Abstract - This article describes the research carried out for the detection of viruses responsible for VHS, IHN and IPN diseases in farmed rainbow trout (Oncorhynchus mykiss) in Kosovo for the three-year period between 2006 and 2008. Losses are often reported in trout fingerlings, but no virus has ever been isolated in the rainbow trout in Kosovo. A research project was carried out to determine the occurrence of VHSV, IHNV & IPNV from the samples of fish tissue and ovarian fluids from mature broodfish. In total, 467 fishes from 113 (pools) in 10 rainbow trout aquaculture facilities were screened. Laboratory analysis was performed at the TGD (Tiergesundheitsdienst Bayern e. V) laboratory in Germany using the biomolecular method of RT-PCR and nested-PCR. The Infectious Pancreatic Necrosis virus was detected in seven trout farms, and prevalence from total samples (pools) was 11.5 %. This is the first research and report for IPN virus diagnosis in farmed rainbow trout fry, on-growing fish and broodfish in Kosovo.

Keywords: Rainbow trout, viral diseases, IPN, RT-PCR, nested PCR

Kratak sadržaj - Rad se odnosi na otkrivanje virusa odgovornih za VHSV, IHNV i IPNV bolesti kalifornijske pastrmke na Kosovu tokom trogodišnjeg perioda između 2006-2008. Gubici u mladi su često prijavljivani, mada virus nikad nije izolovan na Kosovu. Istraživanja su provedena da se odredi pojava virusne hemragične septikemije pastrmki (VHSV), zarazne hematopoetske nekroze (IHN) i zarazne nekroze gušterače (IPNV) iz uzoraka tkiva jedinki i uzoraka ovarijsalne tečnosti polno zrelih jedinki. Ukupno 467 riba, iz 113 zbirnih uzoraka, sa 10 ribljih farmi je pretraženo. Laboratorijske analize su izvršene u TGD (Tiergesundheitsdienst Bayern e. V) laboratoriji u Njemačkoj.
Introduction

Table size trout of around 350 g is commonly cultured in the concrete walled raceways with concrete bottoms. In the vast majority of trout farms in Kosovo, the fresh water is sourced directly from the natural springs without undergoing a pre-treatment, maintaining a constant temperature of around 11 °C. Trout farming has become a rapidly growing industry in Kosovo, but several disease problems have developed. Recently, the import of rainbow trout summer eggs to Kosovo has increased, about 500,000 eggs were imported from Germany and the United States of America in the years 2007 and 2008.

Viral hemorrhagic septicemia virus (VHSV) and infectious haematopoietic necrosis (IHNV) are both important disease agents in farmed rainbow trout, and the disease outbreaks cause major losses in fish farming worldwide. Both VHSV and IHNV are viruses of the Rhabdoviridae family, genus Novirhabdovirus (2). Diseases may infect salmonid fish of all ages and cause 80% to 100% mortality (13). Disease outbreaks are mainly in fingerlings less than 6 months of age. Transmission of virus occurs horizontally, however vertical transmission has been evidenced for IHNV (8). Infectious pancreatic necrosis virus (IPNV) belongs to the Birnaviridae family, genus Aquabirnavirus (9), which can cause high mortality in the salmonids. In rainbow trout, only young fish become clinically ill and as such, it has been classified as a disease of first feeding fry (9). The disease can be spread horizontally as well as vertically through ovarian fluids (4, 10). Horizontal spread of IPN disease depends on the age of fish and management/husbandry conditions (13). In farms with IPN outbreaks, lifelong asymptomatic fish with latent infection may carry the virus in adult stages without showing any evidence of disease (9, 12). The highest mortality for all three diseases occurs when the water temperature is between 10 °C to 14 °C (8, 10).

Sano (11) considers viral diseases in fish very serious due to the fact that their diagnosis is difficult, in a significant number of cases they are acute or sub-acute diseases. Noga (8) described viral disease characteristics as a temperature-depended pathogenicity, host-specific, with a higher sensitivity relating to young fish and virus carriers after infection, and without a possibility to be treated adequately by medication. The three viruses investigated in this study all contain a RNA viral genome and therefore RT-PCR (reverse transcriptase PCR) is used for RNA transcription and cDNA amplification (1).

The viral disease status of cultured rainbow trout in Kosovo had been unknown until this research commenced. This study is important for the development of aquaculture and fisheries in Kosovo, from both a fish health and economic aspect.
Material and method

Samples for laboratory analysis were collected from: complete fry visceral organs (liver, kidney and spleen) from table size fish, and ovarian fluid from female broodfish. Pools of 3-5 fishes or organs from the same fish farm have been preserved in plastic tubes in ethanol 70% and isopropanol 98%, and ovarian fluids pools are saved frozen in 1.5 ml tubes. In total, 113 pools were sampled containing 467 fish, from 10 rainbow trout farms over the three year period between 2006 and 2008, in five sampling periods (Table 1).

