Efficacy and safety of a modified-live cyprinid herpesvirus 3 vaccine in koi (*Cyprinus carpio koi*) for prevention of koi herpesvirus disease

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Objective—To investigate safety and efficacy of a cyprinid herpesvirus type 3 (CyHV3) modified-live virus vaccine for the prevention of koi herpesvirus disease (KHVd).

Animals—420 healthy koi (*Cyprinus carpio koi*).

Procedures—Fish were vaccinated with a 1X dose or 10X overdose of CyHV3 modified-live virus vaccine or a placebo through bath exposure in tanks at 22°C. Horizontal transmission of vaccine virus was evaluated by commingling unvaccinated and vaccinated fish. Efficacy was evaluated by challenge exposure of vaccinated and naïve fish to a wild-type virus. Fish that died were submitted for quantitative PCR assay for CyHV3 and histologic evaluation.

Results—The CyHV3 vaccine was safe and efficacious, even at a 10X overdose. Vaccine-associated mortality rate was inversely associated with body weight, with a cumulative mortality rate of 9.4% (18/192) in fish weighing ≤ 87 g and no deaths in fish weighing > 87 g (0/48). Horizontal transfer of vaccine virus from vaccinates to naïve fish was negligible. For efficacy, the vaccine provided a significant reduction in mortality rate after challenge exposure to a wild-type virus, with a prevented fraction of 0.83 versus the placebo control fish.

Conclusions and Clinical Relevance—KHVd is highly contagious and commonly leads to deaths in 80% to 100% of exposed fish, representing a major threat to koi and common carp populations throughout the world. The CyHV3 modified-live virus vaccine had a favorable safety profile and was an effective vaccine for the control of KHVd in koi weighing > 87 g. (*Am J Vet Res* 2014;75:899–904)

Koi herpesvirus disease is caused by infection of koi (*Cyprinus carpio koi*) or common carp (*Cyprinus carpio*) with CyHV3.[1] The first reported outbreaks of KHVd were in Europe in 1997[2] and in Israel and the United States in 1998.[3] The viral agent was isolated in 2000[3] and has now been identified in fish of 5 continents.

Cyprinid herpesvirus 3 may infect a fish through several ways, including via the gills, ingestion, or contact with skin, especially when the integument is abraded.[4,5] Most KHVd outbreaks have been reported among captive populations of carp, but there have been mass deaths in wild common carp populations in the United States,[3] Canada,[6] the United Kingdom,[7] Japan,[8] Indonesia,[9] and South Africa.[10] Israel and the United Kingdom (and, unofficially, all affected regions) have acknowledged that the disease is widely distributed within their countries. Current preventative medicine protocols include screening with a quantitative PCR assay[11] and for antibodies against CyHV3 with an ELISA[12] to detect active KHVd or previous exposure, respectively. Limited regulation has resulted in subclinically affected CyHV3-carrier fish being shipped around the world, which has facilitated the spread of KHVd and endemic maintenance of the virus.[13]

The increasing economic value of koi and common carp has prompted efforts to develop an effective vaccine to control KHVd. In an early attempt to reduce the impact of the disease, naïve fish were cohabited with sick fish at water temperatures that permitted viral replication (permissive temperature) and were then transferred to a tank with a nonpermissive water temperature. This reduced the mortality rate from 80% to 100% to approximately 40% to

Abbreviations

Ct	Cycle threshold
CyHV3	Cyprinid herpesvirus 3
KHVd	Koi herpesvirus disease
MEM	Minimum essential medium
PFU	Plaque-forming unit

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60%, which still represented a considerable loss. This procedure also carried the risk that the virus could reemerge and induce disease.[14] Research efforts have focused on the development of an attenuated live-virus vaccine. When attenuated viruses were used previously, the mortality rate after challenge exposure was reduced to < 30%.[14–16] The purpose of the study reported here was to evaluate the safety and efficacy of an attenuated strain of CyHV3 as a vaccine to protect against KHVd in koi.[15,16]

Materials and Methods

Animals—Koi (n = 420) with a mean body weight of 73.4 g were purchased from a closed-system commercial producer[a] with no history of CyHV3 infection. Fish were transferred to the Fish Health Laboratory at the University of California-Davis, maintained for 37 days in three 650-L holding tanks at 21° to 23°C, and fed a commercial koi diet at 1% of body weight/d. Fish (n = 60) were euthanized and screened for CyHV3 DNA or antibodies against CyHV3 by means of a quantitative PCR assay[11] and ELISA,[12] respectively. Parasitic or bacterial infections were assessed by microscopic analysis of fresh mounts of gill biopsy specimens and skin scrapings or microbial culture of a kidney swab specimen on sheep blood agar at 22°C. Fish had no detectable CyHV3 DNA or serum antibodies against CyHV3 and no detectable parasitic or bacterial infections. Fish were euthanized with methane tricaine sulfonate[b] (500 µg/mL) buffered with sodium bicarbonate[c] (500 µg/mL) for the screening and when required throughout the study. This research was conducted in accordance with an animal care and use protocol approved by the University of California-Davis Institutional Animal Care and Use Committee.

