コイヘルペスウイルス(KHV)の効果的な分離培養手法

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Effective Procedures for Culture Isolation of Koi Herpesvirus (KHV)

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ABSTRACT—Isolation of pathogens is essential for studies on infectious diseases. Present study aimed to develop an effective method for culture isolation of koi herpesvirus (KHV). KHV was effectively isolated using CCB cells only from fish with clinical signs. KHV survived longer at 4°C or –30°C than at 23°C in fish tissues. These results suggest that KHV can be isolated from symptomatic fish kept at the low temperature. To increase isolation efficiencies, the brain as well as the gills and kidney should be included in target organs for examination. In addition, freezing of the organs at –30°C or –85°C is recommended when samples are preserved for a long period before isolation.

Key words: virus isolation, koi herpesvirus, KHV, CCB, sensitivity

Koi herpesvirus (KHV) disease caused by cyprinid herpesvirus 3 (CyHV-3) is an emerging disease causing mass mortality in koi and common carp, *Cyprinus carpio* worldwide[1]. The disease is listed by the OIE (World Organisation for Animal Health) and was designated a "specific disease" by Japanese law in 2003[2]. It is described in the manual of diagnostic tests for aquatic animals (OIE, 2009) that, except for KHV disease, culture isolation of pathogenic viruses with established cell lines is the best method for the diagnosis and surveillance of all viral fin-fish diseases due to the availability, utility, and diagnostic specificity and sensitivity of culture-based methods[3]. Although several cell lines such as KF-1 cells[4], CCB cells[5] and KFC cells[6] have been developed for culture isolation of KHV, PCR assay is frequently used for diagnosis of KHV disease due to low susceptibility of those cell lines to KHV. Also in Japan, we have to use PCR assay for diagnosis of KHV disease according to the law for ensuring sustainable aquaculture production[2]. On the other hand, there is no doubt in the value of being able to isolate infectious virus, which is very useful for future investigation on comparison of pathogenicity among KHV isolates from different epidemics, etc.

In the present study, we investigated sensitivity of KHV culture-isolation using CCB cell line, one of the most susceptible cells to KHV, and also influence of storage condition of fish samples for KHV isolation in future epidemiological studies.

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Materials and Methods

Virus

CCB cells kindly provided by Dr. Neukirch were maintained in minimum essential medium (MEM, Gibco) supplemented with 5% fetal bovine serum for propagation of KHV NRIA0301[7]. After inoculated with KHV, CCB cells were incubated at 20°C for 10 days, and the culture supernatant was harvested to stock at –85°C until use.

Experimental infection

Total 50 of common carp (body weight: 30–50 g), bred under KHV-free condition in the National Research Institute of Aquaculture (NRIA), were immersed into a 1:1000 dilution of the KHV stock solution at 23°C for 1 h. Fish were reared in two aquaria with 60 L and 200 L of flowing water at 23°C; the former aquarium contained 10 fish for mortality observation, whereas the latter aquarium contained 40 fish for fish sampling. The fish were fed with a diet of commercial dry pellets.

Culture-isolation of KHV from infected fish

Three surviving fish were randomly collected from the fish in sampling aquarium at 3, 7, 14 and 21 days post exposure (dpe), and the gills, kidney and brain were dissected with sterilized scissors for culture-isolation of KHV. The organs were homogenized with 10 volumes of MEM containing 10 × concentration of Antibiotic-Antimycotic liquid (Gibco) using disposal homogenizer (BioMassher II®, Sarstedt), and supernatants of the homogenates were obtained by centrifugation (2,000 *g*, 5 min). After reaction at 15°C for 2 h, 50 μL of the supernatants were inoculated to 24-hour old CCB cells seeded in 24-well plates, and incubated at 20°C to observe cytopathic effect (CPE) for 3 wks. The viral titer in each sample was estimated as 50% tissue culture infectious dose ($TCID_{50}$)/g tissue weight.

