Aquaculture xxx (xxxx) xxxx

Contents lists available at ScienceDirect

Aquaculture

journal homepage: www.elsevier.com/locate/aquaculture

Red sea bream iridovirus disease (RSIVD) outbreak in Asian seabass (*Lates calcarifer*) cultured in open estuarine cages along the west coast of India: First report

S.K. Girisha[a,*,1], T.G. Puneeth[a,1], M.S. Nithin[a], B.T. Naveen Kumar[b], S.K. Ajay[a], T.N. Vinay[c], T. Suresh[a], M.N. Venugopal[a], K.S. Ramesh[a]

[a] College of Fisheries, Karnataka Veterinary, Animal and Fisheries Sciences University, Matsyanagar, Mangalore, 575002, Karnataka, India
[b] College of Fisheries, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab, India
[c] Indian Council of Agricultural Research -Central Institute of Brackishwater Aquaculture, MRC Nagar, Chennai, Tamil Nadu, 600028, India

ARTICLE INFO

Keywords:
Asian seabass
Iridoviridae
Megalocytivirus
Red sea bream iridovirus
Diagnosis

ABSTRACT

In the present study, moribund Asian seabass (*Lates calcarifer*) fish samples were collected from open estuarine cages and observed for clinical signs followed by molecular diagnosis using primers targeting DNA polymerase gene of RSIV reported from OIE. The 568 bp amplicon confirmed the 100% sequence similarity with the reported sequences of RSIV from other countries. It formed the four clusters (RSIV, ISKNV, TBIV, and SDDV) with showing the close relation to Korean RSIV isolate than the RSIV isolate of Japanin the phylogenetic tree and multiple alignment analysis. The histopathogical examinations revealed, necrosed cellular material, melanomacrophage centers, RBC proliferation, lymphocytic infiltration, increased vacuoles and irregular intracytoplasmic viral inclusion bodies were observed in infected fish gill and spleen tissues. The challenge test with tissue homogenate reproduced 100% mortality in the healthy seabass by 6th day post infection (dpi). The first report of the virus in cultured Asian seabass from the region has been notified to OIE.

1. Introduction

Asian seabass *(Lates calcarifer)* is an economically important food fish cultured in several Asian countries (FAO, 2012) and the global production was reported to be 72000 tons in 2014 (FAO, 2017). India produces 6000–8000 tons of seabass in the open sea/estuarine cages in the coastal areas worth INR 2 billion (AQUASTAT-CIBA, 2018). Infectious diseases are a major concern in modern-day aquaculture as the production technologies focus on intensification to meet the increasing demand of quality protein for human consumption. Further, the unscientific management practices are increasing the risk of diseases due to emerging and re-emerging pathogens leading to huge economic losses (Qin et al., 2006). Hence, a national level disease surveillance and monitoring programme cum diagnosis and reporting will become a prerequisite to control and prevent the diseases in aquaculture.

The emerging diseases due to infectious pathogens like viral nervous necrosis (VNN) (Banerjee et al., 2014), scale drop disease virus (SDDV) (Senapin et al., 2019), infectious spleen and kidney necrosis virus (ISKNV) (Dong et al., 2017), turbot reddish body iridovirus (TBIV) (Shi et al., 2004) and red sea bream iridovirus (RSIV) genotype I and II (Inouye et al., 1992; Kurita and Nakajima, 2012) are known to be devastating on marine finfish farming in Asian countries. RSIV belongs to the genus *Megalocytivirus* in the family *iridoviridae*, with *Iridovirus, Ranavirus, Lymphocystivirus* and *Chloriridovirus* falling under the same family (Jancovich et al., 2012). Further, *Megalocytivirus* is classified into RSIV genotype I and genotype II, ISKNV and turbot reddish body iridovirus (TBIV) (Fu et al., 2011; Kurita and Nakajima, 2012). RSIV has been reported from several Asian countries including Taiwan, China, Hong Kong, Korea, Japan, Malaysia, Singapore and Thailand (OIE, 2012). It is known to infect several brackish and marine fin-fish species including *Lates calcarifer* (OIE, 2012; Wu et al., 2013). The clinical signs include lethargy, slow movement, enlarged basophilic cells in the target organs like gills, kidney, heart, liver and spleen (Inouye et al., 1992; Jung et al., 1997; Nakajima et al., 1998) with 100% mortality. RSIV has not been reported from any species till date from India.

