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Study of viral diseases in some freshwater fish in the Republic of Kosovo

Agim Rexhepi[1]*, Kristaq Bërxholi[2], Peter Scheinert[3], Afrim Hamidi[1], and Kurtesh Sherifi[1]

[1]*Faculty of Agricultural and Veterinary, Prishtina, Kosovo*

[2]*Agricultural University of Tirana, Faculty of Veterinary Medicine, Albania*

[3]*Animal Health Service (Tiergesundheitsdienst -TGD), Bayern E. V., Germany*

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ABSTRACT

This research was carried out to study the occurrence of viruses causing diseases in fish, namely Viral Haemorrhagic Septicaemia (VHS), Infectious Haematopoietic Necrosis (IHN), Infectious Pancreatic Necrosis (IPN) in wild brown trout and Spring Vireaemia of Carp (SVC) in carp and silver crucian carp in Kosovo. Laboratory analyses were performed at the animal health service laboratory in Munich in Germany using the biomolecular method of RT-PCR and nested-PCR. Sampling sites included one carp farm, one lake, one river for common carp, as well as natural habitats from five rivers populated with brown trout in Kosovo. In the period from 2006 to 2008, 255 fish organs and whole fish (fry) were collected and pooled (five fish per pool) in 51 pools. The results of the laboratory analysis from the research showed that 25% (8 of 32) of brown trout sampled were IHNV positive in pools originating from three rivers and 3.1% (1 of 32) were IPNV positive from one river. Pools of cyprinids were 15.7% (3 of 19) SVCV positive originating from one carp farm. These results are indicative of a significant distribution of IHNV in brown trout, the presence of IPNV in brown trout and SVCV in cyprinids.

Key words: viral diseases, brown trout, carp, PCR, Kosovo

Introduction

Viral diseases in fish are very serious due to the fact that their diagnosis is difficult and in a significant number of cases they are acute or sub-acute diseases (SANO, 1995).

VHSV and IHNV are viruses of the *Rhabdoviridae* family, genus *Novirhabdovirus* (BRUDESETH et al., 2002). Both viruses may infect salmonid fish of all ages and can cause

*Corresponding author:

Dr. Agim Rexhepi, DVM, MSc, PhD, Department of Veterinary Medicine, Faculty of Agriculture and Veterinary in Prishtina, Kosovo, Phone: +377 44 171 780; E-mail: agimrexhepi@yahoo.com

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80% to 100% mortality (WILLIAMS et al., 1999). Disease outbreaks are found mainly in fingerlings under 6 months of age. Transmission of viruses occurs horizontally, however vertical transmission has been evidenced for IHNV (NOGA, 2000). The mortality rate of brown trout after IHNV infection was no more than 50%, reported in a study by LA PATRA and FRYER (1990).

Infectious pancreatic necrosis virus (IPNV) belongs to the *Birnaviridae* family, genus *Aquabirnavirus* (ROBERTS, 2001). The disease can be spread horizontally as well as vertically through ovarian fluids (LOPEZ-LASTRA et al., 1994; SAINT-JEAN et al., 2001). In farms with IPN outbreaks, lifelong asymptomatic fish with latent infection may carry the virus in adult stages without showing any evidence of disease (NOGA, 2000; SAINT-JEAN et al., 2001). Infectious pancreatic necrosis virus (IPNV) and infectious haematopoietic necrosis virus (IHNV) are both important disease agents, and disease outbreaks cause losses in fish farming worldwide among wild and cultured salmonid fish (LA PATRA et al., 2001).

Spring viremia of carp virus (SVCV) is a fish rhabdovirus that causes severe disease in wild and cultured common carp (*Cyprinus carpio*) with a very high mortality rate among infected fish. SVCV is a member of the family *Rhabdoviridae*, genus *Vesiculovirus* (ORESHKOVA et al., 1999).

VHSV, IHNV and SVCV have been registered in the list of contagious diseases notifiable to the OIE (Office International des Epizooties (2011): Diseases notifiable to the OIE. http://www.oie.int/eng/maladies/en_classification2011.htm?e1d7), and diagnostic tests have been described in the OIE manual of diagnostic tests for aquatic animals (Office International des Epizooties (2010): Diagnostics manual for aquatic animal diseases, http://www.oie.int/eng/normes/fmanual/A_summry.htm).

In Kosovo, prior to this study, which began in 2006, no fish disease research had been carried out and there was no available information based on fish virus diagnosis. During the same time period of this research project, fish viral diseases in Kosovo were reported in REXHEPI et al. (2009), where a high number of IPNV-positive farmed rainbow trout sites were identified. 11.5% (13 of 113 samples) of the IPNV positive pools were detected, originating from 7 sites (7 from 13 sites) or 53.8%, with no VHSV or IHNV detected in rainbow trout.