Influence of storage temperature and period on KHV infectivity

Twelve fish sampled from the aquarium were randomly collected at 7 dpe and sacrificed on ice. Fish were separately stocked into three plastic bags (4 fish

in each bag), and kept at 23°C, 4°C or –30°C. Gill, kidney and brain (a total 100 mg of each tissue) were aseptically taken from four fish kept at 23°C at 0, 3, 6, 12, 18 and 24 h after storage. The same weight of samples were taken from the three organs kept at 4°C and –30°C at 0, 1, 3, 7 and 14 days after storage. Preparation and inoculation of viral supernatants were prepared by the same method as described above, and passed through 0.45 μm cellulose acetate membrane filter to avoid bacterial contamination. The viral titers were estimated as the same method described above.

Influence of freeze-thaw on KHV infectivity

The gills of a moribund fish were sampled at 8 dpe from the aquarium, and dissected to obtain 5 pieces of gill lamellas (approx. 80 mg in each weight). Before freezing, one piece of the gill lamellas was subjected for estimation of KHV infectivity titer. Other four pieces were kept in plastic bags at –30°C. After repeated with 1, 3, 5 or 7 times of freeze-thaw cycles, the samples were subjected to estimation of KHV infectivity titer as the same method described above.

Results and discussion

In the aquarium for monitoring, mortality of common carp occurred within 2 wks of post-exposure, and the cumulative mortality was 80% (Fig. 1). Gross signs of KHV infection such as hemorrhaging of the skin, congestion of the fins, exfoliation of the epidermis and sluggishness were observed from 3 to 14 dpe, but survivors subsequently recovered. KHV was culture-isolated from gills of the infected fish at 3–14 dpe and from kidney and brain of the fish at 7–14 dpe (Table 1). In the fish at 3 dpe, KHV infectivity titers ranged from $10^{2.05}$ to $10^{3.55}$ $TCID_{50}$/g was detected from gill, but not from kidney or brain. In the fish at 7 dpe, infectivity titers of KHV from gills and kidney ranged from $10^{5.05}$ to $10^{5.55}$ $TCID_{50}$/g, whereas those from brain were from $10^{3.05}$ to $10^{3.55}$ $TCID_{50}$/g; there was no significant difference in viral titer between the gills and kidney, but those in the brain were lower. In the fish at 14 dpe, KHV infectivity titers ranged from $10^{3.8}$ to $10^{4.55}$ $TCID_{50}$/g, and which titers were equal or higher than those in the gills and kidney (Table 1). These results indicate that gills and kidney are the best target organs for KHV isolation from moribund fish, although brain is recommended for sampling after the peak of mortality.

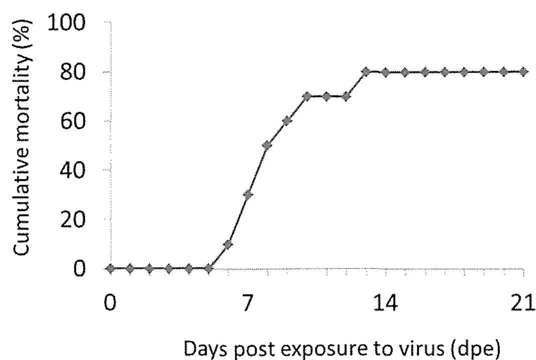

Fig. 1. Cumulative mortality of common carp infected with KHV (NRIA0301) in the aquarium for mortality observation.

We investigated influence of storage temperature and period against KHV infectivity in fish samples (Table 2). When samples were kept at 23°C, KHV was detectable by culture isolation within 12 h, but it was

Table 1. Infectivity titer of KHV in the three origins from infected fish sampled at each day post exposure (dpe) to KHV

Days post exposure to KHV	Infectivity titer of KHV (log $TCID_{50}$/g)		
	Gills	Kidney	Brain
3	2.05	–*	–
	2.8	–	–
	3.55	–	–
7	5.55	5.05	3.05
	5.55	5.05	3.05
	5.55	5.3	3.55
14	3.8	2.8	3.8
	4.05	3.3	4.05
	4.3	3.3	4.55
21	–	–	–
	–	–	–
	–	–	–

* Under the detection limit.

