

## **Field Investigations on a Serious Disease Outbreak among Koi and Common Carp (*Cyprinus carpio*) in Indonesia**

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### **ABSTRACT**

Since March 2002, episodes of mass mortality have occurred in cultured *Cyprinus carpio* (koi and common carp) from Java Island, Indonesia. The disease outbreaks caused significant economic losses and social impacts. For instance, in Blitar regency, East Java, koi carp from 5,000 fish farmers suffered economic losses totaling more than Rp. 5 billions (US\$ 0.5 millions) within a 3 month period. To prevent the spread of the outbreak to other island, the government of Indonesia has closed Java Islands from any movement of koi and common carp. This paper described the clinical history, gross signs, histopathology, PCR detection and experimental infection of the disease. The outbreak occurred after heavy rain, movement of adult fish to other ponds or transport of fry to other areas. This phenomenon leads to the hypothesis that a virus was latent and became active under these particular circumstances, such as stress of transportation and handling and environmental changes, particularly water temperature fluctuations. Another possibility is that the disease resulted from trans-boundary movement of infected koi carp from Hongkong. The disease occurred in on-growing fish of all ages and in all culture systems including static, flow through and cage culture. A variety

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of symptoms have been reported from infected fish. However, the only consistent clinical sign of the disease is gill necrosis. Based on the clinical history, gross signs and histopathological changes, experimental infection and polymerase chain reaction (PCR) detection of naturally and experimentally diseased fish, it is strongly suspected that koi herpesvirus (KHV) is involved in the serious outbreaks among koi and common carp in Indonesia. This is the first KHV outbreak reported in Asian region.

## INTRODUCTION

Common carp (*Cyprinus carpio*) is the main freshwater fish cultured in Indonesia. Annual production of cultured common carp is 75,322 metric tonnes (Anon, 2002). Fifty percent of this annual production is contributed by West Java through its intensive running water and floating netcages culture systems. However, since April 2002, disease outbreaks that caused massive mortality in cultured common carp have occurred in West Java. This first major outbreak occurred at the end of April, 2002 in cultured common carp in Subang regency, West Java. Economic losses included more than 450 metric tons of common carp cultured in running water systems. Within 2 weeks, the outbreak had spread to the regency of West Java and the western part of Central Java. Interestingly, a month before the disease outbreaks in common carp in West Java, there was massive mortality in koi carp in Blitar regency, East Java, the central region for koi carp production in Indonesia. The head of the local Association of Ornamental Fish Culture estimated that the outbreak destroyed high quality koi carp belong to 5,000 fish farmers with economic losses more than Rp. 5 billions (US\$ 0.5 millions) within a 3 month period.

In response to the outbreak, a national task force to control the disease has been developed that included scientists from Bogor Agricultural University, Central Research Institute for Aquaculture, staffs of the Directorate General of Aquaculture and the provincial fisheries services. In cooperation with fish farmers and local fisheries services, the task force have been investigating the recent disease outbreak in koi and common carp in Java islands, Indonesia. In this paper, we present the characteristics of the disease outbreak and the results of parasitological, microbiological and virological investigations into the causes of the epizootic.

## MATERIALS AND METHODS

### Diseased fish in natural outbreak

Koi and common carp were collected from koi farms and common carp farms (running water and floating net cage culture systems) located in East, Central and West Java. We also received samples send by fish farmers and fisheries officers. In order to examine the nature of the disease outbreak, general signs of diseased fish, clinical history of each case and general inspection of diseased fish were recorded.

### Gross signs and parasitological examination

In order to examine the general signs of the disease, the entire body surface including tail, fin and gills were examined. Wet mount of the gills and skin scraping were directly examined under the light microscope for the presence of ectoparasites. After external examination,

the body surface of the fish was disinfected with 70% ethanol. Standard necropsy procedures were applied and pathological-anatomical (PA) changes of internal organs namely gills, liver, spleen, intestine, gall bladder, kidney, heart and brain were recorded.

### **Microbiological studies**

Fungi were isolated from skin ulcers using Glucose Peptone Oxolinic Acid (GPOC) agar plate. Skin ulcers, gills filaments, liver, spleen and kidney were used for the bacterial isolations. The tissues were streaked onto Tryptic Soy Agar (TSA, Difco) and incubated at 27°C for 24-48 hours. The colonies were then identified based on morphological, physiological and biochemical properties according to standard criteria.