The main purpose of this study was to determine the occurrence of VHSV, IHNV and IPNV in brown trout and SVCV in the common carp and silver crucian carp.

Materials and methods

Fish sampling. Samples were collected from fish internal organs (average weight 120 g) and whole fish or fry (average weight 5 g). Sections of liver, spleen, kidney from each fish were taken and pooled (five fish per pool) in a small plastic tube. This was carried

out on three separate occasions in the period from 2006 to 2008. A total of 32 tissue pools originating from brown trout and 19 cyprinids (13 common carp, 6 silver crucian carp) were sampled. The samples were kept in ethanol 70% and isopropanol 98%.

The sample sites for cyprinids were a carp farm, Lake Badovci and River Drini for common carp (*Cyprinus carpio)* and a carp farm (unwanted fish species on a fish farm) for silver crucian carp (*Carassius carassius gibelio*). The brown trout (*Salmo trutta*) sample sites were natural habitats selected throughout the country to include all major rivers as follows; Drini (spring), Lumëbardhi i Pejës, Lumëbardhi i Deçanit, Lepenci and Neredimes. Brown trout were caught by electrofishing with a backpack electroshocker (Hans Grassl IG200/2, 150 - 200 V).

RNA extraction, reverse transcription and cDNA amplification. Total RNA was extracted from 30 mg fish tissue, from pooled organs (liver, kidney, spleen) or from whole fish (fry) using RNasy Mini Handbook, (Qiagen).

The RT-PCR mixture (Abgene) contained: Reverse IT Master mix (Thermoprime plus DNA polymerase 1.25 U/50 µL, optimized reaction buffer dNTP 0.2 mM, $MgCl_2$ 1.5 mM, RTase Blend (50 U/µL) including RNase inhibitor), 1 µL (50 pmol/µL) of sense and antisense primer, 1µL reverse IT Blend, 19 µL distilled water and 3 µL from sample. The nested PCR mixture (Abgene) contained: Master Mix contained Thermoprime plus DNA polymerase 1.25 U/50 µL, 75 mM Tris-HCl, 20 mM $(NH_4)_2SO_4$, 2.5 mM $MgCl_2$, 0.01% Tween, 0.2 mM of each dATP, dCTP, dGTP and dTTP; 0.5 µL (50 pmol/µL) of sense and antisense primer and 3 µL of the amplification products obtained by RT-PCR.

Primers. Primers (Abgene) were selected on the basis of published sequences of the cDNA of virus genome (Table 1).

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 a thermal cycler: 47 °C for 60 min and 94 °C for 5 min followed by 40 amplification cycles of denaturation at 94 °C for 30 s, annealing at 60 °C for 30 s, extension at 72 °C for 90 s, and final extension at 72 °C for 10 min.

Table 1. The primers used for VHSV, IHNV, IPNV and SVCV detection

PCR	Sequences	Base pairs	Reference
VHS RT	5'- ATGG GAA TGG AAC ACT TTT TTC- 3' 5' - TCA GAC CGT CTG ACT TCT GGA- 3'	1524 bp	MILLER et al. (1998)
Nested	5'- TCC CGT TAT CAG TCA CCA G - 3' 5'- TGT GAT CAT GGG TCC TGG TG - 3'	440 bp	
IHN RT	5' GTTCAACTTCAACGCCAACAGG -3' 5' TGAAGTACCCCACCCCG AGCATCC 3'	371 bp	WILLIAMS et al. (1999)
IPN RT	5' CCGCAACTTACTTGAGATCCATTATGC - 3' 5' CGTCTGGTTCAGATTCCACCTGTAGTG - 3'	206 bp	WILLIAMS et al. (1999)
SVC RT	5'-GCC TAA ATG TGT TGA TGG AAC G-3' 5'-GGA TAA TAT CGG CTT GGA AAG C-3'	470 bp	KOUTNA et al. (2003)
Nested	5'-CAA GAG AAG CTG ACA TCA GTG G-3' 5'-GAC AAT AGG TCC CTC TAC TTC G-3'	141 bp	

Reverse transcription of SVCV RNA and amplification of cDNA were performed in a thermal cycler by the following program: 47 °C for 45 min and 95 °C for 2.5 min followed by 35 amplification cycles of denaturation at 94 °C for 30 s, annealing at 50 °C for 1 min, extension at 72 °C for 1min, and final extension at 72 °C for 7 min. SVC nested PCR was performed in a thermal cycler; 92 °C for 2 min, following 25 amplification cycles of denaturation at 95 °C for 30 s, annealing at 55 °C for 30 s, extension at 72 °C for 30 s, and final extension at 72 °C for 7 min

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. On each gel, negative controls from uninfected material and positive controls were also run.

