

SUSCEPTIBILITY OF DIFFERENT CELLS TO RED SEA BREAM IRIDOVIRUS (RSIV)

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ABSTRACT

RSIV is an isolate virus in the genus *Megalocytivirus* (family Iridoviridae) that has been reported to be pathogen in more than 31 marine fish species in East Asia. The aim of study was to know the susceptibility of several cultured cells to RSIV. RSIV inoculum was inoculated onto cultured cells and then incubated in 25°C. Routine observation of cytopathic effect (CPE) was carried out for 7 days and harvested cells were prepared for virus titration and electron microscopy (EM). The result showed that RSIV grew and propagated in GF (grunt fin), KF-1 (koi fin) and BF-2 (barfin flounder) which caused cytopathic effect as cells enlargement. However, RSIV did not propagated on EPC (epithelioma papulosum cyprini), FHM (feathered minnow) and EK-1 (eel kidney) cells. The virus titer were $10^{5.3}$ TCID₅₀/mL in GF cells, $10^{3.8}$ and 4.3 TCID₅₀/mL in KF-1, $10^{3.6}$ and 3.8 TCID₅₀/mL in BF-2, and $7 \times 10^{2.1}$ TCID₅₀/mL in EPC, FHM and EK-1. The EM observation revealed formation of enlarged cells containing hexagonal virus particles with 140-160 nm in diameter. These results indicated that GF was cultured cell to be optimal for replication of isolate RSIV derived from Ise bay, Mie, Japan.

KEYWORDS: RSIV, cells culture, enlarged cells, virus particles

INTRODUCTION

Members of the family *Iridoviridae* infect a diverse array of invertebrate and cold-blooded vertebrate hosts and are currently viewed as emerging pathogens of fish and amphibians (Chinchar *et al.*, 2007). The family *Iridoviridae* is currently classified into five genera (*Iridovirus*, *Chloriridovirus*, *Ranavirus*, *Megalocytivirus*, and *Lymphocystivirus*) each consisting of one or more species and strains (Chinchar *et al.*, 2005). *Megalocytivirus* encompasses isolates of RSIV (red sea bream iridovirus), SBIV (sea bass iridovirus), GSDIV (grouper sleepy disease iridovirus), ALIV (African lampeye iridovirus), DGIV (dwarf gourami iridovirus), TGIV (Taiwan grouper iridovirus), TBIV (turbot iridovirus), ISKNV (infectious

spleen and kidney necrosis virus), etc. Their genetic homology has been confirmed in ATPase and major capsid protein (MCP) of SBIV, RSIV, GSDIV, ALIV, and DGIV (Sudthongkong *et al.*, 2002b).

Outbreak of RSIV began in cultured red sea bream (*Pagrus major*) in Shikoku Island, Japan in 1990, and since 1991, the disease has involved morbidity and mortality up to 60% of fish population in western part of Japan, primarily in summer. The disease fish were lethargic and showed severe anemia, petechiae of gills and enlargement of spleen (Nakajima & Sorimachi, 1994; Inouye *et al.*, 1992). The disease was histopathologically characterized by formation of inclusion body-bearing cells (IBCs) and necrotic cells with intracytoplasmic virus

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propagation, which both were observed as enlarged cells (Jung *et al.*, 1997; Miyazaki, 2007; Mahardika, 2009). These enlarged cells were deeply stained with Giemsa solution (Inouye *et al.*, 1992). Moreover, Jung *et al.* (1997) reported that the enlarged cells usually had intensively basophilic and Feulgen positive cytoplasm, and an expanded nucleus with a prominent nucleolus. Hexagonal virus particles, 150–200 nm in diameter, were observed in both of productive IBCs and necrotic cells (Inouye *et al.*, 1992; Jung *et al.*, 1997; Sudthongkong *et al.*, 2002a; Miyazaki, 2007; Mahardika, 2009). The RSIV has been shown to have a wide geographical distribution and host range (Matsuoka *et al.*, 1996).

Nakajima & Sorimachi (1994) reported that the isolat RSIV from Shikoku Island was grown in some fish cell lines with typical cytopathic effect (CPE) is characterized by cell rounded and enlargement. BF-2 (bluegill fry-2) cell line and KRE-3 (a hybrid of kelp and red spotted grouper embryo cells) cell lines were demonstrated to be optimal for cultured of RSIV virus and proliferation. In the present study was carried out to improve the isolation of isolat RSIV derived from a culture farm at Ise bay in Mie Prefecture, Japan and describe the susceptibility of 6 fish cell lines to the virus with observation of CPE, virus titer and electron microscopy.

