

SURVEY AND DIAGNOSTICS OF FISH DISEASES IN THE REPUBLIC OF SERBIA DURING THE PERIOD 2011-2012

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NADZOR I DIJAGNOSTIKA BOLESTI RIBA NA PODRUČJU REPUBLIKE SRBIJE U PERIODU OD 2011. DO 2012.GODINE

Apstrakt

Tokom dvogodišnjeg ispitivanja, preko 5000 uzoraka riba je ispitano na prisustvo bolesti. Tom prilikom je obuhvaćeno 56 šaranskih i 32 pastrmska ribnjaka. Na ribnjacima je vršen klinički pregled i odabir uzoraka za laboratoriju. Bakteriološka ispitivanja obavljena su zasejavanjem iz promjenjenih unutrašnjih organa, kože i škrga na standardne i specifične hranljive podloge. Nakon 48^h vršen je pregled kolonija na oblik, boju, granulisanost, mukoznost, hrapavost i hemolitičnost. Determinacija izolovanih bakterija izvršena je na osnovu morfoloških i biohemijskih karakteristika. Za virusološka ispitivanja su korišćeni homogenati bubrega, slezine, jetre i škrga. Pulirani parenhimatozni organi i škrge su homogenizovani i centrifugirani na 2500 x g, 20 minuta. Za izolaciju, supernatanti su inkulirani na 24 sata stare kulture EPC i BF-2 ćelijskih linija. Inokulisane kulture su inkubirane na 15 - 20°C, tokom 7 dana i svakodnevno posmatrane na pojavu citopatogenog efekta. Identifikacija virusa je vršena PCR, ELISA testom i testom fluorescentnih antitela. Kao materijal za PCR je uziman homogenat organa i prva ili druga pasaža odgovarajuće ćelijske linije. DNK je ekstrahovana pomoću DNA mini kit prema uputstvu proizvođača (Qiagen, Nemačka). RNK je ekstrahovana pomoću RNA mini kita prema uputstvu proizvođača (Qiagen, Nemačka). PCR produkti su sekvencirani direktno, pomoću Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, SAD) i ABI PRISM 3100-Avant Genetic Analyzer (Applied Biosystems). Dobijene sekvence su analizirane pomoću Sequencing Analysis Software 5.1 (Applied Biosystems). Tokom dvogodišnjeg ispitivanja utvrdili smo prisustvo zarazne nekroze gušterače (ZNG) i evropskog virusa somića (ECV). Utvrđena je pojava sindroma crvenih pega (RMS), jersinioze, renibakterioze, furunkuloze, septikemije izazvane pokretnim aeromonadama i sporadične pojave drugih oboljenja bakterijske i gljivične etiologije. Najčešće su utvrđene infestacije sledećim parazitima: *Trichodina domerguei*, *Chilodonella ciprini*, *Dactilogirus Girodactylus sp*, *Ichthyophthirius multifiliis*, *Diplo-*

stomum spathaceum, *Caryophyllaeus fimbriceps* i *Eustrongilus* sp. U radu su opisane najvažnije bolesti riba dijagnostikovane su tokom 2011 i 2012 godine na ribnjacima u Srbiji.

Ključne reči: bolesti riba, dijagnostika

Keywords: fish diseases, diagnostics

INTRODUCTION

The intensive aquaculture is often characterized by high density of fish, poor water quality, 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 (Jeremic, 2003). The appearance and development of fish diseases is a consequence of the interaction of pathogen, host and environment. 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. Many diseases affecting farmed fish also represents a threat to natural fish populations (Thoesen, 1994). The relatively small number of pathogenic bacteria is responsible for major economic losses in cultured fish (Toranzo et al., 2005). In addition to the release of active substances in aquatic ecosystems, the usual therapeutic interventions in aquaculture can lead to antibiotic resistance in bacterial pathogens of fish, but also in other bacteria present in the environment (Alderman and Hastings, 1998). In this paper, the diseases diagnosed in fish farms in Serbia during the period 2011 - 2012 are described.

MATERIAL AND METHODS

During two year period more than 5000 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. Liver, kidney and spleen were inoculated in trypticase soy agar (TSA), blood agar (BA), furunculosis agar, Mueller-Hinton agar (MH), Rimler-Shotts agar (RS), KDM-2 agar and brilliant green agar (BG), depending on the origin of fish. Samples from external lesions, were inoculated on cytophaga agar and trypticase soy agar. After incubation at 20-25°C for 4 days or at 10-15°C for 10 days, isolated bacteria were subcultivated in order to test the purity of isolates. Pure culture of isolated colonies were biochemically characterized using API 20E, API 20NE (Biomérieux), and following biochemical tests were performed: Gram stain, cytochrome oxidase, catalase, beta-galactosidase, arginine dihydrolase, lysine decarboxylase, ornithine decarboxylase, citrate utilization, H₂S production, urease, tryptophan deaminase, indole production, Voges-Proskauer, gelatinase, fermentation of glucose, mannitol, inositol, sorbitol, rhamnose, sucrose, amygdalin, methyl red, arabinose, lactose, esculin, xylose, mobility, and oxidative / fermentative test. The isolates were identified to the genus or species level based on standard bacterial taxonomic procedures (Austin and Austin, 2007). The isolates were stored in broth

with the addition of 15-20% glycerol at -80°C. For confirmatory identification immunofluorescence (FAT) and PCR were used.

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 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. For virus identification, ELISA, FAT, PCR, RT-PCR and sequencing were performed. 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, USA). RNA was extracted using RNA mini kit according to the manufacturer (QIAGEN, USA). PCR products (for SVC, IPN, ECV) were sequenced directly using Big Dye Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, 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

Each year national surveillance programmes are carried out for viral diseases i.e. infectious haemopoietic necrosis (IHN), viral haemorrhagic septicaemia (VHS), infectious pancreatic necrosis (IPN), spring viremia of carp (SVC), koi herpesvirus (KHV) and the bacterium *Renibacterium salmoninarum*. Testing for other infectious agents was also performed in various research projects, and on private basis, as part of the biosecurity program in some farms.