Vaccination and challenge exposure—Fish were transferred from holding tanks into 6 vaccination tanks (each vaccination tank contained 130 L of water and 60 fish) and allowed to acclimatize for 10 to 15 minutes. Mean ± SD body weight of fish on the day of vaccination was 78.2 ± 15 g. Tanks were drained until each contained only 30 L of water. The ratio of fish biomass to water did not exceed 20% (wt/vol).

Fish were vaccinated with the CyHV3 vaccine (1X dose or 10X overdose of each of 2 serial lots [B0110-1 and B0110-2]) or a placebo (1X or 10X placebos). Serial lots of attenuated CyHV3 modified-live virus[15,16] vaccine (viral titers, 1.3×10^6 PFUs/mL for B0110-1 and 1.6×10^6 PFUs/mL for B0110-2) and the placebo (medium used to grow uninfected cells) were supplied by the vaccine manufacturer.[d] The vaccine serials were diluted (volumes of 0.22 and 2.2 mL of B0110-1 and 0.17 and 1.7 mL of B0110-2 for the 1X dose and 10X overdose, respectively) into the vaccine baths to achieve 10 PFUs/mL for the 1X dose and 100 PFUs/mL for the 10X overdose. Similar volumes of MEM culture medium (0.19 and 1.9 mL, respectively) were diluted for the 1X and 10X placebos. Vaccination was via bath exposure for 45 to 60 minutes in tanks at a permissive water temperature of 20° to 24°C, which was used throughout the study. Oxygen concentration was maintained at > 6 µg/mL, and pH was maintained between 6.8 and 7.4.

A wild-type CyHV3 challenge exposure virus (KHVp8 F98-50) was maintained as described elsewhere.[3] Culture medium was harvested at 14 days after inoculation (viral concentration, 1.4×10^4 PFUs/mL) and immediately used for challenge exposure of the fish during the efficacy portion of this study.

Safety evaluation—After vaccination, koi from each of the 6 designated vaccination tanks (two 1× and two 10× groups) or 2 sham-vaccinated tanks (1X and 10X MEM) were placed respectively in a 2-row bank of five 130-L tanks/side (4 observation tanks and 1 sample tank; 12 fish/tank), thus providing replicates for each treatment group. Three fish from the sample tanks for each vaccination dose by lot number and for each placebo were euthanized and evaluated on days 5, 14, 21, and 28 after vaccination. All tanks were monitored twice daily for 28 days; dead or moribund fish were removed and samples collected.

Horizontal transmission of vaccine virus—After the 28-day safety phase, 6 koi from each vaccinated group (1X and 10X) were commingled with 10 fin-clipped koi from the 10X placebo group. The 2 groups (16 fish/group) were housed in separate 130-L tanks. A third 130-L tank comprised 16 placebo control fish (6 fish from the 10X placebo group and 10 fin-clipped fish from the 10X placebo group). Fish were observed daily for 4 weeks; dead and moribund fish were removed and samples collected. All surviving fish were subsequently euthanized and samples collected.

Efficacy evaluation—Following the safety phase, fish in the 1X vaccinated group (n = 60; equal numbers for both serial lots), 1X placebo group (48), and 10X placebo group (12) entered the efficacy phase of the study. Fish were allocated into 2 groups (30 vaccinated and 30 placebo-treated [naïve] fish/group). One group was challenge exposed to a wild-type CyHV3 via a bath, whereas the other group was exposed to MEM (culture media devoid of CyHV3; sham challenge-exposure control group). For each group, fish were placed in three 130-L observation tanks (10 fish/tank). Tanks were drained until each contained only 15 L of water. Virus challenge exposure tanks received 38 mL of wild-type CyHV3 containing 35.5 PFUs/mL, and sham challenge-exposure control tanks received 38 mL of MEM. One hour later, water flow (3.8 L/min) was resumed. Tanks were monitored twice daily for 28 days; dead and moribund fish were removed and samples collected.