MATERIALS AND METHODS

Cell Culture

GF (grunt fin), KF-1 (koi fin), BF-2 (barfin flounder), EPC (epithelioma papulosum cyprini), FHM (feathered minnow) and EK-1 (eel kidney) cell lines were continuously cultured in Eagle's minimum essential medium (EMEM: Nissui, Japan) supplemented with fetal bovine serum (FBS), L-glutamin, and buffered with NaHCO to pH 7.2–7.4. For routine passage and virus inoculation, 10% and 2% FBS was added to the medium (EMEM-10 and 2), respectively.

Virus Sample

RSIV isolates were prepared from tissues of the moribund red sea bream affected by RSIV (positive RSIV by PCR assay) from a culture farm at Ise bay in Mie Prefecture, Japan in 2007. The spleens was homogenized in approximately 10 volume of EMEM-2 containing penicillin (50 IU/mL) and streptomycin (50 mg/mL). The tis-

sue homogenates were centrifuged (CT4D Himac, Hitachi) at 1,200 x g for 15 min. Supernatant as a virus sample were filtered (450 nm) and stored at -80°C until examined.

Virus Titration

Viral infectivity was determined by limiting dilution assay at 7 days post infection. Briefly, 0.1 mL of each cell line in EMEM-2 was dropped into 8 wells of a 96-well plate and incubated at 25°C for 1–2 days. Then, 50 mL of 10-fold dilutions of virus sample were prepared in other a 96-well plate with EMEM-2 and added into 4 wells. Plates were incubated at 25°C for all cell lines and examined daily for CPE. Each cell line was treated by virus in two time repeated. The virus titers were expressed as the 50% tissue culture infectious dose (TCID₅₀), determined by the method of Reed & Muench (1938).

Transmission Electron Microscopy (TEM)

To determine the virus can be propagated in the cell lines, monolayer cells were harvested by scraper and centrifuged at 1200 x g for 10 min. The precipitates were twice washed with PBS and fixed with 70% Karnovsky's solution, post-fixed with 1.0% osmium, dehydrated stepwise in a graded series of ethanol and embedded in Epon-812. The blocks were sectioned using glass knife and diamond knife on Leica Ultracut microtome. Ultra-thin sections were stained with uranyl acetate and lead citrate, then examined with a H-7,000 electron microscopy at 75 kV (Mahardika & Miyazaki, 2009).

RESULTS

Susceptibility of Cultured Cells to RSIV

Rounded and enlarged cells as CPE was observed in GF, KF-1 (Figure 1) and BF-2 cells. Some rounded cells were first observed at one day post infection. At day 2 post infection, enlarged cells appeared in part of monolayer and became frequent by day of incubation. Rounded and enlarged cells were present in the infected cells but not in the controls (Figure 1-A & B3). On the other hand, small number of rounded cells were observed in EPC (Figure 2-A1 and 2-A2) and EK-1 cells within 6 days after inoculation. Its might be caused by cells became confluent by day incubation, which resulted in cells suicide as well as those in

