A Primary Study on Oral Vaccination with Liposomes Entrapping Koi Herpesvirus (KHV) Antigens Against KHV Infection in Carp

T. MIYAZAKI[1], S. YASUMOTO[1], Y. KUZUYA[1] and T. YOSHIMURA[2]

[1]Graduate School of Bioresources, Mie University, Tsu, Mie Japan
[2]Graduate School of Engineering, Mie University, Tsu, Mie, Japan

ABSTRACT

"Koi herpesvirus (KHV)" is an emerging piscine herpesvirus that only infects koi carp *Cyprinus carpio koi* and common carp *C. carpio carpio* causing mass mortalities with severe economic losses worldwide since 1998. Therefore, protection of carp with KHV vaccines is urgently needed. We developed an improved liposome-vaccine containing KHV antigens within the liposomal membrane compartment for oral vaccination to carp. Carp immunized by 3 day oral administrations of the liposome-KHV vaccine showed 77% survival against a challenge with $10^{1.3}$ $TCID_{50}$ /100 μL of KHV while unvaccinated control fish showed 10% survival. The relative percent survival (RPS) was 74%. This demonstrated that oral immunization with the liposome-KHV vaccine was efficacious against KHV infection in carp.

Miyazaki, T., Yasumoto, S., Kuzuya, Y. and Yoshimura, T. 2008. A primary study on oral vaccination with liposomes entrapping koi herpes virus (KHV) antigens against KHV infection in carp, pp. 99-184. *In* Bondad-Reantaso, M.G., Mohan, C.V., Crumlish, M. and Subasinghe, R.P. (eds.). Diseases in Asian Aquaculture VI. Fish Health Section, Asian Fisheries Society, Manila, Philippines. 505 pp.

Corresponding author: T. Miyazaki, miyazaki@bio.mie-u.ac.jp

INTRODUCTION

Koi herpesvirus (KHV) is an emergent piscine herpesvirus that only infects koi carp *Cyprinus carpio koi* and common carp *C. carpio carpio* causing mass mortalities with severe economic losses in Japan and other parts of the world such as Israel, the USA, Germany, Netherlands, Taiwan, Indonesia and South Africa since 1998 (Hedrick *et al*., 2000; Gray *et al.,* 2002; Ronen *et al*., 2003; Perelberg *et al*., 2003; Sano *et al*., 2004; Schlotfeldt, 2004; Haenen *et al*., 2004). Protection of carp with KHV vaccines is much needed. In order to prevent KHV infection, a live vaccine using attenuated KHV has been developed for the intramuscular injection (Ronen *et al*., 2003). Vaccination by injection is time-consuming and labor intensive, and subjects the fish to severe stress by handling. Oral vaccination, in which vaccine is mixed with food, is a more practical method because it requires little labor and causes no stress to fish. Our goal was to develop an efficacious oral vaccine to protect carp against KHV infection. In oral vaccination, the uptake of antigens is expected to occur in the intestine. Previous study has shown that fishes took up both crude lipids and proteins in the posterior intestine (Miyazaki and Fujiwara, 1988). The nature of lipid uptake in the intestine is common in juvenile as well as grow-out fishes. This finding suggests that antigen contained in lipid particles can be used not only to deliver antigens to the posterior intestine but also facilitate their uptake. Liposomes are known to be easily taken up into cells (Matyas *et al*., 2003; Kamps and Scherphof, 2004). We have developed improved liposomes that can exist as suspension in water, so that it can be mixed with moist pellet-diets or soaked into dry pellet-diet for field application (Yoshimura *et al*., 2004).

In the present study, we formulated a liposome-vaccine entrapping formalin-killed KHV antigens and examined its ability to stimulate immunity in common carp by oral administration. We evaluated both antibody titers in orally vaccinated fish and protection against experimental infection with KHV.

MATERIALS AND METHODS

KHV and liposome-vaccine preparation

KHV (isolate: NYKK0411) was originally isolated from a diseased koi carp in Japan in 2004 and cultured using KF-1 cell (koi fin cell, given by Dr. Hedrick, University of California, USA) according to Hedrick *et al*. (2000). After fragmentation of all of KHV-infected cells, the viruses were inactivated by adding formalin in 0.3% (v/v) final concentration for 48 h at 24^{o}C. The mixture was centrifuged at 3,000×g, for 15 min and ultrafiltrated (450 nm) to remove cellular debris. After ultra-centrifugation (200.000xg for 60 min), the resulted pellet of KHV was resuspended in phosphate-buffered saline (PBS, pH 7.2), and the washing was repeated three times to remove the formaldehyde. The recovered formalin-killed KHV was suspended in 1 ml PBS. The formalin-killed KHV suspension and live KHV were used for liposome-vaccine preparation and challenge tests in carp, respectively.