In the study, dead and moribund Asian seabass showing the

* Corresponding author. Department of Aquatic Animal Health Management (Formerly Fisheries Microbiology), Karnataka Veterinary, Animal and Fisheries Sciences University, Matsyanagar, Mangalore, 575002, Karnataka, India.
E-mail address: skgirisha@gmail.com (S.K. Girisha).
[1] contributed equally.

https://doi.org/10.1016/j.aquaculture.2019.734712
Received 5 August 2019; Received in revised form 6 October 2019; Accepted 6 November 2019
Available online 09 November 2019
0044-8486/

abnormal behavior were collected from the open cages located in the Seetha River Estuary, Haradi, Udupi (Taluk & District), Karnataka, India (Along the west coast of India; Latitude: 13^0 26′ 8.917" (13.4358102) Longitude: 74^0 43' 0.228" (74.71673)) The collected samples were diagnosed to find the causative agent.

2. Materials and methods

2.1. Preliminary diagnosis and sample collection

The preliminary diagnosis was carried out to observe fish behavior and clinical signs such as slow-moving, lethargy, reduced feeding, movement of fish close to walls and surface of the water in cages. Pathological changes in spleen, kidney and gill were observed in the dead and moribund fish. Moribund and dead fishes were brought to the laboratory in ice and dead samples were stored at −80 °C for pathogen isolation and confirmation.

2.2. Amplification and sequencing of viral genes and phylogenetic analysis

The total DNA was extracted from the spleen, kidney, liver, and gill tissues from infected fishes as described by (Otta et al., 2003) with slight modifications. Briefly, the digested homogenate was centrifuged at 10,000 × *g* for 10 min at room temperature and 300 µl of supernatant was mixed with the same volume of phenol: chloroform: isoamyl alcohol (25:24:1). The DNA was precipitated from the aqueous phase with 3 vol of absolute ethanol followed by centrifugation at 14,000 × *g* for 10 min. DNA pellet was washed and dissolved in 50 µl of TE buffer (pH 8.0). The samples were screened for iridovirus and nodavirus by using the pathogen-specific primers (Table 1; Kurita and Nakajima, 2012; Parameswaran et al., 2008), following the standard protocols and visualized on 1.5% agarose gel. The amplified DNA polymerase genes of RSIV isolates obtained from diseased Asian seabass were sequenced and the accession numbers were submitted to GenBank. The sequences were aligned with other viruses belonging to genus *Megalocytivirus* and a phylogenetic tree was constructed using MEGA10.

2.3. Histopathological examination

Spleen and gill tissues from infected and control fishes were collected and fixed in Davidson's fixative (Humason, 1972) for 48 h. All the fixed tissues were processed, embedded in paraffin, sectioned and stained with haematoxylin and eosin (HE). The prepared slides were observed under the light microscope to examine the histopathological changes.

2.4. Challenge experiment

In vivo challenge experiment was conducted to test the Koch's postulate theory. Briefly, healthy Asian seabass weighing 16.21 ± 1.67 g were procured from the commercial fish farm. After 10 days of acclimatization at the laboratory, a total of 60 RSIV negative fishes were selected and randomly distributed into two experimental groups as control and challenge group with three replicates each. 0.1 ml viral homogenate prepared from the RSIV infected spleen tissue and TNE buffer was injected (Intraperitoneal injection) to each fish in the challenge group (n = 30) and control group respectively. During the challenge experiment, seawater quality was maintained at ambient range for the Asian seabass culture (Temp 28 °C ± 2 °C; Salinity- 20 ppt). The experiment was conducted for 10 days with daily recording of behavioral changes and mortality pattern. Finally, RSIV specific PCR assay mentioned in the section 2.2 was carried out to confirm the mortality due to RSIV.

3. Results

3.1. Description of the disease history and preliminary diagnosis

Unknown disease in Asian seabass with abnormal behavior and mass mortality was reported by farmers since 2018, from the west coast of India causing severe mortalities. The disease affected grow-out culture in open cages in the brackishwater environment. The infected/moribund fishes typically showed pale body coloration Fig. 1A and slow movement in cages, often not feeding. The pathological changes included a pale gill coloration Fig. 1B and enlarged spleen Fig. 1C. Running mortality leading to mass mortality, up to 80–90% was recorded. The clinical signs were typical of a viral infection.

