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ISOLATION AND IDENTIFICATION OF CARP INTERSTITIAL NEPHRITIS AND GILL NECROSIS VIRUS (CNGV) IN FINGERLING COMMON CARP (*CYPRINUS CARPIO* L.)

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ABSTRACT. High mortality in traditional carp farms and warm water cage farms was observed in Poland. Mortality rates were consistently above 50-80% in ponds. A preliminary study suggested that carp nephritis and gill necrosis virus/koi herpesvirus (CNGV/KHV) is the pathogen inducing this high mortality. The diagnosis of CNGV infection in common carp fingerlings based on viral isolation and current PCR assays has limited sensitivity. The aim of the current study was to develop more sensitive methods for the isolation and identification of CNGV in common carp, *Cyprinus carpio* L., fingerlings by cloning the virus gene encoding thymidine kinase (TK) and then using it for highly sensitive PCR diagnosis. Common carp fingerlings weighing 60 g that had survived mass mortality at a temperature of 22°C were obtained from two carp farms. The gills and kidneys from 20 diseased carp from each farm were used for virus isolation and identification. Koi fin cell (KFC) cultures were used for virus cultivation and propagation. DNA extraction was performed using thermal lysis. The supernatant containing the DNA was used in PCR assays. The virus isolated and identified from diseased carp fingerlings was used to experimentally challenge healthy carp fingerlings weighing 20 g by injection or bathing. The results of this study indicated that the TK-based PCR assay is simple to perform and is sensitive in detecting CNGV infection. The cytopathic effect (CPE) in KFC from 5 to 7 days after infection was observed. Cells became enlarged and developed abundant endoplasmic vacuoles. The results of the challenge test indicated that the isolated virus induced the clinical symptoms of CNGV infection. The mortality percentage was higher in the group of fish infected by injection (93%) in comparison to that infected by bathing (72%). Until the conclusion of the experiment (day 30), mortality was not observed in the control groups. The results of this study verified that CNGV is highly pathogenic for carp fingerlings and causes high mortality.

Key words: COMMON CARP (*CYPRINUS CARPIO*), CARP INTERSTITIAL NEPHRITIS AND GILL NECROSIS VIRUS, TK-BASED PCR, PATHOGENICITY

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INTRODUCTION

During the summer of 2002, numerous deaths of common carp, *Cyprinus carpio* L., were observed in several Polish fish farms. Mortality rates were consistently above 50-80% in ponds. The preliminary clinical, anatomopathological, histopathological, and microbiological studies suggested that koi herpesvirus (KHV) was the pathogen inducing this high mortality in common carp. The disease has now spread to numerous farms throughout the country. Clinical signs of KHV are often non-specific and mortality may occur rapidly at a temperature between 18 to 22°C. Discoloration and several necroses of the gills are the most consistent symptom of infection, with disorientation and erratic swimming prior to death. Several closely-related species cultivated with common carp (European perch, *Perca fluviatlis* L., silver carp, *Hypophthalmichthys molitrix* (Val.), grass carp, *Ctenopharyngodon idella* (Val.)) in similar ponds did not present any symptoms of disease and were found to be fully resistant to it. Poland is the largest producer of carp in Europe, and this high mortality induced by KHV is causing substantial financial losses (Siwicki et al. 2004, 2005).

A herpes-like virus designated koi herpesvirus (KHV) was first isolated in the USA in 1998 following outbreaks in koi and common carp in Israel and the USA (Hedrick et al. 2000). This disease has been observed in many farms in European, African, and Asian countries (Bretzinger et al. 1999, Choi et al. 2004, Haenen et al. 2004, Hedrick et al. 2000, Miyazaki et al. 2003). In Poland, KHV in warm water and traditional carp farms has also been isolated (Antychowicz et al. 2005, Bergmann et al. 2006).

Koi herpesvirus is a highly contagious viral disease which causes significant morbidity and mortality in common carp and koi carp, its ornamental domesticated form (Hedrick et al. 2000, Perelberg et al. 2003, Haenen et al. 2004). Although the virus is currently regarded as a DNA-virus of the family Herpesviridae (Gilad et al. 2002), this classification is disputed in some reports, in which it has been renamed Carp Nephritis and Gill Necrosis Virus (CNGV). Pikarsky et al. (2004) proposed that the etiologic agent of this disease may represent an as yet unclassified virus species that is endemic in common carp. In concurrence with previous studies of KHV, the virus that Pikarsky et al. (2004) isolated and designated carp interstitial nephritis and gill necrosis virus (CNGV) has an icosahedron-shaped core of 100 to 110 nm, is an enveloped virus, and bears thread-like structures on the core surface resembling those of the herpesvirus.

