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POLYMERASE CHAIN REACTION FOR IDENTIFICATION OF CYPRINID HERPESVIRUSES IN UKRAINE

Yu. P. Rud[1]
M. I. Maistrenko[2]
L. P. Buchatskiy[1, 2]

[1]Institute of Fisheries of the National Academy of Agriculture Sciences of Ukraine, Kyiv
[2]ESC Institute of Biology and Medicine, Taras Shevchenko Kyiv National University, Ukraine

E-mail: rud.yuriy.univ@gmail.com

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The aim of the research was to investigate diseased fish species Labeo (*Labeo bicolor*) and Sack-gill Catfish (*Heteropneustes fossilis*) by testing several diagnostic systems targeting fish herpesviruses in purpose to determine the etiology of the outbreak infection in aquarium fishes at the Kyiv Zoo during summer 2017. During a fish health inspection of aquariums in Kyiv Zoo, an *Cyprinid herpesvirus 3* — CyHV-3 was detected in Labeo and Sack-gill Catfish. Preliminary examination of infected fish revealed a range of lesions particularly in internal organs and tissues. The gills of diseased fish were characterized by hyperaemia and necrosis. The infringement of liver color and structure, kidney swelling and gallbladder necrosis were observed in both fish species. The virus did not grow in fish cell lines of RTG-2, FHM and EPC, that was evident by absence of any morphological changes in appropriate cell lines. Also initially fish were tested for parasitic and bacterial infections and they were determined to be non-infected. Our results demonstrated that specific oligonucleotide primers for thymidine kinase gene of CyHV-3 were successfully amplified the specific DNA fragments. The length of polymerase chain reaction product, as expected, was 264 nucleotide pairs. The amplified specific fragments were identical to the area of thymidine kinase gene CyHV-3, as was shown by sequence analysis. The identity of nucleotide sequences composed 97–99%. The same positive results were obtained using primers that recommended by the International Epizootic Bureau, fragments in size of 409 and 292 were also obtained. In our opinion, CyHV-3 was brought to Ukraine by import of aquarium fish avoiding sanitary control of transboundary transportation. Therefore, the uncontrolled import of aquarium and cultured fish species is a serious problem because imported fish could be as a source of highly pathogenic infections for industrial aquaculture.

Key words: polymerase chain reaction, CyHV-3 fish herpesvirus of *Labeo bicolor* and Sack-gill Catfish.

Taxonomically, members of the family *Herpesviridae* consist of a linear, double-stranded DNA genome with an icosahedral capsid that is finally surrounded by a host-derived envelope containing virus glycoproteins. Phylogenetically, herpesviruses divided on three major clades that are related at the sequence level. Herpesviruses can infect both vertebrates: mammals, birds, reptiles, amphibians and fish, and a single virus that infects an invertebrate, the oyster [1].

Only three fish herpesviruses, ictalurid herpesvirus or channel catfish virus (IcHV-1, CCV), Koi herpesvirus (KHV) and anguillid herpesvirus (AngHV-1) have been sequenced completely. KHV is related closely to cyprinid herpesviruses CyHV-1 and CyHV- 2, the agents associated with carp pox and hematopoietic necrosis in goldfish, respectively [2–4]. The estimated genome size of KHV is 277 kbp, which is greater than that observed previously among all herpesviruses (125 to 245 kbp). Also KHV is distantly related to IcHV-1 and ranid herpesvirus (RaHV-1). Therefore, KHV has been proposed formally as a member of the *Alloherpesviridae*

under the species name *Cyprinid herpesvirus 3* (CyHV-3) [5].

CyHV-3 was first identified in 1998 as the cause of mass mortality among juvenile and adult koi and among common carp cultured in Israel, the United States, and Germany [6]. The disease had spread to other countries, leading to substantial financial losses. The virus has now been found associated with mass mortality events on most continents, including countries throughout Europe, North America, Asia and South Africa. Considerably more information has since been obtained on the properties of this virus, including its host range, the effects of water temperature on disease outcome, development of detection methods, and novel attempts at control [7].

