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Application on Microwave Energy in the Preparation of Fish Samples for Electron Microscopic Observation

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Chemotherapy can not be applied for the control of fish viral diseases because viruses depend on host machinery for their replication. Although new control strategies including vaccination are under development, avoidance of virus introduction by rapid and correct diagnosis is the best way of fish viral disease control. Although observation of virus particles with an electron microscope is an easy method for virus detection, it take a few days for the sample preparation. In order to shorten the sample preparation time, microwave radiation was applied in the procedure. With this method, 15 seconds was enough for fixation of virus infected fish samples or cultured cells inoculated with infectious hematopoietic necrosis virus, which takes 2~4 hours with routine methods. Also four minutes was enough for polymerization of embedding resin which takes 24~48 hours with routine methods. Samples prepared with microwave were good enough for direct electron microscopic observation and immunogold labeling assay.

Key words: fish virus diagnosis, electron microscope, IHNV, lymphocystis, microwave, immunogold labelling assay

Introduction

Viruses, being obligate parasites, require live hosts and use host's machinery for their replication. So, chemotherapeutical control methods such as application of antibiotics can not be used for the control of viral diseases. In contrast with viruses of land animals that transmitted mostly by insect vectors, fish viruses can be easily spread in water and can cause serious economic loss in very short time.

Although new control methods such as vaccine are under development, avoidance of virus introduction of is the best way of viral disease control at this moment. In order to achieve this, rapid and sensitive diagnostic techniques are required. Some viruses cause specific symptom, and this can be used for diagnosis. Diseased tissue can be observed with either light or electron microscope. Cultured fish cell can be inoculated with ground and filtered samples and cytopathic effect (CPE) can be observed. This procedure also takes few days to a week, and some fish viruses replicate only in specific cell line or others do not grow in cultured cell at all. Viral proteins can be detected by enzyme linked immunosorbent assay (ELISA) (Dixon and Hill, 1984; Way and Dixon, 1988), Western blot (Hse and Leong, 1985), and a fluorescent antibody test (LaPatra et al., 1989).

Although vius particles can be observed with an electron microscope, it takes a few days to prepare the samples for observation. The tissue must be fixed with glutaraldehyde or formaldehyde for the conservation of the structure. Sometimes protein or lipid leaks from the cell during this process (Hopwood, 1972). Also, the fixatives penetrate very slowly and the tissue must be cut in small pieces, and it usually takes more than 2 hours for fixative penetration (Hopwood, 1967). After fixation and dehydration by serial changes of increasing concentration of ethanol, the tissue is embedded in resin such as Spurrs and Epon. Usually these resins are polymerized in high temperature, over 60℃, for 24~72 hours. This process can destroy antigens that are required for immuno-cytochemistry.

Microwave energy has been successfully used for the fixation and embedding of animal and plant

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tissues (Boon et al., 1986; Hopwood et al., 1984; Login and Dvorak, 1988; Westcot et al., 1993). With this method, the tissue could be prepared within a day and the antigenicity of the sample has not been changed. Therefore, this method could be used for the rapid detection of fish viruses by direct electron microscopic observation or after immunogold labeling.

In this study, microwave energy was applied during the fixation and embedding processes of fish samples infected with lymphocystis virus and cultured fish cells inoculated with infectious hematopoietic necrosis virus (IHNV).

Materials and Methods

Cell line and virus

The chinook salmon embryo (CHSE-214) cell line was propagated in minimum essential medium (MEM, GIBCO BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (FBS), 50 I.U/mℓ penicillin, 50 μg/mℓ streptomycin and buffered to pH 7.4 with sodium bicarbonate and Tris-HCI. IHNV Round Butte strain (RB-76) was kindly provided by Dr. M. A. Park of National Fisheries Research and Development Agency. Cells were grown at 20℃ for routine propagation and kept 5~7 days at 18℃ after virus inoculation. Cultured rockfish infected with lymphocystis virus was obtained from mari-floating netcage in Namhae.

Equilibration of microwave oven

A commercial home microwave oven from Samsung company (Model RE-551B, Maximum output : 700W) was used for the fixation and embedding. A wooden block of 100×85×43 mm was placed at the center of the chamber. Six holes of ϕ15×15 mm were made on the wooden block to hold sample vials of ϕ14×40 mm. A 500 mℓ glass beaker filled with 300 mℓ water was placed at the corner as water heat sink. A sample vial with 2 mℓ fixative (0.05 m cacodylate buffer, pH 7.0, 0.02% glutaraldehyde, 4% paraformaldehyde) was placed without a cap in the hole of the wooden block. The vial was radiated for 5 to 40 second and the temperature of fixative and heat sink was measured by inserting a thermometer into them. New fixative and water in the heat sink was used for each measurement.