Although they believe CNGV and KHV to be the same, they have found that CNGV contains an asymmetrical electron-dense region within the viral core, which is probably the genomic nucleoprotein complex with a double-stranded DNA molecule of 270 to 290 kbp (Hutoran et al. 2005). The genome of the CNGV is larger than those of the other members of Herpesviridae. The authors suggested that CNGV is similar to the previously described KHV (Hedrick et al. 2000), and although the morphology of CNGV is similar in some aspects to that of Herpesviridae, for the time being they prefer to designate it carp interstitial nephritis and gill necrosis virus (CNGV), according to its pathological manifestation rather than its phylogenetic classification, which has yet to be determined (Pikarsky et al. 2004, Hutoran et al. 2005).

The diagnosis of CNGV infection in common carp fingerlings based on viral isolation and current PCR assays is of limited sensitivity, which is why it is imperative to develop new tools for the diagnosis of CNGV infection. The aim of the current study was to isolate and identify CNGV in common carp fingerlings by cloning the CNGV gene encoding thymidine kinase (TK) and using it in highly sensitive PCR-based diagnosis in accordance with the method presented by Bercovier et al. (2005).

MATERIAL AND METHODS

Common carp fingerlings (one-year old) with an average body weight of 60 g that had survived mass mortality connected with a CNGV/KHV infection were obtained from two carp farms. The water temperature was maintained at 20 to 22°C. The gills and kidneys from 20 carp from each farm were used for virus isolation and identification.

CELL CULTURE

The koi fin cell (KFC) cultures used for virus propagation were kindly supplied by Professor Moshe Kotler of the Department of Pathology, Hebrew University-Hadassah Medical School in Jerusalem, Israel. The cells were cultivated in a culture medium containing 60% Dulbecco's modified Eagle's medium, 20% Leibovitz (L15) medium, 10% fetal bovine serum (Sigma), 10% tryptose phosphate (Difco), and supplemented with 1% HEPES and antibiotics to form of monolayer over a period of 10-14 days in a 22°C incubator supplemented with 5% CO_2. The monolayer cultures were trypsinized and transferred into new flasks with fresh medium. The KFC was inoculated with supernatants

prepared from gills and kidneys, respectively. The infected KFC were incubated at 22°C for 10 days and monitored for cytopathic effect (CPE). The virus was released into the culture medium during the appearance of a CPE, but a significant amount remained associated with the cell. When CPE was observed in the KFC, the PCR method was used for virus identification. The purification of the virus from the culture medium was done according to the protocol in Pikarsky et al. (2004). The direct PCR method was also used to identify the CNGV from the gills and kidneys of the experimentally infected carp.

DNA EXTRACTION AND PCR ASSAY

DNA extraction was performed using thermal lysis. Tissue samples (approximately 0.1 g of the gill and kidney) were homogenized with PBS (Phosphate Buffered Saline) followed by centrifugation at 1677 × g for 4 min (mini Spin plus, Eppendorf AG Hamburg, Germany).

The supernatant was placed in 1.5 microfuge tubes with an equal volume of Tris-EDTA buffer (AppliChem GmbH Darmstadt, Germany) and heated at 99°C for 2 min (Thermomixer comfort, Eppendorf AG Hamburg, Germany). DNA extraction was completed by centrifugation at 10000 × g for 3 min.

The supernatant containing the DNA was used in the PCR assay. TK-based amplification was performed according to Bercovier et al. (2005). The specific primers (TKf 5'-GGGTTACCTGTACGAG-3' and TKr 5'-CACCCAGTAGATTATGC-3') were derived from the KHV thymidine kinase gene and resulted in a 409 bp amplified fragment (Bercovier et al. 2005). PCR was done using the above primers for 35 cycles under the following conditions: initial denaturation 95°C 5 min; denaturation 95°C 30 s; annealing 52°C 30 s; extension 72°C 1 min; final extension 72°C 10 min.