Liposomes containing KHV antigens were produced using the procedure described below. A chloroform solution containing phosphatidylserine, phosphatidylcholine and cholesterole at a molar ratio of 1:10:5 (total 16 μmol) was evaporated in a tube to produce phospholipid thin films on the internal surface of the tube). One ml of the formalin-killed KHV suspension (corresponding to a protein concentration of 10 g) was presonicated with a probe type sonicator SONIFIER 250 (Branson) and was added to the tube with lipid films and agitated for 30 sec. The lipid and protein solution was resonicated several times. The resulting semi-transparent solution was centrifuged at 750xg for 5 min at 4°C. The supernatant contained liposomes fused KHV antigens in the liposomal membrane compartment and were about 5-10 m in diameter with a confocal fluorescence microscope (Carl Zeiss) (data not shown). The provided liposomes were used as the liposome-KHV vaccine.

Vaccination, serum antibody assay and challenge tests

All common carp used in the experiment were offspring of the same parent fish. The fish were randomly divided into 7 groups: 4 vaccinated groups and 3 unvaccinated control groups (n=5, 25-30 g body weight). They were held in tanks (25 °C, 30 l) with a water filtrate system. The liposome-KHV vaccine was completely absorbed within dry pellet-diet and given to fish in vaccinated groups for 3 days at a 24-hr interval. Each fish daily received a total of 20 μL of the liposome-KHV vaccine. After third administration of the liposome-KHV vaccine, fish were nursed for the following 21 days with normal dry pellets. Two vaccinated fish jumped out of their tanks and died. The control groups received dry pellets absorbed the same amount of PBS without liposomes for 3 days and normal dry pellets for the following 21 days. To evaluate the serum KHV-antibody titers on day 22, the blood was taken from one group of vaccinated fish (5 fish) and one group of control (5 fish) while fish were anesthetized with 1.5% carbamic acid ethyl esthyl. The sera were processed in a routine way. KHV neutralization assay was performed using a 96-well tissue culture plate method with KF-1 cells and KHV ($10^{1.25}TCID_{50}$/50μL), and neutralization titers of the anti-sera were determined.

To determine the efficacy of the liposome-KHV vaccine, all fish must be challenged at the same dose of live KHV. We established a novel challenge method based on the mode of KHV natural infection which occurred in a pond, i.e., KHV invaded respiratory epithelial cells of gills. This finding has been confirmed by other researchers (Hedrick *et al*., 2000; Gray *et al.,* 2002; Perelberg *et al*., 2003; Sano *et al*., 2004; Schlotfeldt *et al*., 2004; Haenen *et al*., 2004). We inoculated KHV by putting the virus suspension on the gill surface while fish were anesthetized with 1.5% carbamic acid ethyl esthyl. The fish were wrapped with wet papers and kept in the air for 5 min to complete KHV adhesion on gill cells. Fish were then returned to the experimental tanks. To determine the challenge dose of live KHV, we applied this method and obtained data determining success of the artificial infection with KHV. Mortality was correlated to inoculated level of KHV: 100% mortality at $10^{3.3}TCID_{50}$/100μL, 80% at $10^{2.3}TCID_{50}$/100μL and 40% mortality at $10^{1.3}TCID_{50}$/100μL. The moribund fish and fish just after death showed definite changes of gill tissues. We determined the challenge dose at $10^{2.3}TCID_{50}$/100μL of KHV. In the challenge trials, a total

of 13 vaccinated fish and 10 unvaccinated fish were inoculated with KHV by dropping a total of the 100μL KHV suspension on both sides of the gills through the gill cavity with a 1 ml syringe. The fish were returned in water and observed the onset of KHV infection and mortality at 21-22°C for 21days.

Polymerase chain reaction (PCR) assay for KHV

Polymerase chain reaction (PCR) for detection of a KHV DNA fragment was performed according to Gray *et al*. (2002). PCR assay was performed using DNA extracts from tissues of the gill and kidney, which was a representative of visceral organs in all of moribund and survived fish in both vaccinated and unvaccinated groups.

RESULTS

Orally-vaccinated fish showed high levels of the antigen specific antibody ($2^{5.75}\pm2^{5.48}$; mean±SEM), which was significantly ($P<0.01$) higher than that of unvaccinated group ($2^{3.59}\pm2^{2.09}$).

In challenge trials, unvaccinated control groups showed 90 % mortality (4/5, 5/5 fish) within days 7-16 post challenge (Fig. 1). Moribund fish and fish just after death showed dark-body coloration and many swollen cells of gill epithelia. Most fish of the vaccinated groups were weakened showing dark-body coloration but only 23% mortality (0/5, 1/4 and 2/4 fish) occurred within days 7-9 post challenge. The remaining fish fully recovered several days later. The moribund fish displayed many swollen cells in the gill epithelium