Observation and sample collection procedures—Throughout the study, fish were monitored twice daily to detect dead or moribund fish. All such fish were assessed for parasites in skin scrapings and gill biopsy specimens. After challenge exposure, dead or moribund fish were removed, euthanized if necessary, and examined for gross and microscopic lesions.[3,17] Swab specimens were aseptically collected from the trunk kidney and processed for aerobic bacterial culture on sheep blood agar at 22°C.

Tissue samples of the gills, skin, liver, kidneys, spleen, and intestines were immersed in neutral-buffered 10% formalin for histologic evaluation; tissues were processed routinely and embedded in paraffin, sectioned at a thickness of 3 µm, and stained with H&E. For the safety

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phase, histologic evaluation included all live-sampled fish and dead fish that underwent necropsy before substantial decomposition. For the efficacy phase, histologic evaluation included 1× vaccinates that died and fish from the wild-type CyHV3 challenge-exposure placebo control group that died. Histologic evaluation was not performed for the horizontal transmission experiment. One pathologist (GDM) performed all histologic evaluations; that investigator did not have knowledge of vaccination history of the koi or the hypotheses being tested. Fish were assessed for condition variables (ie, food in the gastrointestinal tract, glycogen stores in the liver, and mesenteric fat content) and lesions. Scores for histopathologic findings were assigned as follows: 0 = none, 1 = mild or small amounts, 2 = moderate, or 3 = severe or abundant.

Sample preparation and quantitative PCR assay—Pooled samples of the gills, trunk kidney, and spleen from each necropsied fish were stored at –20°C in 70% ethanol.[18] Genomic DNA was extracted by use of a commercially available kit.[e] Concentrations of DNA were normalized to 50 ng/μL for all samples. Quantitative PCR assay was performed with specific primers (KHV-86f/KHV-163r) and a CyHV3-specific hydrolysis probe (KHV-109p)[11] that amplifies a 78-bp fragment (GenBank accession No. AF411803). Reactions (12 μL) were conducted with a 48-well real-time PCR assay system[f] and cycling conditions reported elsewhere.[11] A plasmid carrying a 484-bp insert was prepared from a standard CyHV3 strain (KHV-U) as previously described[18] and cloned into a thymine-adenine cloning vector.[g] Serial dilutions (1:10) of the plasmid DNA were used to generate a standard curve. Samples were assayed with 10^6 to 10^2 copies of plasmid standards to allow for quantification of CyHV3 DNA per microgram of tissue. The quantitative PCR assay Ct was used to group fish on the basis of viral load as follows: not detected, 39.0 to 28.0 (weak positive), 27.9 to 20.0 (strong positive), and < 20.0 (very strong positive). Detection limit for the quantitative PCR assay was 10 copies/μg of DNA.[11] For samples with < 10 CyHV3 copies/μg of DNA, extrapolation based on the standard curve was used. However, the copy number in those samples was not reliable because it was not within the range of the standard curve.

Statistical analysis—Receiver operating characteristic curve analysis was performed to evaluate the validity of body weight to discriminate survival and mortality rates for the safety trial. The Mann-Whitney Wilcoxon test was used to compare continuous data for outcomes between groups. A Fisher exact test was used to compare 2 binomial proportions between groups. Values of $P < 0.05$ were considered significant. A simple linear regression was used to compare the date of death after vaccination and the $\log_{10}$ CyHV3 copy number for dead fish from which samples were collected. To illustrate efficacy, a Kaplan-Meier survival curve was created of the cumulative aggregate mortality rate of koi after challenge exposure to a wild-type CyHV3 and MEM sham challenge exposure for 1× vaccinates and 1× placebo control fish and their associated 95% confidence intervals for the survivor function of the 2 groups.