The amplification of target DNA was carried out in a 20 µl reaction using water, 1 µl of total DNA extracted from the gills, 10x Taq Buffer, 25 mM $Mg(OA_C)_2$, 200 µM each dNTP and 2U Taq polymerase (Eppendorf, AG Hamburg, Germany), 1 µM each of the primers (oligonucleotide synthesis – Institute of Biochemisty and Biophysics Polish Academy of Sciences).

The PCR products were detected in 1% agarose gel with ethidium bromide staining and UV illumination (MINI-SUB CELL GT, PowerPac Basic, Molecular Imager Gel Doc TM, Biorad Laboratories Inc.).

CHALLENGE TEST

Healthy common carp fingerlings with an average body weight of 20 g were used in the challenge test. The virus isolate from common carp fingerlings was used for experimental infection by injection or bathing. The KFC culture was used for virus cultivation. Ten days after the infection of the cell line with the isolate virus, a complete cytopathic effect (CPE) was observed. Virus concentrations were estimated by $TCID_{50}$ using the method by Reed and Meunch (1938) as presented for fish herpesvirus by Hedrick et al. (2000).

Challenge by injection. One hundred twenty fish were stocked into a 300 l tank with aerated, filtrated water heated to a temperature of 22°C. The virus isolated from common carp fingerlings was used for experimental infection, and healthy fish were injected intraperitoneally (i.p.) with 0.2 ml of isolate virus suspension at a concentration pf 4 $TCID_{50}$ ml^{-1} as previously described by Pikarsky et al. (2004). The control group (120 fish) was stocked into a 300 l tank with water at a similar temperature, and these specimens were injected intraperitoneally with 0.2 ml of medium harvested from uninfected KFC culture (no virus). On days 3 and 7 after experimental infection 10 fish from each the experimental and control groups were euthanized by immersion in Propiscin (5 ml l^{-1}; Inland Fisheries Institute in Olsztyn, Poland), and the kidneys and gills were removed in order to identify CNGV with the PCR method.

Challenge by bathing. One hundred twenty fish were stocked into a 150 l tank with water at a temperature of 22°C and were exposed to a virus suspension at a concentration of 4 TCID ml^{-1}. After 50 minutes of exposure to the virus, the fish were transferred to a 300 l tank. The control group (120 fish) was bathed in water at a similar temperature that contained medium harvested from uninfected KFC culture. On days 3 and 7 following infection 10 fish from experimental and control groups were euthanized by immersion in Propiscin (5 ml l^{-1}), and the kidneys and gills were removed in order to identify CNGV with the PCR method.

RESULTS AND DISCUSSION

Since common carp is the most widely cultivated fish in Poland and Poland is the largest carp producer in Europe, the high mortality observed in traditional carp culture has a direct negative financial impact on this branch of aquaculture. The rapid spread of CNGV/KHV is probably due to the intensive worldwide trade of these splendid fish,

which occurs mostly without veterinary suspension. The disease appears in ponds only at the permissive temperature of 18 to 28°C, but the highest mortality was observed within the temperature range of 22 to 24°C. The diagnosis of CNGV infection in fingerling carp based on viral isolation and current PCR assays is of limited sensitivity; therefore, it is necessary to develop new tools for the early diagnosis of CNGV/KHV infection. The current study focused on developing a robust, sensitive PCR assay based on a defined KHV gene sequence which was applied to improve the diagnosis of CNGV infection in common carp fingerlings. This was based on the method presented previously by Bercovier et al. (2005), who reported that a hypothetical thymidine kinase gene (TK) was identified, subcloned, and expressed as a recombinant protein. The TK-based PCR assay did not amplify the DNA of other fish herpesviruses and is specific for the detection of KHV (Bercovier et al. 2005); these authors showed that the TK-based PCR assay is more sensitive in the detection of KHV than the previously described PCR assay. In the current study indicated that the TK-based PCR assay is simple and sensitive for the detection of CNGV infection. Under optimal conditions, this PCR assay amplified a 409 bp fragment when CNGV/KHV DNA was used as the template (Fig. 1).

