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First Detection of Spring Viremia Of Carp Virus in Cyprinus Carpio, In Lake Shkodra/Scadar Albania

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ABSTRACT: Spring viremia of carp is a highly contagious viral disease caused by *Rhabdovirus carpio* which is a single strand RNA virus. A combination of one step RT-PCR (reverse transcription) and Semi-nested PCR was used to detect spring viremia of carp virus in infected cell cultures (EPC) and fish tissues. All the fish samples were collected from Lake Shkodra and from artificial ponds in different region of Albania. Total RNA was extracted from 500 µl of fish tissue extract or from 100 µl of supernatant from infected EPC cell cultures. One step RT-PCR is performed using a modification of the methods of Stone *et al.*(1) in a 15 µl reaction mix volume. We used SVCV F1 (primer forward) 5'- TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' and SVCV R2 (primer reverse) 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-CAN*-CAY*-3 primers for the amplification of 714 bp fragment of SVCV cDNA (2). The reaction mix was subjected to thermal cycles (2). Amplified cDNA was analyzed in agarose gel electrophoresis. For the second round of amplification we used a semi-nested PCR with SVCV F1 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' and SVCV R4 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3* primers (1). Amplified fragment cDNA (606 bp) is analyzed by agarose gel electrophoreses (2).The results of gel electrophoreses was positive for three fish samples (*Cyprinus carpio*) collected from Lake Shkodra. This is the first report of the detection of spring viremia of carp virus in Albania in a natural habitat like Lake Shkodra.

KEYWORDS: SVCV, RT-PCR, Semi-nested PCR, cDNA, gel electrophoreses.

I. INTRODUCTION

Lake Shkodra/Skadar (named respectively in littoral countries) is located on the border between Albania and Montenegro at latitude 40º 10' N, longitude 19º 15' E. The lake, which is located in karst terrain on the outer part of the southeastern Dinaric Alps (Fig. 1). The lake is the largest of the Balkan lakes and has a surface area that fluctuates seasonally from approximately 370 to 600 km2. Its water level also varies seasonally from 4.7 m (summer) to 9.8 m (winter) above sea level. The mean depth of the lake is 5 m and the total volume is 1890x106 m3. The direct drainage basin of Lake Shkodra is 5490 km^2, of which 4460 km^2 are in Montenegro and 1030 km^2 are in Albania. The largest inflow is the Morača River (Montenegro), which provides more than 62% of the lake water. The outlet river is the Buna River, which converges with the River Drin just a few hundred meters downstream of the outlet. The Drin has a total drainage area of about 14,000 km^2, which is almost three times larger than the catchment of Lake Shkodra. It is therefore not surprising that during high water discharges the Drin system impedes the outflow from the lake. Lake Shkodra is a subtropical water body lying in an area that has an extremely high evaporation rate. The considerable amount of precipitation, limited mostly to winter months, coupled with high summer temperatures contributes to chemical weathering and the development of the karst landscape in the area. Mountains rise steeply from the southwestern shores of the lake, while its northern and northeastern shores are flat, thus providing an extensive semi-littoral zone with dense macrophyte cover. Frequent winds and shallow depths do not permit the formation of permanent thermal stratification. The lake is regarded as mesotrophic with a tendency to become eutrophic in the summer months. Lake Shkodra has pronounced water-level fluctuations (high levels during winter and low during summer), which has resulted in a large wetland area sustaining rare and endangered bird species. In 1996, Lake Shkodra was included in the Ramsar list of wetlands of international importance by the Ramsar Convention on Wetlands.