Limited information is available on the ways of CyHV-3 spreading in nature. Possibly, the source of infection (except for the ornamental koi fish) could be the water containing waste products from contaminated fish farm or fisheries equipment. It was established that the virus can penetrate through the fish skin. An important role in virus transmission is attributed to waterfowl birds. CyHV-3 could save its infectivity in the water during several days. Polymerase chain reaction (PCR) analysis identified the CyHV-3 DNA in carp without clinical signs of the disease [8]. Such fish with chronic infection could also be as a source of infection at fish-breeding farms.

CyHV-3, was firstly described in Ukraine in 2013 [9]. There is a danger of this highly pathogenic virus spreading through fish farms in Ukraine. One of the proposed preventative measures to stop the spreading of CyHV-3 is strict compliance with veterinary regulations. Transportation of carp fry in the European Union is allowed only from certified CyHV-3-free farms. Other bio safety measures include quarantining of imported fish for two months in compatible cultivation with sensitive fish at temperatures that are optimal for CyHV-3 reproduction.

Particular attention must be given to the sanitary control of transboundary transportation of aquarium fishes that could be as a source of highly pathogenic infection for industrial aquaculture. In this report we investigated diseased fish species Labeo (*Labeo bicolor*) and Sack-gill Catfish (*Heteropneustes fossilis*) by testing several diagnostic systems targeting fish herpesviruses in purpose to determine the etiology of the outbreak infection in aquarium fish at the Kyiv Zoo during summer 2017.

Materials and Methods

In 2017, a total of 15 fish samples were continuously collected from the moribund and dead labeo and sack-gill catfish in aquariums of Kyiv Zoo. The samples of internal organs were removed from individual fish and placed into a 1.5 ml microcentrifuge tube. Samples were transported on ice to the laboratory and processed immediately.

RTG-2, FHM and EPC cell lines were maintained in MEM medium (PAA) supplemented with 100 U ml^{-1} penicillin, 100 µg ml^{-1} streptomycin and 10% fetal bovine serum (FBS Gold, PAA). The samples of internal organs were homogenized with MEM and filtred through the 0.45 µm membrane (Sarstedt). Then the filtrate was inoculated onto 24-hours cell monolayers growing in 25 cm^2 flasks. After absorption for 60 min at 20 °C, MEM medium supplemented with 2% of FBS was added to the monolayers. All flasks with control and inoculated cells were observed daily using light microscopy.

DNA samples were extracted from gills, kidney, spleen, brain and gut providing relevant information about the virus tropism in labeo and sack-gill catfish. About 100 microliters (µl) of tissue homogenate was prepared in the PBS (pH= 7.4), followed by the addition 500 µl lysis buffer (10 mM TRIS-HCl pH = 8.0, 0.1 M NaCl, 25 mM EDTA, 0.5% SDS) and proteinase K. The mixture was incubated for 2 hours at 37 °C. DNA was extracted with phenol and centrifuged for 5 min at 10500 g. DNA from the supernatant was extracted with chloroform:isoamyl alcohol mixture (24:1). The supernatant was then supplemented with 0.1 volume of 3M sodium acetate solution (pH 5.2) and 2.5 volumes of chilled (−20 °C) ethanol. DNA was precipitated at room temperature for 1 h, followed by centrifugation at 10500 g for 10 min. DNA precipitate was washed with 70% ethanol and dissolved in TE buffer (10 mM TRIS-HCl, 1 mM EDTA, pH = 7.5) or deionized water [10].

For PCR assay we used several sets of oligonucleotide primers. According to OIE recommendations we applied specific primers for the thymidine kinase (TK) gene [11]. The primers sequences were as follows: forward 5′-GGG-TTA-CCT-GTA-CGA-G-3′ and reverse 5′-CAC-CCA-GTA-GAT-TAT-GC-3′ with expected product size of 409 bp. In addition we used primers with product size of 292 bp [12]. The sequences were as follows: forward 5′-GAC-ACC-ACA-TCT-GCA-AGG-AG-3′and reverse 5′-GAC-ACA-TGT-TAC-AAT-GGT-

CGC-3′. Using software VectorNTI11 and online service BLAST we design our own oligonucleotide primers specific to TK gene of CyHV-3. We determined the specificity and its physical properties. The sequences of primers were For 5′- ATG-CAG-CGT-CTG-GAG-GAA-TAC -3′ and Rev 5′-ATT-CTG-ACG-GTG-AAG-GGT-GCG -3′.

