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NOTE

Characterization of an iridovirus detected from cultured turbot *Scophthalmus maximus* in Korea

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ABSTRACT: Juvenile turbot *Scophthalmus maximus* that became sick during an outbreak of disease at mariculture facilities at Go-Chang, Korea, in 2003, were examined to identify the cause of the disease. The fish had pale body color, an enlarged abdomen, protruding eyes, an enlarged spleen and kidney, and pale gills and/or liver. Histopathogical examination revealed basophilic enlarged cells in the kidney, spleen, gills, heart, stomach, intestine, liver, pancreas and skin. Hexagonal viral particles with a diameter of 136 to 159 nm were observed in the enlarged cells. A specific 1299 bp fragment of the major capsid protein (MCP) gene of the turbot iridovirus (TBIV) was amplified by PCR. Sequence homology was greater than 93.76% between the MCP gene in TBIV and the same gene in 5 viruses in the tentatively proposed genus *Tropivirus* (family *Iridoviridae*): red sea bream iridovirus, sea bass iridovirus, grouper sleepy disease iridovirus, African lampeye iridovirus and dwarf gourami iridovirus. These results suggest that the virus detected from turbot is similar to the proposed genus *Tropivirus*.

KEY WORDS: Turbot · *Scophthalmus maximus* · Iridovirus · RSIV · MCP · *Tropivirus*

INTRODUCTION

Turbot *Scophthalmus maximus* is a commercially important fish species that occurs mainly in the eastern North Atlantic and the Mediterranean Sea. Catches have declined markedly during the last decade, and as a result aquaculture has become an important source of turbot in several countries, including Spain, France, Norway and China.

In Korea, more than 245 000 tons of marine fishes and shellfish were cultured in 2001 (based on the official statistics issued by the National Fisheries Research & Development Institute in 2003), but the aquaculture industry is now facing difficulties due to over-production and the importation of live fishes. To overcome these problems, fish farmers have switched to more economical species, and turbot was selected because it is fast-growing and in high demand due to the popularity of white meat fish in Korea.

In 2003, an outbreak of disease at aquaculture farms in Go-Chang on the west coast of Korea caused a high rate of mortality (50 to 70%) in cultured juvenile turbot. During histopathological examination of the diseased fish, enlarged cells were observed in various internal tissues, which is suggestive of red sea bream iridovirus (RSIV) disease; this also occurs in red sea bream *Pagrus major*, grouper *Epinephelus ocellata* (L.), striped beakperch *Oplegnathus fasciatus* and red drum *Sciaenos ocellata* (L.) (Jung et al. 1997, Jung & Oh 2000, Sano et al. 2002, Weng et al. 2002). In addition, a partial iridovirus genome has been detected in the spleens and kidneys of diseased turbot using PCR described by Kurita et al. (1998). In this paper, we report the histopathological characteristics, electron microscopic features and molecular characters of the virus detected in the diseased turbot.

MATERIALS AND METHODS

Epizootics. Turbot eggs were imported from France and hatched at an aquaculture farm in Go-Chang,

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Korea. Juvenile turbot (mean weight 9.0 ± 1.9 g, mean length 7.9 ± 0.5 cm) began to show signs of disease in June 2003. Affected fish were lethargic and had a reduced appetite, pale body color, enlarged abdomen and protruding eyes. The key internal characteristics of the diseased fish were an extremely enlarged spleen and kidney, and pale gills and/or liver (Fig. 1).

The fish farmers misdiagnosed the disease as hirame rhabdovirus (HRV) disease or viral hemorrhagic septicemia (VHS) disease, and increased the water temperature from 17–18°C to 20–23°C in an attempt to control the disease. The mortality increased rapidly when the water temperature was increased. Total mortality was about 50 to 70%. After sampling some of the diseased turbot, the remaining fish were killed, and all facilities were disinfected with sodium hypochlorite.

Histology. The kidney, spleen, gills, heart, stomach, intestine, liver, pancreas, skin, eye and brain were removed from diseased fish and immediately fixed in 10% neutral buffered formalin (NBF). After fixation, standard histological procedures were used for tissue dehydration and paraffin embedding. Tissue sections were stained with haematoxylin and eosin (H&E).