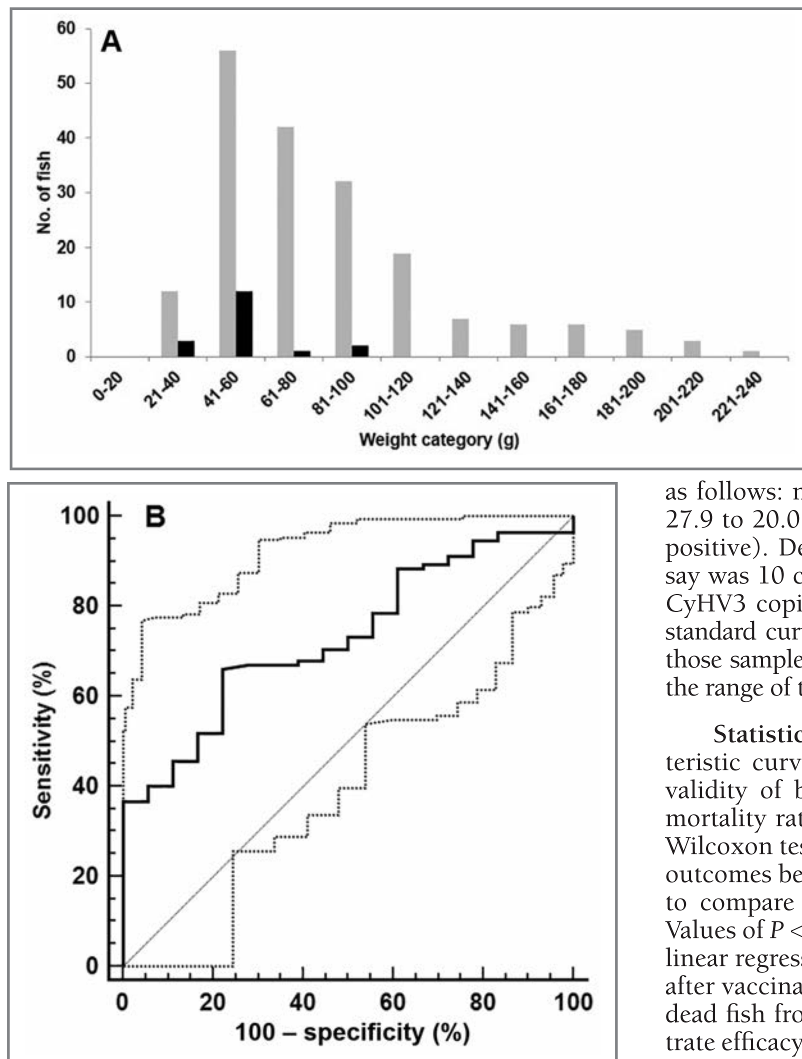

Figure 1—Results for koi (*Cyprinus carpio koi*) after vaccination with a modified-live CyHV3 vaccine or sham vaccination. A—Distribution of body weight of koi at the end of the safety phase in which fish were vaccinated with a modified-live CyHV3 vaccine. Survivors (gray bars) and koi that died (black bars) are separated by weight category. B—Receiver operating characteristic curve (solid black line) and 95% confidence interval (dotted lines) of the relationship between vaccine-associated mortality rate and body weight. Sensitivity represents the true positive rate, and 100 – specificity represents the false-positive percentage. The solid gray diagonal line with a zero intercept indicates no association between mortality rate and body weight; area under the curve for that line is 0.5. Area under the curve that approaches 1.0 (top left corner) is increasingly indicative of an association between mortality rate and body weight. The area under the curve for the receiver operating characteristic curve is 0.732.

Results

Mortality rate—Eighteen fish died following vaccination (ten 1× vaccinates and eight 10× vaccinates); mean time to death was 21.8 days after vaccination. There were no significant differences in postvaccination survival rates between serials and dose rates within

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serials. Among the 15 fish from which tissue samples were collected, the first fish that died (day 13 after vaccination) had the highest viral copy number. For the remaining 14 fish that were tested, all had positive results for CyHV3, and day of death after vaccination correlated significantly ($P < 0.001$; linear regression) with a decrease in copy number over time.

Postvaccination mortality rate was greater among smaller fish. The proportional mortality rate was significantly ($P = 0.01$) greater in fish that weighed < 80 g than in fish that weighed ≥ 80 g (16/112 [14.3%] vs 2/80 [2.5%]; **Figure 1**). Receiver operating characteristic curve analysis indicated a direct relationship between vaccine-associated mortality rate and body weight, with fish that weighed ≤ 87 g having a mortality rate of 9.4% (18/192) and with a subset of those fish weighing > 87 g having a mortality rate of 0% (0/48) .

Mortality rate during the safety phase appeared to be associated more with secondary disease than with CyHV3 copy number. Seven of 15 fish that were evaluated did not die until ≥ 26 days after vaccination, and CyHV3 copy number in all 15 of these fish was < 2,000 copies/µg of DNA.

