Characterisation of Iridovirus Isolated from Diseased Marbled Sleepy Goby, *Oxyeleotris Marmoratus*

PONGPUN PRASANKOK, MALINEE CHUTMONGKONKUL
Department of Biology, Chulalongkorn University, Bangkok

SOMKIAT KANCHANAKHAN
Aquatic Animal Health Research Institute, Department of Fisheries, Bangkok

ABSTRACT

High mortalities of cultured marbled sleepy goby or sand goby, Oxyeleotris marmoratus, occurred in Nakonpathom province, Central Thailand in March 2000. The diseased fish had minor ulcers on the body and around the mouth. No external parasites or blood parasites were observed and no bacteria were isolated from the liver, kidney or spleen. Three diseased fish were used for virological investigation. The tissue extracts were inoculated on to *Epithelioma papulosum cyprini* (EPC) cells at 25°C inducing round plaques. Electron microscopy showed the presence of numerous icosahedral cytoplasmic particles averaging 132 ± 7.8 nm in diameter. Virus titres were over 6 log10 $TCID_{50}$/ml lower when incubated with IUdR or chloroform indicating the particles possessed a DNA genome and an envelope. The virus isolate was sensitive to heat at 56°C. These properties indicate that the new virus isolate can be classified as a virus member of the family *Iridoviridae*. This virus propagated well in fish cell lines, BF-2, EPC, FHM, BB, SSN-1 and discus tail (DT), and 2 reptile cell lines, Siamese crocodile embryo (SCE) and soft-shelled turtle embryo (STE) at 25-30°C. The highest virus titre, 9.2 $\log_{10}$ $TCID_{50}$/ml, was obtained from the BF-2 line. New virions were released from EPC cells about 15 h post-infection at 25°C. PCR amplification of the new isolate and four other previous isolates of frog iridoviruses in Thailand using specific primers designed from the major capsid protein gene of ranavirus FV-3 gave predicted PCR products of 300 bp. Sequence analysis of the PCR products found 98-99% nucleotide homology to FV-3 and *Rana tigrina* ranavirus. The marbled sleepy goby iridovirus is proposed as Oxyeleotris marmoratus ranavirus or OMRV. Virulence and pathogenicity of OMRV are yet to be clarified.

INTRODUCTION

Marbled sleepy goby or sand goby, *Oxyeleotris marmoratus*, is a freshwater fish cultured for food in Thailand and neighboring countries. It has a high commercial value and is exported to Japan, China P.R., Chinese Taipei, Hongkong China, Singapore and Malasia. In Thailand, the goby is raised in floating cages and in earthern ponds in Nakornsawan, Uthaithani, Nakonpathom, Ayuthaya and Pathumthani and elsewhere. Goby seed is mainly collected from the wild, as seed production from hatcheries is limited. A number of pathogens have been found in the fish including parasites, bacteria, and fungi that can cause great

Prasankok, P., M. Chutmongkonkul, and S. Kanchanakhan. 2005. Characterisation of iridovirus isolated from diseased marbled sleepy goby, *Oxyeleotris Marmoratus. In* P. Walker, R. Lester and M.G. Bondad-Reantaso (eds). Diseases in Asian Aquaculture V, pp. 197-206. Fish Health Section, Asian Fisheries Society, Manila.

losses to fish farmers (S. Kanchanakhan, unpublished data). An aquabirnavirus, the first virus isolated from the goby, was associated with epizootic ulcerative syndrome (EUS) in Ayuthaya province in 1986 (Hedrick *et al*., 1986).

In 2000, high mortalities of marbled sleepy gobies occurred in Nakonpathom province, Central Thailand. The diseased fish exhibited minor ulcers on the body and around the mouth. No external parasites or blood parasites were observed and no bacteria could be isolated from internal organs, liver, kidney or spleen. Three viral agents were isolated from diseased fish using *Epithelioma papulosum cyprini* (EPC) fish cell line at the Aquatic Animal Health Research Institute (Prasankok *et al*., 2002). The objectives of the present study were to characterise and identify one virus isolate (code AV2008).

