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Note

Effect of virus infectivity titer following centrifugation and filtration during virus extraction from fish samples

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A 0.45-μm membrane filter is generally used to remove bacterial contamination during virus extraction from fish samples. However, the number of fish viruses is drastically reduced after filtration with a 0.45 μm filter. In this study, we investigated the effect of filters on virus infectivity titer and the change in virus titer and bacterial number following different centrifugation conditions to determine a suitable procedure for virus extraction from fish samples. $10^{4.05}$ and $10^{5.05}$ $TCID_{50}$/ml of infectious hematopoietic necrosis virus (IHNV) and $10^{4.05}$ and $10^{4.55}$ $TCID_{50}$/ml of *Oncorhynchus masou* virus (OMV) were not detectable after filtration with two types of 0.45-μm filters, except the IHNV titer was reduced by about 10 fold after filter use (company A). No significant difference was found in the virus titer following centrifugation at 880 × *g* (30 min) or 3,500 × *g* (30 min), whereas IHNV and OMV titers were reduced by about 10 and 10–1000 fold by centrifugation at 14,000 × *g* (30 min) and 14,000 × *g* (10 and 30 min), respectively. A total of 97.7–99.9% *Escherichia coli* were eliminated by centrifugation at 880 × *g* (30 min) and 3,500 × *g* (30 min). These results show that fish viruses were affected by filtering, even though the effect differed by virus species and filter type. Therefore, centrifugation at 3,500 × *g* (30 min) and use of medium with antibiotics may be useful for virus extraction along with a reduction in bacteria.

Key words: Fish virus, Centrifugation, 0.45 μm filter, Virological examination

Aquaculture has grown rapidly over the past few decades. However, because of high culture densities and environmental pollution, various infectious diseases have appeared (Wolf, 1988; Austin and Austin, 1999; Woo and Bruno, 1999). In particular, viral diseases are a threat to aquaculture because of the lack of available treatments. Infectious haematopoietic necrosis (IHN), viral hemorrhagic septicemia, red sea bream iridovirus disease, and viral nervous necrosis are representative viral diseases (Wolf, 1988; Essbauer and Ahne, 2001).

A virological examination is conducted using a fish cell line to diagnose a viral disease in diseased fish (Oh *et al*., 2001; Kim *et al*., 2009; OIE, 2012). Generally, fish samples are transported on ice, and internal organs are aseptically collected and homogenized with nine volumes of Hanks' balanced salt solution (HBSS). After centrifugation (2,000－4,000 × *g*, 10－20 min, 4°C), the supernatant is filtered through a 0.45 μm membrane filter, and 100 μl is inoculated onto fish cell lines in 24-well plates. The cell cultures are incubated at 15－25°C and examined for the development of cytopathic effects.

We determined that virus titers decreased dramatically after filtration of virus supernatants with 0.45 μm filters. Thus, using a filter may be a dangerous procedure for virus extraction from fish samples.

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Thus, we investigated the effect of 0.45 μm filters on virus infectivity titer as well as the change in virus titer and bacteria number by different centrifugation conditions to determine a suitable virus extraction procedure.

Materials and methods

Viruses and bacterium

Two viruses (IHN virus [IHNV]: rhabdovirus and *Oncorhynchus masou* virus [OMV]: herpesvirus) and one bacterium (*Escherichia coli*) were used in this study. IHNV (strain: IHNV-ChAb7601) and OMV (OMV00-7812) were isolated from chum salmon, *Oncorhynchus keta,* and masu salmon, *O. masou*, respectively (Kimura *et al*., 1981; Yoshimizu *et al*., 1988), whereas *E. coli* (BL21) was obtained from Novagen (Darmstadt, Germany). Epithelioma papulosum cyprini (EPC) and rainbow trout gonad (RTG-2) cells were used for the virus culture and infectivity assays. The cell lines were maintained at 15°C in Eagle's minimum essential medium (Gibco, USA) supplemented with 10% fetal bovine serum, 100 IU/ml penicillin G, and 100 μg/ml streptomycin sulfate. *E. coli* was incubated at 37°C in Luria–Bertani medium (Difco, USA). After 24 h, the bacteria were collected by centrifugation (6,000 × *g*, 20 min) and suspended in sterile phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4 and pH 7.2) for testing.

Change in virus infectivity titer with different filters

Two different filters were used in this study (Table 1): 0.45 μm filters made by companies A and B. One ml aliquots of IHNV ($10^{4.05–5.05}$ 50% of the tissue culture infectious dose [$TCID_{50}$]/ml) and OMV ($10^{4.05–4.55}$ $TCID_{50}$/ml) were filtered through the above filters and the infectivity titers of the filtrates were measured by the $TCID_{50}$ method (Reed and Muench, 1938).