Viral load—Quantitative PCR assay of samples collected from 48 vaccinates revealed that 21 (44%) had negative results, 24 (50%) had weak positive results, and 3 (6%) had strong positive results. Each of the 24 placebo control fish from which samples were collected had negative results for the quantitative PCR assay. Peak viral load was detected at 13 and 14 days after vaccination in all live and dead fish, respectively (**Table 1**); copy numbers then decreased from 14 to 28 days after vaccination. There was no significant difference in Ct value or total CyHV3 copy number in fish vaccinated with a 1X dose or 10X overdose, but there was a significant ($P < 0.001$) difference in total CyHV3 copy number and Ct value between live and dead fish.

Fish from which samples were collected—For each vaccination group, all 48 fish from which samples were collected were alive and had no gross signs of disease when euthanized on days 5, 14, 21, and 28 after vaccination. Prevalence of *Gyrodactylus* spp was similar in vaccinates and placebo control fish. *Ichthyobodo necator* was detected only in vaccinates (2/48 [4.2%]). Differences in microscopic findings were more distinct between dead and live fish from which samples were collected than between vaccination groups of live fish from which samples were collected (**Table 2**).

Table 1—Mean viral load and number of koi (*Cyprinus carpio koi*) with positive test results (> 10 copies/µg of DNA) on days 5, 14, 21, and 28 after vaccination.

Day after vaccination	Mean ± SD viral load ($\log_{10}$ copies/µg of DNA)	Fish with positive results for viral DNA*
5	1.01 ± 0.79	9/12
14	2.29 ± 2.28	8/12
21	1.43 ± 1.40	7/12
28	0.36 ± 0.82	3/12

*Value reported is number of fish with positive results/number of fish tested.

There were no observable gross or histopathologic differences between live-sampled vaccinates and live-sampled placebo fish. A high copy number of CyHV3 was associated with 6 microscopic findings (increased hematopoietic cells in the kidneys, inflammatory cells in gill arches, gill lamellar hyperplasia or hypertrophy, intestinal bacterial overgrowth, decreased number of immature renal tubules, and splenic vascular congestion). For dead fish from which samples were collected, those with the highest copy number of CyHV3 typically had more tissue autolysis; fewer immature renal tubules; less splenic vascular congestion; and higher scores for branchial monogenean parasites, renal tubular intraluminal necrotic cells, dilated renal tubules, and intestinal bacterial overgrowth. Low hepatocellular glycogen concentration was associated with a poor outcome. All 7 fish with no hepatocellular glycogen died, and 11 of 15 dead fish had less than moderate amounts of hepatocellular glycogen. Among 72 live-sampled fish, only 4 (5.6%) had less than moderate amounts of hepatocellular glycogen. Two fish (one live and the other dead) had branchial necrosis with filamentous bacteria; both had high copy numbers of CyHV3. *Aeromonas hydrophila* was cultured from 7 of 15 dead fish. One fish with a high CyHV3 copy number (2.8×10^6 copies/µg of DNA) had intranuclear inclusions that were distinct enough to diagnose as viral inclusion bodies during routine light microscopy.

Horizontal transmission of vaccine virus—No deaths occurred and signs of disease were not detected in cohabited vaccinated or naïve fish. In 1 tank, one of six 1X vaccinates had marginally positive results for CyHV3 (45.2 copies/µg of DNA), but none of the 10 naïve fish had detectable CyHV3. In the second tank, two of six 10X vaccinates had marginally positive results for CyHV3 (12 and 51 copies/µg of DNA); 3 of the 10 naïve fish also had marginally positive results for CyHV3 (range, 5.8 to 24.7 copies/µg of DNA). In the placebo control tank, 1 of 6 placebo-treated fish (393

Table 2—Mean ± SEM scores for histopathologic findings in tissues obtained during the safety phase of the study from vaccinated koi that died or from all live-sampled fish from which samples were collected.