Infectious pancreatic necrosis (ZNG/IPN) - The disease was first noticed in Serbia in 1989 (Jeremic, 1989) and did not emerge until 2007, when the disease was confirmed at trout farm in Mačva district, in diseased rainbow trout, derived from imported fertilized eggs. The number of IPN affected localities in 2011 and 2012 was similar to previous years i.e. slightly over 10. All rainbow trout outbreaks were experienced during the juvenile stage. The disease is almost certainly under-diagnosed and therefore under-reported.

Viral Hemorrhagic Septicaemia (VHS) - All salmonid aquaculture facilities have been tested for VHS-virus every year as part of a national surveillance programme. The virus has not yet been detected. Serbia has therefore been considered a VHS-virus free zone.

Infectious Hematopoietic Necrosis (IHN) - All salmonid aquaculture facilities have been tested for IHN-virus every year as part of a national surveillance programme. The virus has not yet been detected. Serbia has therefore been considered a IHN-virus free zone.

European Catfish Virus (ECV) - The disease was first noticed in Serbia in 2008 and it is diagnosed every year since then in brown bullhead (*Ameiurus nebulosus*) and black bullhead (*Ameiurus melas*). Diseased fish showed erratic swimming. Slightly swollen abdomen and haemorrhagic spots on the external surface of the fish were present. Internally, liver ischemia, splenomegaly and frequently petechial haemorrhaging of mesenteric fat and internal organs. Based on the sequence analysis of the highly conserved major capsid protein, a positive diagnosis of epizootic hematopoietic necrosis caused by ECV was established.

Sleeping Disease (SD) - Sleeping disease is currently a matter of concern for salmonid fish farmers in most parts of the world. SD is characterized by a very particular "sleeping" behaviour with some of the fish in the affected group resting on their sides at

the bottom of the tank. Histological observations of diseased fish show a chronological appearance of lesions in the pancreas, in the heart, and in the muscle at the last stage of the disease. Suspicious cases with characteristic signs of disease were recorded in two trout farms in 2012, but no virus was isolated, and no PCR positive samples were detected.

Spring Viremia of Carp (SVC) - Acute contagious viral disease caused by the *Rhabdovirus carpio*. Carp of all ages are affected and also other cyprinid fish species (Ahne et al. 2002). 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. No positive cases of SVC were detected during two year period in carp farms in Serbia.

Koi Herpes Virus (KHV) - All cyprinid aquaculture facilities have been tested for KHV-virus every year as part of a national surveillance programme. The virus has not yet been detected. Serbia has therefore been considered a KHV-virus free zone.

Furunculosis is a contagious disease caused by gram-negative bacteria *Aeromonas salmonicida subsp. salmonicida*. In 2011, the disease was diagnosed in fingerlings in five trout farms. In 2012 furunculosis with characteristic clinical symptoms was detected in six trout farms. *Aeromonas salmonicida subsp. salmonicida* was isolated from spleen and kidney of diseased fish on furunculosis agar.

Bacterial kidney disease (BKD) is characterised by a chronic progression with development of granuloma in various organs. In 2011, the disease was diagnosed in four trout farms. In 2012 BKD with characteristic clinical symptoms was detected in three trout farms. Infected fish showed pale gills, exophthalmia, and abdominal distension. Turbid fluid was present in abdominal and pericardial cavities, and creamy-white granulomatous lesions were present in the kidney and, less frequently, in the spleen and the liver. Direct immunofluorescence test and PCR showed the presence of *R. salmoninarum*, a bacterium was isolated from the kidneys of diseased trout in KDM-2 agar.

Yersiniosis - In 2011, yersiniosis was detected in 8, and in 2012 in 11 fish farms. Several outbreaks have been associated with poor quality fish, high biomass, high water temperatures and low water availability.

Red mark syndrome (RMS) - In 2011, RMS was detected in 5, and in 2012 in 7 fish farms. **Other bacterial infections** - Outbreaks of septicemia caused by motile aeromonads were registered in 15 carp farms. All cases were associated with increased mortality. Detection of *Pseudomonas fluorescens* in fish farms is not unusual, and is often associated with poor water quality. In 2011, a higher number of such diagnoses were made compared to previous years and the situation will be monitored to establish whether this bacterium is increasing in virulence. A few cases of infection with *Flavobacterium psychrophilum* were identified in rainbow trout fry.

Parasitic diseases - The following parasites were recorded most frequently: *Trichodina domerguei*, *Chilodonella cyprini*, *Dactylogyrus sp.*, *Gyrodactylus sp.*, *Ichthyophthirius multifiliis*, *Diplostomum spathaceum*, *Caryophyllaeus fimbriceps* and *Eustrongilus sp.*

Fungal diseases - diseases caused by fungus, such as mycotic nephritis, swimbladder mycosis (various fungal spp.) and gill mycosis are registered sporadically. *Sapro-*

legnia sp. in eggs, gills and skin of carp and trout is a not uncommon finding and work continues towards prevention and treatment of these conditions.

Antibiotic resistance - routine testing of fish pathogenic bacteria isolated from fish in aquaculture during 2011-2012 identified new instances of reduced sensitivity to antibiotics licensed for use in Serbian aquaculture. Isolates of *Aeromonas hydrophila*, *Yersinia ruckeri* and *Pseudomonas spp.* with reduced sensitivity to antibiotics continue to be isolated from fish farms.

CONCLUSIONS

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.

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