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Surveillance of Emerging Fish Viral Pathogens in Some Southeast Asian Countries

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Key words: koi herpesvirus (KHV), spring viremia of carp virus (SVCV), grass carp reovirus (GCRV), surveillance, Cambodia, Lao PDR, Myanmar, Philippines, Vietnam

Abstract

Preventing the transboundary movement of fish viral pathogens in a global environment requires active surveillance. This study examined the presence of three emerging viral pathogens among koi, common, grass, and silver carp in Cambodia, Lao PDR, Myanmar, Philippines, and Vietnam. The studied viruses included koi herpesvirus (KHV), spring viremia of carp virus (SVCV), and grass carp reovirus (GCRV). Detection methods consisted of virus isolation by cell culture, infection assay in naive fish, polymerase chain reaction (PCR), and reverse-transcriptase PCR (RT-PCR). Tissues were collected and pooled from 193 fish samples in Dec. 2004 to Feb. 2005, 406 in Sep. 2005 to Feb. 2006, and 1302 in Oct. 2006 to Feb. 2007. For cell culture, tissue filtrates were prepared from pooled spleens, kidneys, livers, and gills and inoculated onto koi fin (KF-1), grass carp kidney (GCK), and fat head minnow (FHM) cells. For infection assay, tissue filtrates were injected intraperitoneally to healthy, naive common carp. No virus was detected after three cell culture passages and the infection bioassays. One-step and nested-step PCR was used to detect KHV in gills of fish samples. One-step and semi-nested RT-PCR was used to detect SVCV and GCRV in the spleens, kidneys, and livers of fish samples. Samples from all three years from all five countries yielded negative results for all three viruses, indicating that KHV, SVCV, and GCRV were not present in these five countries during the period of the study although KHV outbreaks had been detected in Indonesia, Taiwan, Japan, Thailand, China, and Malaysia.

Introduction

Fish culture in Southeast Asia has developed rapidly in the past two decades. With success stories of fish culture ventures, came the inevitable introduction of non-indigenous fish species that were potential sources of viral pathogens. A case in point is the rash of outbreaks of koi herpesvirus (KHV) in Israel, Indonesia, China, Taiwan, and Japan that significantly impacted aquaculture of koi and common carps (Sano et al., 2004; Tu et al., 2004; Sunarto et al., 2005; Ilouze et al., 2006). Workshops focusing on this pathogen were conducted in the UK, Japan (Matsusato

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et al., 2005; Miyazaki, 2005), Denmark (Haenen and Hedrick, 2006), Italy in Sept 2007, and Israel (Kotler and Bergman, 2008). The KHV outbreaks reported in the USA, Israel, UK, and Europe were among the earliest cases (Hedrick et al., 2000; Perelberg et al., 2003).

Other viral pathogens in Asia are the grass carp reovirus (GCRV) and the spring viremia of carp virus (SVCV). GCRV was endemic to China as early as 1953 (Nie and Pan, 1985) and to Vietnam before this study was initiated (T.H. Tran, pers. comm). SVCV is an important viral infection in Europe and a potential pathogen in Southeast Asia (Fijan, 1999).

The pattern of viral disease outbreaks and the fact that koi carp had been actively traded worldwide indicate the transboundary movement of the viruses. The imminent danger posed by these pathogens can affect vulnerable cultured carps in Asia. Prevention of transboundary movement of the pathogens should be a high priority. Surveillance of the viruses is necessary for early detection and prompt warning of concerned Asian countries, particularly in developing countries where diagnostic capabilities are either nil or just beginning. The objectives of this study were to survey the presence and distribution of KHV, GCRV, and SVCV in Cambodia, Lao PDR, Myanmar, Philippines, and Vietnam and to prevent the transboundary movement of these viruses in Southeast Asia by building fish virology capacities in those countries in which they are lacking.