Histopathologic finding	Live fish (n = 72)	Dead fish (n = 15)
Quality control		
Liver autolysis	0.6 ± 0.06	1.5 ± 0.27
Measures of physiologic condition		
Hepatocellular glycogen	2.6 ± 0.07	0.8 ± 0.22
Relative area of hematopoietic cells in kidneys	1.1 ± 0.03	1.7 ± 0.12
Immature renal tubules	0.9 ± 0.04	0.5 ± 0.13
Lesions and other microscopic findings		
Inflammatory cells in gill arches	0.8 ± 0.06	1.4 ± 0.17
Gill lamellar hyperplasia or hypertrophy	0.1 ± 0.04	0.5 ± 0.22
Hepatocellular cytoplasmic pigment	0 ± 0	0.3 ± 0.15
Hepatocellular single-cell necrosis	0 ± 0.02	0.2 ± 0.14
Cytoplasmic vacuoles in exocrine portion of pancreas	0 ± 0.02	0.4 ± 0.19
Renal tubular intraluminal necrotic cells	0 ± 0.02	0.3 ± 0.15
Renal tubular luminal dilation	0 ± 0.01	0.2 ± 0.14
Renal tubular epithelial protein droplets	0.3 ± 0.07	0.1 ± 0.09
Intestinal bacterial overgrowth	0.2 ± 0.06	0.5 ± 0.24

Scores were assigned as follows: 0 = none, 1 = mild or small amounts, 2 = moderate, or 3 = severe or abundant.

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copies/μg of DNA) and 1 of 10 fin-clipped fish (14,863 copies/μg DNA) had positive results. These 2 samples were also analyzed independently[h]; the placebo-treated fish had 3 of 3 wells with positive results (10,000 copies/μg of DNA/well), whereas the fin-clipped fish had 1 of 3 wells with positive results (33 copies/μg of DNA/well). Copy numbers of CyHV3 were not significantly different between the placebo control tank and the 1X dose (P = 1.00) or 10X overdose (P = 0.39).

Efficacy—After challenge exposure to a wild-type CyHV3, group aggregate mortality rate among 1X vaccinates was 16.7% (5/30; **Figure 2**). Only fish that weighed < 70 g died, and all 5 dead fish had high copy numbers of CyHV3 (mean, 5.3×10^6 copies/μg of DNA). Moribund fish had clinical signs associated with KHVd, which was corroborated by histopathologic findings in five 1X vaccinates that died and 4 fish from the wild-type CyHV3 challenge-exposure placebo control group that died. At 28 days after vaccination, 15 of the remaining 25 (60%) vaccinates had negative results for CyHV3 on quantitative PCR assay, whereas the other 10 (40%) fish had very low viral copy numbers (mean, 19 copies/μg of DNA).

For the 30 placebo-treated fish after challenge exposure, group aggregate mortality rate was 96.7% (29/30; Figure 2). Prior to death, fish had clinical signs of KHVd, which was confirmed histologically in 3 fish submitted for necropsy. The lone surviving placebo-treated fish after challenge exposure had 88 copies/μg of DNA.

After sham challenge exposure, all thirty 1X vaccinates survived and had no clinical or pathological signs consistent with KHVd (Figure 2). All fish tested (n = 10) had negative quantitative PCR assay results (5 had no reaction, and the other 5 had < 10 copies/μg of DNA). Similarly, all 30 placebo-treated fish survived after sham challenge exposure, and none of them had clinical or pathological signs consistent with KHVd. Quantitative PCR results were negative in each of the 10 placebo-treated fish tested.

Comparative mortality rates after wild-type CyHV3 challenge exposure—The aggregate proportional mortality rate was significantly ($P < 0.001$) less for 1X vaccinates versus 1X placebo-treated fish (0.167 vs 0.933). The calculated prevented fraction was 0.83. The prevented fraction for each observation tank, compared with that for each of the 3 placebo control replicates, ranged from 0.56 to 1.0. Proportional mortality rate was significantly ($P < 0.001$) less in 1X vaccinates that weighed ≥ 70 g (0/14) than in placebo control fish that weighed ≥ 70 g (10/10).

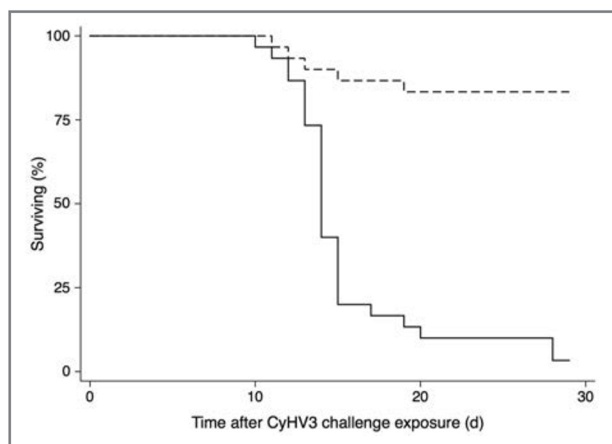

Figure 2—Kaplan-Meier curve of the cumulative aggregate mortality rate of koi after challenge exposure to a wild-type CyHV3 for 1X vaccinates (dashed line) and sham-vaccinated control fish (solid line).