### **Histopathological studies**

For histological examinations, tissue samples of skin ulcer, gill, liver, spleen, kidney, intestine and heart were fixed in 10 % Neutral Buffered Formalin (NBF) and transferred to the Pathology Lab., Faculty of Veterinary Medicine, Bogor Agricultural University. Upon arrival, the fixed tissues were processed according to routine histological techniques, sectioned at 3-4 µm in thickness and stained with hematoxylin and eosin (H & E)

### **Experimental infection**

Healthy common carp collected from Cijeruk Research Station (CRS) where the disease outbreak was not recorded, were used for an experimental infection study. The 90 fishes were acclimatized for 2 weeks and distributed into 9 equal groups of 10 fishes. The first three groups were designated for the co-habitation test, in which 5 diseased fish of common carp originated from Cirata Reservoir were added to each of the three groups. The diseased fish of common carp (*Cyprinus carpio*) from Cirata Reservoir were tagged. The second three groups of carp from CRS were injected intraperitoneally with 200 µl of a filtered homogenate made from diseased fish. Control groups were injected intraperitoneally with 200 µl of filtrate homogenate of healthy fish. The filtrates were prepared from pooled gills, spleen and kidney. The tissues were homogenized with cold sterile mortar, diluted to 1:10 with Hank's balanced salt solution (HBSS) and centrifuged at 3,000 rpm for 10 min. The supernatant was filtered through a 0.45 µm membrane filter (Millipore). The fish were held in aquaria with aeration. Dead and moribund fish were removed from the aquaria and their tissues were preserved in 10% NBF and 95% ethanol for histopathology and PCR detection of KHV, respectively.

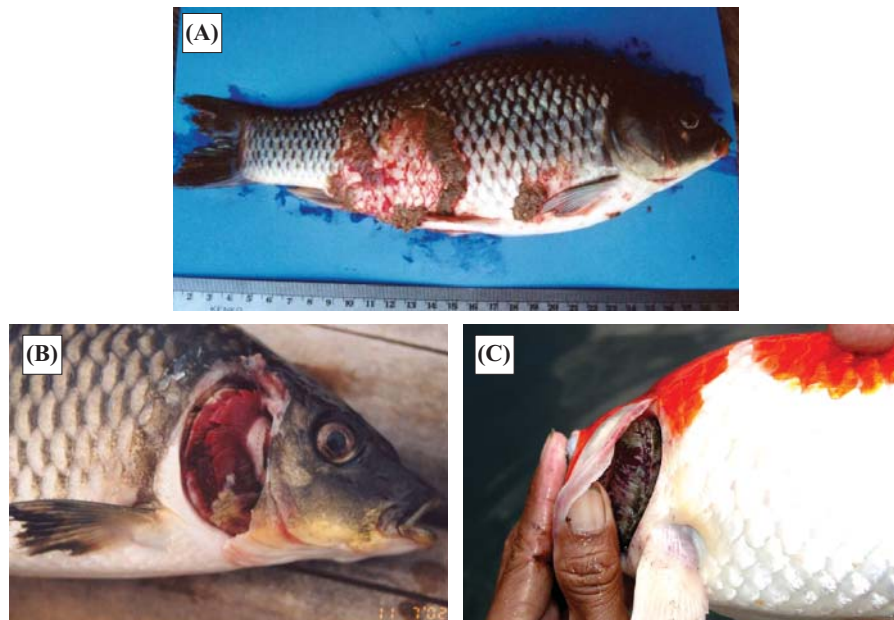
### **Polymerase chain reaction (PCR) assays**

Gills, spleen and kidney of infected fish were preserved in 95% ethanol and used for DNA extraction using DNAzol® according to manufacturer's protocols. Fifty to one hundred milligrams of the tissue was homogenized in 1 ml of DNAzol® and centrifuged at 14,000 rpm for 10 min at room temperature (± 27°C). DNA was precipitated in 0.5 ml of 100% cold ethanol. After centrifugation at 10,000 rpm for 5 min at room temperature, the DNA pellet was washed three times with 1 ml of cold 90% ethanol. The DNA was then air-dried and suspended in 50 µl TE buffer.