The PCR mixture consisted of 12,5 µl of DreamTaq Green PCR MasterMix (ThermoScientific), 20 pmol of each primer, 1 µl of cDNA and the nuclease-free water up to a total volume of 25 µl. The amplification was conducted with 35 temperature cycles of: 1 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C followed by a final extension step of 10 min at 72 °C. PCR was performed on 96 Universal gradient peqSTAR (PEQLAB, Biotechnologie GmbH). The products were analyzed by 2.0% agarose gel electrophoresis. The gel was stained with ethidium bromide and results were documented under UV transilluminator. Positive and negative assay controls were included. Control PCR assay for other fish viruses were also assessed.

The PCR products were purified with the Silica Bead DNA Gel Extraction Kit (ThermoScientific) and subjected to nucleotide sequence analysis using a 3130 Genetic Analyzer (Applied Biosystems). The sequences were aligned with sequences available in the GenBank database (NCBI) according to the CLUSTAL W by the software of Molecular Evolutionary Genetics Analysis (MEGA) version 6.0.

Results and Discussion

The 2017 aquarium fish infection outbreak at the Kyiv Zoo resulted in mass mortality (up to 85%). Among the infected species were cyprinid fish, such as redfin tetra (*Hyphessobrycon anisitsi*) and labeo (*Labeo bicolor*), perciformes (red-throated *Cichlasoma meeki*) and sack-gill catfish (*Heteropneustes fossilis*). In order to establish the etiologic agent of infection the gills, kidney, spleen, brain and gut were examined.

The gills of diseased fish were characterized by hyperaemia and bright pink coloration. Dissection of sack-gill catfish revealed the infringement of liver color and structure, kidney swelling and overflow of the gallbladder. The same pathological changes were observed in red-tailed black labeo. Initially fish were tested for parasitic and bacterial infections and they were determined to be non-infected by mentioned pathogens (Fig. 1).

Our results demonstrate that specific oligonucleotide primers for thymidine kinase gene of CyHV-3 were successfully amplified the specific DNA fragments. The length of the amplified PCR product using primers designed by us, as expected, was 264 nucleotide pairs (Fig. 2). The same results were obtained using primers that recommended by OIE. The amplified specific fragment was identical to the area of TK gene of CyHV-3, as was shown by sequence analysis.

a *b*

Fig. 1. **The diseased labeo (*Labeo bicolor*) and sack-gill catfish (*Heteropneustes fossilis*):** clinical symptoms of disease included gill edema, internal organ deformation, and tissue necrosis (indicated by arrow)

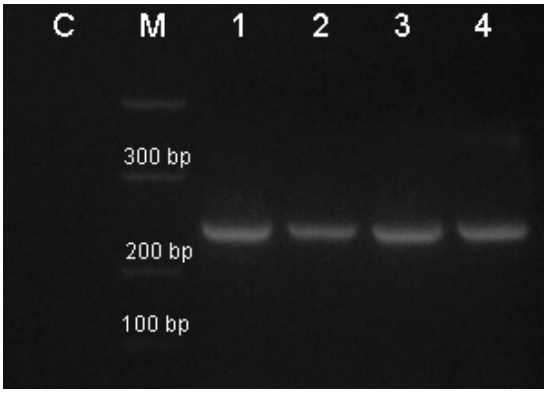

Fig. 2. **Gel electrophoresis of PCR products to identify CyHV-3:**
C — control (all reaction components except DNA); 1 — DNA samples isolated from red-tailed black labeo (*Labeo bicolor*); 2 — DNA samples isolated from Sack-gill Catfish (*Heteropneustes fossilis*); 3–4 — DNA samples of CyHV-3 isolated from diseased carp *Cyprinus carpio* (isolate Volyn-1); M — DNA marker ladder

According to the literature data, CyHV-3-specific DNA is detectable by PCR in kidneys and blood of infected carp as early as one day after infection [13]. We identified the virus DNA in the gills, liver, intestines and skin. It can also be detected in the brain tissue, but we did not identify the virus in brain samples. The data in recent reports shows, this viral infection leads to nephritis within 2 days, which can last up to ten days. In investigated fish the gills were affected, as evidenced by the loss of villi and inflammation of gill rakers. The main symptoms of the disease in tested fish were tiredness, loss of coordination, sunken eyes, spots on the skin and gill necrosis. The vast majority of mentioned above symptoms were evident in both labeo and catfish species cultivated in aquariums of the Kyiv Zoo.