RNA extraction, reverse transcription and amplification: Total RNA was extracted from the fish tissue collected from the whole fish (fry), pooled organs (liver, kidney, spleen) and ovarian fluids using RNasy Mini Handbook, (Qiagen, June 2001) following Rnasy Mini protocol for isolation of total RNA from animal tissue. RT-PCR mixture contained: Reverse IT Master mix (Thermoprime plus DNA polymerase 1.25U/50 μl, optimized reaction buffer dNTP 0.2 mM, MgCl₂ 1.5 mM, RTase Blend (50U/μl) including RNase inhibitor), 50pmol/μl of sense and antisense primer, 1μl reverse IT Blend, 19 μl distilled water and 3 μl from sample. Nested PCR mixture contained: Master Mix contained Thermoprime plus DNA polymerase 1.25U/50 μl, 75 mM Tris-Cl, 20 mM (NH₄)₂SO₄, 2.5 mM MgCl₂, 0.01% Tween, 0.2 mM of each dATP, dCTP, dGTP and dTTP; 50 pmol/μl of sense and antisense primer, and 3 μl of the amplification products obtained by RT-PCR.

Primers: The aquatic IPNV-specific primers, 1 μl sense primer WB1 (CCGCAACTTATGGATCCTATG) and 1 μl antisense primer WB2 (CGTCTGTTCAGATCCCTGTAGT) which recognize a 206-bp cDNA fragment within the VP2 gene of IPNV (13). The IHNV-specific primers, 1 μl sense primer IHN3 (GTTCACTTCCAACGCAACAGG) and 1 μl antisense primer IHN4 (TGAAGTACCCCACCCCGAGCATCC) recognize a 371-bp cDNA fragment within the N gene of IHNV (13). The VHSV specific primers for RT PCR amplification 1 μl sense VG1 (ATGGGAATGGAACACTTTTT) and 1 μl antisense VGR (TGAGACGTCAGTTCGATTCCGTGAA) which recognize a 1524-bp cDNA fragment within the G gene of VHSV. For Nested PCR primers, 0.5 μl sense VD5 (TCCGTATCATACGTATTCCAGAGG) and 0.5 μl antisense VD3 (TGATCAGGTTCCCTGTGGA) which recognize a 440-bp cDNA fragment within the G gene of VHSV (7).

RT PCR and Nested PCR thermal cycler programs: Reverse transcription of VHSV RNA and amplification of cDNA were performed in a thermal cycler by the following program: 42 °C for 60 min and 95 °C for 1 min followed by 40 amplification cycles of denaturation at 95 °C for 30 s, annealing at 52 °C for 40 s, extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. VHSV nested PCR was performed in a thermal cycler; 95 °C for 1 min, following 25 amplification cycles of denaturation at 93 °C for 30 s, annealing at 52 °C for 40 s, extension at 72 °C for 40 s, and final extension at 72 °C for 5 min. Reverse transcription of IHNV and IPNV RNA and amplification of cDNA were performed in the thermal cycler: 47 °C for 60 min and 94 °C for 5 min followed by 40 amplification cycles of denaturation at 94 °C for 30s, annealing at 60 °C for 30 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min.
On each gel, negative controls from uninfected material and positive controls were run as well.

Gel electrophoresis: PCR products were analysed on a 2% agarose gel by electrophoresis in 120 V for 60 min in TAE buffer (sterile water, Tris, acetic acid and EDTA) loaded with 8 to 10 μl of PCR sample. A PCR marker of 100-bp DNA ladder molecular weight was also loaded and run on each gel. The gels were stained in 1% ethidium bromide and a UV transilluminator (Biorad) was used to visualize the bands, and results were recorded by photography.

Results

Clinical signs: At the time of sampling, a peculiar swimming motion was observed in fry positive with IPNV (2006, 2007 and 2008): fish were swimming on their sides or with slow spiral movements. The fry were also darker in skin pigmentation and had swollen abdomens, as previously described (8, 9). According to the fish farm site managers, mortalities with similar signs had occurred in previous years. During the sampling period, the mortality rate ranged from 10% up to 90% in fry stage. The fish sampled in 2008, weighing about 100 g, resulting as IPNV positive were notably darker in colour and appeared weak and lethargic. No visible signs of disease are seen in broodfish (2006 and 2007) positive with IPN.

Table 1. Number of samples and results of research of IPNV in rainbow trout farms over a three-year period

<table>
<thead>
<tr>
<th>Year</th>
<th>Number of examined</th>
<th>Number of IPNV positive</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Fry</td>
<td>Brood-fish</td>
</tr>
<tr>
<td>Pools</td>
<td>Fish</td>
<td>Pools</td>
</tr>
<tr>
<td>2006</td>
<td>4</td>
<td>20</td>
</tr>
<tr>
<td>2007</td>
<td>1</td>
<td>20</td>
</tr>
<tr>
<td>2008</td>
<td>13</td>
<td>138</td>
</tr>
</tbody>
</table>

The Table 1 shows the results of the 2006, 2007 and 2008 sample number and laboratory results on the fry, fish tissue and ovarian fluid.