Change in virus infectivity titer and bacteria number following different centrifugation conditions

Four different centrifugation conditions were used in this study (Tables 1 and 2): 880 × *g* (30 min), 3,500 × *g* (30 min), 14,000 × *g* (10 min), and 14,000 × *g* (30 min). One ml aliquots of IHNV ($10^{4.05–5.05}$ $TCID_{50}$/ml), OMV ($10^{4.05–4.55}$ $TCID_{50}$/ml), and *E. coli* ($8.1\times10^3 – 1.9\times10^5$ colony-forming unit (CFU)/ 100 μl) were centrifuged under the above conditions at 4°C. After centrifugation, a 100 μl aliquot of each supernatant was carefully collected, and the virus titer and bacteria number were measured by $TCID_{50}$ and CFU, respectively.

Results and Discussion

Changes in IHNV and OMV infectivity titers by

Table 1. Changes in virus infectivity titer following different centrifugation conditions and filters

Centrifugation condition and filter types	IHNV $logTCID_{50}$/ml			OMV $logTCID_{50}$/ml		
	Exp. 1	Exp. 2	Exp. 3	Exp. 1	Exp. 2	Exp. 3
Control	4.05	5.05	4.55	4.05	4.55	4.55
880 × *g*, 30 min	4.05	4.55	NT	4.05	4.55	NT
3,500 × *g*, 30 min	4.55	5.05	NT	3.55	3.8	NT
14,000 × *g*, 10 min	3.8	4.3	NT	3.05	3.05	2.8
14,000 × *g*, 30 min	3.3	3.8	3.8	1.55	1.05	< 0.8, < 0.8
0.45 μm filter (Company A)	< 0.8	< 0.8	NT	< 0.8	< 0.8	NT
0.45 μm filter (Company B)	NT	3.55	2.8, 3.3	< 0.8	< 0.8	< 0.8

NT, not test

< 0.8, detection limit

filter type are shown in Table 1. The $10^{4.05}$ and $10^{5.05}$ $TCID_{50}$/ml of IHNV and $10^{4.05}$ and $10^{4.55}$ $TCID_{50}$/ml of OMV were not detectable after filtration with the 0.45 μm filter (company A). The $10^{4.55}$ and $10^{5.05}$ $TCID_{50}$/ml of IHNV was reduced about 10-fold by the filter (company B), even though the $10^{4.05}$ and $10^{4.55}$ $TCID_{50}$/ml of OMV were not detected after filtration. These results show that the infectivity titers of IHNV and OMV were affected by filter and that the effect was different by virus species or filter type. The difference in the reduction of titer between the viruses might be related to virus size; IHNV virions are 170 nm (150～190) long and 70 nm (65～75 nm) in diameter, whereas OMV particles are 220～240 nm (Wolf, 1988).

The changes in IHNV and OMV infectivity titers under different centrifugation conditions are shown in Table 1. No significant difference was found for the IHNV virus titer under the three centrifugation conditions of 880 × *g* (30 min), 3,500 × *g* (30 min) and 14,000 × *g* (10 min): $10^{4.05}$ $TCID_{50}$/ml (control) and $10^{3.8}-10^{4.55}$ $TCID_{50}$/ml (centrifuged groups) in experiment 1, and $10^{5.05}$ $TCID_{50}$/ml (control) and $10^{4.3}-10^{5.05}$ $TCID_{50}$/ml (centrifuged groups) in experiment 2. In contrast, the $10^{4.05}$ and $10^{5.05}$ $TCID_{50}$/ml of IHNV decreased $10^{3.3}$ and $10^{3.8}$ $TCID_{50}$/ml, respectively following centrifugation at 14,000 × *g* (30 min). No difference was found in OMV virus infectivity titer after centrifugation at 880 × *g* (30 min), but the titers decreased subsequently following higher centrifugation speed and longer duration: $10^{4.05}$ $TCID_{50}$/ml titer (control) was reduced to $10^{3.55}$ $TCID_{50}$/ml (by 3,500 × *g*, 30 min), $10^{3.05}$ $TCID_{50}$/ml (14,000 × *g*, 10 min) and $10^{1.55}$ $TCID_{50}$/ml (14,000 × *g*, 30 min) in experiment 1, and the $10^{4.55}$ $TCID_{50}$/ml titer (control) was reduced to $10^{3.8}$ $TCID_{50}$/ml (3,500 × *g*, 30 min), $10^{2.8}$ and $10^{3.05}$ $TCID_{50}$/ml (14,000 × *g*, 10 min), and $< 10^{0.8}$ and $10^{1.05}$ $TCID_{50}$/ml (14,000 × *g*, 30 min) in experiments 2 and 3, respectively. In particular, the reduction of the OMV titer was more drastic than that of IHNV following centrifugation at 14,000 × *g* (30 min). These results show that IHNV and OMV infectivity titers were affected by centrifugation conditions, as indicated by a reduction of about 10-fold in the IHNV infectivity titer at 14,000 × *g* (30 min) and the 10－1000 fold reduction in OMV infectivity titer following 14,000 × *g* (10 and 30 min). Moreover, it was confirmed that the virus titer was almost unaffected below 3,500 × *g* (30 min).