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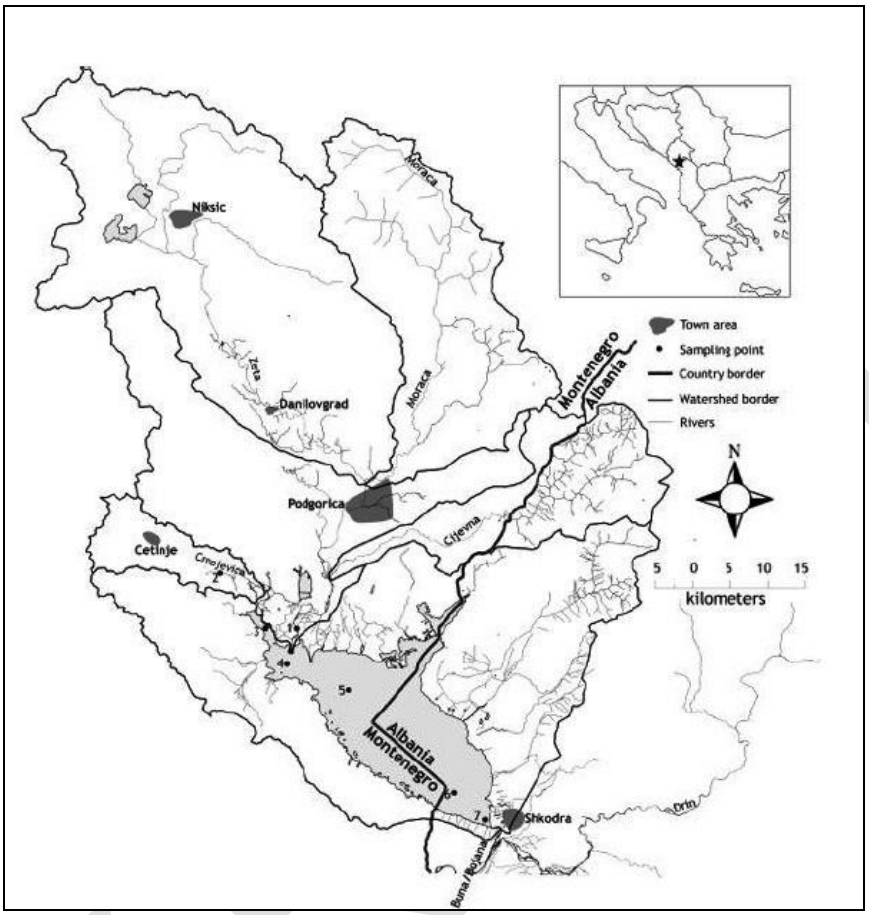

Fig. 1. Transboundary Shkodra/Scadar Lake (Albania-Montenegro border)

The effects of spring viraemia of carp (SVC), a rhabdoviral disease of cyprinids which manifest as an acute haemorrhagic and highly contagious viraemia, has been recognized by the European carp farmers many decades ago [1] [2]. Following the history of disease trucking the disease affects predominantly common carp (*Cyprinus carpio*). Among the Albanian fish farmers the carp has been expanding in the last five decades almost in all territories, while in the naturala big lakes it has well established populations, forming in some like Shkodra/Scadar Lake up to 90% of yearly fish production [3]. Besides common carp, SVC infections have been detected in crucian carp (*Carassius carassius*), sheatfish (*Silurus glanis*), silver carp (*Hypophthalmichthys molitrix*), bighead carp (*Aristichthys nobilis*), and tench (*Tinca tinca*) [4] [5] [6] [7]. All these species has been introduced in Albanian in the last decades [8]. Accordingly, there is a high probability that the other species of Cyprinidae may also be susceptible to this infection under particular conditions. The disease occurs during spring at water temperatures between 10°C to 17°C, affecting fish of all age categories independent of their health status, virulence of the infectious agents, the environment and fish density [9] [10] [11] [12] [13] [14] [15]. Susceptibility of a fish population to the infectious agent is influenced by low humoral immunity. At higher temperatures, the infected carp develop humoral antibodies that can neutralize the spread of virus and protect against re-infection. The disease is generally associated with high mortality; often approaching 70% in young fish (Ahne et al., 2002). Consequently, introduction of the virus into a commercial hatchery or aquaculture facility can have profound economic effects. SVCV has been registered in the List of Contagious Diseases notifiable to the OIE (see www.oie.int), and diagnostic tests have been described in the OIE Manual of Diagnostic Tests for Aquatic Animals [16].