3.2. Confirmation of RSIV by PCR and sequence analysis

Among viral pathogens, the PCR assay amplified the product size of 568 bp corresponding to the DNA polymerase gene of RSIV (Fig. 2A). The nucleotide sequence (Submitted to NCBI) of the DNA polymerase gene showed 100% sequence similarity and clustered with RSIV, reported from other countries (Fig. 2B). Also, the phylogenetic tree constructed using MEGA software revealed that DNA polymerase gene formed the four clusters such as RSIV, ISKNV, TBIV, and SDDV. RSIV isolated in this study is grouped into a single clade with the RSIV reported from Korea and Japan. Moreover, on the basis of the sequence similarity, Indian isolate is closely related to the Korean RSIV isolate than the RSIV isolate of Japan (Fig. 2C). Finally, reconfirmation of the RSIV infection in the suspected samples were performed at two national referral laboratories i.e. ICAR-National Bureau of Fish Genetic Resources (NBFGR), Lucknow and ICAR- Central Institute of Brackishwater Aquaculture (CIBA), Chennai.

3.3. Histopathological examination

Histopathological observations such as increasded RBC proliferation, lymphocytic infiltration, fused secondary lamellae (SL), necrosed cellular material, reduced SL height were observed in the RSIV infected gill tissue (Fig. 3 A-B). The RSIV infected spleen showed leucocytic depopulation in the white pulp, melanomacrophage centers, increased vacuoles and irregular intracytoplasmic viral inclusion bodies were observed compared to control tissue (Fig. 3 C-D).

3.4. Challenge experiment

The fish group injected with virus started showing clinical signs similar to the naturally diseased fish from second day post infection and

Table 1
PCR primers and cycling conditions used to detect the targeted virus.

Sl. No	Primer Name	Targeted virus	Sequence (5′-3′)	Cycling conditions			No. of cycles	Product size	Reference
				Denaturation	Annealing	Extension			
1	4F 4R	Iridovirus	CGGGGGCAATGACGACTACA CCGCCTGTGCCTTTTCTGGA	95 °C for 60s	58 °C for 60s	72 °C for 60s	35	568 bp	Kurita et al. (1998)
2	LCNV-F LCNV-R	Nodavirus	GTTCCCTGTACAACGATTCC GGATTTGACGGGGCTGCTCA	95 °C for 60s	48 °C for 60s	72 °C for 60s	35	294 bp	Parameswaran et al. (2008)