Laboratory analyses were performed at the animal health service laboratory in Munich in Germany.

Results

From the 32 pools of brown trout, only one pool (3.1%) was IPNV positive, and eight pools were IHNV positive (25.0%). No positive pool with VHSV was detected. In

pools from cyprinids (13 common carp, 6 silver crucian carp), three positive pools were identified (15.8%), two in common carp and one in silver crucian carp.

Clinical signs. At the time of sampling, no visible signs of disease are seen in IHNV-positive and IPNV-positive brown trout or SVCV-positive cyprinids (common carp and silver crucian carp).

Laboratory results. All brown trout samples were analyzed for VHSV, IHNV and IPNV, with IHNV and IPNV being detected. IHNV was detected in eight tissue pools (brown trout), in three sampling sites namely the Neredimes (2 positive pools), Lepenci (3 positive pools) and Lumëbardhi i Pejës (3 positive pools) rivers (Fig.1). Only one pool was IPNV positive from the Lumëbardhi i Pejës river (Fig. 2).

From the cyprinids samples, 3 pools were identified as positive for the SVC virus. SVCV was detected in two pools collected containing market size common carp (about 1.5 kg) and one pool from silver crucian carp (Fig. 3). SVCV was not detected from any of the fish screened from either Lake Badovci or the River Drini.

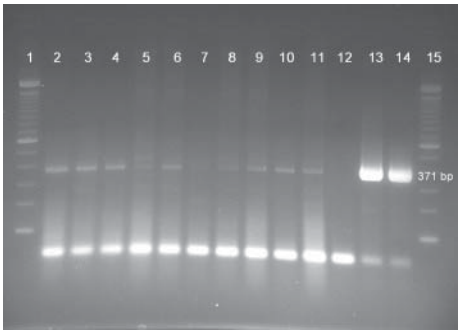

Fig. 1. Detection of the infectious haematopoietic necrosis virus (IHNV) in brown trout samples by reverse transcription PCR (RT-PCR). Lane 1 and 15: 100 bp ladders (Abgene); lane 2, 3, 4, 6, 8, 9, 10, 11 positive IHNV; lane 7 negative IHNV; lane 12 negative control of RT-PCR; lane 13 and 14, positive IHNV-positive control at 371 bp.

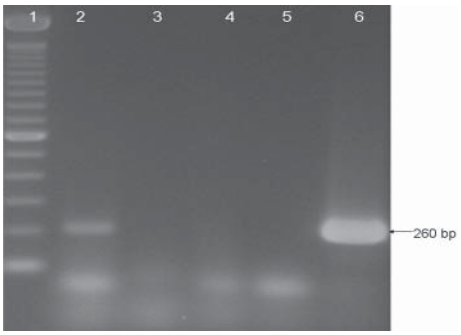

Fig. 2. Detection of the infectious pancreatic necrosis virus (IPNV) in brown trout by reverse transcription PCR (RT-PCR). Lane 1 and 6: 100 bp ladders (Abgene); lane 2, positive IPN; lane 3 and 4 negative IPNV; lane 5 negative control; and lane 6 IPNV-positive control at 260 bp.

Fig. 3. Detection of the spring viremia of carp virus (SVCV) in cyprinids by reverse transcription PCR (RT-PCR). Lane 1 and 16: 100 bp ladders (Abgene); lane 2 and 3 negative PCR control; lane 4, SVCV positive in silver crucian carp; lane 6 and 7 SVCV positive in common carp; lane 5, 7-14 negative SVCV; lane 15 SVCV positive control at 141 bp.

Discussion

The aim of this study was to obtain an overview of the presence of VHSV, IHNV and IPNV in wild brown trout and SVCV in common carp and silver crucian carp during the period from 2006 to 2008 in Kosovo.

The detection of IHNV in 25.0% of pools originating from three rivers is indication of a significant distribution of viruses in wild brown trout in Kosovo rivers. A mortality rate of up to 50% in IHN disease outbreaks in brown trout is reported (LA PATRA and FRYER, 1990). The presence of IHNV in brown trout is reported to have a significant impact on commercial and recreational fishing activities as well as efforts to rebuild threatened or endangered fish stocks (MORZUNOV et al., 1995). Contact between farmed and wild fish is inevitable, therefore the presence of IHNV on wild brown trout should be considered as a permanent threat to the health of farmed rainbow trout, because of the high mortality rate of 80% to 100% (WILLIAMS et al., 1999) in disease outbreaks.