Materials and Methods

Sampling. The presence of KHV, GCRV, and SVCV were monitored through *in situ* samplings of pond-cultured, cage-cultured, and wild koi, common carp (*Cyprinus carpio*), silver carp (*Hypophthalmichthys molitrix*), and grass carp (*Ctenopharyngodon idellus*). Fish samples were collected in Cambodia, Lao PDR, Myanmar, Philippines, and Vietnam during Dec. 2004-Feb. 2005, Sep. 2005-Feb. 2006, and Oct. 2006-Feb. 2007 following the guidelines of Cameron (2002; Table 1).

Cell lines. Cells of koi fin (KF-1), grass carp kidney (GCK), and fat head minnow (FHM) were obtained from Drs. R. Hedrick (USA), Y.L. Jiang (PR China), and A. Goodwin (USA), respectively. The cells were used for the detection and isolation of the target viral pathogens. The cells were propagated and maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) and an antibiotic (penicillin-streptomycin) at the Fish Health Laboratory of the Aquaculture Department of the Southeast Asian Fisheries Development Center (SEAFDEC AQD).

Virus isolates/isolations. Sections of gills, kidneys, spleens, and livers were pooled from 1-10 fish samples, diluted to 10% with MEM supplemented with 10 x penicillin-streptomycin, and incubated overnight at 4°C. The organs were then homogenized, centrifuged at 2500 rpm at 4°C, and filtered in a 0.45 µm filter. Aliquots of each filtrate were kept in a -80°C ultralow freezer until viral assay. Isolates of KHV, GCRV, and SVCV were requested from Drs. R. Hedrick/M. Sano, Y.L. Jiang, and A. Goodwin, respectively, as positive controls, and stored in the -80°C ultralow freezer. Tissue filtrates from common carp and koi carp were assayed for KHV, from grass carp for GCRV, and from all carp samples for SVCV. Virus detection assays for KHV, GCRV, and SVCV were conducted by inoculating tissue filtrates onto monolayers of KF-1, GCK, and FHM cells, respectively, in 24-well plates. Inoculated cells were incubated at 21°C for KHV, 15°C for SVCV, and at 28°C for GCRV. The cells were monitored daily for development of cytopathic effects (CPE) until three weeks after inoculation. Blind passages were conducted at least twice for each sample.

Experimental fish. Specific virus-free, naive common carp were obtained from the hatchery of the Bureau of Fisheries and Aquatic Resources (BFAR) in Tanay, Rizal, and transported to SEAFDEC, Iloilo. Experimental fish were acclimated for one week at 20-23°C before initiating pathogenicity experiments. Injected fish were stocked in 5-l glass aquaria containing UV sterilized fresh water with aeration and kept at 20-24°C throughout the duration of the experiment.

Table 1. Samples of common, koi, silver, and grass carp, collected in five Asian countries from 2004 to 2007 (total = 1901).

Country	*Carp*	*Dec. 2004-Feb. 2005*	*Sep. 2005-Feb. 2006*	*Oct. 2006-Feb. 2007*
Cambodia	Common	4	16	36
	Koi	7	0	0
	Grass	1	12	41
	Silver	3	27	61
Subtotal		15	55	138
Lao PDR	Common	11	21	145
	Koi	0	0	0
	Grass	8	6	48
	Silver	0	3	0
Subtotal		19	30	193
Myanmar	Common	6	15	95
	Koi	8	0	33
	Grass	30	13	10
	Silver	4	12	0
Subtotal		48	40	138
Philippines	Common	10	0	0
	Koi	69	179	462
	Grass	0	0	0
	Silver	0	0	0
Subtotal		79	179	462
Vietnam	Common	18	39	81
	Koi	5	45	176
	Grass	9	5	55
	Silver	0	13	59
Subtotal		32	102	371
Total		193	406	1302

Infection/bioassays. Naive carp were injected intraperitoneally with tissue filtrates and monitored twice daily for three weeks for development of pathological signs. Inoculated fish that showed signs of morbidity or mortality were examined by polymerase chain reaction (PCR) and reverse-transcriptase PCR (RT-PCR) to determine the presence of the three target viruses.