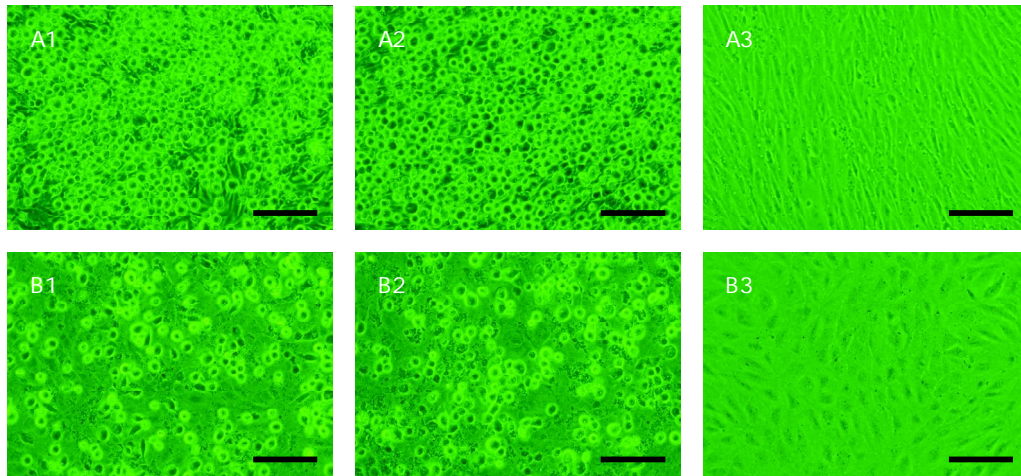


Figure 1. (A1 and A2) Cytopathic effect (CPE) of RSIV-infected GF cells at 6 d p.i. shows many varieties of enlarged cells. Most of them were separated from the cellular monolayer. (A3) Normal GF cells as control at 5 d p.i. (B1 and B2) CPE of RSIV-infected KF-1 at 6 d p.i. shows many enlarged cells, many of which separated from cellular monolayer. (B3) Control KF-1 at 3 d p.i. Scale bar = 50 μ m

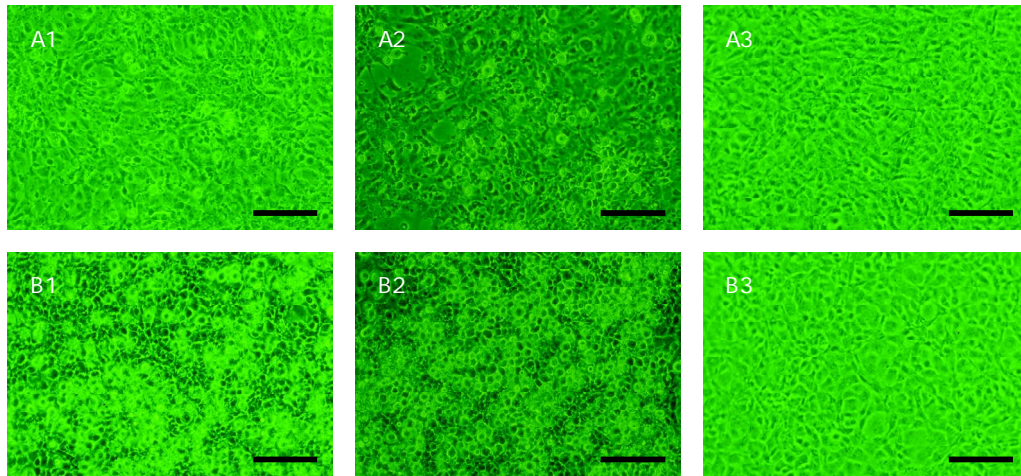


Figure 2. (A1 and A2) EPC cells inoculated RSIV at 6 d p.i. shows a few number of rounded cells. (A3) Control EPC cells at 3 d p.i. (B1 and B2) FHM cells inoculated RSIV at 6 d p.i. shows resembled of many rounded cells which mostly became fragmented. (B3) Control FHM cells at 3 d p.i. shows small syncytia. Scale bar = 50 μ m

control cultured cells (2-A3). While FHM cells showed many rounded cells resembled in some parts which most of them became fragmented (Figure 2-B1 and 2-B2), but the other part showed no cells. It also might be caused by day incubation in which cells grew faster resulting in moved of many cells and resembled to some parts of growth media.

The GF cells gave the highest titers of about $10^{5.3}$ TCID₅₀/mL in 7 days (Table 1). In the virus titration, our observation were similar with observation of Nakajima & Sorimachi (1994) that the CPE was clear in the wells were low dilutions of the inoculated virus sample, but the CPE was obscure in the wells with high virus-dilutions.

Table 1. Cell susceptibility of 6 cultured cells to RSIV

Cultured cell		Virus titer (TCID ₅₀ /mL)
GF	I	10 ^{5.3}
	II	10 ^{5.3}
KF-1	I	10 ^{3.8}
	II	10 ^{4.3}
BF-2	I	10 ^{3.6}
	II	10 ^{3.8}
EPC	I	7 10 ^{2.1}
	II	7 10 ^{2.1}
FHM	I	7 10 ^{2.1}
	II	7 10 ^{2.1}
EK-1	I	7 10 ^{2.1}
	II	7 10 ^{2.1}