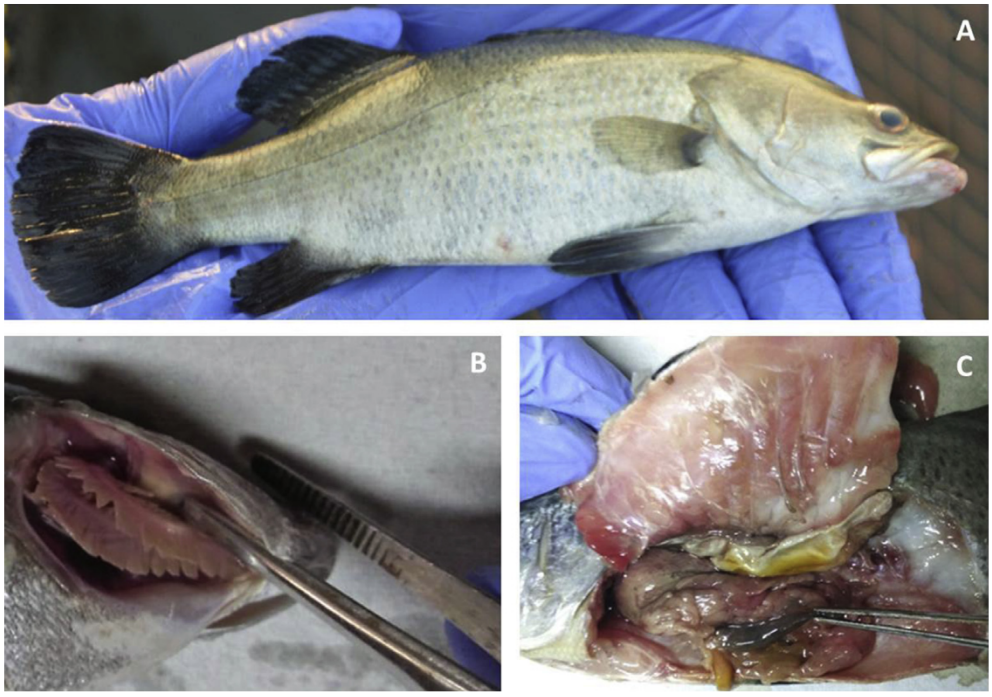

Fig. 1. A Diseased Asian seabass; **B** Infected Asian seabass showing pale gills **C** Enlarged spleen of diseased Asian seabass.

cumulative mortality was observed till 5th day post infection followed by 100% mortality by 6th day post infection (Fig. 4). While no clinical signs and mortality was recorded from fish in the control group. The internal examinations of the dead fish from the infected group showed enlargement of spleen and kidney tissue. Also, tissue samples of spleen and kidney from dead fish were found RSIV positive by PCR assay and no viral DNA was detected in the control group fish.