PCR results: All samples were analysed for VHSV, IHNV and IPNV, with only IPNV being detected (table 1). From the samples obtained in 2006 from one fish farm, IPNV was detected in 3 of 48 pools. Detection was in fingerlings but not in table size trout or ovarian fluids taken from broodfish.
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Figure 1. First detection of the infectious pancreatic necrosis virus (IPNV) in fingerlings tissue samples by reverse transcription PCR (RT-PCR). Lane 1 and 8: 100 bp ladders (Abgene); lane 2, negative control of RT-PCR; lane 3, negative control of RNA extraction; lane 6 negative IPNV; lanes 4 and 5 positive IPN and lane 7 IPNV-positive control.

Slika 1. Prva detekcija virusa zarazne nekroze gušterače (IPNV) u uzorcima tkiva mladi kalifornijske pastrmke tehnikom RT-PCR (reverse transcription polymerase chain reaction). Trake od 1 do 8 : 100 bp skala (Abgene); traka 2, negativna kontrola RT-PCR tehnike; traka 3 negativana kontrola RNA ekstrakcije; linije 4 i 5 pozitivno na IPN virus; linija 6 negativna kontrola IPN virusa i linija 7 IPN virus pozitivna kontrola.

From the samples in the year 2007 from 51 pools, 8 pools were positive for IPNV on 5 trout farms. In 2008, from 14 pools, IPNV was detected in two pools from two trout farms. The total number of IPNV positive pools over the three year investigation was 13, or prevalence was 11.5%. Seven of the 10 trout farms (70.0%) investigated had at least one IPNV positive pool. In all cases with IPNV positive results from fry, increased mortalities were reported on site (personal communication), and signs of IPN disease outbreaks were observed. In broodfish screened as IPNV positive carriers, no clinical signs of IPN disease were observed. In all fish farms where IPN virus was detected by PCR, the water temperatures ranged from 10 °C to 11 °C. VHSV and IHNV were not detected from any of the fish screened and no gross external signs of VHS and IHN disease were observed during this study.

Discussion

The aim of this study was to provide an overview on the status of virus presence in rainbow trout in Kosovo. In five surveys of farmed rainbow trout in Kosovo in period 2006-2008, a high number of sites with virus-positive occurrence is found among the fish farms investigated (total 7 sites). Within the 113 tissue samples analyzed, 13 were positive for IPNV, mostly from rainbow trout fingerlings and reproductive fluid.
The importance of making accurate and rapid IPNV detection is significant due to the high number of losses, which can be associated with IPNV outbreaks and also because survivors may become lifelong asymptomatic carriers, continuously shedding virus in their faeces, urine and reproductive fluids: transmitting it to their progeny and/or other susceptible fish. The finding of IPNV in broodfish supports the hypothesis of latent carriers for IPNV infections in rainbow trout without signs of disease (4, 10). Milne et al. (6) has reported that growth performance is negatively affected in carrier populations, which is another very important economical factor concerning IPNV.

Recently, the fish farmers have been importing eggs from the European countries and some live fish movements occurred from the neighbouring countries (including Bosnia and Herzegovina) within the region. Similar to other Balkan countries, namely Turkey and Greece, IPNV was detected in fry and fingerling stages (3, 12).

In none of the trout farms where virus was detected from broodfish, was IPNV detected from fry or fingerlings. Based on these results, it may be deduced that the most probable spread of IPN virus in Kosovo is more likely to be due to horizontal transmission with regard to equipment, movement of ova/fish and by the fish farm personnel/visitors.

This RT-PCR method proved to be a rapid, reliable and sensitive technique for the identification of the IPN virus from infected fish tissue showing clinical signs and from IPNV infected ovarian fluids. Lopez-Vazquez et al. (5) reported that RT-PCR has become a commonly used tool for detection of viral fish pathogens. Williams et al. (13) reported the successful amplification of IPNV from virus suspension using the WB1 and WB2 primer set used in our research.

Unfortunately, in the absence of very efficient prevention and control measures, IPN disease may cause high loses in trout farms and be very difficult to eradicate. It is ultimately a time to perform regular health checks on the trout farms, which include routine inspection of broodfish without neglecting asymptomatic carriers of virus with lower titres of virus, as reported in Milne et al. (6). This will minimise the spread of IPNV when fish are moved to different locations, and wild trout infections.

The trout farms where IPNV has been detected are situated in three different regions in Kosovo. One of the trout farms that tested IPNV positive has a water source from an adjacent freshwater river system and the rest of the farms have direct water inlet from the natural springs, with no other fish present within inlet water environment. All water outlets without any treatments are connected to the river systems.

Conclusion

This is the first research carried out for detection of IPNV from farmed trout fingerlings, on-growing fish and broodfish ovarian fluids.

The main conclusion that can be drawn from this study is that IPNV is present in trout farms in Kosovo with a significant occurrence.

IPNV was detected on fingerlings with clinical signs of IPN disease, and in broodfish as a latent infection.

This RT-PCR proved to be a rapid and sensitive technique for the identification of the IPNV from the infected fish tissue and ovarian fluids.
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