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II. MATERIALS AND METHODS

The main objective of our study was to detect SVCV in common carp (*Cyprinus carpio*) samples collected from Lake Shkodra and artificial pond culture from different region of Albania like Elbasan, Fier, Tapize by using molecular methods like RT-PCR (reverse transcriptase) and semi-nested PCR.
Virus isolate SVCV S30 used as positive control was kindly provided by Dr. D.Stone of the Centre for Environment, Fisheries and Aquaculture Science (Cefas).

From the fish samples we extracted liver, kidney and spleen. Organs homogenate were centrifuged at 3000 ×g for 15 min at 4 °C. We used 500 µl of the supernatants for the total RNA extraction. Also we make a serial tenfold dilution of organs homogenate supernatants to inoculate to a 24 hours EPC cell monolayer. For the EPC cell we use at first L-15 medium with 7.5 % fetal bovine serum. After 24 hours we drain the cell monolayer from their medium and we add an appropriate amount (200 µl) of the serial dilution of the organ homogenate that we made from every samples. For the tenfold serial dilution we use 20 µl of organ homogenate treated with 1% antibiotic and 180 µl of L-15 medium with 2% fetal bovine serum. After one hour of adsorbtion at 15°C. After we withdraw the inoculum we add in the cell monolayer 500µl of L-15 medium with 2% of fetal bovine serum. It was incubated at 20°C. Inoculated cell culture were observed over a period of time between 4 to 7 days. We observed the first cytopathic effect after 24 hours. Cytopathic effect was observed in same samples collected from Lake Shkodra (samples number 5, 32 and 44) and from artificial pond in Elbasan (samples number 47 and 51)

The total RNA was extracted from 500µl of supernatant of fish organs homogenate or from 500µl of supernatant from cell culture exhibiting cytopathic effects (CPE). The method used for RNA extraction is SIGMA GEL-Elute Total RNA Mammalian Extraction Kit.

For the Reverse-Transcription polymerase chain reaction (RT-PCR) we use primers deriving from the region deriving from glycoprotein gene: 5'- TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' primer forward (SVCV F1) and 5'-AGA-TGG-TAT-GGA-CCC-CAA-TAC-ATH*-CAN*-CAY*-3, primer reverse (SVCV-R2)(1). The one step RT-PCR thermal cycles protocol consisted of: First stage 30 min at 45 °C and 2 min at 95°C; Second stage 40 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C; Third stage 10 min at 72 °C (2). The RT-PCR product (amplified DNA 714 bp fragment) was visualized using GelRed™ with 1% agarose gel electrophoresis

For the second round we performed a Semi-Nested PCR using primers: 5'-TCT-TGG-AGC-CAA-ATA-GCT-CAR*-R*TC-3' (SVCV F1) and 5'-CTG-GGG-TTT-CCN*-CCT-CAA-AGY*-TGY*-3* (SVC R4) (1). The thermal cycle's protocol consisted of: First stage 30 min at 45 °C and 2 min at 95°C; Second stage 40 cycles of 1 min at 95°C, 1 min at 55°C and 1 min at 72°C; Third stage 10 min at 72 °C (2). The Semi-Nested PCR product (amplified DNA 606 bp fragment) was visualized using GelRed™ with 1% agarose gel electrophoresis (2).

III. RESULTS

The total number of samples analyzed with RT-PCR and semi-Nested PCR was 75. Only three of them show a positive result of Semi-Nested PCR. These samples were all collected in Lake Shkodra. Samples labeled number 5, 6 and 7 show no signs of SVC disease. We observed a full lysis of the EPC cell monolayer when we infected them (EPC cell) with the supernatant of the organ homogenate (liver, spleen and kidney) of samples number 5. Cell culture infected with the supernatant of the samples number 6 and 7 did not exhibiting cytopathic effects (CPE).