Electron Microscopy Observation

Electron microscopic (EM) observation of RSIV-infected GF, KF-1 and BF-2 cells revealed enlarged cells allowing virus propagation within the intracytoplasmic virus assembly site (VAS). Many enlarged cells had disappearance of the nuclei. These cells developed intracytoplasmic VASs, within which many virions propagated. The VAS replaced a large proportion of the cytoplasm, which abundant in ribosomes, sER, mitochondria, vacuoles, and masses of granules. The VAS had an electron-lucent matrix with abundant fine granules, microfilaments, and multiple virus particles at various stages of morphogenesis, such hexagonal mature virions, partially-filled virions, and empty virions. Mature virions were 140-160 nm in size, comprise, in turn, a complete package of an electron-dense core within the capsid. Some enlarged cells had an enlarged nucleus and a cytoplasm abundant in mitochondria, ribosome, rER and sER without development of VAS.

On the other hand, some enlarged cells were derived from RSIV-inoculated EPC, FHM, and EK-1 cells. These enlarged cells had enlarged or piknotik or disappearance nuclei without virus propagation. Some cells in FHM showed degeneration and fragmentation of the intracytoplasmic organelles, resulting in necrotic which might be caused by suicide process. Enlarged cells in EK-1 look like contained VAS with an electronlucent matrix with

abundant fine granules, microfilaments and coarse granules, however its developed without virus propagation.

DISCUSSION

The rapidity of CPE appearance and final titer obtained are important for the choice of a cultured cell for the detection of fish viruses (Nakajima & Sorimachi, 1994). From the results obtained, we suggested that GF was the optimal cultured cell among those tested for the isolation and proliferation of RSIV. Previously reported that BF-2 and GF cell lines were demonstrated to be optimal for RSIV infection and proliferation (Nakajima & Sorimachi, 1994; Imajoh *et al.*, 2004). TGIV was successfully cultured in KRE cells derived from hybrid groupers (Chou *et al.*, 1998). However, viral infectivity rapidly declines according to serial passage. Therefore, for obtaining susceptible cultured cells, Imajoh *et al.* (2007) developed cultured CRF-1 cells derived from a tail fin of red sea bream. On the other hand, some virus isolat within genus *Megalocytivirus* were difficult to cultivate in cultured cells, such as DGIV (Go *et al.*, 2006), GSDIV (Mahardika, unpublished data). For better isolation and replication of *Megalocytivirus*, it need to develop primary cell line from each host fish.

By EM observation, the enlarged cells as CPE under light microscopy were enlarged cells contained enlarged nucleus and enlarged cells allowed propagation of RSIV viral particles. Intensive electron microscopic observations led by Mahardika & Miyazaki (2009) revealed continual nuclear degeneration of the enlarged cells. In the early stage of RSIV infection, the nucleus became hypertrophic and was filled with masses of electron-dense granules. Soon after this, the nuclear membrane ruptured, releasing the masses of granules into the cytoplasm. Then, the nuclear components were assimilated with the cytoplasm, resulting in the disappearance of the nucleus. The enlarged cells that lost the nucleus and developed intracytoplasmic VASs with multiple virus particles. Imajoh *et al.* (2004) proposed that RSIV-infected cells displayed fragmentation and a disappearance of the nucleus and, therefore, they underwent the naturally occurred apoptosis. Moreover, Chinchar *et al.* (2005) reported that synthesis of viral DNA is known to occur within the nucleus and the cytoplasm. Viral DNA replication commences within the nucleus and then newly-synthesized viral DNA