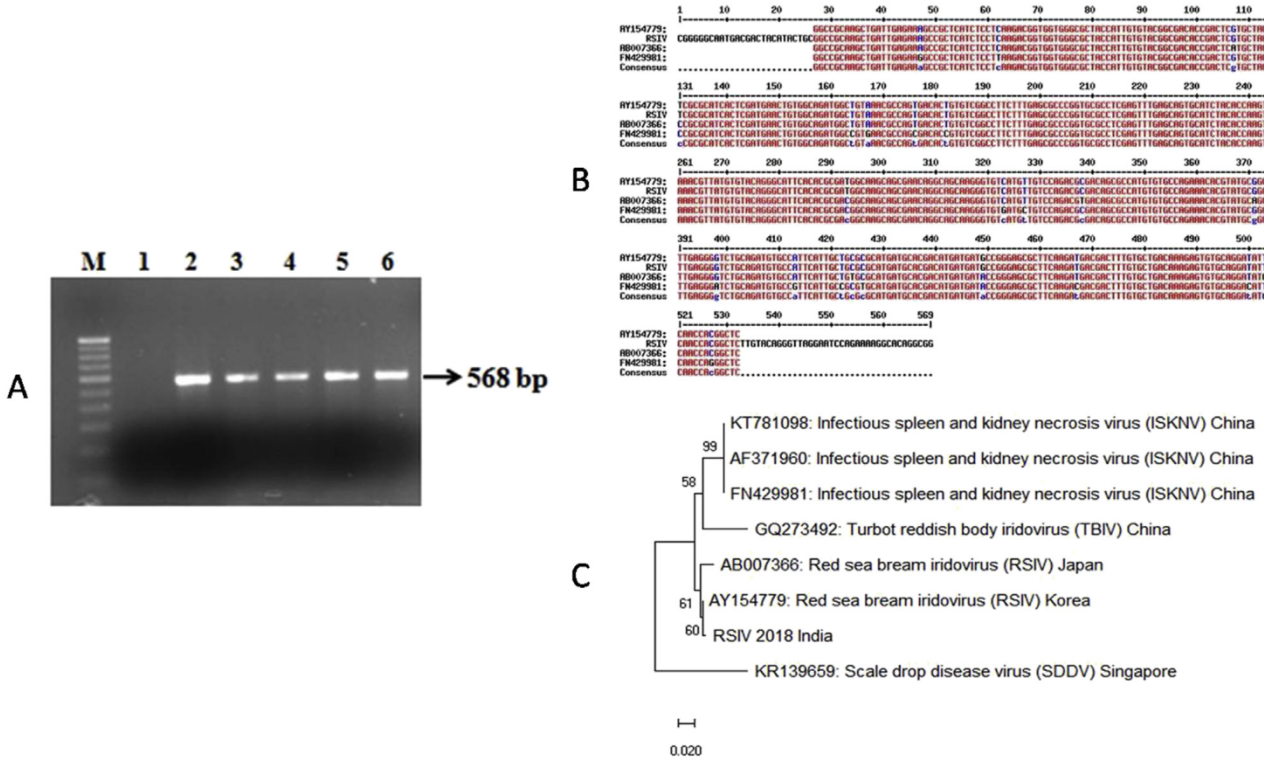

Fig. 2. Molecular diagnosis of RSIVD in Asian Seabass cultured along the west coast of India. A. Agarose gel electrophoresis showing amplification of DNA polymerase gene (4F/4R). Lane M: 100 bp DNA ladder; Lane 1: Negative control; Lane 2: Positive control; Lane 3: Gill tissue; Lane 4: Liver tissue; Lane 5: Kidney tissue; Lane 6: Spleen tissue.; **B.** Multiple alignments between the sequenced DNA polymerase gene with RSIV reported from other countries**; C.** Neighbor-joining phylogenetic tree showing the relationship among nucleotide sequence of DNA polymerase of Megalocytivirus.

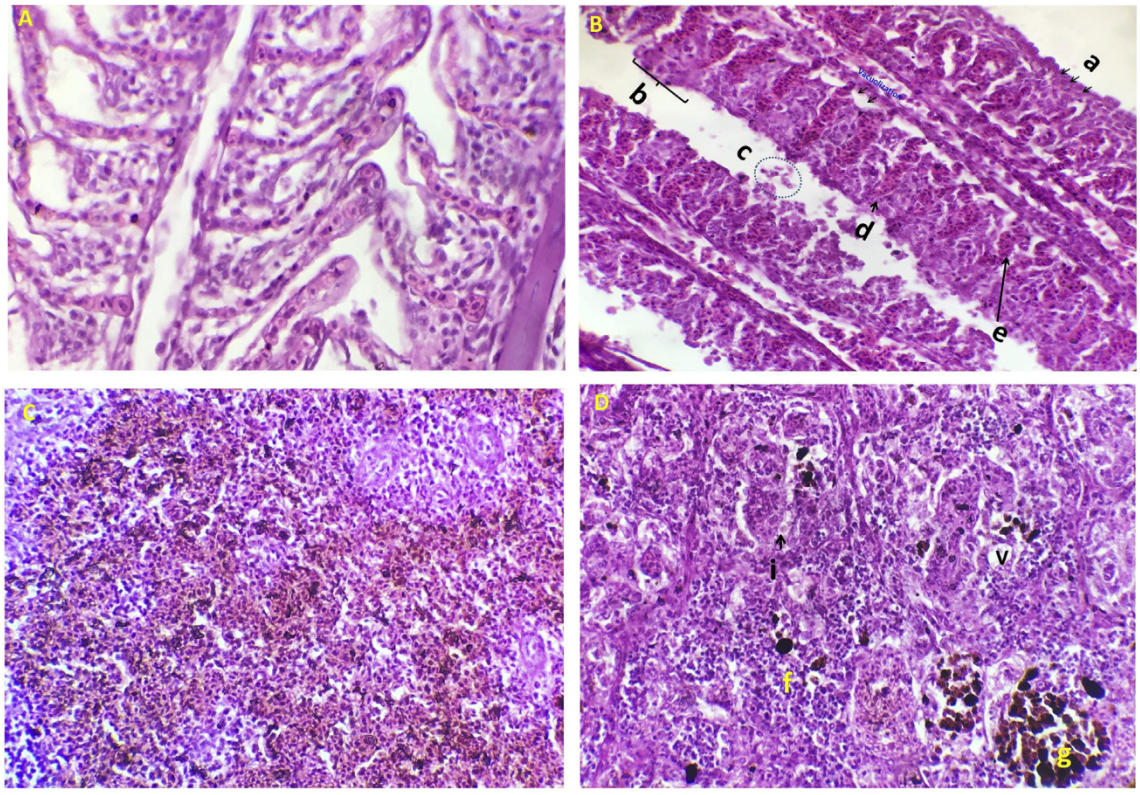

Fig. 3. The histopathology of gill (A = control & B = infected) and spleen tissues (C = control & D = infected) infected with RSIV. Gill tissue infected with RSIV (B) showed Lymphocytic infiltration (a), fused secondary lamellae (SL) (b), necrosed cellular material (c), reduced SL height (d) RBC proliferation (e) and spleen tissues infected RSIV (C) showed leucocytic depopulation in the white pulp (f), melanomacrophage centers (g), vacuole (h) and irregular intracytoplasmic viral inclusion bodies (i).