MATERIALS AND METHODS

Cell culture and virus isolation

The virus had been isolated from diseased marbled sleepy goby that were cultured in an earthen pond farm in Nakonpathom province in March 2000. Briefly, 1 g of pooled organs, liver, kidney, spleen and ulcer, was homogenised, diluted 1:10 with Hank's balanced salt solution (HBSS), and centrifuged for 15 min at 2000 rpm at 4°C. The extracts were further diluted 1:5, and then filtered through 0.45 µ disposable filters. The extracts were simultaneously inoculated on to EPC cells at 25°C. The cells were maintained in maintenance medium or L-15 supplemented with 2% FCS, penicillin 100 units/ml and streptomycin 100 µg/ml. Viruses were obtained from three diseased fish. The amount of virus was determined by tissue culture infective dose with 50% end point ($TCID_{50}$/ml) and calculated according to Karber (1931).

Virus morphology

Viruses were propagated in 25 cm^2 flasks of EPC cells. The cell sheet was fixed on day 2 post-infection using 2.5% glutaraldehyde and rapidly transferred to a tube then spun at 1,200 rpm for 20 min at 4°C. The packed cells with viruses were processed for transmission electron microscopy according to Hayat (1970). Specimens were observed using a GEM-200 CX transmission electron microscope at 80 kV.

Physico-chemical properties of virus

The type of nucleic acid was determined by incubating the virus with 50 µM of 5-iodo-2'-deoxyuridine (IUdR). An organic solvent, chloroform, was used to test the envelope property of the virus. Viral stability at 56°C was also examined by heating for 30 min and 60 min. These tests were performed according to Rovozzo and Bruke (1973). The stability in glycine-HCl buffer pH 3 was tested according to Kasornchandra *et al*. (1991).

Cell line susceptibility

The following cell lines were used; BB (brown bullhead caudal trunk) (Wolf and Quimby, 1969), BF-2 (bluegill fry) (Wolf *et al*., 1966), EPC (*Epithelioma papulosum cyprini*) (Fijian *et al*., 1983), FHM (fathead minnow tail) (Gravell and Malsberger, 1965), SSN-1 (striped snakehead whole fry tissue) (Frerichs *et al*., 1991) and three un-established cell lines, STE

(soft-shelled turtle embryo), DT (discuss tail) and SCE (Siamese crocodile embryo). The amount of virus produced in 8 cell lines was determined and calculated as $TCID_{50}$/ml unit, and results were ranked to indicate the susceptibility of the cell lines to the virus.

Optimum growth temperature

EPC cells were grown in growth medium (L-15 containing 10% FCS, penicillin 100 units/ml and streptomycin 100 µg/ml) in eight 25 cm^2 tissue culture flasks at 25(C for 48 h. The medium was removed and 4 flasks of EPC cells received 1 ml of virus at 10^2 $TCID_{50}$/ml while the other 4 flasks received 1 ml of maintenance medium. The viruses were allowed adsorb at 25°C for 1 h. The cell monolayer was rinsed twice with maintenance medium to remove the un-attached viral particles and then 7 ml of maintenance medium was added to each flask. Each flask of virus-infected cells and an uninfected control was incubated at 15, 20, 25 and 30°C. 200 µl supernatant fluid samples were taken at 0 h, 2 h, and on days 1, 2, 3, 4, 5, 6 and 7 post-infection. The amount of virus was determined as tissue culture infective dose ($TCID_{50}$/ml).

One-step growth cycle

The EPC cells were grown in growth medium in two 25 cm^2 tissue culture flasks at 25°C for 48 h. The tissue culture medium was removed and one flask of EPC cells received 1 ml of virus at 10^3 $TCID_{50}$/ml while the other received 1 ml of maintenance medium. The viruses were allowed to adsorb at 25°C for 1 h. The cell monolayer was rinsed twice with maintenance medium to remove the un-attached viral particles and then 7 ml of maintenance medium was added. Flasks were incubated at 25°C. 200 µl samples of supernatant fluid were collected at 0, 2, 4, 7, 10, 15, 20, 30, 40 and 50 h post-infection. The amount of virus was determined as $TCID_{50}$/ml.