Fixation and embedding

Rockfish fin tissue showing typical lymphocystis symptom was cut into about 2×2 mm size in fixative placed on a slide glass. CHSE-214 cells infected with IHNV were harvested 4 days after inoculation by scraping the bottom of culture vessel. The cells were centrifuged for 15 min at 1,000×g. The pellet was used for fixation. A few cubes of the dissected tissue or cell pellet were transferred to sample vial containing 2 mℓ of fixative and radiated for 15 seconds. A water heat sink was placed as above and one vial was radiated each time. The fixative was removed immediately after radiation and the sample was washed three times by replacing the fixative with wash buffer (0.2 M cacodylate buffer, pH 7.0). The cell pellet dispersed during the fixation and washing, and it was recovered by centrifuging for 2 min at 15,000×g between each washing step.

The fixed samples were dehydrated with serial changes of ethanol. The dehydration step was as follows; 10 min in 60, 70, 80, 90% ethanol followed by 15 min in 100% and 30 min in fresh 100% ethanol. As a bridging step, the dehydrated samples were dehydrated 30 min in 50 : 50 mixture of 100% ethanol and LR white medium grade, infiltrated for 30 min in 100% LR white, 30 min in fresh 100% LR white again, then stored in fresh LR white overnight. After two infiltrations steps of 2 hrs in fresh LR white, the samples were embedded and polymerized. The setting of microwave for embedding was as follow; A glass plate (ϕ10 min) filled with 60 mℓ of water was placed at the center of the microwave oven chamber. Two slide glasses (25×75 mm) were placed on the top of the glass plate to hold a embedding mold tray (Easy-Mold™, Electron Microscopy Sciences, Fort Washington, PA). Among 20 block molds on the tray, 3~4 molds were filled with the specimen and LR white for each cycle of embedding. There was no water heat sink placed in the embedding process but the water in the glass plate was replaced with fresh water after each embedding. The samples were radiated for 4.5 min for polymerization, then removed from the oven and let cool down to room temperature. The sample blocks were removed from the mold and the hardness was tested with a razor blade.

Ultramicrotomy and immunogold labeling

Thin section of 50~70 mm were made with a LKB ultramicrotome. For direct observation of lymphocystis virus from rockfish tissue, the sections were transferred onto a grid and double stained with 5% uranyl acetate and 0.04% lead citrate for 30 min and 2~5 min, respectively. Thin sections from IHNV inoculated CHSE-214 cells were

observed after immunogold labeling. The grid was placed on a drop of 5% FPG [5% fetal bovine serum (FBS) in phosphate buffered saline (PBS, pH 7.0) containing 0.02 M glycine] to block non-specific binding. The grid was transferred on a drop of 1 : 10 diluted rabbit anti-IHNV polyclonal antibody diluted in 5% FPG and incubated for 40 min at room temperature. The unbound antibody was washed off four times by transferring the grid on a drop of 10 % FPG and incubating for 5 min. Excess liquid was removed from the grid by touching a dry filter paper. Then the grid was placed on a drop of protein A-gold conjugate 1 : 15 diluted in 5% FPG and incubated for 40 min at room temperature. The grid was washed four times by placing the grid on drop of PBS for 5 min. The excess liquid was removed and the grid was double stained as above. The grid was observed with a transmission electron microscope.

Results and Discussion

Equilibration of the microwave oven

The temperature of the fixative was 16℃ at the beginning and reached 83℃ after 40 sec irradiation. Login and Dvorak (1988) reported that optimal integrity of animal tissue was achieved when the final temperature of the fixative ranged from 40~50℃. Similar range of fixative temperature was used for the fixation of tobacco leaf tissue infected with tobacco mosaic virus (Benhamou et al., 1991)

The temperature of fixative reached 43℃ after 15 sec irradiation (Fig. 1). Therefore, all the samples were irradiated for 15 sec for fixation in the following experiments. Although the exact physicochemical mechanism of rapid fixation by microwave irradiation has not been explained, thermal effect of the microwave irradiation and direct effect on the electric field could help the penetration of the fixative into tissue and cells.