Electron microscopy. The spleens were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer (pH 7.2) at 4°C. After several rinses with 0.1 M phosphate buffer, each sample was post-fixed with 1% OsO_4 for 1 h. Subsequently, the tissue was dehydrated in an ethanol series and embedded in Epon 812. Ultra thin sections were prepared using an RMC-MTX ultramicrotome (SIMS). Sections were stained with lead citrate and uranyl acetate. Stained grids were observed under a Hitachi-7000 electron microscope.

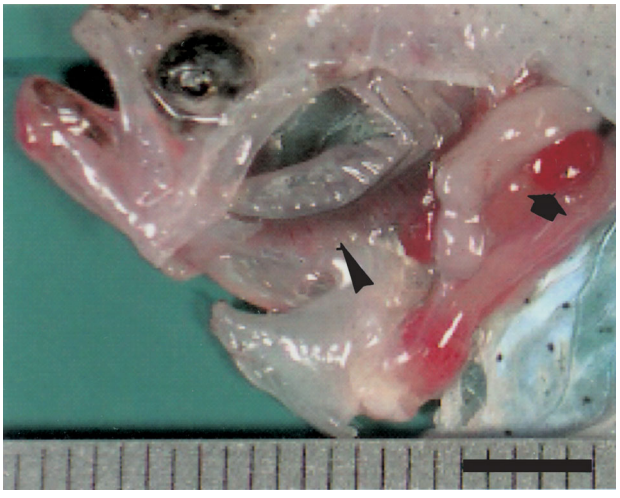

Fig. 1. *Scophthalmus maximus*. Diseased turbot with a severely enlarged spleen (arrow) and pale gills (arrowhead). Scale bar = 5 mm

DNA extraction. In preliminary investigations, the thick PCR product appeared from spleen and gills samples, although a thin band was amplified from some organs including stomach, intestine, liver and kidney. Therefore, the spleen and gills of diseased turbot were chosen for DNA extraction in this study. These tissues were homogenized in a 4-fold volume of TE buffer (10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and centrifuged at 2500 × *g* for 10 min at 4°C. A 20 µl aliquot of Proteinase K (1 mg/ml; TaKaRa) was added to 200 µl of the supernatant. The mixture was incubated at 55°C for 2 h. DNA was isolated using phenol and chloroform. Nucleic acids were precipitated with isopropanol, resuspended with distilled water and stored at –20°C until use.

PCR amplification. The PCR primer set of the major capsid protein (MCP) gene was designed from the sequence of red sea bream iridovirus MCP gene in the GenBank DNA database (accession number AY310918). The primer position in the deposited MCP gene is as follows: the forward primer (TBIVMCP-1F) is at bases 12 through 30 (5′-CTCAGGTGCGAACG-TAACC -3′) and the reverse primer (TBIVMCP-1R) is at bases 1288 through 1310 (5′-TTGACTGCAATAAC-GACCAGTTC-3′). This primer set covers 1299/1376 bp (94.4%) of the MCP gene. The PCR reactions were performed in a final volume of 20 µl of reaction mixture containing 50 mM KCl, 10 mM Tris-HCl (pH 9.0), 1.5 mM $MgCl_2$, 0.1% Triton X-100, 100 pM of each primer, 0.2 mM of each dNTP, 1U *Taq* DNA polymerase and template DNA. PCR amplification was performed using a GeneAmp 2400 thermal cycler (Perkin Elmer) with 30 cycles (95°C for 1 min, 59°C for 1 min, and 72°C for 1 min). The PCR products were analyzed in 1.5% agarose gels containing ethidium bromide and visualized under UV light.

Cloning of the PCR product and sequencing. The 1299 bp specific PCR product was purified using the QIAquick Gel Extraction Kit (Qiagen). The purified PCR product was cloned into the pCR 2.1 vector system (Invitrogen), and then transformed to *Escherichia coli* strain TOP10 (Invitrogen) using standard protocols. The vector was extracted from grown *E. coli* TOP10 and used for sequencing.