Polymerase chain reaction and sequence analysis

Polymerase chain reaction. Viral DNA was extracted from 15-45% sucrose-gradients of viral pellet using QIAmp DNA Mini Kit (Qiagen). DNA template (1 µl) was added to Taq polymerase buffer containing 10 mM Tris-HCl, 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 200 pM of each primers, 2 unit *Taq* DNA polymerase (Promega) heated at 95°C for 3 min and amplification was carried out in 30 cycles (1 min 95°C, 1 min 55°C and 2 min 72°C) plus 5 min 72°C extension. The primers used were mc-1 5'-GTTTCATCGACTTGGCCACT and mc-3 5'-ATGTTGTGCATGGGGTTCTT, which designed from the known nucleotide sequences of the major capsid protein gene of the Ranavirus FV-3 (Mao *et al*., 1996). The same PCR protocols were also used to amplify DNA from frog iridoviruses, Rana tigrina ranavirus (RTRV) Thailand isolate, which had been isolated from diseased cultured frogs from different provinces, Bangkok (AV9803), Songkhla (N2001), Roy-et (AV9944) and Pichit (AV9922) (Kanchanakhan *et al*., 1999; Kanchanakhan *et al*., 2002). The PCR products were analysed by gel electrophoresis in 1% agarose gel with ethidium bromide staining.

Sequence analysis. The PCR products were cut from 1% low-melting point agarose gel then purified by silica adsorption using a QIAquick gel extraction kit (Qiagen). The purified DNAs were sequenced using primer mc-1 and an ABI cycle sequencer dRhodamine Big

Dye kit. The reaction products were analysed on an ABI model 377 automated sequencer at the Bioservice Unit, NSTDA, Bangkok. Nucleotide sequences were compared to the known sequences published the GenBank using the Blast program (Altschul *et al*., 1997). Nucleotides were aligned using the Genetyx program.

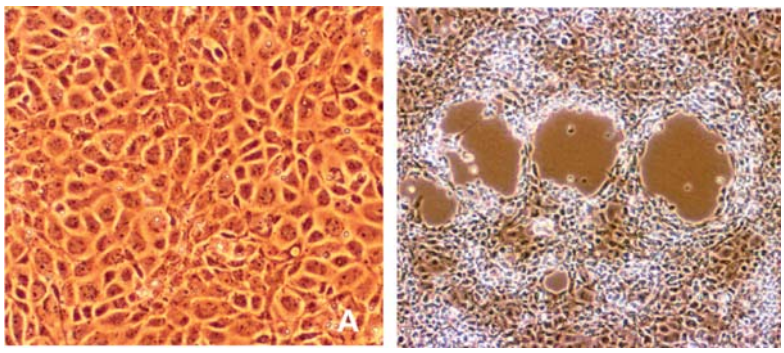

Figure 1. Cytopathic effect of marbled sleepy goby iridovirus in EPC cells. (A). Normal EPC cells: (B). Infected EPC cells on day 3 after inoculation with virus.

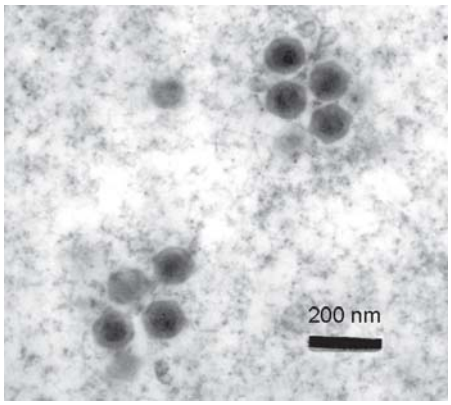

Figure 2. Electron micrograph of an ultra-thin section of EPC cells infected with the marbled sleepy goby virus showed iridovirus particles with 132.5 nm in diameter presented in the cytoplasm.

RESULTS

The marbled sleepy goby virus induced small foci in the cell monolayer followed by progressive rounding, detachment and degeneration of the cells. The cytopathic effect (CPE) appeared as spherical plaques within a few days post-infection (Fig. 1). The cell sheet was completely destroyed in days 3-5. The marbled sleepy goby virus had icosahedral symmetry with a hexagonal nucleocapsid. The diameter of nucleocapsid was 132.5 ± 7.8 nm vertex to vertex (Fig. 2). Viral propagation in EPC cells was reduced over 3 $\log_{10}$ $TCID_{50}$/ml when they had been treated with 50 μM IUdR. This finding indicates that the virus has a DNA genome. The virus lost infectivity when incubated with chloroform indicating the presence of an envelope. The marbled sleepy goby iridovirus lost all infectivity after incubation at 56°C for 30 min or in buffer pH 3 at room temperature for 4 h.