Undoubtedly, many infections in aquarium fish may be asymptomatic. It has been shown that CyHV-3 is capable of producing a latent form of infection in carp fish, which is influenced by environmental factors and has the potential to turn into acute infection [14]. Therefore, the uncontrolled import of aquarium fish is a serious threat to commercial fish farms.

Outbreaks continue to occur among cultured populations of koi and also epidemics have been observed among wild common carp populations in the United Kingdom and Japan in recent years. Until recently, the only host for the carp herpes virus of the third type (CyHV-3) was thought to be the common carp (*Cyprinus carpio carpio*) and its subspecies, the Koi carp (*Cyprinus carpio koi*). It was accepted that some fish species, including *Carassius auratus* and other members of the cypriniformes family are resistant to the disease even during a long stay in the same water tank with infected fish [15].

However, the presence of CyHV-3 was recently confirmed by PCR in other cypriniformes fish that were grown in polyculture with the infected fish: in common carp (*Cyprinus carpio carpio*), grass carp (*Ctenopharyngodon idella*), spotted silver carp (*Aristichthys nobilis*), silver carp usual (*Hypophthalmichthys molitrix*), tench (*Tinca tinca*), zanthe (*Vimba vimba*). Moreover, it appears that CyHV-3 is capable of infecting the sheatfish (*Silurus glanis*) and sturgeons, such as Russian (*Acipenser gueldenstaedtii*) and Atlantic (*Acipenser oxyrinchus*) sturgeon and sterlet (*Acipenser ruthenus*). The CyHV-3 DNA was detected in the Rotifera by Japanese researchers [16, 17].

The rapid spreading of virus around the world could be attributed to the fact that ornamental koi carp and another species are in high demand among hobbyists and is widely traded. World Organization for Animal Health (OIE) recognized this emergent infection in 2007 as a threat and subject to declaration and eradication. The absence of reports for the virus spreading in Eastern Europe is one of the reasons for the ban on carp imports to the European Union.

As a conclusion, herpesvirus of the third type was discovered during an outbreak of viral infection in sack-gill catfish and red-tailed black labeo at the Kyiv Zoo. The developed diagnostic PCR assay together with diagnostics which are recommended by OIE could be useful for virus detection. The amplified specific fragment in size of 264 nucleotide pairs was identical to the area of TK gene of CyHV-3, as was shown by sequence analysis.

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ПОЛІМЕРАЗНА ЛАНЦЮГОВА РЕАКЦІЯ ДЛЯ ІДЕНТИФІКАЦІЇ ВІРУСУ ГЕРПЕСУ КОРОПОВИХ В УКРАЇНІ

Ю. П. Рудь[1], *М. І. Майстренко*[2], *Л. П. Бучацький*[1, 2]

[1]Інститут рибного господарства НААНУ, Київ
[2]ННЦ Інститут біології та медицини, Київський національний університет імені Тараса Шевченка, Україна

rud.yuriy.univ@gmail.com

Метою роботи було дослідити хворих риб видів лабео двокольорового (*Labeo bicolor*) та сома мішкозябрового (*Heteropneustes fossilis*) тестуванням декількох діагностикумів для виявлення вірусу герпесу та визначення етіології спалахів інфекції серед акваріумних риб у Київському зоопарку влітку 2017 року. Під час досліджень захворювань акваріумних риб у Київському зоопарку знайдено високопатогенний вірус *Cyprinid herpesvirus 3* – CyHV-3 у лабео двокольорового та мішкозябрового сомика. Аналіз інфікованих риб виявив низку уражень, передусім у внутрішніх органах і тканинах. Зябра хворих риб характеризувалися гіперемією та некрозом. У обох видів риб спостерігали порушення структури печінки та зміну її кольору, набряки нирок і крововиливи жовчного міхура. Вірус не репродукувався у перевивних клітинних лініях риб RTG-2, FHM та EPC, про що свідчила відсутність будь-яких морфологічних змін у досліджуваних культурах клітин. В організмі інфікованих риб не було виявлено збудників паразитарних або бактеріальних інфекцій.