The PCR detection of koi herpesvirus (KHV) was conducted using the specific primer set developed by Gray *et al* (2002). The SphI-5 primer set (5' GACACCACATCTGCAAG-GAG-3') and (5'GACACATGTTACAATGGTGGC-3') was designed to amplify a 290 bp amplicon. DNA amplification was carried out in a 25 µl reaction mixture that consisted of 2 µl of template DNA, 0.25 µg of primers and one bead of RTG (Ready To Go PCR bead, Amersham). Polymerase chain reaction was performed in a programmable thermocycler PTC-200 (MJ Research, USA). Without pre-denaturation, the DNA was directly amplified by 30 successive cycles of denaturation at 94°C for 1 min, annealing at 55°C for 2 min and extension at 72°C for 3 min. Final elongation at 72°C for 7 minutes was done for completion of the reaction. The amplicons were resolved by 2% agarose gel electrophoresis in 1x TBE buffer, then stained with 0.05% ethidium bromide for 4 min and visualised by UV trans-illuminator. The specificity of the amplified product was confirmed through appearance of single band fragment at molecular weight of 290 bp. A hundred DNA ladder (Promega) was used as a marker.

## RESULTS

The first episode of mass mortalities of cultured koi (*Cyprinus carpio koi*) was recorded at the end of March 2002 in Blitar, East Java. It occurred after heavy rains among new fishes introduced from Surabaya, the capital city of East Java. The fish were imported from China through Hongkong in December 2001 and January 2002. The outbreak, occurred in koi carp of all ages, caused a total mortality of up to 80-95%. However, big fish were more susceptible than small fish. Infected fish were lethargic, showed loss of balance and gasped for air. Common gross sign including sloughing off the epithelium with loss of mucus and



**Figure 1.** Clinical signs of affected fish. Blister-like lesion on the skin (A). Severe gill necrosis in common carp (B) and koi (C).

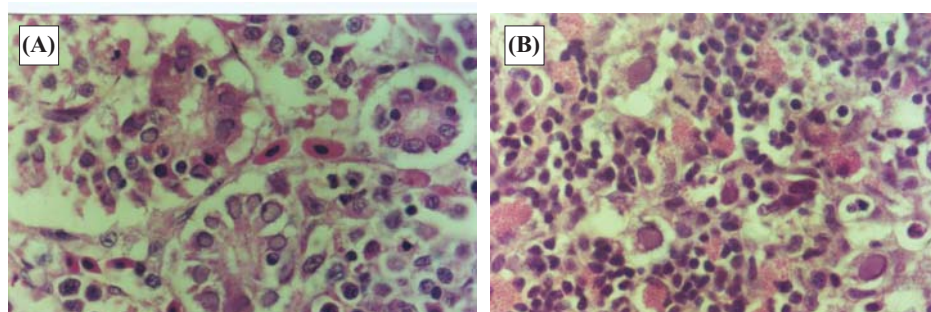
rough appearance of the skin, haemorrhages of operculum, fins, tail and abdomen and severe gill damage. Some diseased fish showed a blister-like lesion on the skin (Figure 1), so called 'penyakit melepuh' in Indonesian language (DGA, 2002).

The second disease outbreak occurred in cultured common carp (*Cyprinus carpio carpio*) during the end of April, 2002 in Subang regency, West Java. The gross signs of the diseased common carp were extremely similar with that observed previously in koi carp. Due to immediate harvest, there was an over supply of fish in the region. Therefore, farmers sold the infected fish at a very low price (Rp 3,000/kg; normal price Rp 7,000/kg). Economic losses included more than 450 metric tons of common carp cultured in running water systems.

The third episode of the outbreak occurred in May to early June 2002 in cultured common carp in floating netcage at Citarum river system. The system is composed of the Saguling reservoir in upper reaches, Cirata in the middle, and Jatiluhur in the down stream. There are 4,425 units floating net cages of mostly common carp in Saguling reservoir. There are no data on the losses, but it was estimated that 40-50% of the cages were affected. Weeks before the outbreak, farmers introduced common carp from Subang region due to the low price of fish.