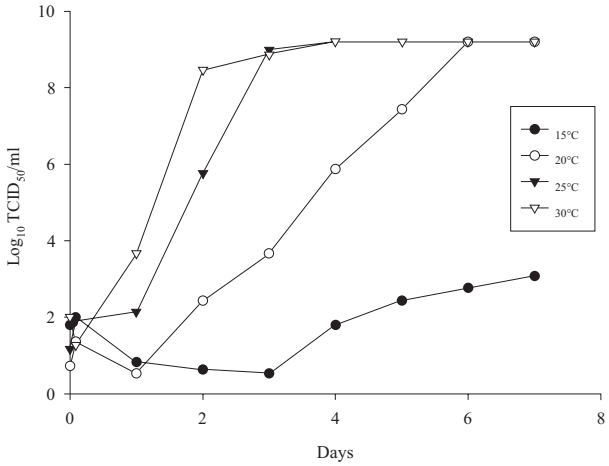

Figure 3. Optimum growth temperatures of marbled sleepy goby iridovirus in EPC cells at 15, 20, 25 and 30°C.

The marbled sleepy goby iridovirus propagated rapidly in EPC cells at 25-30°C (Fig. 3). The amount of viruses reached a maximum at ~10^9 $TCID_{50}$/ml within 3-4 days. This iridovirus had a slow growth and caused only minor changes in ECP cells when incubated at 15°C. The one round infection cycle or one step growth cycle of marbled sleepy goby iridovirus was determined (Fig. 4). The new virions began to release from the host cells at ~15 - 20 h post-infection at 25°C.

Cell line susceptibilities

The marbled sleepy goby iridovirus induced CPE in all fish and reptiles cell lines tested. The degree of susceptibility was measured and ranked as the amount of viruses produced at 25°C, and was 9.2, 9.09, 9.09, 9.07, 8.93, 8.10, 5.53 and 5.53 $\log_{10}$ $TCID_{50}$/ml for BF-2, EPC, STE, SCE, FHM, BB, DT and SSN-1, respectively.

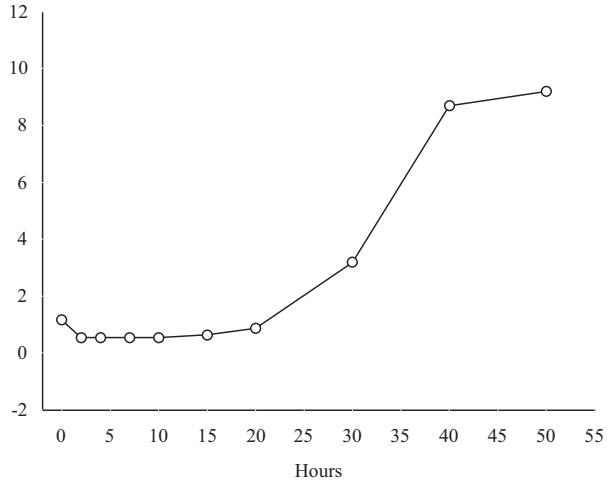

Figure 4. One-step growth cycle of marbled sleepy goby iridovirus in EPC cells at 25°C.