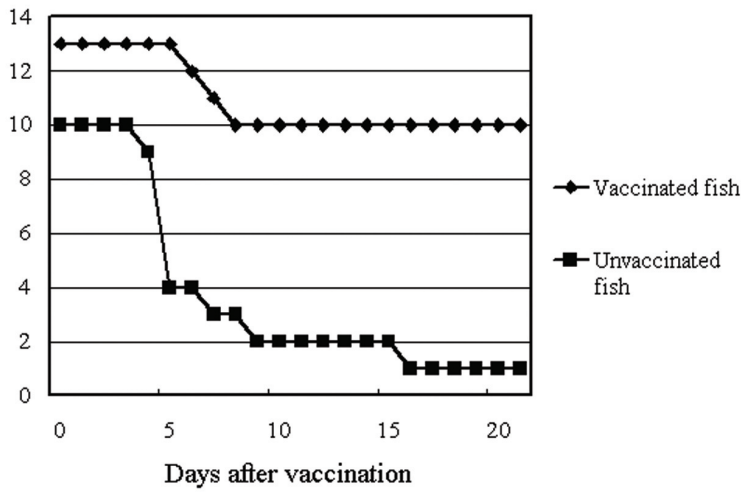

Figure 1. Mortality graph of the vaccinated koi (□ n=13) and unvaccinated carp (■ n=10) after challenge with live KHV at $10^{1.3}$ $TCID_{50}$/100mL on day 22 post-vaccine administration. For the following 21days, vaccinated carp show a 77% survival rate while unvaccinated carp showed a 90% mortality rate. The average relative percent survival was 74%.

while all of survivors showed no abnormal cells in gills. The resulting relative percent survival (RPS) was 74%. In the PCR assay, the putative PCR amplicons were derived from DNA extracts from the gills and kidney of moribund fish in both unvaccinated and vaccinated groups. On the other hand, except for one fish, PCR resulted negative on the gills and kidney of 9 survivors in vaccinated groups (data not shown).

DISCUSSION

In the present study, we developed an improved liposome-vaccine entrapping KHV-antigens, which could exist as suspension in water and be soaked into dry pellet-diet. Oral administration of the liposome-KHV vaccine for 3 days revealed that it could induce high titer of the specific KHV antibody. Challenge trials resulted in high survival of vaccinated fish (RSP 74%), indicating that vaccinated fish were protected against KHV. Based on the PCR results on survivors, all except one, vaccinated fish were protected from KHV infection. Even if they allowed KHV to infect cells of gill epithelia just after inoculation, they recovered from KHV infection. Thus, oral vaccination with the liposome-KHV vaccine indicated the induction of immunity against KHV infection in carp.

CONCLUSIONS

Three conclusions can be drawn from this study: (a) an improved liposome-KHV vaccine was developed, (b) it was effective in oral vaccination and induction of antibody against KHV infection and (c) it will be a breakthrough in mass vaccination for farmed fishes.

REFERENCES

Sommerville, C., Lester, R.B. and Walker, P. 1982. The pathology of *Haplorchis pumilio* (Loos, 1896) infection in cultured tilapias. *J. Fish Dis.* 5: 243-250.

Gray, W., Mullis, L., LaPatra, S.E., Groff, J.M. and Goodwin, A. 2002. Detection of koi herpesvirus DNA in tissues of infected fish. *J. Fish. Dis.* 25**:**171-78

Haenen, O.L.M., Way, K., Bergmann, S.M. and Ariel, E. 2004. The emergence of koi herpesvirus and significance to European aquaculture. *Bull. Euro. Asso. Fish Pathol.* 24: 293-307

Hedrick, R.P., Gilad, O., Yun, S. and Spangenberg, J. V. 2000. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of a common carp. *J. Aquat. Anim. Health* 12: 44-57.

Kamps, J.A. and Scherphof, G.L. 2004. Biodistribution and uptake of liposomes *in vivo*. *Methods in Enzymology* 387: 257-266.

Matyas, G..R., Muderhwa J.M. and Alving C.R. 2003. Oil in water liposomeal emulsions for vaccine delivery. *Methods in Enzymology* 373: 34-50.

Miyazaki, T. and Fujiwara, K. 1988. Histological studies on yolk utilization and digestive function in larvae and juvenile of red sea bream and black sea bream. *Bull. Fac. Bioresour. Mie Univ.* 1:15-27.

Perelberg, A., Smirnov, M., Hutoran, M., Diamant, A., Bejerano Y. and Kotler, M. Epidemiological description of a new viral disease affecting cultured *Cyprinus carpio* in Israel. *Israel J. Aquacul*. 55:5-12.

Ronen, A., Perelberg, A., Abramowitz, J., Hutoran, M., Tinman, S., Bejerano, I., Steinitz, M. and Kotler, M. 2003. Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine* 21:4677-4684.

Sano, M., Ito, T., Kurita, J., Yanai, T., Watanabe, N., Miwa S. and Iida, T. 2004. First detection of koi herpesvirus in cultured common carp *Cyprinus carpio* in Japan. *Fish Pathol.* 39:165-67.

Schlotfeldt, H. F. 2004. Severe losses of common carp in Germany due to Koi Herpesvirus (KHV). *Bull. Euro. Asso. Fish Pathol.* 24**:**216-217.

Yoshimura, T., Takagi, T., Tsumoto, K., Shono, M. and Miyazaki, T. 2004. Development of orally administrated liposome vaccines against bacteria- and virus-infectious disease in cultured fishes, pp. 225-228. *In* Nicole, A.S. (ed.). 12th International Congress of Immunology and 4th Annual Conference of FOCIS. Montreal, Canada.