Результати досліджень показали, що специфічні олігонуклеотидні праймери до гена тимідинкінази CyHV-3 успішно ампліфікували фрагмент ДНК вірусу. Довжина продукту полімеразної ланцюгової реакції, як і очікували, становила 264 пари нуклеотидів. Ампліфіковані специфічні фрагменти були ідентичні ділянці гена тимідинкінази CyHV-3, як показав аналіз послідовності ДНК. Ідентичність нуклеотидних послідовностей становила 97–99%. Використовуючи як контроль праймери, рекомендовані Міжнародним епізоотичним бюро, було отримано фрагменти розміром 409 і 292, що також свідчило про ідентифікацію CyHV-3. На нашу думку, CyHV-3 потрапив в Україну через нелегальний імпорт акваріумних риб, а також через відсутність належного санітарного контролю під час транскордонних перевезень. Неконтрольоване імпортування акваріумних і культивованих видів риб є серйозною проблемою, оскільки імпортована риба може бути джерелом високопатогенних інфекцій для промислової аквакультури.

Ключові слова: полімеразна ланцюгова реакція, вірус герпесу CyHV-3, лабео двокольоровий, мішкозябровий сомик.

ПОЛИМЕРАЗНАЯ ЦЕПНАЯ РЕАКЦИЯ ДЛЯ ИДЕНТИФИКАЦИИ ВИРУСА ГЕРПЕСА КАРПОВЫХ В УКРАИНЕ

Ю. П. Рудь[1], *М. И. Майстренко*[2], *Л. П. Бучацкий*[1, 2]

[1]Институт рыбного хозяйства НААНУ, Киев
[2]ННЦ Институт биологии и медицины, Киевский национальный университет имени Тараса Шевченко, Украина

E-mail: rud.yuriy.univ@gmail.com

Целью работы было исследовать больных рыб видов лабео двухцветного *(Labeo bicolor)* и сома мешкожаберного (*Heteropneustes fossilis)* тестированием нескольких диагностикумов для выявления вируса герпеса и определения этиологии вспышек инфекции среди аквариумных рыб в Киевском зоопарке летом 2017 года. Во время исследования заболеваний аквариумных рыб в Киевском зоопарке обнаружен вирус *Cyprinid herpesvirus 3* – CyHV-3 у лабео двухцветного и мешкожаберного сомика. Анализ инфицированных рыб выявил ряд поражений, прежде всего во внутренних органах и тканях. Жабры больной рыбы характеризовались гиперемией и некрозом. У обоих видов рыб наблюдалось нарушение структуры печени и изменение ее цвета, отеки почек и кровоизлияния желчного пузыря. Вирус не репродуцировался в перевиваемых клеточных линиях рыб RTG-2, FHM и EPC, о чем свидетельствовало отсутствие каких-либо морфологических изменений в исследуемых культурах клеток. В организме инфицированных рыб не было обнаружено возбудителей паразитарных или бактериальных инфекций.

Результаты исследований показали, что специфические олигонуклеотидные праймеры к гену тимидинкиназы CyHV-3 успешно амплифицировали фрагмент ДНК вируса. Длина продукта полимеразной цепной реакции, как и ожидалось, составила 264 пары нуклеотидов. Амплифицированные специфические фрагменты были идентичны участку гена тимидинкиназы CyHV-3, о чем свидетельствовал анализ нуклеотидной последовательности ДНК. Идентичность нуклеотидных последовательностей составляла 97–99%. При использовании в качестве контроля праймеров, рекомендованных Международным бюро эпизоотий, были получены фрагменты размером 409 и 292, что также свидетельствовало о наличии CyHV-3. По нашему мнению, CyHV-3 был занесен в Украину путем нелегального импорта аквариумных рыб, а также из-за отсутствия надлежащего санитарного контроля при трансграничных перевозках. Неконтролированное импортирование аквариумных и культивируемых видов рыб является серьезной проблемой, поскольку импортируемая рыба может быть источником высокопатогенных инфекций для промышленной аквакультуры.

Ключевые слова: полимеразная цепная реакция, вирус герпеса CyHV-3, лабео двухцветный, мешкожаберный сомик.