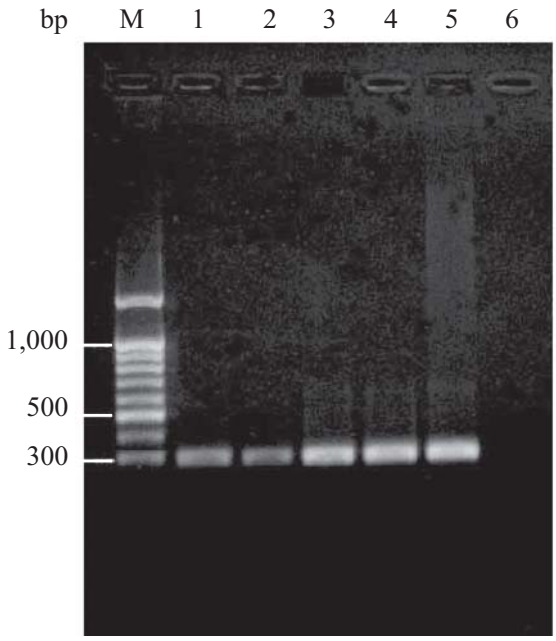

Figure 5. PCR amplification of MCP gene of marbled sleepy goby iridovirus and 4 frog iridoviruses. Marker (lane M), OMRV (lane 1), AV9803 (lane 2), N2001 (lane 3), AV9922 (lane 4), AV9944 (lane 5) and negative control (lane 6).

PCR amplification of viral DNA

PCR amplification of marbled sleepy goby iridovirus using a specific primer set for the major capsid protein gene of ranavirus FV3 exhibited a product a of 300 bp which was similar in size to PCR products generated from DNA templates of four frog iridovirus isolates (Fig. 5).

Sequence analysis

The PCR products of marbled sleepy goby iridovirus and 4 frog iridovirus isolates were directly sequenced. About 284 bp of 300 bp PCR products could be determined from the sequencing gel. The 5 partial sequences of the MCP gene were almost identical. The nucleotide sequences exhibited 98 and 99% nucleotide homology to the MCP gene of ranavirus type genus FV-3 and *Rana tigrina* ranavirus China PR isolate (He *et al*., 2002), respectively (Fig. 6).