Ultrastructure of lymphocystis virus

In order to test possible application of microwave fixation and embedding in fish virus diagnosis, the lymphocystis virus which causes typical symptom and has distinctive structural feature was chosen. The virus is the largest of the iridoviridae family and the average particle size is about 200 ± 50 nm although it is affected by the preparation procedures. The virus has electron dense core enclosed by viral shell composed of two unit membranes called inner membrane and outer membrane. There are

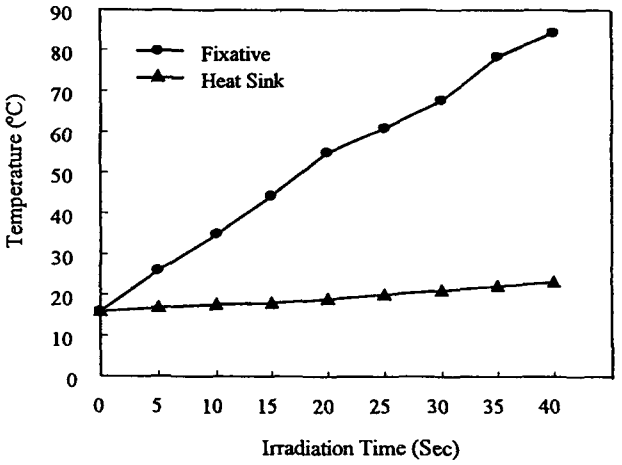

Fig. 1. Equilibation of a commercial microwave oven for sample preparation. The temperature of 2 mℓ fixative in a sample vial placed on a wooden block and heat sink of 300 mℓ water in a 500 mℓ beaker was measured after irradiation at the maximum output to determine the optimum irradiation time for sample fixation. The results are the averages of three replications.

globular subunits on the surface of the particle, and filaments attached to the subunits form fuzzy fringe in electron microscope observation (Wolf, 1988).

well-conserved virus particles of about 200 nm were found in the cell from the infected tissue (Fig. 2A). A hexagonal shell surrounding the electron dense core was clear and fuzzy fringe was also observed. The shape of the virus was equally well conserved compared to particles shown in other publications (Wolf, 1988; Berthiaume et al., 1984). This result suggests that the microwave irradiation method could be used for rapid preparation of samples for electron microscopic observation of fish viruses without causing severe structural changes of the virus particles, which could be useful for rapid diagnosis of fish viruses.

Immunocytochemical observation

In routine embedding process, samples ae treated with heat over 60℃ for at least 24 hrs, and the chemicals and heat can destroy the antigenicity of proteins in the samples. Although other methods such as cryo-immunocytochemical method can solve this problem, this technique requires lengthy process and can not be used for large number of samples. Microwave energy has been used to solve the problem and has been proven to be effective in animal and plant samples (Login et al., 1987; Westcot et al., 1993).

