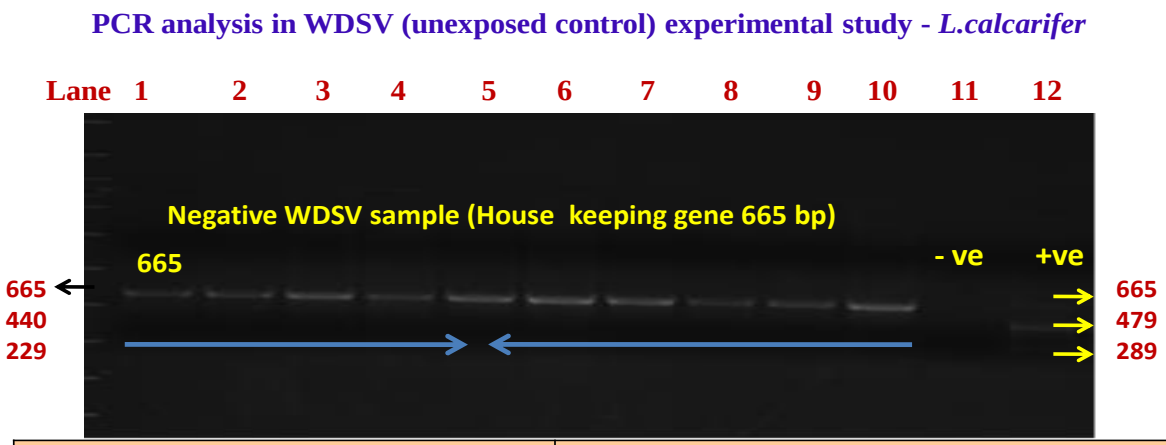
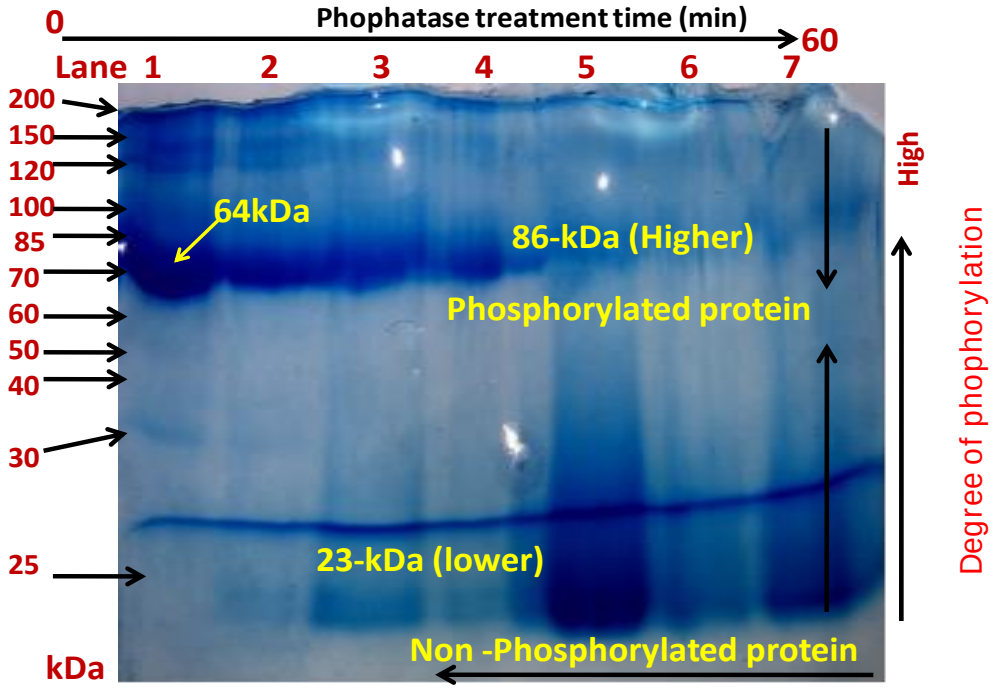


A. *L.calacarifer* – Control tank-B			
Lane 1	1st to 10th day WDSV Negative -ve sample (665bp) - composite sample	Lane 7	50th d-55th d WDSV Negative -ve sample (665bp) – Tank-A-composite sample
Lane 2	15th to 20th day WDSV Negative -ve sample (665bp) – Tank-A-composite sample	Lane 8	60th d-75th d WDSV Negative -ve sample (665bp) – Tank-A-composite sample
Lane 3	25th d-30th d WDSV Negative -ve sample (665bp) – Tank-A-composite	Lane 9	78th -80thd- WDSV Negative -ve sample (665p) – Tank-A
Lane 4	35th d-38th d WDSV Negative -ve sample (665bp) – Tank-A-composite sample	Lane 10	82nd d WDSV Negative -ve sample (665bp) – Tank-A
Lane 5	40st d-42nd d WDSV Negative -ve sample (665bp) – Tank-A-composite sample	Lane 11	Negative control (yeast tRNA)
Lane 6	45th d-48th d WDSV Negative -ve sample (665bp) – Tank-A-composite sample	Lane 12	Positive control (665, 479 and 289bp)

Fig. 4: PCR analysis in WDSV (unexposed control) experimental study – *L.calcarifer*



Different proteins are phosphorylated in isolated *L. calcarifer* serum. Phosphorylation assays were done using identical protein contents for Phospho protien.

Fig. 5: SDS-PAGE detection of phosphoprotein (64kDa) in WDSV by using BSA prortein marker for comparison of sensitivity and specificity major phosphorylation products.