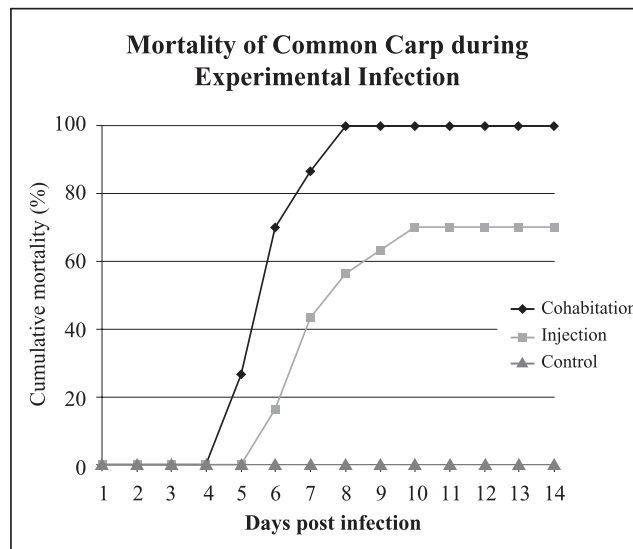
Presently there are about 33,000 cages in operation in Cirata reservoir. There was no history of a disease outbreak of this nature but there are cases of mortality due to up-welling which occur every year, particularly during the beginning of the rainy season (October, November, December). The floating netcage applied a double cage culture system; the upper cage measures 7 x 7m with 2.5 m water depth were used for common carp and the second layer for tilapia. The first recorded outbreak of disease under investigation in common carp was in early July 2002. Clinical signs include reduced feed consumption, lethargy, and other inconsistent clinical abnormalities except for the characteristic gill necrosis/rot (Figure 1).

Mortalities occurred only among common carp but not tilapia despite the double cage system. Mostly big fish were affected with characteristic gill rot; small fish in separate cages looked healthy; but the cages with small and big fish, both sizes of fish were affected. In big fish, mortality was up to 15 kg/cage/day. At the beginning of the outbreak, farmers harvested fish when they found 4-5 fishes dying in the cage. The fish were sold at half price (Rp 3,000/kg; normal price Rp 7,000/kg). However, as the outbreak progressed farmers faced marketing problems due to the over supply of dead fish and the prohibition to send common



**Figure 2.** Basophilic intranuclear inclusion bodies in the kidney (A) and in the gills (B) of infected fish.





**Figure 3.** Cumulative mortality among common carp during experimental infection by either cohabitation or injection of filtrate homogenate.

carp from Java islands to other islands within the country. The last reservoir in Citarum river system is Jatiluhur reservoir. There are 2,000 cages in the reservoir and no diseases outbreaks have occurred in common carp here have been reported yet.

Screening for parasites, fungi and bacteria showed multiple infections on affected fish. *Dactylogyrus* sp., *Trichodina* sp, *Ichthyophthyrus multifiliis* and *Argulus* sp were found in some debilitated fish. *Flexibacter columnaris* and *Aeromonas hydrophila* were isolated from necrotic gill filaments and skin ulcers of affected fish, respectively. *Saprolegnia* sp and *Achyla* sp were found in some fish showing blister-like lesions. Attempts have been made to treat the infected fish with potassium permanganate and antibiotics (Enrofloxacin, Erythromycin, Amoxycilin and Oxytetracyclin), but with no success.

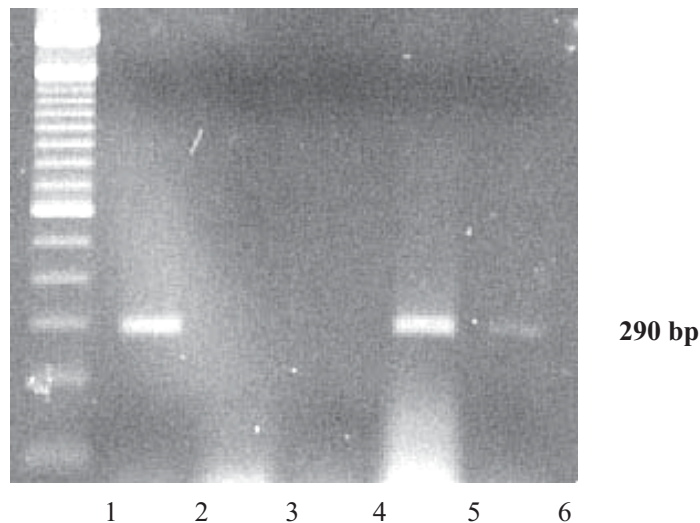
The histopathological studies revealed necrotic changes in the gill, fin, skin, kidney, spleen, liver, heart and intestine. Marked tissue changes were observed in the gills. Hyperplasia and hypertrophy of epithelial cells was severe and fusion of adjacent secondary lamellae was common. Some parasite cysts were occasionally observed in the fused secondary lamellae. Prominent eosinophilic intranuclear inclusions were observed in the gill and kidney of infected fish (Figure 2).