Sequencing was performed based on the dideoxynucleotide termination method in an automated ABI PRISM 310 DNA sequencer (PE Biosystems) using the ABI PRISM Big Dye Terminator Cycle Sequencing FS Ready Reaction Kit (PE Biosystems) and the M-13 forward and reverse universal primer set. To obtain complete sequencing data, another primer set was designed in the inserted gene and used for sequencing. The sequence of this primer set was as follows: TBIVMCP-2F (5′-CTGGTCTCCGGTATCACCA-3′) and TBIVMCP-2R (5′-TGACGGGATGACTGAACCT-

3′). Analysis of the results was performed using Genetyx Win Ver. 5.1 software. The nucleotide sequence of the partial MCP gene of turbot iridovirus (TBIV) was submitted to GenBank (accession number AB166788).

The TBIV MCP gene was compared with the MCP genes of 2 genera of iridoviruses — (1) genus *Lymphocyctivirus* (lymphocystis disease virus 1 [LCDV-1; L63545, Tidona & Darai 1997]), (2) genus *Ranavirus* (grouper iridovirus [GIV; AF364593, Murali et al. 2002] and frog virus 3 [FV3; U36913, Mao et al. 1996]), and the genus *Tropivirus*, tentatively proposed by Sudthongkong et al. (2002) (red sea bream iridovirus [RSIV; AY310918, Sudthongkong et al. 2002], sea bass iridovirus [SBIV; AB109372, Sudthongkong et al. 2002], dwarf gourami iridovirus [DGIV; AY285744, Sudthongkong et al. 2002], grouper sleepy disease iridovirus [GSDIV; AY285746, Sudthongkong et al. 2002] and African lampeye iridovirus [ALIV; AY285745, Sudthongkong et al. 2002]).

RESULTS

Histopatholgy and electron microscopy

All moribund fish had enlarged cells in the kidney, spleen, gills, heart, stomach, intestine, liver, pancreas and skin. The enlarged cells had an extremely basophilic and granular cytoplasm, and their nuclei were often hypertrophic, fragmented or not visible.

In the spleen, large numbers of enlarged cells appeared mainly in the splenic pulp and sheathed tissue accompanied by hemorrhages (Fig. 2A). The kidney had enlarged cells in the glomerulus (Fig. 2B) and haematopoietic tissue together with necrosis and infiltration of inflammatory cells. The tubular epithelium of the kidney occasionally showed degeneration. In the gills, many enlarged cells were found in the branchial arteries, venous sinus and inter-lamellar epithelia of the filaments. The filaments showed necrosis in the inter-lamellar epithelia and hyperemia in the lamellar capillaries. In the skin, many enlarged cells were located adjacent to adipose tissue, and infiltration of inflammatory cells occurred around the adipose tissue and muscle fibers. In the heart, enlarged cells occurred in the bulbus arteriosus, atrium, ventricles and bulbus endocardium (Fig. 2C). The epicardium was infiltrated by inflammatory cells. In some fish, necrosis was observed in the myocardium. In the stomach, the lamina propria, submucosa, muscle layer and serous membrane contained enlarged cells and inflammatory cells (Fig. 2D). Some fish also had enlarged cells in the epithelial layer. In the intestine, numerous enlarged cells occurred in the lamina propria and submucosa but not in the muscle layer. Inflammatory cells and necrosis were observed in the lamina propria and submucosa. In the liver, enlarged cells and inflammatory cells were observed in the sinusoids and adjacent parenchymal cells, where multiple discrete necrotic foci were observed. In the pancreas, enlarged cells occurred adjacent to the acinar cells, and necrosis was visible in the acinar cells. In the eyes, inflammatory cells and necrosis appeared in the choroid, but enlarged cells were not observed. The brain showed no histopathological changes. No histopathological changes including enlarged cells were observed in the any control tissues (Fig. 2E,F).

Electron microscopy revealed many hexagonal-shaped virions in the enlarged cells and necrotic area. Each virion consisted of a central, electron-dense core surrounded by an electron-translucent zone, and measured 136 to 159 nm from vertex to vertex (the size was calculated by measuring 50 virions) (Fig. 3).