Discussion

The modified-live virus vaccine used in the present study had a favorable safety profile in the population of koi (mean body weight, 73.4 g), even when used at a 10X overdose, and was efficacious for protecting against KHVd following challenge exposure to a wild-type CyHV3. Safety of the vaccine was supported by mortality rate analysis, histologic evaluation, cohabitation experiments, and molecular diagnostic testing.

In the present study, this vaccine conferred a significant reduction in mortality rate for vaccinates after challenge exposure to wild-type CyHV3 (16.7%) when compared with the mortality rate of unvaccinated controls (96.7%). This reduction in the mortality rate attributed to vaccine protection was greater than when compared with mortality rates previously reported for a recombinant vaccine (40% to 80%)[19] and oral administration of CyHV3 antigens encased in liposomes (74%).[20] However, fish in those studies[19,20] were not the same size or strain of koi, nor were they challenge exposed to the same wild-type CyHV3 strain used in the study reported here. Low CyHV3 copy numbers after viral challenge exposure suggested that the wild-type virus was unable to consistently establish infection in vaccinates; most vaccinates had copy numbers below the detection limit. Residual traces of vaccine, rather than the wild-type CyHV3, likely accounted for low amounts of viral DNA in 1X vaccinates after viral or sham challenge exposure, but further analysis would be needed to confirm this because the assay we used detected both wild-type CyHV3 and the vaccine strain.

Histopathologic changes and parasitic infections were associated more closely with CyHV3 status or death than with vaccination status; severe lesions were more common in fish with high viral loads. Fish death and a high copy number were associated with microscopic evidence of kidney inflammation or anemia, which are indicative of cessation of growth or decreased feed intake. Infection with *Aeromonas hydrophila* is common during stress, and this likely was the situation for the fish in the present study. The lack of definitive viral inclusion bodies in most fish in the study is consistent with results of other studies.[3,20] These findings further illustrated the limitations of relying solely on the presence of intranuclear inclusion bodies for diagnosis of KHVd.

For a modified-live virus vaccine to be protective, the modified-live virus must propagate in fish at least briefly after vaccination in conditions that are permissive for wild-type CyHV3 infection.[16] In the present study, propagation was confirmed when CyHV3 DNA increased (propagation) and then peaked at approximately day 13 or 14 after vaccination.

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Horizontal transfer of the vaccine virus was negligible. Detection of CyHV3 in samples collected from 2 placebo control fish might have indicated a false-positive result, which had a very low copy number of CyHV3 (ie, close to the detection limit). Other scenarios might have been a contaminated sample or an accidently misidentified vaccinate. On the basis of pathogenesis of CyHV3, a naïve fish would have had clinical signs of disease and would have transferred virus to other fish in this cohort during the observational period. No prior exposure to CyHV3 was likely in the population, given results of prescreening testing of the fish, history of the farm source, their maintenance in permissive temperatures with no deaths in the control fish, and negative test results for CyHV3 DNA in samples obtained from placebo control fish in the safety phase with a mortality rate of 96.7% after challenge exposure.

Vaccination with a modified-live CyHV3 vaccine resulted in a favorable safety profile and was effective in koi that weighed > 80 g. Vaccine-associated deaths occurred only in fish that weighed ≤ 87 g, even at a 10X overdose. Higher viral copy numbers were detected in fish that died, compared with viral copy numbers for live fish from which samples were collected, and most of the histopathologic changes and parasitic infections were in fish that died. Horizontal transmission of vaccine virus was negligible between vaccinated and placebo-treated fish. Analysis of these data indicated that the modified-live CyHV3 vaccine could be a useful tool in preventing KHVd in koi for management on farms, at retailers, or in hobbyist collections.

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a. Koi Enterprise, West Sacramento, Calif.
b. Western Chemical, Ferndale, Wash.
c. Arm and Hammer, Princeton, NJ.
d. KoVax Ltd, Jerusalem, Israel.
e. DNeasy blood and tissue kit, QIAGEN Inc, Valencia, Calif.
f. StepOne real-time PCR system, Applied Biosystems, Grand Island, NY.
g. Promega, Madison, Wis.
h. Real-time PCR Research and Diagnostics Core Facility, Department of Medicine and Epidemiology, School of Veterinary Medicine, University of California-Davis, Davis, Calif.