DNA extraction. DNA was extracted from gill tissues preserved in 95% ethanol using the protocol of DNAzol (Molecular Research Center, OH, USA). Briefly, approximately 50 mg of gill tissue was blotted in tissue paper, washed with TE buffer to remove excess alcohol, homogenized in 500 µl DNAzol, and centrifuged for 10 min at 14,800 x *g* at 4°C. The supernatant was transferred to a new tube. DNA was precipitated by the addition of 500 µl 100% ethanol. Pelleted DNA

was washed twice with 95% ethanol by centrifugation and air-dried for a few seconds. The dried DNA pellets were suspended in 200 µl of 8 mM NaOH and incubated at 45°C for 15 min, after which 20 µl of TE buffer was added. The extracted DNA was diluted 100-fold in TE buffer and kept at -20°C until use.

RNA extraction. Total RNA was extracted from 0.1 g tissue samples of livers, kidneys, or spleens and from 100-1000 µl of the control viral supernatant, depending on the quantity of the RNA pellet produced. Extraction was done using standard procedures. The sample was homogenized in 1 ml of Trizol Reagent™ and inverted 10 times before being centrifuged at 12,000 rpm for 10 min at 4°C. The clear supernatant was mixed with 0.2 ml chloroform and shaken vigorously by hand for 15 s. The tube was centrifuged at 2000 rpm for 10 min at 4°C to separate the phases. Approximately 0.6 ml of the clear aqueous phase was transferred to a separate tube to which 0.5 ml isopropanol was added, mixed by inversion 10 times, and incubated for 10 min at 15-30°C to precipitate RNA. The precipitate was collected by centrifugation at 12,000 rpm for 10 min at 4°C. The supernatant was discarded and the pellet was washed with 75% ethanol with vortexing. The tube was centrifuged again at 7500 rpm and the supernatant discarded. The tube was finally spun and the remaining ethanol was pipetted. The pellet was dried for 5 min, dissolved in 40 µl of double distilled water by passing the solution through a pipette tip a few times, and incubated 10 min at 55-60°C. Extracted RNA was either used immediately or stored at -80°C until needed.

PCR for detection of KHV. The virus was detected by PCR using the IQ2000™ KHV Detection and Prevention System Kit (Farming IntelliGene Tech. Corp., Taiwan). Briefly, 2 µl of the diluted DNA extract was added to the prepared reaction mixtures containing 7.5 µl of First PCR PreMix and 0.5 µl of IQzyme DNA Polymerase. PCR amplification was performed in a programmable thermal cycler (Eppendorf Mastercycler, Germany) with the following parameters: 15 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, extension at 72°C for 30 s, and ending with a final extension at 72°C for 30 s and 20°C for 30 s. The first PCR product was then added to 14 µl of Nested PCR PreMix and 1 µl of IQzyme DNA Polymerase, followed by nested PCR amplification with the following parameters: 30 cycles of denaturation at 94°C for 20 s, annealing at 60°C for 20 s, extension at 72°C for 30 s, and ending with a final extension at 72°C for 30 s and 20°C for 30 s. Amplified products were separated in 2% agarose gels, stained with ethidium bromide, and visualized using a gel documentation system (Syngene, GeneGenius, denature dsRNA and UK). Amplified KHV products were evaluated as: 229 bp for samples with light infection and 229 and 440 bp for samples with severe infection.

Reverse transcription. For SVCV detection, cDNA synthesis was carried out in a 20-µl reaction volume consisting of 1X MMLV reverse transcription reaction buffer (50 mM Tris-HCl pH 8.3, 75 mM KCl, 10 mM DTT, 3mM $MgCl_2$) containing 1 mM dNTP, 100 pmol SVCV R2 primer, 20 units MMLV reverse transcriptase (Promega), and 1/10 total RNA extract. Reverse transcription was performed at 37°C for 1 h. For GCRV detection, the RNA extract was incubated in boiling water for 10 min to denature dsRNA and then cooled on ice. Five µl RNA was combined with 1 µM of each primer. A reverse transcription mix consisting of 50 mM Tris-HCl, pH 8.3 at 42°C, 10 mM $MgCl_2$, 50 mM KCl, 10 mM DTT, 1 mM dNTP, and 8 units MMLV reverse transcriptase (Promega) was added. Reverse transcription was performed in a final volume of 20 µl at 42ºC for 45 min.