Table 2. Influence of storage temperature and period on KHV infectivity

Temperature	Time	Infectivity titer (log $TCID_{50}$/g)	
		gills	kidney
23°C	0 h	4.55	4.3
	6 h	4.3	4.05
	12 h	2.55	3.55
	18 h	–*	3.3
	24 h	–	–
4°C	0 h	4.55	4.3
	1 d	4.55	4.3
	3 d	3.8	4.05
	7 d	–	3.8
	14 d	–	–
–30°C	0 h	4.55	4.55
	1 d	4.55	4.3
	3 d	4.55	4.3
	7 d	4.55	4.3
	14 d	4.8	4.3

* Under the detection limit.

under the detection limit in the gills kept for more than 18 h and in the kidney kept for 24 h. In the samples kept at 4°C, infectivity titers of KHV were stable within 3 days in the gills and for 7 days in kidney, but after then KHV infectivity titers decreased under the detection limit. When the samples were kept at –30°C, KHV infectivity titers in both gill and kidney were stable at least for 14 days (Table 2). The results strongly suggest that temperature control for sample stock is important for culture isolation of KHV. Regarding KHV infectivity in fish rearing water, Shimizu *et al.*[8] reported that KHV infectivity is lost quickly in environmental water compare than that in sterilized water, and which was due to presence of bacteria. Similarly, loss of KHV infectivity in fish tissues could be influenced by growth of bacterial in the samples. Thus, sampled fish should be kept on ice during transportation to laboratory; or if possible we recommend that dissected sample-tissues should be immersed into iced-cold antibiotics solution[9]. Generally, KHV disease occurs in fields at 18°C–28°C of temperatures[1]. Based on our results (Table 2), KHV infectivity in dead fish samples kept without cool-down may be lost within a day. Therefore, we would recommend that symptomatic diseased fish, surviving fish or freshly dead fish with reddish-colored gills should be used for KHV isolation.

In the fresh sample, no significant difference was observed in the infectivity titer of KHV between gill and kidney tissues (Table 2). And the KHV titers in the kidney kept at 23°C for 18 h and at 4°C for 7 days were lower than the initial KHV infectivity titers, but still detectable ($10^{3.3}$ and $10^{3.8}$ $TCID_{50}$/g, respectively), whereas KHV titer in the gills kept at the same condition were under the detectable level (Table 2). These data also suggest that KHV in kidney is more stable compare than that in gill, because gill tissue could be contaminated with bacteria from fish environment as mentioned above. It is therefore considered that kidney could be suitable for culture-isolation of KHV, when only dead fish are available to subject to culture-isolation of KHV in field investigations.

Infectivity titers of KHV in the gills and kidney were very stable under the storage at –30°C for 14 days (Table 2). Thus, we investigated influence of freeze-thaw to KHV infectivity titer in tissue samples. KHV titers in the gills with 0, 1, 3, 5 and 7 cycles of freeze-thaw were $10^{5.8}$, $10^{5.8}$, $10^{5.55}$, $10^{5.8}$ and $10^{5.3}$ $TCID_{50}$/g tissue weight, respectively, suggesting that KHV infectivity in tissue samples could be stable against freeze-thaw cycles. It was reported that frozen tissues are unsuitable for virus isolation[10], and which was not agreed with the present results. This could be due to difference in size of the samples; actually in the present study we used only gill lamellas, which could be frozen and thawed quickly. In our separated experiments, cultured KHV in MEM is quite sensitive to freeze-thaw cycles (data not shown), moreover, KHV frozen at –80°C was more stable compare than that at –20°C[*1]. It is therefore considered that samples for culture-isolation of KHV should be frozen as tissues at –80°C.

In conclusion, KHV is possible to culture-isolate with CCB cells by using gill, kidney and brain of moribund or freshly dead fish; the best tissues are gills and kidney for moribund samples, whereas kidney is recommended if samples are dead and brain may be useful for sampling after the peak of mortality. Sample tissues for culture-isolation of KHV should be maintained at 4°C, –30°C or lower temperature; KHV can be isolated from samples kept at 4°C within 3 days, and also from samples kept at –30°C within at least 14 days. Moreover, KHV infectivity in frozen tissue is stable, and it is not influenced by freeze-thaw cycles of samples. The present protocol would facilitate not only culture-isolation of KHV from fish samples more easily but also studies on changes of KHV pathogenicity on the most recent global epidemic.