References

1. Waltzek TB, Kelley GO, Stone DM, et al. Koi herpesvirus represents a third cyprinid herpesvirus (CyHV-3) in the family *Herpesviridae*. *J Gen Virol* 2005;86:1659–1667.
2. Bretzinger A, Fischer-Scherl T, Oumouma M, et al. Mass mortalities in koi, *Cyprinus carpio*, associated with gill and skin disease. *Bull Eur Assoc Fish Pathol* 1999;19:182–185.
3. Hedrick RP, Gilad O, Yun S, et al. A herpesvirus associated with mass mortality of juvenile and adult koi, a strain of common carp. *J Aquat Anim Health* 2000;12:44–57.
4. Costes B, Raj VS, Michel B, et al. The major portal of entry of koi herpesvirus in *Cyprinus carpio* is the skin. *J Virol* 2009;83:2819–2830.
5. Raj VS, Fournier G, Rakus K, et al. Skin mucus of *Cyprinus carpio* inhibits cyprinid herpesvirus 3 binding to epidermal cells. *Vet Res* 2011;42:92.
6. Garver KA, Al-Hussinee L, Hawley LM, et al. Mass mortality associated with koi herpesvirus in wild common carp in Canada. *J Wildl Dis* 2010;46:1242–1251.
7. Taylor NG, Dixon PF, Jeffery KR, et al. Koi herpesvirus: distribution and prospects for control in England and Wales. *J Fish Dis* 2010;33:221–230.
8. Iida T, Sano M. Koi herpesvirus disease. *Uirusu* 2005;55:145–151.
9. Bondad-Reantaso MG, Sunarto A, Subasinghe RP. Managing the koi herpesvirus disease outbreak in Indonesia and the lessons learned. *Dev Biol (Basel)* 2007;129:21–28.
10. Pokorova D, Vesely T, Piackova V, et al. Current knowledge on koi herpesvirus (KHV): a review. *Vet Med Czech* 2005;50:139–147.
11. Gilad O, Yun S, Zagmutt-Vergara FJ, et al. Concentrations of a koi herpesvirus (KHV) in tissues of experimentally infected *Cyprinus carpio koi* as assessed by real-time TaqMan PCR. *Dis Aquat Organ* 2004;60:179–187.
12. Adkison MA, Gilad O, Hedrick RP. An enzyme linked immunosorbent assay (ELISA) for detection of antibodies to the koi herpesvirus (KHV) in the serum of koi *Cyprinus carpio*. *Fish Pathol* 2005;40:53–62.
13. Pearson H. Carp virus crisis prompts moves to avert global spread. *Nature* 2004;427:577.
14. Ronen A, Perelberg A, Abramowitz J, et al. Efficient vaccine against the virus causing a lethal disease in cultured *Cyprinus carpio*. *Vaccine* 2003;21:4677–4684.
15. Perelberg A, Ilouze M, Kotler M, et al. Antibody response and resistance of *Cyprinus carpio* immunized with cyprinid herpes virus 3 (CyHV-3). *Vaccine* 2008;26:3750–3756.
16. Perelberg A, Ronen A, Hutoran M, et al. Protection of cultured *Cyprinus carpio* against a lethal viral disease by an attenuated virus vaccine. *Vaccine* 2005;23:3396–3403.
17. Pikarsky E, Ronen A, Abramowitz J, et al. Pathogenesis of acute viral disease induced in fish by carp interstitial nephritis and gill necrosis virus. *J Virol* 2004;78:9544–9551.
18. Gilad O, Yun S, Andree KB, et al. Initial characteristics of koi herpesvirus and development of a polymerase chain reaction assay to detect the virus in koi, *Cyprinus carpio koi*. *Dis Aquat Organ* 2002;48:101–108.
19. Costes B, Lieffrig F, Vanderplasschen A. *A recombinant koi herpesvirus (KHV) or Cyprinid herpesvirus 3 (CyHV-3) and a vaccine for the prevention of a disease caused by KHV/CyHV-3 in* Cyprinus carpio carpio *or* Cyprinus carpio koi. European patent EP2031065 (A1). Mar 4, 2009.
20. Miyazaki T, Yasumoto S, Kuzuya Y, et al. A primary study on oral vaccination with liposomes entrapping koi herpes virus (KHV) antigens against KHV infection in carp. In: Bondad-Reantaso MG, Mohan C, Crumlish M, et al, eds. *Diseases in Asian aquaculture VI*. Manila, Philippines: Asian Fisheries Society, 2008;99–184.

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