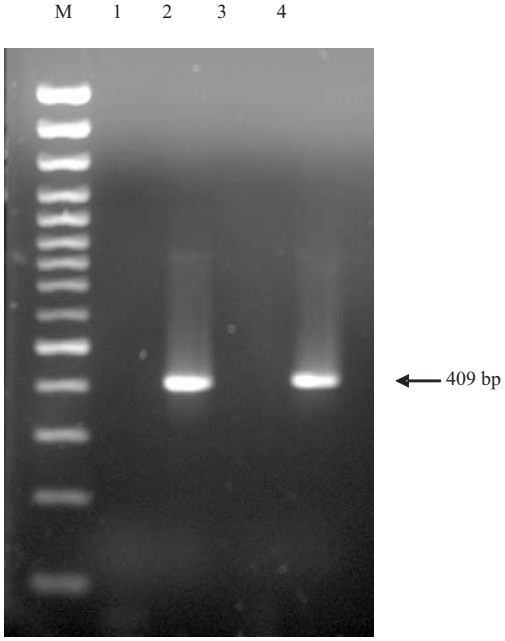

Fig. 1. Results from a thymidine kinase (TK)-based polymerase chain reaction (PCR) assay amplified a 409 bp fragment for the detection of CNGV in common carp fingerlings. Lanes: (M) – 100 bp DNA molecular weight standard, (1) – negative control (without target DNA), (2) – CNGV positive standard from Israel, (3) – CNGV negative sample, (4) – positive sample with CNGV from kidneys of infected fish.

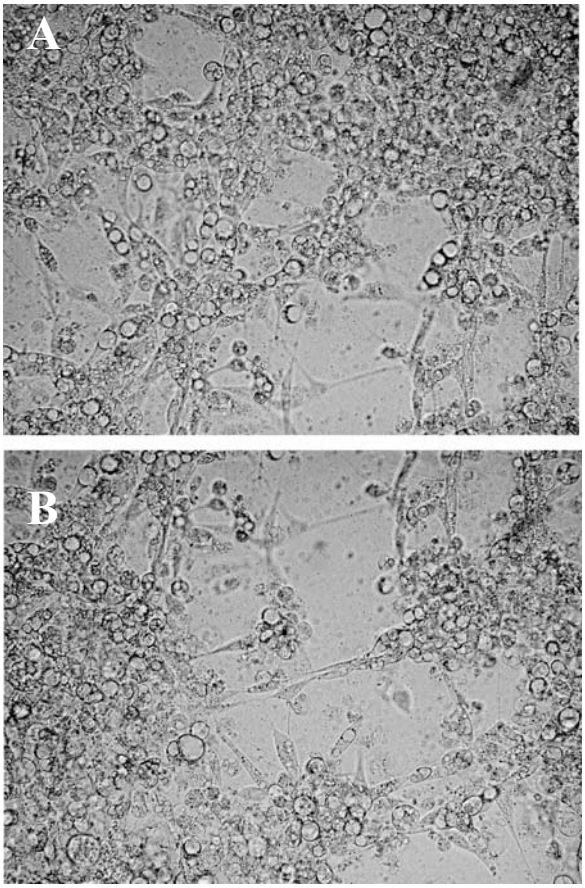

Fig. 2. Cytopathic effect (CPE) induced by CNGV from gills (A) and kidneys (B) of diseased carp fingerling on koi fin cell (KFC) cultures at temperature of 22°C. In comparison to the uninfected KFC, the cytopathic effect was observed from 5 to 6 days post-infection when cells became enlarged and developed abundant endoplasmic vacuoles.

The CNGV was isolated from sick carp fingerlings by cultivating KFC cultures with cells from the gills (Fig. 2A) and kidneys (Fig. 2B) of sick fish, and homogenates (aliquots) from these organs were used to infect the cell cultures. The cytopathic effect (CPE) in KFC cultures was observed between 5 to 6 days after infection (Fig. 2 A, B) as compared to the uninfected cells. The cells became enlarged and developed abundant endoplasmic vacuoles. A similar cytopathic effect induced by CNGV in KFC culture at a temperature of 22°C was observed by Pikarsky et al. (2004). The authors reported that at 2 to 3 days post infection (p.i.), the volume of the infected cells increased and