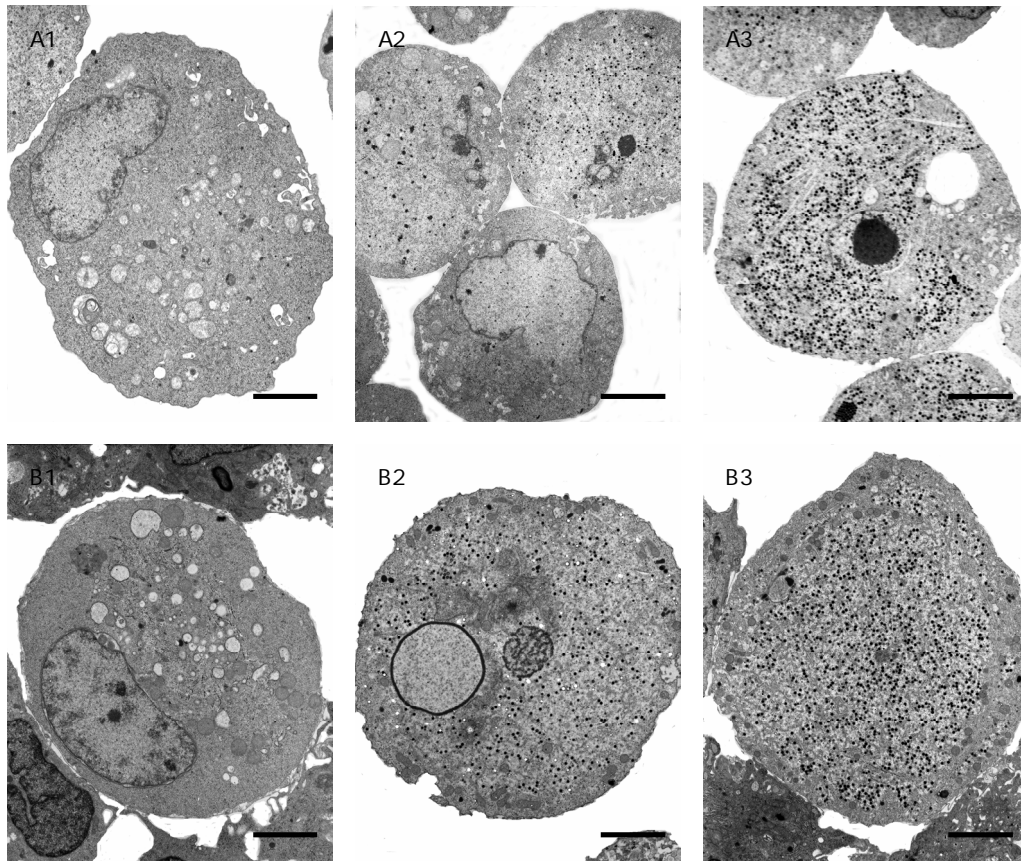


Figure 3. (A1-3) Electron micrograph of enlarged cell derived from RSIV-infected GF cells at 7 d p.i. (A1) Enlarged cell has an enlarged nucleus with a cytoplasm abundant in mitochondria, ribosomes, rER and sER (scale bar = 2500 nm). (A2) Morphogenesis of enlarged cells infected RSIV. One (bottom) has enlarged nucleus with partial rupture of the nuclear membrane. Top-left has a fragmented nucleus and develops an intracytoplasmic VAS in which a small number of virus particles are present. Top-right has a developed intracytoplasmic VAS with propagation of many virus particles (scale bar = 1300 nm). (A3) Two cells (center and bottom) had disappearance of the nuclei and development of intracytoplasmic VAS that propagation of multiple virions takes place (scale bar = 2800 nm). (B1-3) Electron micrograph of enlarged cell derived from RSIV-infected KF-1 cells at 7 d p.i. (B1) Enlarged cell has an enlarged nucleus (scale bar = 2600 nm). (B2 and 3) enlarged cells had disappearance of the nuclei and development of intracytoplasmic VASs with multiple virus particles (scale bar = 3000 and 3300 nm)

molecules are subsequently transported to the cytoplasm where they serve as templates for the formation of concatameric DNA, that is, second stage DNA synthesis. The enlarged cells seen in RSIV-infected GF, KF-1 and BF-2 cells were similar to necrotic cells in RSIV-infected red sea bream, *in vivo*, which observed as enlarged cells under light microscopy (Jung *et al.*, 1997; Miyazaki, 2007; Mahardika, 2009).

However, in the present study, the IBCs were not found. IBCs were reported to be developed in RSIV-infected GF cells (Mahardika & Miyazaki, 2009) with small in the number.

In other cultured cells (EPC, FHM, and EK-1), some enlarged cells were appeared, but it developed without virus propagation. This indicated that these cultured cells were not susceptible for RSIV isolation and proliferation.