PCR amplification

The results of PCR using the TBIVMCP-1F and TBIVMCP-1R primer set are shown in Fig. 4. The specific PCR products corresponding to the 1299-bp fragment of the MCP gene were detected in the spleen and gills, and the negative control using distilled water showed no amplification.

Analysis of the MCP nucleotide sequence

The homology of the MCP gene between TBIV and other reported iridoviruses is shown in Table 1. The nucleotide sequence of TBIV MCP was highly homologous with viruses in the tentatively proposed genus *Tropivirus* (SBIV, 94.38%; RSIV, 94.30%; GSDIV, 94.23%; ALIV, 93.92%; and DGIV, 93.76%). When the deduced amino acid sequences were compared, the homology between TBIV and the genus *Tropivirus* was more than 97.92% (data not shown). On the other hand, the homology between TBIV and the other 2 genera was less than 56.35% for nucleotide sequences and less than 49.2% for deduced amino acid sequences.

DISCUSSION

We observed enlarged cells in various tissues during histopathological observations and many hexagonal virions (136 to 159 nm) in the enlarged cells and necrotic areas. The histopathological signs and virion size are similar to those of red sea bream iridovirus (RSIV) disease in red sea bream (Inouye et al. 1992)

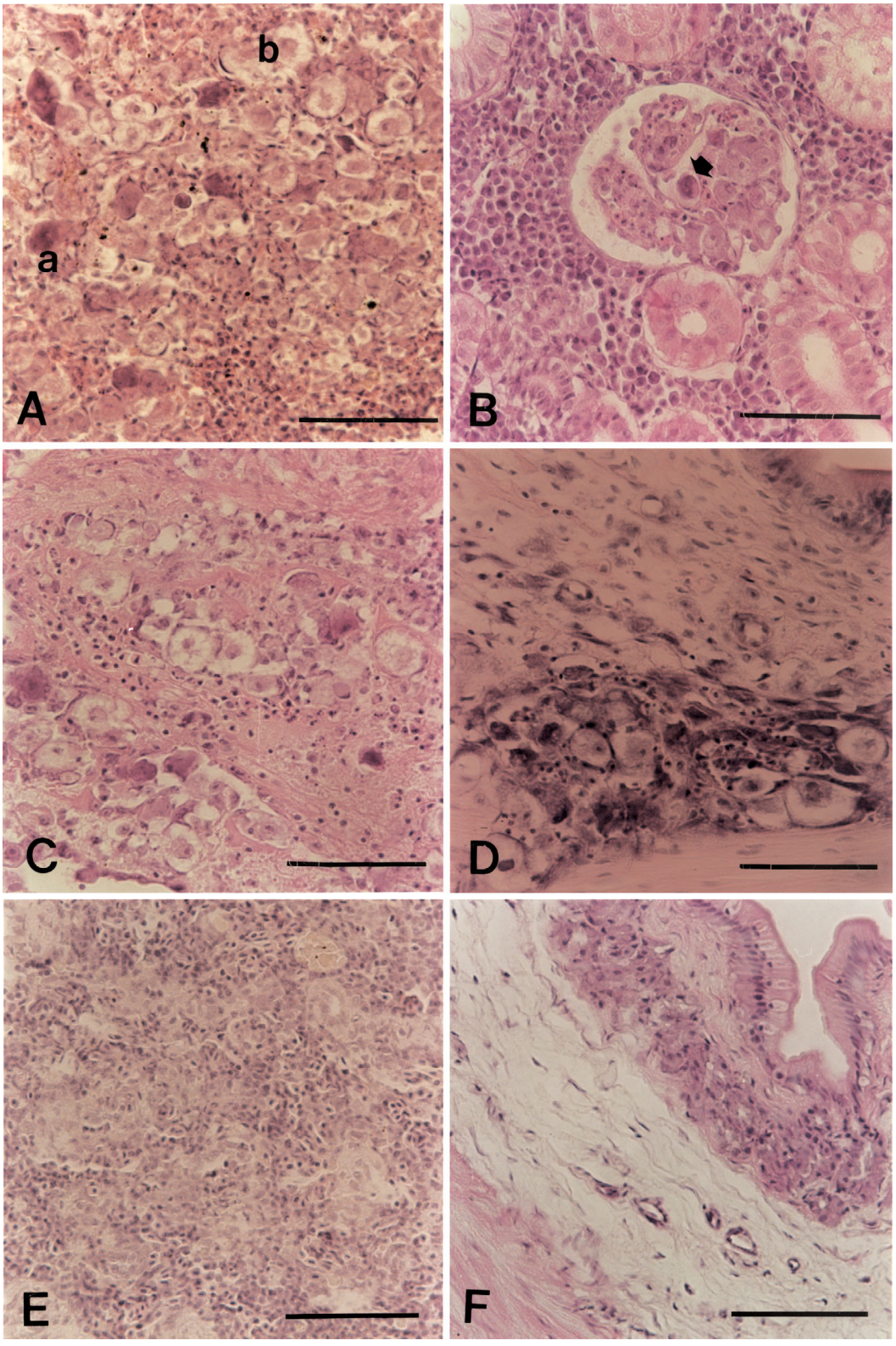

Fig. 2. *Scophthalmus maximus*. Histopathology of turbot infected with the turbot iridovirus (TBIV). (A) Many enlarged cells with an extremely basophilic (a) and granular cytoplasm (b) in the spleen. (B) Enlarged cell in the glomerulus of the kidney (arrow). (C) Necrosis and many enlarged cells in the bulbus endocardium of the heart. (D) Many enlarged cells in the submucosal layer of the stomach. (E) Spleen and (F) stomach of control tissue. Scale bars = 50 µm

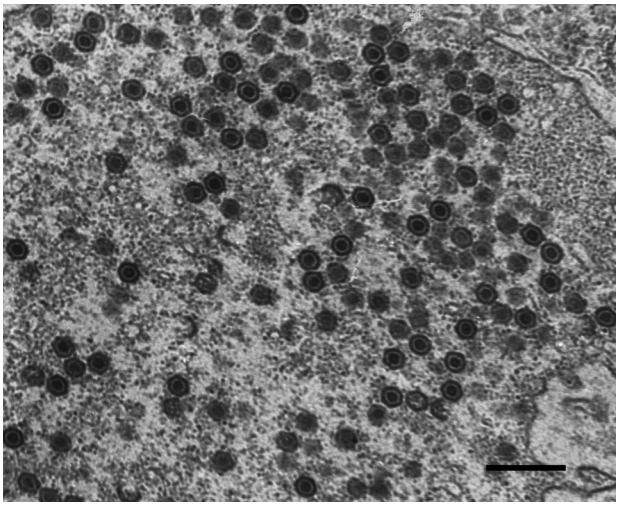

Fig. 3. Iridovirus particles of infected turbot ranging from 136 to 159 nm in the cytoplasm of enlarged cells. Scale bar = 500 nm

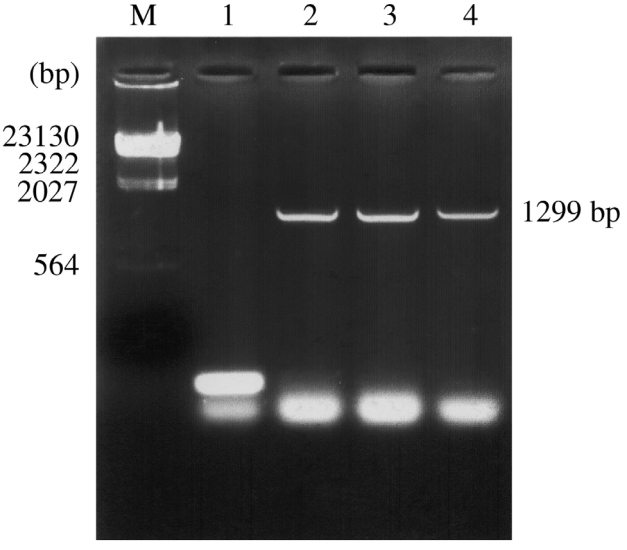

Fig. 4. Detection of TBIV MCP gene from the gills and spleen of diseased turbot by PCR. Lanes are M: molecular marker; 1: negative control without template; 2: gills of diseased turbot; 3: spleen of diseased turbot; 4: positive control (red sea bream iridovirus, RSIV)