Semi-nested PCR for detection of SVCV. Following reverse transcription, semi-nested PCR tests for SVCV were done using OIE protocol for SVCV (OIE, 2003). A 714-bp fragment of SVCV cDNA was amplified using primers derived from sequences of the region coding for the glycoprotein genes: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-ACN*-CAY*-3' (SVCV R2), using a modified method of Stone et al. (2003). To avoid non-generation of bands in a single round of amplification, a semi-nested assay amplifying the 606 bp product was performed using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3'

(SVC R4), with the product of the first reaction as the template. Degenerate bases (in asterisks) were included in the primers to detect different SVCV strains. First-step PCR was carried out in a 50-µl reaction volume consisting of 1X Go Taq Flexi buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 50 pmol each of SVCV R2 and SVCV F1 primers, 1.25 units of Go Taq Flexi DNA polymerase (Promega), and 2.5 µl RT reaction mix. The reaction was subjected to 35 cycles of denaturation (1 min at 95°C), annealing (1 min at 55°C), extension (1 min at 72°C), and final extension (10 min at 72°C). Amplified DNA (714 bp) was analyzed by agarose gel (2%) electrophoresis and bands visualized with a gel documentation system. The semi-nested step was set up in a 50-µl reaction volume containing 1X Go Taq Flexi buffer, 2.5 mM $MgCl_2$, 200 µM dNTP, 50 pmol each of SVCV R4 and SVCV F1 primers, 1.25 units of Go Taq Flexi DNA Polymerase (Promega), and 2.5 µl of the product of the first round. Amplification conditions and gel analysis of the amplified fragment (606 bp) were as in the first-step PCR.

Single-step PCR for GCRV. Following reverse transcription, single-step PCR was performed using the method of Li et al. (1997) to detect GCRV. The 320-bp product was amplified using primers designed from sequences of the GCRV capsid polypeptide gene (Wang et al., 1993): Reverse 5'AGT-TCT-CAA-AGC-TGA-GAC-GG 3' and Forward 5' ACG-TGC-GAT-TGG-AAG-AGC-TT 3'. Five µl of cDNA product was added to 45 µl of the PCR mixture: 1X Go Taq Flexi buffer, 2.0 mM $MgCl_2$, 200 µM dNTP, 0.25 µM primers, and 2 units Go Taq Flexi DNA polymerase (Promega). Amplification was conducted as follows: predenaturation for 3 min at 94ºC, 30 cycles of denaturation (30 s at 94ºC), annealing (30 s at 58ºC), elongation (60 s at 72ºC), and final extension (5 min at 72ºC). Gel electrophoresis and analysis were as with SVCV.

Detection of parasites and systemic bacterial infection. Samples of fish gills were examined for the presence of parasites using a compound microscope. At the same time, bacterial isolations from the kidneys of representative fish samples using trypticase soy agar (TSA) were conducted to detect the presence of systemic bacterial infection.

Passive surveillance. Passive surveillance was conducted through *in situ* interviews with the staffs of fisheries in the five target countries during sample collection. Data were collated and summarized.

Results

Data are summarized in Table 1. In Cambodia, sampling sites included ponds near Phnom Pehn, Takeo, and Kandal Provinces where most of the carp aquaculture was located. In Lao PDR, samples were collected from Nam Ngum reservoir, Kham Bao, Namsong Lake, Vientiane, Luang Prabang, and Pakse. In Myanmar, collection sites included carps ponds in Hlawga, Mandalay, and Twanthay. In the Philippines, most fish samples consisted of koi from Laguna, Pampanga, Cebu, and Davao and common carp from Baguio. In Vietnam, carp samples were collected from Nam Dinh, Hanoi, Haiphong, Bac Giang, Ho Chi Minh, Bin Hoa, and Long Xuyen. To increase the likelihood that viruses, if present, would be detected, fish were sampled during the months of September to February when water temperatures are generally cooler and viral replication should be optimum. Overall, 1901 carp samples were examined and tested.