Table 1: Isolation of the spring viremia of carp virus in same samples using EPC cell culture. The virus SVCV was confirmed by RT-PCR and Semi-Nested PCR.

Sample	**1° passage**	**2° passage**	**Tissue**	
			RT-PCR	Nested PCR
1	1:10 Toxic effect 1:100	negative	Negative	
2	negative	negative	Negative	

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3	negative	negative	Negative	negative
4	negative	negative	Negative	negative
5	Full lysis PCR neg nPCR pos	negative	Negative	positive
6	1:10 Toxic effect 1:100	negative	Negative	positive
7	negative	negative	Negative	positive
8	negative	negative	Negative	
9	negative	negative	Negative	negative
10	negative	negative	Negative	
11	negative	negative	Negative	
12	negative	negative	Negative	
13	partial cell detachment	negative	Negative	
14	negative	negative	Negative	Negative
15	negative	negative	Negative	
23	negative	negative	Negative	
24	negative	negative	Negative	
25	negative	negative	Negative	
26	negative	negative	Negative	
31	negative	negative	Negative	Negative
32	negative	mild lysis PCR neg nPCR neg	Negative	Negative
33	negative	negative		
34	negative	negative	Negative	
35	negative	negative	Negative	
36	1:10 Toxic effect 1:100	negative	Negative	
37	negative	negative		
38	slight alteration of the monolayer	negative	Negative	Negative
39	negative	negative	Negative	
44	negative	mild lysis PCR neg nPCR neg	Negative	Negative
47	negative	mild lysis PCR neg nPCR neg	Negative	Negative
48	negative	negative	Negative	
49	negative	negative	Negative	
50	negative	negative		
51	negative	mild lysis PCR neg nPCR neg	Negative	Negative
58	negative	negative	Negative	Negative

Spring viremia of carp virus was isolated in EPC cell culture exhibiting a cytopathic effect when we infected them with the supernatant of the organ homogenate for each samples. Only samples labeled number 5, 36, 44, 47, 51 exhibit a cytopathic effect CPE. Then the isolate virus was identified with molecular biological methods such as RT-PCR and Semi-Nested PCR. The result of the gel-electrophoresis for the semi-nested PCR is positive for samples 5, 6, 7. Total RNA was extracted from fish tissue and from EPC cell culture that show a cytopathic effect. Confirmation of SVC virus with Semi- nested was positive for the samples number 5 when the RNA was extracted from the inoculum of the EPC cell, first passage.

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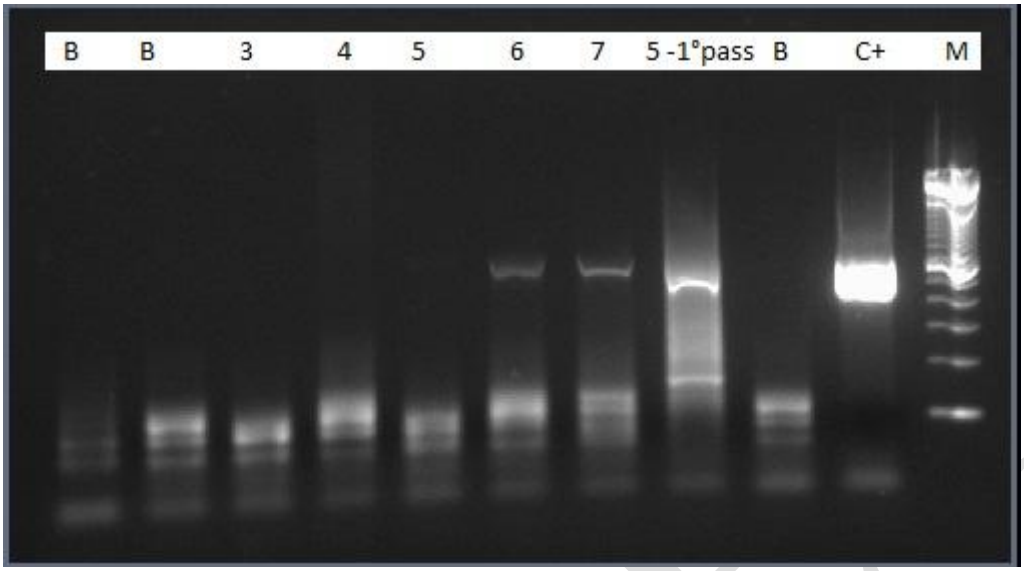