Table 1. Comparative analysis (%) of the MCP nucleotide sequence between TBIV and other iridoviruses

	TBIV	SBIV	RSIV	GSDIV	ALIV	DGIV	FV3	GIV	LCDV-1
TBIV		94.38	94.3	94.23	93.92	93.76	56.35	52.06	53.06
SBIV			99.3	99.85	94.84	94.84	56.44	51.35	54.08
RSIV				99.31	95.07	95.07	56.62	51.65	53.7
GSDIV					94.84	94.84	56.36	51.28	54
ALIV						99.62	57.1	51.95	53.02
DGIV							56.88	51.8	52.95
FV3								69.83	52.18
GIV									54.67
LCDV-1									

and striped beakperch (Jung & Oh 2000). Also, turbot iridovirus (TBIV) induced 60% of turbot mortality at 23°C in experimental infection, although no mortality was observed in the non-injection group (W. S. Kim, M. J. Oh, S. T. Jung, S. I. Kitamura unpubl. data). This result suggests that the mass mortality of turbot was caused by this virus.

The outbreak of TBIV disease in Go-Chang in 2003 occurred at 17 to 18°C, and the mortality increased rapidly when the water temperature was increased to 20–23°C. The optimum temperature for maintaining turbot is around 17°C, and a temperature of 20 to 23°C may cause stress in these fish. We suspect that the increased temperature triggered an increase in mortality. Examination of how temperature affects the physiological condition of turbot and TBIV replication is needed.

Bloch & Larsen (1993) reported the occurrence of an iridovirus-like agent in cultured turbot fry in Denmark. This agent measured 160 to 185 nm in diameter and was detected in the fin, gills, liver, kidney, spleen, heart, pancreas, and intestinal collagen. Clinical signs included abnormal movements, spasm swimming and darkened pigmentation in the tail and fins. However, enlarged cells were not observed in any diseased turbot tissues reported by Bloch & Larsen (1993). TBIV-infected fish showed no specific signs in their fins, and the main signs were pale body color, an enlarged abdomen and protruding eyes. These findings suggest that TBIV is different from the iridovirus-like agent reported by Bloch & Larsen (1993).

The family *Iridoviridae* comprises 4 genera: *Iridovirus*, *Chloriridovirus*, *Ranavirus* and *Lymphocystivirus* (Williams et al. 2000), and RSIV belongs to the genus *Ranavirus* (The Universal Virus Database of the International Committee on Taxonomy of Viruses). Recently, Sudthongkong et al. (2002) proposed a new genus, *Tropivirus*, in this family for tropical iridoviruses including RSIV, SBIV, GSDIV, ALIV and DGIV based on nucleotide sequencing of the MCP and ATPase genes. We found that sequence homology in the MCP gene of TBIV was high (>93.75%) with the MCP genes of these viruses and low with the MCP genes of the genera *Ranavirus* (FV3; 56.35%, GIV; 52.06%) and *Lymphocystivirus* (LCDV-1; 53.06%), suggesting that TBIV is similar to the tentatively proposed genus *Tropivirus*.

RSIV-like viruses have been detected from many fish species in several Asian countries, including Korea, Japan, China, Thailand and Indonesia. Kawakami & Nakajima

(2002) reported that RSIV-like disease occurred in 31 cultured fish species including the Perciformes, Pleuronectiformes and Tetraodontiformes; however, this is the first report in turbot. It is unclear whether TBIV was introduced to Korea through imported eggs or whether it is an endemic virus. However, similar viruses have been detected in Korea, and this disease has not been reported in Europe, including France from where the eggs were imported, which suggests that the infection occurred in Korea.

In conclusion, we observed enlarged cells in various turbot tissues and many hexagonal virions (136 to 159 nm) in the enlarged cells and necrotic areas that resembled RSIV. The sequence analysis of TBIV MCP suggests this virus is similar to the tentatively proposed genus, *Tropivirus*, in the family *Iridoviridae*.

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