STUDY OF VIRAL DISEASES OF FISH IN THE REPUBLIC OF SERBIA DURING THE *PERIOD 2005-2010.*

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ISPITIVANJE VIRUSNIH BOLESTI RIBA NA PODRUČJU REPUBLIKE SRBIJE U PERIODU OD 2005. DO 2010.GODINE

Abstrakt

Tokom petogodišnjeg istraživanja prisustva virusnih bolesti riba kao što su PVŠ, VHS, ZHN, EHN, ZNG ispitano je 56 šaranskih i 32 pastrmska ribnjaka. Virusne bolesti riba su dijagnostikovane pomoću modernih laboratorijskih metoda: izolacija virusa na kulturi tkiva, PCR, ELISA i test fluorescentnih antitiela. U radu su opisane bolesti riba koje su dijagnostikovane pre 2005., kao što je prolećna viremija šarana, ali i pojava novih bolesti, nakon uvoza živih riba i njihovih proizvoda u Srbiju, i to zarazne nekroze gušterače pastrmki i epizootske hematopoezne nekroze američkog somića.

Ključne reči*: ribe, virusne bolesti, izolacija i identifikacija*

INTRODUCTION

Aquaculture is one of the fastest growing food production industries with an average growth rate of 9.2% per year. Fish diseases represents significant obstacle for development of aquaculture, which annually causes losses that are measured in billions of dollars. The intensive aquaculture process is often characterized by high density of fish, poor water quality, and accumulation of pathogens in the production systems and in the environment. As a result, most populations of fish from intensive rearing systems are characterized by chronic stress. Stress leads to increased susceptibility to disease, and prevalence of disease depends on the interaction between fish pathogens and the environment (Jeremić, 2003). Also, international trade of live fish and their products is a major hidden cause of many outbreaks. Damages caused by the disease significantly delay the development of fisheries and prevent its transition to modern forms of intensive

aquaculture. This paper describes the most common and also the latest viral diseases diagnosed in fish farms in Serbia during the period 2005 - 2010.

MATERIAL AND METODS

During the five years 7000 samples of all fish categories from 56 carp and 32 trout farms were examined for the presence of viral diseases. Clinical examination and selection of samples for laboratory was done on the fish farms. For histopathological examination, altered organs were stained with hematoxylin and eosin. For virological investigation, homogenates of kidney, spleen, liver and gills were used. Pools of parenchymatous organs and gills were homogenized with MEM and centrifuged at 2500 x g, 20 minutes. For isolation, supernatants were inoculated at 24 hours old culture of EPC, FHM, RTG-2 and BF-2 cell lines. Inoculated cultures were incubated at 15 - 20°C for 7 days and observed daily by the appearance of cytopathic effect. Virus was identified performed by PCR, ELISA and fluorescent antibody test. As a material for PCR extracted organ homogenate and the first or second passage of the appropriate cell lines were used. DNA was extracted using a DNA mini kit according to the manufacturer (QIAGEN, Valencia, CA, USA). RNA was extracted using RNA mini kit according to the manufacturer (QIAGEN, Valencia, CA, USA). PCR products (for SVC, IPN, ECV) were sequenced directly using Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). The obtained sequences were analyzed using Sequencing Analysis Software 5.1 (Applied Biosystems).

RESULTS AND DISCUSSION

Viral infections represent a constant danger for fish breeding. These diseases are often related to stressful situations and spread to a greater or lesser extent in all ponds (Jeremić and Radosavljević 2007). After wintering, in early spring and summer period, mortality often occurs in juvenile trout and carp caused by various infectious agents, due to the unfavorable conditions of environment and international trade in live fish and their products. During five years following diseases were identified:

Spring viremia of carp - acute contagious viral disease caused by the *Rhabdovirus carpio*. Carp of all ages are affected and also other cyprinid fish species. Due to seasonal occurrence of the disease, the most vulnerable are 9-12 and 21-24 months old carp. Physiological status of carp after wintering significantly contributes to the occurrence of disease in the spring, having in mind that at a similar temperature conditions are present in autumn, but without big losses. Mortality caused by spring viremia occurs from November to July with a peak between April and June. The disease usually occurs at temperatures between 11-17°C, and very rarely at temperatures lower than 10°C. SVC outbreaks first occurred in the spring of 1986 in Serbia, with water temperature between 13 and 15°C. Severity of disease varied from pool to pool in the same pond, or from pond to pond, and some ponds were viral positive in two consecutive years. Previous investigations of prevalence and incidence showed that in 1950 samples from 38 carp farms in Serbia, *Rhabdovirus carpio* was determined in 256 samples as follows: 124 samples in April, 112 in May, 19 in June, 4 in December and 3 samples in February (Jeremic et al. 1997). Keeping fish during the winter in large densities of fish without food, during the initial rise of temperature in spring, with all the factors that provoke stress, favoring the occurrence of this disease and high mortality (Jeremić et al. 2005). The occurrence of

mortality with clinical symptoms of the disease is found in 1 pond with a two-year old carp in 2005, reared in intensive production, after the transport from the winter quarters (Jeremić et al. 2008). Clinical and pathomorphological examination of the skin and gills of diseased and moribund fish revealed an increased amount of mucus, exoftalmia, enlarged abdominal cavity and reddening of the vent. Gills were ishemic, with petechial haemorrhages on fin and gills. In internal organs, notable changes were found: peritonitis, petechial haemorrhages and miliary necrosis of the liver, hemorrhages and necrosis of the kidneys, bleeding in the anterior part of the air sac, oedema of the spleen. Mucous membranes of the intestine were hemorrhagic, and the lumen filled with a yellow, gelatinous fluid (Jeremić et al. 2005). The most frequent histopathological changes were found in liver, kidney and spleen. In the liver hepatocytes light vacuoles of various sizes were noticed. Besides fatty changes, liver tissue shows a blue dye mononuclear cellular infiltration (hepatitis). In renal interstitium, mononuclear cellular infiltration was clearly evident. Reticular hyperplasia of spleen and discrete blood extravasated were also present (Jeremic et al. 1999).

Rhabdovirus carpio was isolated from the gills and parenchymal organs of diseased carp on EPC and FHM cell lines. In the first passage within 7 days of incubation FHM and EPC cell lines inoculated with filtrate of pooled samples of diseased carp cytotoxic effects occured. Control cells were normal, and cell culture inoculated with the reference virus SVC gave a clear cytopathogenic effect of the CPE. Cytopathic effect appeared 48-72 hours after subcultivation in the form of rounding EPC and FHM cells. After 72 hours good CPE appeared. Identification of virus isolates was performed using ELISA and indirect immunofluorescence test (IFAT). By RT-PCR with specific primers, presence of nucleic acid of the virus of spring viremia of carp was demonstrated. After purification, sequencing of obtained PCR products was done. Comparing the sequences of the glycoprotein with NCBI sequences previously isolated virus showed that the isolates belong to the group Id of the SVCV isolates.

Infectious pancreatic necrosis (ZNG/IPN) - acute, infectious, highly contagious disease of trouts, which are clinically manifested in the first 20 weeks of the feeding, with sudden onset of mortality (10-90%). Mortality depends on many factors, such as virus strain, host and environmental conditions (Dobos & Roberts, 1983). Cause of the disease is Aquabirnavirus, family Birnaviridae (Dobos & Roberts, 1983). The most important source of infection is affected fish and those that remain lifelong carriers after they recover from infection. Carriers excrete virus through feces and sexual products. Detection of such carriers is important for disease control, because the virus in addition to the horizontal is vertically transmitted, via eggs (Jeremic et al. 2008; Dorson & Torchy, 1985), and surface disinfection of eggs is not completely effective in preventing this type of transmission (Bullock et al., 1976).

The disease was first noticed in Serbia in 1989 (Jeremić et al. 1989) and did not emerge until 2007, when the disease was confirmed at a trout farm in Mačva district, in diseased rainbow trout, derived from fertilized eggs imported from USA. In 2008, the disease was diagnosed in three trout ponds in Zlatibor, Mačva and Pirot district, in diseased trout fry. In all cases this was clinically manifest disease with high mortality in rainbow trout (Radosavljević et al. 2009). Diseased fry showed characteristic clinical signs, with dark body pigmentation, swelling of the abdominal region and moderate exophthalmos. In the abdominal cavity red serous fluid was present. Liver and spleen were pale in color, and bile sac was enlarged. The intestines were devoid of food, with a large amount of mucous content.