Changes in *E. coli* number following the different centrifugation conditions are shown in Table 2. Aliquots of 8.1×10^3, 1.7×10^4, and 1.9×10^5 CFU/100 μl of *E. coli* decreased 1.4×10^2 (reduction rate: 98.3%), 3.9×10^2 (97.7%) and 1.4×10^3 (99.3%) CFU/100 μl following centrifugation at 880×*g* (30 min) and < 10 (< 99.9%), 3.0×10^2 (98.2%) and 8.9×10^2 (99.5%) CFU/100 μl following centrifugation at 3,500 × *g* (30 min), respectively. Centrifugation at 14,000 × *g* (10 and 30 min), resulted in a decrease in *E. coli* number (control) by about 100－1000 fold (98.4－99.9%). These results confirm that 97.7－99.9% of bacteria can be eliminated by these centrifugation conditions.

According to the OIE (2012) guide for aquatic animal health, virus extraction from fish samples is conducted using the following procedure. An organ sample in medium (cell culture medium or HBSS) at a

Table 2. Changes in bacteria number following different centrifugation conditions

Centrifugation condition	Colony-formingunit(CFU)/100 μl		
	Exp. 1	Exp. 2	Exp. 3
Control	8.1×10^3	1.7×10^4	1.9×10^5
880 × *g*, 30 min	1.4×10^2	3.9×10^2	1.4×10^3
3,500 × *g*, 30 min	< 10	3.0×10^2	8.9×10^2
14,000 × *g*, 10 min	< 10	2.7×10^2	3.5×10^2
14,000 × *g*, 30 min	< 10	1.5×10^2	1.8×10^2

final dilution of 1/10 is homogenized, centrifuged at 2,000－4,000×*g* for 15 min, and the supernatant is collected. The antibiotic treatment makes filtration through membrane filters unnecessary. In the present study, no significant difference was found in virus titer by centrifugation at 880 × *g* (30 min) or 3,500 × *g* (30 min). Moreover, 97.7－99.9% of bacteria can be eliminated using these centrifugation conditions. These results indicate that centrifugation of about 1,000－3,500 × *g* may be useful for virus extraction together with reducing the number of bacteria, and that a higher centrifugation speed is more effective to reduce bacteria. However, >14,000 × *g* centrifugation should be cautiously handled because IHNV and OMV titers were reduced by centrifugation at 14,000 × *g*.

A 0.45 μm membrane filter is generally used to remove bacterial contamination during virus extraction from fish samples. Unfortunately, IHNV and OMV titers were drastically reduced after filtration with a 0.45 μm filter, and the amount they were reduced depended on the filter type used. Thus, filters should be checked before virus extraction procedures. Antibiotic treatment (suitable concentrations: gentamycin [1,000 μg/ml] or penicillin [800 IU/ml] and streptomycin [800 μg/ml]) makes filtration through a membrane filter unnecessary (OIE, 2012). Moreover, about 99.998% of *E. coli* (BL21) can be eliminated by antibiotic treatment (1,000 IU/ml penicillin and 1,000 μg/ml streptomycin) (data not shown). These results indicate that antibiotics are very useful to reduce bacteria instead of filtration, except antibiotic-resistant bacteria.

In conclusion, a 0.45 μm membrane filter is generally used to remove bacterial contamination during virus extraction from organ samples. However, fish viruses were drastically reduced after filtration with a 0.45 μm filter. Therefore, centrifugation at 3,500 × *g* (30 min) and use of medium with antibiotics may be useful for virus extraction together with reductions in bacteria. If contamination is expected in a centrifuged sample, the filter should be used after checking for no reduction in virus titer.

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