The IPNV-positive sample from wild brown trout detected in this survey was taken from the same water body (river) where IPNV was detected in samples taken from farmed rainbow trout reported in REXHEPI et al. (2009). The wild fish were either previously infected by a virus released from an infected farm or infected with a virus originating from free ranging fish, representing a potential source of re-infection for the farmed fish.

IPNV in wild salmonid species has not been reported as a serious problem (EVENSEN et al., 2003).

Both IHNV and IPNV were detected in brown trout asymptomatic carriers, with no visible sign of disease. The spread of IPNV from infected fish in farms to wild fish populations may be assumed and as such brown trout may become asymptomatic carriers and a reservoir for fish viruses (EVENSEN et al., 2003). The previous year's fish fingerlings from IPNV positive farms reported in REXHEPI et al. (2009) were used by sport fishermen for fish stocking of rivers and lakes (personal communication).

The clinical signs for SVC disease have been reported in previous years on a detected SVCV positive carp farm (personal communication), but no laboratory analysis was carried out. SVC outbreaks are most common in farmed carp but can also occur in wild carp as reported by GRAVER et al. (2007). SVCV has caused serious losses in carp farming in central and eastern parts of Europe, as well as mass mortalities in wild common carp in North America. The SVCV in this study was detected in asymptomatic carrier fish reported previously from AHNE et al. (2002). The results showed that besides common carp, other cyprinid species, such as silver crucian carp, are a potential asymptomatic carrier species for SVCV.

Virus asymptomatic carrier fish may introduce the virus into healthy stocks. Fish of different sizes from the SVCV positive farm in this study are used by sports fishermen for fish restocking of rivers and lakes (personal communication), and this is an important way for the virus to spread to the wild carp population.

The RT-PCR and Nested PCR method proved to be a rapid, reliable and sensitive technique for the detection of the viruses from asymptomatic carrier fish. Similar results were reported previously by KOUTNA et al. (2003), and LOPEZ-VAZQUEZ et al. (2006).

These results are indicative of the significant distribution of IHNV in brown trout, the presence of IPNV in brown trout and SVCV in cyprinids. Restocking measures carry the risk of virus introduction via carrier farmed fish into wild fish populations, especially if the fish originate from a farm of unknown virus status. The results of this research indicate that it is finally time to perform regular health checks on farmed and wild fish, which should include the routine inspection of brood fish, without neglecting asymptomatic carriers of the viruses. This will minimize the spread of fish viruses when fish are moved to different locations and minimize wild fish infections.

References

AHNE, W., H. V. BJORKLUND, S. ESSBAUER, N. FIJAN, G. KURATH, J. R. WINTON (2002): Spring viremia of carp (SVC). Dis. Aquat. Org. 52, 261-272.

BRUDESETH, B. E., J. CASTARIC, Ø. EVENSEN (2002): Studies on pathogenesis following single and double infection with viral hemorrhagic septicemia virus and infectious hematopoietic necrosis virus in rainbow trout (*Oncorhynchus mykiss*). Vet. Pathol. 39, 180-189.

EVENSEN, Ø., B. SKJELSTAD, E. RIMSTAD, E. BRUN, L. JOHANSEN, R. STAGG, P. MIDTLYNG, I. JENSEN (2003): IPN in salmonids a review. FHL und VESO, pp. 118.

GRAVER, K. A., A. G. DWILOW, J. RICHARD, T. F. BOOTH, D. R.BENIAC, B. W. SOUTER (2007): First detection and confirmation of spring viraemia of carp virus in common carp, *Cyprinus carpio* L., from Hamilton Harbour, Lake Ontario, Canada. J. Fish. Dis. 30, 665-671.

KOUTNA, M., T. VESELY, I. PSIKAL, J. HULOVA (2003): Identification of spring viraemia of carp virus (SVCV) by combined RT-PCR and nested PCR. Dis. Aquat. Org. 55, 229-235.

LA PATRA, S., J. L. FRYER (1990): Susceptibility of brown trout (*Salmo trutta*) to infectious hematopoietic necrosis virus. Bull. Eur. Ass. Fish Pathol. 10, 125-127.