At the beginning of these unexplained outbreaks, many causative factors were proposed to including water pollution, contaminated feed and bacterial infection. The infectivity trial was then conducted to elucidate the causative agent of the outbreak. Cohabitation of diseased fish with healthy common carp resulted in 100% mortality within an 8 day period. Experimental infections with 0.45  $\mu$ m-filtered homogenates resulted in 70% mortality during a 14 day period (Figure 3). Similar clinical signs i.e. gill necrosis was observed from both groups in the cohabitation test and injection of filtrate homogenate test.

**Table 1.** Results of PCR test against KHV

<b>Fish</b>	<b>Location</b>	<b>Positive results/number of examined fish</b>
Common carp	Cirata Reservoir, West Java	15/17
	Sukabumi, West Java	5/7
	Bogor, West Java	5/6
	Jakarta	2/2
Koi carp	Jakarta	2/4
	Bogor, West Java	2/2
	Sukabumi, West Java	1/1
	Kediri, East Java	1/1
<b>Total</b>		<b>33/40</b>

Polymerase chain reaction detection of KHV was carried out using specific primers set developed by Gray et al., 2000. The results showed some of affected common carp produced a single band 290 base pair (bp) in size, that would be expected in KHV DNA was present. However, other common carp collected from the same pond and koi carp sent by fisheries officers gave negative results (Figure 4). In total, 82.5 % (33/40) of examined fishes showed positive results against KHV (Table 1.)



**Figure 4.** The PCR detection against KHV on koi and common carp in Indonesia. Lane:

1. DNA Marker of 100 bp, 2. Common carp from Bogor/CC01 (positive), 3. Common carp from Bogor/CC05 (negative), 4. Koi carp from Jakarta sent by fisheries officer (negative), 5. Common carp from Cirata/CL04 (positive), 6. Common carp from Cirata/CL09 (weak positive).

## DISCUSSION

As the outbreak continued, various clinical signs were observed or reported. However, the only consistent clinical sign of the outbreak was severe gill necrosis. In the early stage of infections, the gill filaments showed typical focal necrosis. In the late stage, the necrotic gill filaments fused and were badly damaged. However, the symptoms of the disease may be complicated by secondary infection of debilitated fish by opportunistic organisms such as bacteria, fungi and parasites. The outbreak only caused mass mortalities among koi and common carp but not other fishes despite sharing the same water systems such as aquarium, stagnant or running water and floating netcage culture systems. Therefore, it is necessary to establish a 'case definition' of the outbreak i.e. high mortality in koi and common carp with marked gill necrosis. The case definition is much needed to distinguish the outbreak from existing disease problems. The existing disease problems in Indonesian freshwater aquaculture are epizootic ulcerative syndrome (EUS) caused by *Aphanomyces invadans*, red disease due to *Aeromonas hydrophila*, *Flexibacter columnaris* infection, 'periodic mass mortality' and Mycobacteriosis in giant gouramy and Streptococcosis in tilapia (Sunarto, 2002).

Attempts have been made to treat the infected fish with antibiotics, but it has had no success. Some fish farmers have tried to use potassium permanganate (PK) for treatment. However, PK tends to irritate the gills leading to a surging mortality up to 80-90% of affected fish. Attempts to bring healthy-looking fish from an affected area to other 'clean' areas to escape an outbreak were used to control the disease. However, this attempt has 2 major negative impacts. Firstly, the handling and transportation of the fish seems to cause stress to the fish and leads to mass mortality, even if the fish were not going to show mortality in their original pond or cage culture population. Secondly, this practice leads to the rapid spread of the outbreak to other areas. It is noted that the spread of the outbreak from an affected area to other areas by means of fish movement is much faster than by water flow or other ways.

The disease occurred in on-growing fish of all ages and in all culture system including stagnant, running water and cage culture system. However, big fish were more susceptible than small fish. Although this disease process was observed as being highly contagious and extremely virulent, morbidity and mortality were restricted to koi and common carp populations. Several other species including goldfish as well as Nile tilapia stocked within the same ponds remained completely asymptomatic to the disease. However, it is not known yet whether the fish harbour the virus and act as carriers.