During the three-year surveillance of the three target viruses, none of the tissue filtrates from any of the fish samples caused CPE on inoculated cell cultures after three passages. Likewise, the infection bioassay tests detected none of the pathogenic viruses and PCR or RT-PCR were negative for all three viruses. Further, no significant parasitic infestations of the gills or systemic bacterial infections were observed.

Interviews with fish health staff and fish farmers revealed that clinical signs attributed to the three target viruses were not observed during the three-year period of this study. However, GCRV was detected in Northern Vietnam, a few years before this study was conducted (T.C. Tran, pers. commun.).

Discussion

KHV, SVCV, and GCRV were not detected among the carp samples collected from Vietnam, Myanmar, Lao PDR, Cambodia, and Philippines from 2004 to 2007. As of the date of this study, KHV and SVCV have not been reported in these countries although GCRV outbreaks had been reported a few years earlier in Vietnam. In 2006, koi carp purchased in a fish trade show in PR China were intercepted at the international airport in Manila, Philippines. The imported koi were positive for KHV by PCR (Kurita et al., 2009). Since the earlier outbreaks of KHV in Indonesia, Japan, and Taiwan, KHV epizootics were reported in Thailand and Singapore among koi carp (Tandavanitj et al., 2005; S. Kueh, pers. comm.). Occurrences of KHV in Hongkong, PR China, and Malaysia were reported but not associated with heavy mortality (Musa et al., 2005).

During Dec. 2004-Mar. 2007, no major outbreaks of KHV, SVCV, or GCRV were observed by fish farmers. Hence, our samples were collected from apparently normal fish which probably explains why the results of viral cell culture isolations and other tests were negative.

By and large, surveillance of KHV, SVCV, and GCRV in Cambodia, Lao PDR, Myanmar, Philippines, and Vietnam from 2004 to 2007 indicates that these countries are free of these three emerging viruses. In view of recent reports of KHV in neighboring Asian countries such as Thailand, PR China, Taiwan, Malaysia, and Indonesia, it is very fortunate that transboundary transmission of the diseases to the five target countries did not occur.

Our sampling experiences prepared us to respond to any emergency disease event that may occur in SEAFDEC member countries. However, there is need for intensive capacity building of fish health diagnosis services in Cambodia and Lao PDR. For Myanmar, Philippines, and Vietnam, cell culture capability needs to be expanded through training and complementary facilities need building-up to ensure vigilance of these emerging fish viral pathogens and sustainability of aquaculture in Asia.

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References

Cameron A., 2002. *Survey Toolbox for Aquatic Animal Diseases. A Practical Manual and Software Package*. ACIAR Monograph No. 94. 375 pp.

Fijan N., 1999. Spring viremia of carp and other viral diseases and agents of warm-water fish. pp. 177-244. In: P.T.K. Woo, D.W. Bruno (eds.). *Fish Diseases and Disorders, Vol. 3. Viral, Bacterial and Fungal Infections*. CAB International, Wallingford, UK.

Haenen O. and R. Hedrick, 2006. Koi herpesvirus workshop. *Bull. Eur. Ass. Fish Pathol.,* 26:26-37.

Hedrick R.P., Gilad O., Yun S., Spangenberg J.V., Marty G.D., Nordhausen R.W., Kebus M.J., Bercovier H. and A. Eldar, 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.

Ilouze M., Dishon A. and M. Kotler, 2006. Characterization of a novel virus causing a lethal disease in carp and koi. *Microbiol. Mol. Biol. Rev.,* 70:147-156.

Kotler M. and S. Bergman, 2008. *Book of Abstracts - International Workshop on CyHV-3 (KHV)*. Israel, 17-18 Feb.