IPN virus was isolated in 2009 from clinically healthy one year old rainbow trout, which was in quarantine at the trout farm in Mačva district, and in 2010 in the same pond; virus was isolated from rainbow trout that showed no clinical symptoms of disease. In 2010, infectious pancreatic necrosis virus of trout was isolated from clinically diseased rainbow trout in three ponds in the region of Zlatibor. Also in 2010 disease spread to one fish farm in Pomoravski region.

The most frequent histological changes were found in the exocrine pancreas and intestine. Stained sections of the exocrine pancreas showed pronounced signs of necrosis of pancreatic tissue and the presence of inclusions. In the lamina epithelial mucosae tunicae the individual and the multiple cells with eosinophilic cytoplasm were present. These cells were seen in the intestinal lumen as a result of desquamative process (Jeremić et al. 1998). For virus isolation, 24^{h} old RTG-2, EPC, BF2 cell culture were used. Inoculated cultures were incubated at 15^{o}C for 7 days and controlled daily for appearance of cytopathic effect (CPE). After the appearance of CPE identification of the virus was carried out by ELISA and indirect immunofluorescence test (IFAT). RT-PCR demonstrated the presence of the nucleic acid of the virus infectious pancreatic necrosis.

Epizootic haematopoietic necrosis (EHN) - acute viral disease of perch, rainbow trout, catfish and ictalurids caused by Iridoviruses from genus Ranavirus. The disease is caused by three similar viruses: hematopoietic necrosis virus (EHNV), european sheatfish virus (ESV) and european catfish virus (ECV). The disease is characterized by hemorrhages, edema and necrotic changes in liver, spleen, kidney and hematopoetic tissue. Epizootic hematopoietic necrosis was first reported in perch (*Perca fluviatilis*) in Australia (Langdon & Humphrey, 1987). After the outbreak of the diseases caused by the EHNV in Australia, epizootic haematopoietic necrosis was found in France in *Ictalurus melas* (Pozet et al., 1992), in Germany in *Silurus glanis* (Ahne et al., 1990.), Denmark and Finland (Ariel et al, 1999). The disease occurs in all age categories of fish . Cyprinid species are not susceptible to EHN virus. During the August of 2008, high mortality appeared in brown bullhead (*Ameiurus nebulosus*) in one carp farm located in north-western Serbia. The outbreak occurred after a increase of water temperature in the end of July, when the ambient temperature ranged from 30 to 36^{o}C, and water temperatures around 28^{o}C. Mortality lasted until mid-September (Jeremic et al. 2009). Fish presented clinically with exophthalmia, pale gills, and fin and skin haemorrhages. Gross necropsy findings included pale livers, swollen spleens and petechial haemorrhaging of mesenteric fat and internal organs. For virus isolation, 24h old EPC and FHM cell cultures were used. Inoculated cultures were incubated at 20°C during 7 days and observed daily for appearance citopatogenog effect (CPE). An extensive cytopathic effect (CPE) was observed 48^{h} after inoculation. Diagnosis was based on clinical signs, virus isolation in the EPC cell line and polymerase chain reaction (PCR).

CONCLUSIONS

Viral diseases represent a constant threat for fish breeding. Clinically manifested, their participation in the pathology and economical production is of high importance. Their harmful effects are manifested in increased morbidity and mortality, the weakening of the fish, reduced growth, poor feed utilization and lack of breeding material. The appearance of a number of diseases that had not occurred earlier, warns that the measures taken to protect the health of the fish are not enough. Given that most new diseases causes high mortality, it is necessary to invest additional effort in order to maintain the

health status of fish populations through the use of effective biosecurity measures, primarily regardingthe purchase of fertilized eggs and fry outside Serbia. In the carp farms the number of cases of spring viremia of carp is reduced.In the trout farms the number of cases of infectious pancreatic necrosis in trout is increased.

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