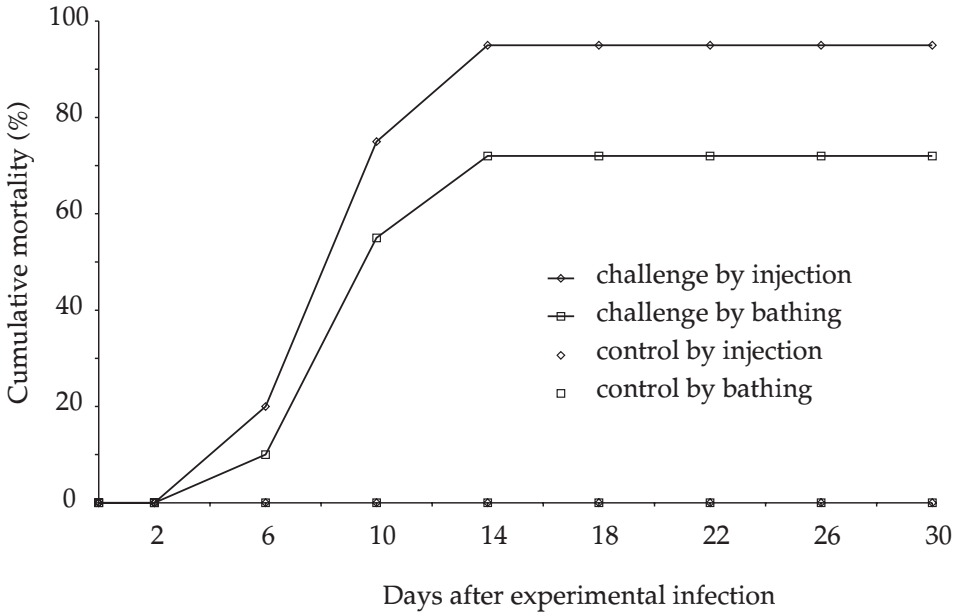

Fig. 3. Cumulative mortality of carp following the challenge with CNGV by injection or bathing and in the control groups. Total mortality was higher in the group of fish infected by injection (93%) as compared to that infected by bathing (72%). The control groups (no mortality observed) remained healthy throughout the study.

abundant endoplasmic vacuoles appeared in the cells, while at a later stage, the cells became round, died, and detached from the substrate. Significant amounts of virus were released into the culture medium at 4 to 5 days p.i.

The virus isolate was propagated, purified, and used in challenge tests by injection or bathing. The mortality rates among the fish following experimental infection through bathing or intraperitoneal injection and in the control groups are presented in Fig. 3. The results showed that the isolated virus induced the disease. All the infected fish showed the classic clinical symptoms of CNGV infection previously presented by Pikarsky et al. (2004) and Hutoran et al. (2005). The virus causes nephritis and gill necrosis as early as 3 days p.i., which intensifies up to 8 days p.i. The first mortality was observed between 6 to 7 days after the challenge, and it was higher in the carp infected by injection. In comparison to the control group, the highest mortality was observed to occur days 9 to 10 days following the challenge. Total mortality was higher in the group of fish infected by injection (93%) than in that infected by bathing (72%). The challenge test demonstrated that the disease is contagious under controlled conditions and that

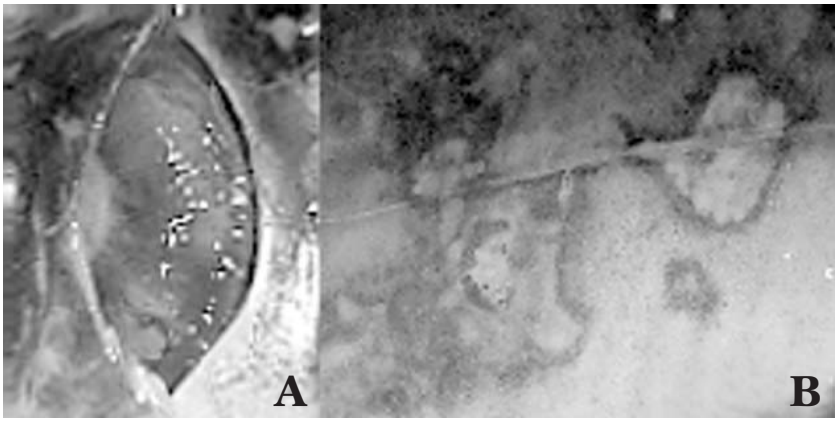

Fig. 4. Gill necrosis (A) and black patches of skin discoloration (B) in carp fingerlings induced by CNGV 7 days after experimental challenge by bathing.