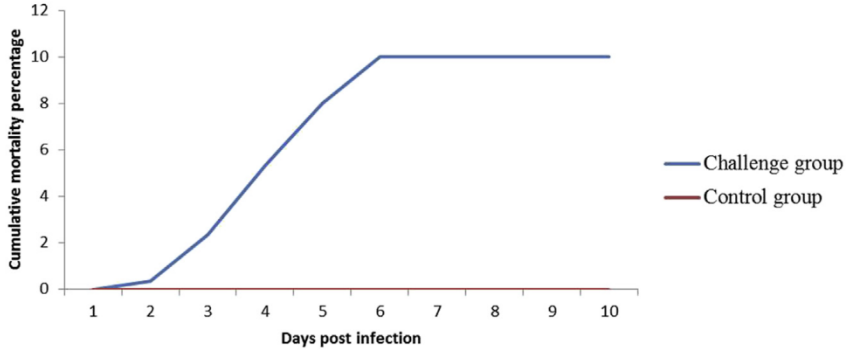

Fig. 4. Cumulative mortality of Asian seabass challenged with the RSIV.

4. Discussion

Recently, a serious infectious disease with running mortality was observed in the open cage cultured Asian seabass in Seetha River Estuary, Haradi, Udupi, Karnataka, from the west coast of India. The preliminary diagnosis followed by hitopathology and molecular techniques confirmed that the disease outbreak and mass mortality is due to RSIV infection. Further, Koch's postulate theory was proved through RSIV challenge experiment. The challenged fishes showed typical clinical signs of RSIV and 100% mortality by 6 days post-infection. This is the first report of RSIV infection in cultured Asian seabass from India and the same has been notified to (OIE, 2019).

We presume that the virus might have been present in the region and have been overlooked due to the absence of proper surveillance and monitoring programs until this work was carried out as a part of "National Surveillance Program for Aquatic Animal Diseases". Since RSIV has a broad host range and has been isolated from fishes in open cages, the risk of spreading the disease to wild fish stock is high. Thus, it becomes very important to screen the broodstock of Asian seabass for pathogen using PCR techniques before seed production, which are intended to be stocked in open cages. Further, the development of prophylactic measures in the form of the vaccine may help to control and prevent the spread of diseases. Several experimental vaccines against RSIV (Jung et al., 2018; Oh et al., 2014) have been developed and evaluated.

In summary, this study reports the emergence of RSIV in India along with other Asian countries. This study will help scientists and aquaculture farmers to strengthen their strategies to control and prevent the spread of disease before it becomes an epidemic.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to

influence the work reported in this paper.

Acknowledgment

This work was carried out under "National Surveillance Program for Aquatic Animal Diseases" (G/Nat.Surveillance/2013/5310). The authors acknowledge National Fisheries Development Board and Department of Fisheries (Previously Department of Animal Husbandry, Dairying and Fisheries), Govt. of India, for the funding support.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2019.734712.

References

AQUASTAT-CIBA, 2018. AQUASTAT India 2018.

Banerjee, D., Hamod, M.A., Suresh, T., Karunasagar, I., 2014. Isolation and characterization of a nodavirus associated with mass mortality in Asian seabass (*Lates calcarifer*) from the west coast of India. VirusDisease 25, 425–429. https://doi.org/10.1007/s13337-014-0226-8.

Dong, H.T., Jitrakorn, S., Kayansamruaj, P., Pirarat, N., Rodkhum, C., Rattanarojpong, T., Senapin, S., Saksmerprome, V., 2017. Infectious spleen and kidney necrosis disease (ISKND) outbreaks in farmed barramundi (*Lates calcarifer*) in Vietnam. Fish Shellfish Immunol. 68, 65–73. https://doi.org/10.1016/j.fsi.2017.06.054.

FAO, 2012. Regional Overview of Fisheries and Aquaculture in Asia and the Pacific 2012.

FAO, 2017. Cultured aquatic information programme. Lates calcarifer. http://www.fao.org/fishery/culturedspecies/Lates_calcarifer/en#tcNA00EA.

Fu, X., Li, N., Liu, L., Lin, Q., Wang, F., Lai, Y., Jiang, H., Pan, H., Shi, C., Wu, S., 2011. Genotype and host range analysis of infectious spleen and kidney necrosis virus (ISKNV). Virus Genes 42, 97–109.

Humason, G.L., 1972. Animal Tissue Techniques, third ed. W.H. Freeman and Company, San Francisco.