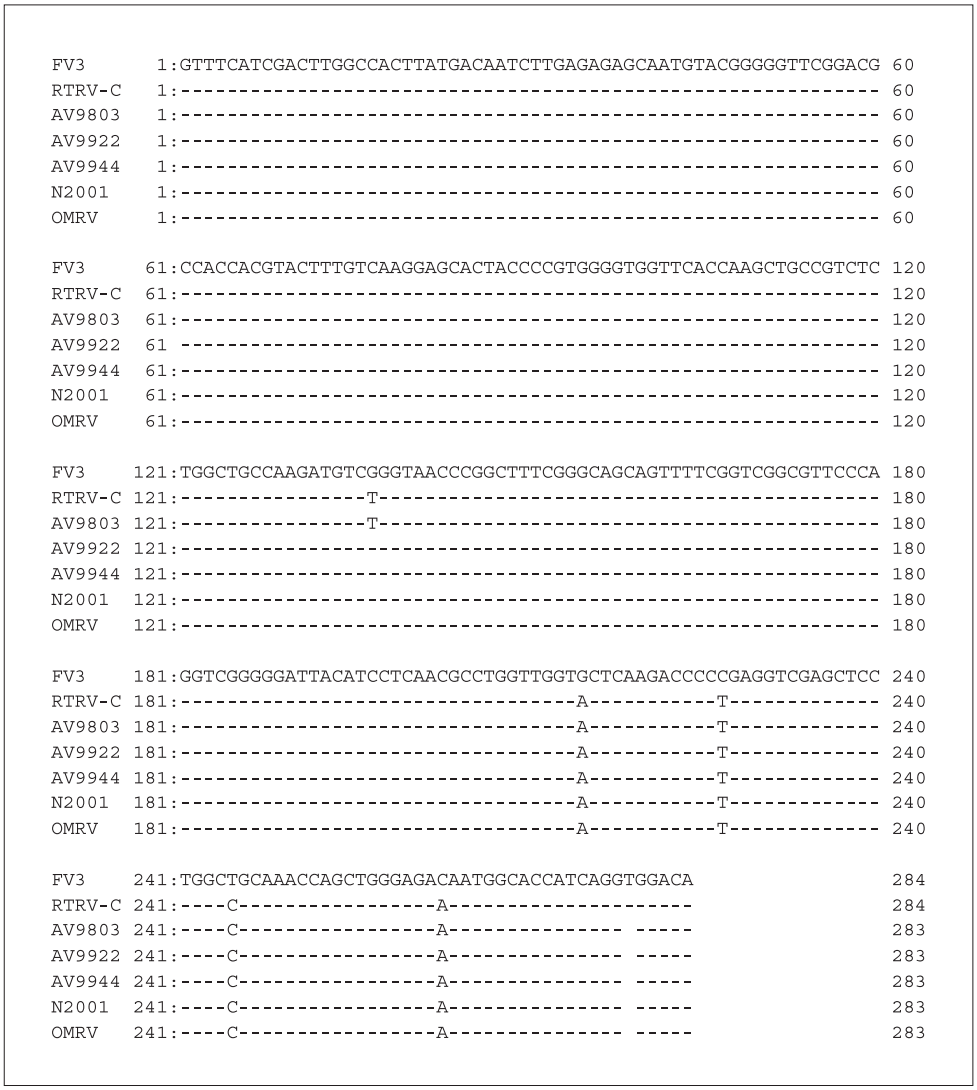

Figure 6. Nucleotide sequences of major capsid protein gene (MCP) of marbled sleepy goby iridovirus (OMRV) and 4 frog iridovirus isolates (AV9803, AV9922, AV9944, N2001) aligned with FV-3 and RTRV-C (RTRV China isolate) from GenBank.

DISCUSSION

Basic viral characterisations indicated that the virus isolated from diseased marbled sleepy goby was an iridovirus. The PCR and sequence analysis indicated that this virus could be classified as a viral member of the genus *Ranavirus*. A partial sequence of the MCP gene had 98-99% nucleotides homology to ranavirus type genus FV3 (GenBank #U36913) and RTRV (GenBank #AF389451). The virus had 96% (268/278) nucleotides homology to epizootic haematopoietic necrosis virus or EHNV (GenBank #AY187045). There is a need to do further gene sequencing of the MCP gene and other genes of the isolate for a more complete.

The marbled sleepy goby, *Oxyeleotris marmoratus*, is a newly recognised host for ranavirus infection. It is therefore proposed that the virus be named *Oxyeleotris marmoratus* ranavirus or OMRV. The present findings and previous reports (Kanchanakhan *et al*., 1999; Kanchanakhan *et al*., 2002) did not enable distinction between ranavirus isolates OMRV and RTRV in Thailand. Ranavirus isolated from diseased frogs and diseased marbled sleepy goby in Thailand are most likely the same viral agent. Fish and soft-shelled turtle and crocodile cell lines supported the growth of ranavirus, which indicate that ranavirus can have wide host ranges or animal carriers in the nature. The multi-host characteristics of ranavirus have been previously recognised (Ahne *et al*., 1997; Ahne *et al*., 1998; Mao *et al*., 1999; Speare and Smith, 1992; Marsh *et al*., 2002).

The ranavirus infection in sand goby fish was found in March 2000, three years after the first ranavirus had been isolated from diseased frogs in Central Thailand (Kanchanakhan *et al*., 1999). It is interesting to note that the clinical signs of the frog and fish ranavirus infection included minor skin ulceration. There were no further cases of ranavirus-infected marbled sleepy goby since the first finding in March 2000, which suggests that marbled sleepy goby is an accidental host. However, ranavirus infection has caused mortality in farmed frog farms since 1998 (Kanchanakhan *et al*., 1999), which suggests that the frog is most likely a natural reservoir. Ranavirus was isolated from a major disease outbreak in tadpoles of cultured tiger frog, Rana tigrina rugulosa, in many farms in Southern China between May and June 2000 (Weng *et al*., 2002) and its genome was completely sequenced (He *et al*., 2002). It is possible that this ranavirus from the tiger frog will be able to infect other fish species in China. Interestingly, an iridovirus was isolated from a diseased soft-shelled turtle in Shenzhen, China (Chen *et al*., 1999). There is a need to compare these reptile and amphibian iridoviruses and to control their spread at national and international levels.

ACKNOWLEDGMENTS

This work was supported by the Aquatic Animal Health Research Institute, Department of Fisheries (DoF), Chulalongkorn University and Fish Disease Project, Government of Japan Trust Fund. The authors would like to thank Dr Ladda Trongwongsa at the National Institute of Animal Health, Department of Livestock Development, for kind assistance in electron microscopy and Mr. Somporn Roongkamnertwongsa, National Institute of Coastal Aquaculture, DoF, for providing frog iridovirus N2001.

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