At the beginning, many factors have been speculated to be responsible for the new outbreak, including water pollution, contaminated feed and bacterial infection. However, based on the clinical signs, the mortality pattern, and mode of spread of the disease, a viral infection was suspected. With knowledge of KHV outbreaks in koi and common carp in Israel, Europe and the USA, a series of laboratory trial with an emphasis on viral disease was carried out. In order to elucidate the causative agent of the outbreak, experimental infection by the co-habitation test and injection of filtrate homogenate with standard virological methods was conducted. Results of co-habitation test i.e. 100 % mortality with clinical sign of gill necrosis suggested that infectious agent or agents are involved on the development of the outbreak. Furthermore, results of injection of filtrate homogenate prepared with standard virological



methods i.e. 70% mortality suggested that viral agent is responsible on the development of disease outbreak. The dead fish were PCR positive of KHV. Lower mortality rate on the group of filtrate homogenate injection suggested viral agent alone might only caused 70% mortality. In the other words, multiple infections with the viral agent and other pathogens such as bacteria, fungi or parasite might lead to 100% mortality.

Carp gill necrosis caused by *Cyprinus carpio* iridovirus (CCIV) has been reported by Popkova and Shchelkunov (1978). However, infectivity trials failed to induce a similar disease in carp, and the agent is no longer considered as the causative agent of the disease (Shchelkunov and Shchelkunov, 1990). Other viruses associated with disease in carp are *Rhabdovirus carpio*, the causative agent of spring viraemia of carp (Fijan, 1972), Cyprinid herpesvirus (CHV) of epidermal hyperplasia/papilloma in goldfish (*Carrassius auratus*) in Japan (Sano et al. 1985) and Malaysia (Samson, 2001), Goldfish haematopoietic necrosis virus (GFHNV) the cause of a herpesviral haemapoietic necrosis in Japan (Jung and Miyazaki, 1995) and Taiwan (Chang et al., 1999) and Carp viraemia associated ana-aki-byo (CVA) (Miyazaki et al., 2000). Recently, another virus known as koi herpesvirus (KHV) has been reported to cause serious disease in koi and common carp. This disease, with typical clinical signs includes high mortality and gills necrosis, was first recognized in Israel in the summer of 1998. Since then the disease has spread to USA (Hedrick et al, 2000), Germany (Hoffman et al., 2002) and United Kingdom (Way et al., 2002).

In the recent outbreak in Indonesia, clinical signs i.e. severe gill necrosis and mortality pattern (80-95%) are similar to KHV infection in koi and common carp in Israel and USA (Hedrick et al., 2000). Prominent basophilic intranuclear inclusion bodies observed in the kidney and the gills of infected fish also resembled cellular changes due to koi herpesvirus (KHV) infection. Furthermore, PCR detection of KHV revealed that the recent outbreak among koi and common carp in Indonesia is strongly suggested to be associated with KHV. In addition, DNA sequence of KHV from Indonesia has high similarity (99.65%) with DNA sequence of KHV from USA (data not shown). This is the first report of KHV in Asian region. However, since May 2003, KHV outbreak has been reported to cause mass mortality in common carp in 24 out of 47 prefectures in Japan (Sano et al, 2004). The disease was also reported to cause serious mortality in koi in Taiwan in fall 2002 (Tu et al, 2004). In 2004, KHV DNA has also been detected in koi in Malaysia, however no mass mortality of koi or common carp was reported in the country (Najiah et al, 2004).

It is difficult to assess the economic impact of the outbreaks. However, it is estimated that the outbreak has caused more than US\$ 0.5 millions within a 3 month period. In order to prevent the spread of the outbreak, the Government under Ministerial Decree No. 28/2002 has officially declared that Java Island is an isolated area with the disease and moving carp and koi from Java Island to other islands is strictly prohibited. In addition, importation of common carp and koi into this country was temporarily not permitted. Three months later, through Ministerial Decree No.40/2002 Bali is pronounced, as infected area and movement of live-fish from Java and Bali Islands to another within the country should follow quarantine check for KHV. Importing koi and common carp is permitted only from free KHV country. In order to control the outbreak, zone mapping of the outbreak and screening of virus carrier are urgently needed.

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