mortality occurs from 6 to 14 days post challenge. The control fish from the injected and bathing groups remained healthy throughout, and mortality was not observed throughout the study (day 30 after infection). In addition, 10 fish were randomly sampled from each challenge group of fish on days 3 and 7 p.i. in order to isolate and identify the virus from the gills and kidneys with TK-based PCR assay. The technique presented by Bercovier et al. (2005) was as sensitive as virus identification from the tissue of infected fishes. On days 3 and 7 post-infection by injection or bathing, the current authors isolated and identified the virus from the gills and kidneys, and all of the fish examined exhibited typical symptoms of CNGV infection, including black patches of skin discoloration and gill necrosis (Fig. 4).

The massive mortality of carp fingerlings in Poland causes serious economic losses to the carp farming industry. This is why it was especially important to determine the disease-causing agent in order to develop effective prophylactic means against this disease. The current study led to the development of a sensitive method for isolating and identifying CNGV with TK-based PCR assay. The results of the challenge test verified that CNGV is highly pathogenic to carp fingerlings. The current authors prefer to designate this disease-causing agent as carp interstitial nephritis and gill necrosis virus (CNGV), according to its pathological manifestation, rather than by its phylogenetic classification of KHV. Future studies will focus on determining the influence of CNGV on the nonspecific cellular and humoral defense mechanism and specific immune

response in order to develop effective prophylactic methods against this disease in common carp fingerlings.

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STRESZCZENIE

IZOLACJA I IDENTYFIKACJA WIRUSA ŚRÓDMIĄŻSZOWEGO ZAPALENIA NEREK I MARTWICY SKRZELI (CNGV) U NARYBKU KARPIA (*CYPRINUS CARPIO* L.)

W ostatnich latach obserwowano w Polsce masowe śnięcia w hodowli karpia, sięgające nawet 80% obsady stawów. Choroba ta pojawia się w okresie czerwiec-sierpień w temperaturze wody powyżej 18°C. Wstępne badania wykazały, że masowe śnięcia są spowodowane przez wirus określany wstępnie jako wirus opryszczki karpia koi (KHV). Wielu autorów sugeruje, że jest zbyt wcześnie, aby sklasyfikować ten wirus filogenetycznie i proponuje nazywać go zgodnie z jego manifestacją patologiczną jako wirus śródmiąższowego zapalenia nerek i martwicy skrzeli (CNGV). Celem prezentowanych badań było doskonalenie metod izolacji i identyfikacji CNGV u jednorocznego narybku karpia. Do badań użyto 40 szt. narybku karpia pochodzącego z dwóch gospodarstw, w których stwierdzono masowe śnięcia ryb. Wirus izolowano z nerki oraz skrzeli chorych ryb oraz zakażano homogenatem z ww. narządów hodowlę komórkową uzyskaną z płetwy ogonowej karpia koi (KFC). Do identyfikacji wirusa z narządów i hodowli KFC zastosowano metodę PCR amplifikując fragment DNA o długości 409 par zasad, kodujący kinazę tymidynową (TK). Wyizolowany z hodowli komórkowej wirus został wykorzystany do eksperymentalnego zakażenia 20 g narybku karpia przez podanie CNGV w iniekcji dootrzewnowej lub 50 min kąpieli. Uzyskane wyniki badań wykazały, że zastosowana metoda PCR z wykorzystaniem genu kodującego TK jest szybką i wysoce czułą techniką identyfikacji CNGV u narybku karpia. Efekt cytopatyczny na hodowli KFC, z charakterystycznymi zmianami w komórkach obserwowano pomiędzy 5 a 7 dniem po zakażeniu. Zakażenia eksperymentalne wyizolowanym wirusem wykazały, że jest on wysoce patogenny dla narybku i indukuje zmiany nekrotyczne w skrzelach oraz postępujące śródmiąższowe zapalenie nerek charakterystyczne dla CNGV. Śmiertelność po eksperymentalnym zakażeniu wyniosła 93% po podaniu wirusa w iniekcji oraz 72% po zakażeniu w kąpieli, przy braku śnięć u ryb grup kontrolnych. Uzyskane wyniki badań jednoznacznie wykazały, że CNGV jest wysoce patogenny dla narybku, doprowadzając do wysokich strat w hodowli.