LA PATRA, S. E., W. N. BATTS, K. OVERTURF, G. R. JONES, W. D. SHEWMAKER, J. R. WINTON (2001): Negligible risk associated with the movement of processed rainbow trout, *Oncorhynchus mykiss* (Walbaum), from an infectious haematopoietic necrosis virus (IHNV) endemic area. J. Fish. Dis. 24, 399-408.

LOPEZ-LASTRA, M., M. GONZALEZ, M. JASHES, M. SANDINO (1994): A detection method for infectious pancreatic necrosis virus (IPNV) based on reverse transcription (RT)- polymerase chain reaction (PCR). J. Fish Dis. 17, 269-282.

LOPEZ-VAZQUEZ, C., C. P. DOPAZE , J. G. OLVEIRA, J. L. BAJRA, I. ANDIN (2006): Development of a rapid, sensitive and non-lethal diagnostic assay for the detection of viral haemorrhagic septicaemia virus. J. Virol. Methods 133, 167-174.

MILLER, T.A., J. RAPP, U. WASLHUBER, R. W. HOFFMAN, P-J. NZMANN (1998): Rapid and sensitive reverse transcriptase-polymerase chain reaction based detection and differential diagnosis of fish pathogenic rhabdoviruses in organ samples and cultured cell. Dis. Aquat. Org. 34, 13-20.

MORZUNOV, S. P., J. R. WINTO, S. T. NICHOL (1995): The complete genome structure and phylogenetic relationship of infectious hematopoietic necrosis virus. Virus Res. 38, 175-192.

NOGA, E. J. (2000): Fish disease diagnosis and treatment. Iowa State University Press, USA, pp. 330.

ORESHKOVA, S. F., I. S. SHCHELKUNOV, N. V. TIKUNOVA, T. I. SHCHELKUNOVA, A. T. PUZYREY, A. A. ILYICHEV (1999): Detection of spring viremia of carp virus isolates by hybridization with non radioactive probes and amplification by polymerase chain reaction. Virus Res. 63, 3-10.

REXHEPI, A., P. SCHEINERT, K. BERXHOLI, A. HAMIDI, K. SHERIFI (2009): Occurrence of infectious pancreatic necrosis (IPNV) in farmed rainbow trout (*Onchorhynchus Mykiss*) in Kosovo. Veterinaria 58, 47-53.

ROBERTS, R. J. (2001): Fish Pathology. W. B. Saunders, London, pp. 473.

SAINT-JEAN, S. R., J. J. BORREGO, S. I. PEREZ-PRIETO (2001): Comparative evaluation of five serological methods and RT-PCR assay for the detection of IPNV in fish. J. Virol. Methods 97, 23-31.

SANO, T. (1995): Viruses and viral diseases of salmonids. Aquaculture 132, 43-52.

WILLIAMS, K., S. BLAKE, A. SWEENEY, J. T. SINGER, B. L. NICHOLSON (1999): Multiplex reverse transcriptase PCR assay for simultaneous detection of three fish viruses. J. Clin. Microbiol. 37, 4139-4141.

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SAŽETAK

U radu je istražena prisutnost virusne hemoragijske septikemije, zarazne hematopoetske nekroze i zarazne nekroze gušterače u potočne pastrve te proljetne viremije šarana u šarana i bijelog glavaša na Kosovu. Uzeti materijal bio je pretražen RT-PCR-om i ugniježđenim PCR-om u laboratoriju službe za životinjsko zdravlje u Münchenu u Njemačkoj. Uzorci šarana i bijeloga glavaša bili su prikupljeni na šaranskom ribnjačarstvu, na jezeru i rijeci, a uzorci potočne pastrve na pet rijeka. U razdoblju od 2006. do 2008. bilo je prikupljeno 255 uzoraka organa i cijelih riba (mlađ) te je po pet uzoraka bilo spojeno u 51 skupni uzorak. Rezultati laboratorijskih pretraga pokazali su da je u skupnim uzorcima potočne pastrve uzetima na trima rijekama 25% (8 od 32) uzoraka bilo pozitivno na virus zarazne hematopoetske nekroze, a na virus zarazne nekroze gušterače bilo je pozitivno 3,1% (1 of 32) iz jedne rijeke. Od skupnih uzoraka organa ciprinida uzetih na ribnjačarstvu, ukupno je 15,7% (3 of 19) bilo pozitivno na virus proljetne viremije šarana. Rezultati ukazuju na znatnu proširenost virusa zarazne hematopoetske nekroze i zarazne nekroze gušterače u potočne pastrve te virusa proljetne viremije šarana u ciprinida.

Ključne riječi: virusne bolesti, potočna pastrva, šaran, lančana reakcija polimerazom, Kosovo