The IHNV particles located by immunogold labe-

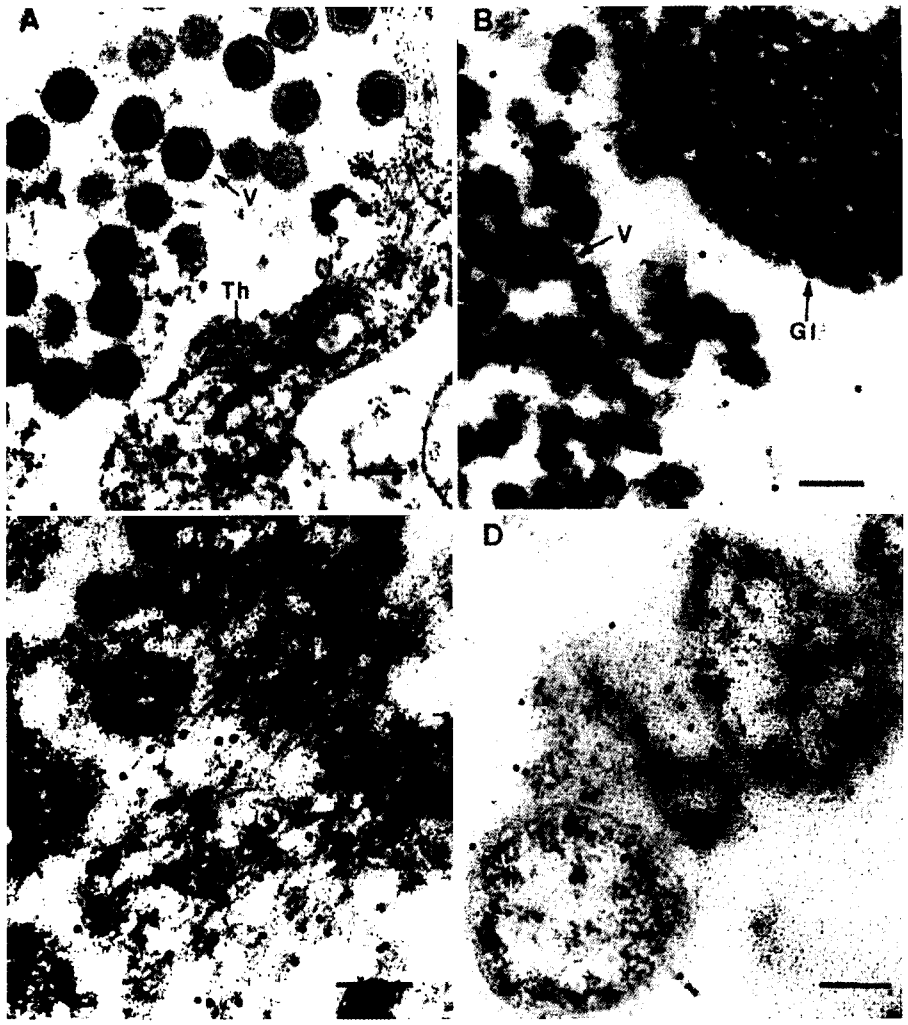

Fig. 2. Electron micrographs of samples prepared with microwave. (A) Rockfish infected with lymphocystis virus. The virus particle (V) and thread-like structure (Th) is indicated. Bar=200 nm. (B) CHSE-214 cells infected with IHNV and immunogold labeled with gold conjugated antibody. Virus particles (V) and granular inclusion body (GI) labeled with gold particles are indicated. Bar=100 nm. (C) Thread-like structures in IHNV infected CHSE- 214 cell are heavily labeled with gold particles. Bae=100 nm. (D) Vesicular structures in IHNV infected CHSE-214 cell labeled with gold particles. Bar=100 nm

ling are shown in Fig. 2. Sometimes it is hard to locate the virus in the cell by regular electron microscopic observation when the virus titer is low. However, it was easy to locate the virus after immunogold labeling (Fig 2B). Cytoplasmic spherical inclusion bodies have been observed in the cells infected with fish rhabdovirus including IHNV (Granzow et al., 1997). These inclusion bodies were granular electron-dense mass. Although these inclusions has been observed in virus infected cells, their origin has not been identified. The spherical inclusion bodies observed in this study and were heavily labeled with gold particle (Fig. 2B), which indicating that this structure was originated from viral proteins. However, it could not be determined which of the viral protein is in the inclusion because the antibody used for the labeling was polyclonal antibody against purified virus. Antibodies specific to each viral protein are being made and the exact compositon of the inclusion could be determined soon.

In addition to the globular inclusions, thread-like structures were detected in IHNV infected cell (Fig. 2C). These structures were also heavily labeled with gold particles, which indicated the viral origin of these structures. Similar structures were observed in cells infected with lymphocystis virus (Fig. 2A,

marked as Th). Viral nucleic acids of enveloped viruses usually exist as complexes of nucleic acids and nucleocapsid proteins in the virus infected cells. Although further identification of these structures is necessary, we assumed these structures are complex of nucleocapsid and nucleic acid, the intermediate of virus replication. Also, vesicular structures labeled with gold particles were found in the infected cell (Fig. 2D), but the exact function or origin of these structures is not clear at this moment.

The overall time required for the sample preparation with common methods and microwave irradiation method were compared. As shown in Table 1, it takes less than 24 hours to prepare the samples with microwave irradiation compared to 3~4 days with routine methods. This technique is rapid and does not destroy the antigen during the process. Therefore, this methods can be very helpful in fish virus diagnosis, especially when combined with immunocytochemistry.

Table 1. Comparison of time required for sample preparation with common methods and microwave irradiation

Processes	Common Methods	Microwave Irradiation
Fixation	2~4 hrs	15 sec
Washing	45 min	45 min
Post-fixation	1~2 hrs	–
Washing	10 min	–
Dehydration	1 hr 40 min	1 hr 40 min
Infiltration	overnight	overnight
Embedding	24~48 hrs	4 min 30 sec

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