Fig. 2. Gel-electrophoresis result of the Semi-Nested PCR for samples number 3, 4, 6, 7 and 5 (RNA in samples 5 were extracted from tissue and from the infected EPC cell first passage).

IV. DISSCUSION

Early detection of a pathogen is an basic component of all surveillance programs aimed at preventing the introduction of a animal disease, particularly at the lakes with transbounday character like Shkodra/Scadar lake (Albanian-Montenegro border). The more rapidly the presence of the pathogen is identified, the more rapidly infected populations can be treated or depopulated [17]. Rapid and accurate detection of the virus is vital to efforts to prevent the further introductions and spread of the virus. In addition to virus culture, several diagnostic tests have been developed to detect SVCV. However, OIE recommends that the detection of virus by any of the serological or PCR based methods be confirmed by virus culture. Based on our studies, virus culture, although very sensitive, can take up to 7 days for detection of very low virus levels. Consequently, it does not meet the standard of necessity for rapid detection. A seven-day delay could have serious economic consequences for a producer that waits to take control measures to prevent further mortality [18] [17]. We compared virus culture with other alternative assays available to detect SVCV.
This study describes for the first time detection of spring viremia of carp virus in common carp n Lake Shkodra by using biomolecular assay such as RT-PCR and Semi-Nested PCR. Spring viremia of carp virus was confirmed by Semi-Nested PCR in three samples labeled number 5, 6 and 7. All these samples (common carp) were collected in Lake Shkodra, March 2013. They do not exhibit any signs of the disease which correlate with observations that SVCV is mostly clinically in apparent [18]. However it is important to mention that SVCV is a contagious viral disease that a great losses in carp pond in Europe [19] [20 [21] and high rate of mortalities in wild common carp in America [14].

It is unclear if the detection of SVCV in Lake Shkodra represents a new introduction or the virus has been there but undetected. The most logical scenario is that the virus was transported via natural movement of fish. There are no data for the SVCV in Albania, while thought to be present in Europe for decades, spring viremia of carp virus (SVCv) was initially diagnosed in Yugoslavia [19]. [22] in their reporting survey for Serbia for the period of 1992-2002 are indicating that thirty-eight hatcheries were encompassed by the 10 year testing. Twelve (31, 57%) out of 38 hatcheries were contaminated by the spring viraemia virus, which is very alarming, as it is a threat to carp production.

Currently no vaccination exists for SVCV. Fish farmer can practice a few good techniques for preventing or stopping the spread of SVCV. It is well known that temperature above 20°C usually prevent or even stop SVC outbreaks so in small carp farming we can use spring or well water. Another preventing measure include the disinfection of eggs by iodophore, regular physical and chemical disinfection of ponds, equipment and a proper disposal of dead fish.

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Following [17] the virus culture is currently required by OIE guidelines to confirm the results of PCR-based assays because of concerns about detecting nonviable viral RNA. The RT-PCR assay could be used as a screening tool to identify SVCV, which could then initiate response activities such as quarantine and strict bio-security protocols. Due to the possibility of RT-PCR detecting nonviable virus, all depopulation events can be deferred until the results of virus isolation is confirmed. Uniform OIE approved test guidelines and test conditions would enhance confidence in test RT-PCR results, and with this standardization the results of this assay could be considered definitive indication that the virus is present. Protocol standardization and a standard procedure for quality assurance assessment of individual laboratories should be developed to ensure diagnostic consistency between laboratories.

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