Kurita J., Yuasa K., Ito T., Sano M., Hedrick R.P., Engelsma M.Y., Haenen O.L.M., Sunarto

A., Kholidin E.B., Chou H.-Y., Tung M.-C., de la Pena L., Lio-Po G., Tu C., Way K. and T. Iida, 2009. Molecular epidemiology of koi herpesvirus. *Fish Path.*, 44(2):59-66.

Li J., Wang T.H., Yi Y.L., Liu H.Q. and H.X. Chen, 1997. A detection method for grass carp hemorrhagic virus (GCHV) based on a reverse transcription-polymerase chain reaction. *Dis. Aquat. Org.,* 29:7-29.

Matsusato T., Hara I., Inouye K., Iida H., Hayase S. and K. Nakajima, 2005. Proceedings of international symposium on koi herpesvirus disease - strategy for koi herpesvirus disease control. *Bull. Fish Res. Agency Suppl.*, 2:1-86.

Miyazaki T., 2005. Symposium the forefront of studies on koi herpesvirus disease. *Jpn. Soc. Fish Pathol.,* 40:201-207 (in Japanese with English abstract).

Musa N., Leong L.K. and A Sunarto, 2005. Koi herpesvirus (KHV) - an emerging pathogen in koi. pp. 146-147. In: *Proc. Colloquium on Viruses of Veterinary & Public Health Importance*. 11-12 January.

Nie D.A. and J.P. Pan, 1985. Diseases of grass carp (*Ctenopharyngodon idellus* Valencienes, 1844) in China, a review from 1953 to 1983. *Fish Pathol.,* 20:323-330.

OIE, 2003. Spring viremia of carp. Chapter 2.1.4. In: *Manual of Diagnostic Tests for Aquatic Animals*.

Perelberg A., Smirnov M., Hutoran M., Diamant A., Bejerano Y. and M. Kotler, 2003. Epidemiological description of a new viral disease afflicting cultured *Cyprinus carpio.* in Israel. *Isr. J. Aquac. - Bamidgeh*, 55:5-12.

Sano M., Ito T., Kurita J., Yanai T., Watanabe N., Miwa S. and T. Iida, 2004. First detection of koi herpesvirus in cultured common carp *Cyprinus carpio* in Japan. *Fish Pathol.,* 39:165-167.

Stone D.M., Ahne W., Denham K.L., Dixon P.F., Liu C.T-Y., Sheppard A.M., Taylor G.R. and K. Way, 2003. Nucleotide sequence analysis of the glycoprotein gene of putative and pike fry rhabdovirus isolates reveals four genogroups. *Dis. Aquat. Org*., 53:203-210.

Sunarto A., Taukhid, Rukyani A., Koesharyani I., Supriyadi H., Gardenia L., Huminto L., Agungpriyoni D.R., Pasaribu F.H., Widodo, Herdikiawan D., Rukmono D. and B Prajitno, 2005. Field investigations on a serious disease outbreak among koi and common carp (*Cyprinus carpio*) in Indonesia. pp. 125-135. In: P. Walker, R. Lester, M. Bondad-Reantaso (eds.). *Diseases in Asian Aquaculture V*. Fish Health Section, Asian Fish. Soc., Manila.

Tandavanitj S., Kanchanakhan S., Polchana J., Puttinaowarat S., Somsiri T., Somjetlertcharoen A., Laoprasert T., Kessuwan K., Wannaprapha M., Panyawachira V. and S. Chinabut, 2005. First occurrence of koi herpes virus disease (KHVD) in Thailand. p. 78. *Abstracts of Diseases of Asian Aquaculture V.* Sri Lanka, 25-28 October.

Tu C., Weng M.C., Shiau J.R. and S.Y. Lin, 2004. Detection of koi herpesvirus in koi *Cyprinus carpio* in Taiwan. *Fish Pathol.,* 39:109-110.

Wang T., Chen H., Liu H. and W. Guo, 1993. Purification of hemorrhagic virus of grass carp and cDNA synthesis, cloning, and sequencing of its genome. pp. 153-156. In: *Annual Report of FEBL*. Int. Acad. Publ., Beijing.