Inouye, K., Yamano, K., Maeno, Y., Nakajima, K., Matsuoka, M., Wada, Y., Sorimachi, M., 1992. Iridovirus infection of cultured red sea bream, Pagrus major. Fish Pathol. 27, 19–27.

Jancovich, J.K., Chinchar, V.G., Hyatt, A., Miyazaki, T., Williams, T., Zhang, Q.Y., 2012. Virus Taxonomy : Ninth Report of the International Committee on Taxonomy of Viruses. Elsevier, San Diego, CA.

Jung, S., Miyazaki, T., Miyata, M., Dayanadol, Y., Tanaka, S., 1997. Pathogenicity of Iridovirus from Japan and Thailand for the red seabream Pagrus major in Japan and histopathology of experimentally infected fish. Fish. Sci. 63, 735–740.

Jung, M.H., Nikapitiya, C., Jung, S.J., 2018. DNA vaccine encoading myristoylated membrane protein (MMP) of rock bream iridovirus (RBIV) induces protective immunity in rock bream *(Oplegnathus fasciatus)*. Vaccine 36 (6), 802–810.

Kurita, J., Nakajima, K., 2012. Megalocytiviruses. Viruses 4, 521–538. https://doi.org/10.3390/v4040521.

Kurita, J., Nakajima, K., Hirono, I., Aoki, T., 1998. Polymerase chain reaction (PCR) amplification of DNA of red sea bream iridovirus (RSIV). Fish Pathol. 33, 17–23.

Nakajima, K., Maeno, Y., Yokoyama, K., Kaji, C., Manabe, S., 1998. Antigen analysis of red sea bream iridovirus and comparison with other fish iridoviruses. Fish Pathol. 33, 73–78. https://doi.org/10.3147/jsfp.33.73.

Oh, S.Y., Oh, M.J., Nishizawa, T., 2014. Potential for a live red seabream iridovirus (RSIV) vaccine in rockbream *Oplegnathus fasciatus* at a low rearing temperature. Vaccine 32, 363–368.

OIE, 2012. Red Sea Bream Iridoviral Disease. https://doi.org/10.8.1.

OIE report, 2019. Red sea bream iridoviral disease. India [WWW Document]. URL. http://www.oie.int/wahis_2/public/wahid.php/Reviewreport/Review?page_refer=MapFullEventReport&reportid=30589 accessed 8.5.19.

Otta, S.K., Karunasagar, Indrani, Karunasagar, Iddya, 2003. Detection of monodon baculovirus and whitespot syndrome virus in apparently healthy *Penaeus monodon* postlarvae from India by polymerase chain reaction. Aquaculture 220, 59–67. https://doi.org/10.1016/S0044-8486(02)00340-X.

Parameswaran, V., Kumar, S.R., Ahmed, I., Hameed, S., 2008. A fish nodavirus associated with mass mortality in hatchery-reared Asian seabass, Lates calcarifer. Aquaculture 275, 366–369.

Qin, Q.W., Wu, T.H., Jia, T.L., Hegde, A., Zhang, R.Q., 2006. Development and characterization of a new tropical marine fish cell line from grouper, *Epinephelus coioides* susceptible to iridovirus and nodavirus. J. Virol. Methods 131, 58–64. https://doi.org/10.1016/j.jviromet.2005.07.009.

Senapin, S., Dong, H.T., Meemetta, W., Gangnonngiw, W., Sangsuriya, P., Vanichviriyakit, R., Sonthi, M., Nuangsaeng, B., 2019. Mortality from scale drop disease in farmed *Lates calcarifer* in Southeast Asia. J. Fish Dis. 42, 119–127. https://doi.org/10.1111/jfd.12915.

Shi, C.-Y., Wang, Y.-G., Yang, S.-L., Huang, J., Wang, Q.-Y., 2004. The first report of an iridovirus-like agent infection in farmed turbot, *Scophthalmus maximus*, in China. Aquaculture 236, 11–25. https://doi.org/10.1016/j.aquaculture.2003.11.007.

Wu, Y.C., Kai, Y.H., Chi, S.H., 2013. Persistently betanodavirus-infected barramundi (*Lates calcarifer*) exhibit resistances to red sea bream iridovirus infection. Dev. Comp. Immunol. 41, 666–674.