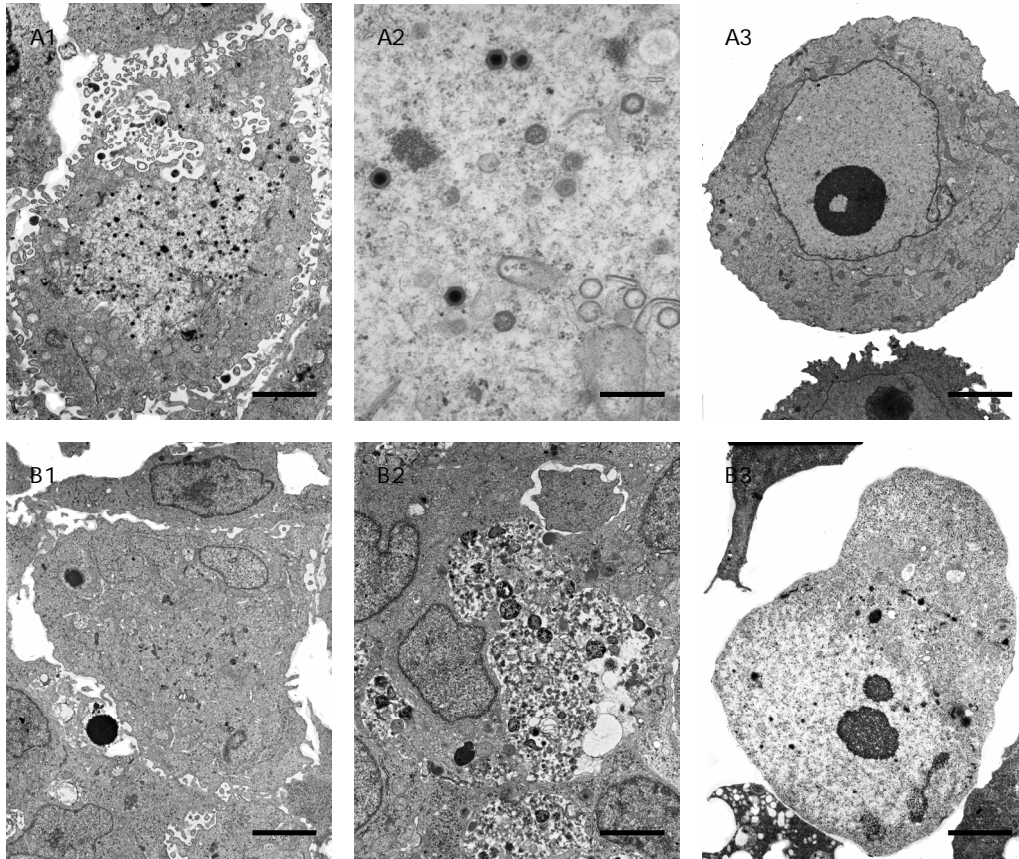


Figure 4. (A1-2) Electron micrograph of enlarged cell derived from RSIV-infected BF-2 cells at 7 d p.i. (A1) Enlarged cell has a developed VAS with multiple virus particles as well as a disappearance of the nucleus (scale bar = 3200 nm). (A2) Detail of an intracytoplasmic VAS. Assembly of RSIV virus particles at all morphogenesis steps is evident. There are empty virions, partially-filled virions and completely filled virions (scale bar = 450 nm). (A3 and B1) Electron micrograph of enlarged cell derived from RSIV-infected EPC cells at 7 d p.i. (A3) Two enlarged cells had enlarged nuclei with nucleoli (scale bar = 1800 nm). (B1) Enlarged cell has a picnotic nucleus with no formation of VAS within its cytoplasm (scale bar = 2600 nm). (B2) Electron micrograph of resembled cells derived from FHM inoculated with RSIV shows necrotic cells with fragmentation of all its organelles (scale bar = 800 nm). (B3) Electron micrograph of enlarged cell derived from EK-1 inoculated with RSIV has a disappearance of the nucleus with developed an intracytoplasmic VAS without virus assembly (scale bar = 2700 nm)

Basic on this result, we brought the healthy cultured cells to Indonesia for cultured of GSDIV (*in vitro*) in Indonesia in order to preparation of GSDIV vaccine.

CONCLUSION

Result of our present study strongly suggest that cultured GF cells was the optimal cultured cells for the propagation and assay

of isolat RSIV from Ise bay, Mie Prefecture, Japan. Under electron microscopy, enlarged cells as CPE in cultured GF, KF-1, and BF-2 cells were cells with disappearance of their nuclei and allowed viral propagation within VAS.

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