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イワナ稚魚期に認められる水腫症の治療および予防対策

石川孝典・生方秀典・間野伸宏
廣瀬一美・中西照幸

イワナ稚魚に水腫を伴う死亡事例が認められた。病魚の鰓を採取し，病理組織学的および走査型電子顕微鏡観察を行ったところ，鰓薄板上皮の増生に伴う癒合がみられたことから，本症例も過去の報告と同様，鰓の病変に起因する水腫症と同一であるものと判断された。この水腫症の対策として，1.0％塩水浴による治療および高濃度アスコルビン酸を添加（10,000 mg/kg 飼料）した市販飼料給餌による予防の有効性について検討を行った。発症確認直後から3日間連続して1.0％塩水浴を実施した結果，明瞭な治療効果が確認された。また注水量を増加させた高濃度アスコルビン酸投与区では，有意な死亡低減効果が認められた。

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コイヘルペスウイルス（KHV）の効果的な分離培養手法

湯浅　啓・佐野元彦・大迫典久

感染症研究に原因病原体の分離は必須である。本研究では，CCB 細胞を用いて KHV を分離培養する際に重要となる条件について検討した。KHV は，症状を示す魚から容易に分離培養できることが分かった。鰓・腎臓・脳の3組織から分離を試みると，分離効率が向上すると考えられた。検体を4°C あるいは−30°C で保存すると，23°C より KHV の生存期間が長いことが確認され，瀕死魚を氷冷して実験室に運ぶ必要性が再確認された。また，分離前の検体を保存する場合は，摘出した組織を−30°C 以下で凍結することが推奨された。

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韓国で1998年に死亡したコイ組織からのコイヘルペスウイルスの検出

N. -S. Lee・S. H. Jung・J. W. Park・J. W. Do

韓国で1998年に発生したコイ大量死亡事例について in situ hybridization によるコイヘルペスウイルス（KHV）の検出を試みた。4地域（湖または貯水池）の9養殖場で採取した KHVD 症状を呈する病魚20尾のパラフィン包埋切片を調べた結果，1養殖場の2尾を除き，それらの鰓や内臓に KHV 陽性細胞が認められた。本結果から，KHV が韓国において1998年に存在したことが明らかとなった。

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Poly(I:C) のヒラメに対する毒性

松井崇憲・呉　明柱・西澤豊彦

ヒラメ（平均 8.2 g）を17°C あるいは13°C で飼育し，800，400，200および 100 μg/fish となるよう Poly(I:C) を筋肉内接種し，その毒性について検討した。17°C 飼育区では，800 μg/fish 接種区で1尾死亡したが，≤400 μg/fish の接種区で死亡は認められなかった。しかし，≥200 μg/fish 接種区の16.7％～33.3％で接種部位周辺の皮膚に赤変および潰瘍が認められた。一方，13°C 飼育区では，100 μg/fish 接種区の33.3％に，また 800 μg/fish 接種区では83.3％に死亡あるいは潰瘍が認められた。以上より，Poly(I:C) は，低温飼育のヒラメでは，比較的高い毒性を示すと考えられた。

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ヒラメの *Streptococcus parauberis* 感染症に対する血清型Ⅰ型およびⅡ型株不活化ワクチンの有効性

森　京子・福田　穣

養殖ヒラメ由来 *S. parauberis* の2種血清型（Ⅰ，Ⅱ型）株それぞれのホルマリン不活化菌体（FKC）ワクチンを試作し，有効性と両血清型間の交差防御効果を検討した。各 FKC を腹腔内接種して3週間後のヒラメに攻撃試験を行った結果，免疫原と同じ株に対して高い予防効果が得られた。しかし，免疫原と異なる血清型に対しては，効果にばらつきが認められ生残魚も高い保菌率を示したことから，十分な交差防御効果は期待できないと思われる。

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