Sel'skokhozyaistvennaya Biologiya [Agricultural Biology], 2013, № 4, p. 53-58

UDC 639.3.09:619:578:616-079.7:577.083.3

INDIRECT ELISA FOR THE DETECTION OF ANTIBODIES IN SERA OF STURGEON FISH SPECIES

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Received January 25, 2013

S u m m a r y

Herpesvirus disease of Siberian sturgeon, caused by SbSHV, is especially severe in the industrial populations. The commonly used method for its specific diagnostics has a sufficient disadvantage because of SbSHV active reproduction in vivo when the water temperature is in the range of 10-18 °C, and therefore, can be applied mainly in spring and sometimes in autumn. At other seasons the amount of the virus in the fish tissues reduces so that it becomes nearly unrevealable. We have developed a method for retrospective diagnosis of herpesvirus disease of Siberian sturgeon using indirect ELISA version. Experimental data on ELISA development for the detection of antibodies against Siberian sturgeon herpesvirus are presented. Procedures for preparing the ELISA specific reagents are described, including the virus reproduction in cell culture, isolation of IgM-like immunoglobulin from hyperimmune antisera of sturgeon, etc. The effectiveness of the proposed method and the reaction of neutralization was compared. It is shown, that by elaborated method the SbSHV antibodies can be detected both in the Siberian sturgeon, and among closely related species of sturgeon, or their hybrids.

Keywords: herpesvirus of the Siberian sturgeon, immunoglobulin, ELISA, serodiagnostics.

Siberian sturgeon herpes virus (SbSHV) is a highly-contagious disease with acute course and mass mortality of mixed-age fingerling of *Acipenser baeri* showing symptoms of hemorrhagic necrosis syndrome. The disease affects industrial populations of sturgeon and develops especially severe in conditions of industrial fish farms. The disease was first diagnosed in Russia in 2006, and, according to the available data, it is quite frequent in sturgeon fish farms (1, 2). The virus was also isolated by the authors in Kazakhstan and Finland. A similar infection of *Acipenser transmontanus* is known in North America and Western Europe (3). Its pathogen – DNA containing virus of the family *Alloherpesviridae* – was also isolated from bester, Russian sturgeon, and Russian-Lena sturgeon (1, 2). Susceptibility to experimental infection was revealed in sturgeon and hybrid sturgeon ½ beluga ½ sterlet (SBS).

Disease control of SbSHV is focused primarily on its specific diagnostics. In guidelines of the World Organization for Animal Health, direct identification of a pathogen or its components is recommend as preferable method of diagnostics. Currently, there is the only such method for diagnosing SbSHV – classical isolation in cell culture with subsequent identification by neutralization reaction. PCR method for detection of SbSHV genome is under development (4).

However, the major disadvantage of the commonly accepted technique is that active reproduction of SbSHV in vivo occurs at the water temperature within the range of 10-18 °C. Therefore, this method is applicable mainly in spring and sometimes in fall; in other times of the year, the virus content in tissues of fish is too low to be detected.

At the same time, summer temperatures of water (above 20 °C) provide conditions for active production of anti-viral antibodies in the blood of infected fish; these antibodies can be detected by neutralization reaction for at least 7 (sterlet) - 13 (Siberian sturgeon) months, which allows using indirect methods of retrospective diagnostics of the disease based on detection of antiherpetic antibodies. Such methods can be applied throughout the summer season and even longer, which greatly increases timeframe of diagnostic tests and efficiency of seroepizootic survey of fish farms.

The reaction of antibody neutralization is one of classic methods of detecting antiviral antibodies, though it is time consuming in contrast to such promising instrumental methods as solid-phase enzyme-linked immunosorbent assay (ELISA).

The purpose of this study was to develop the method for retrospective diagnosis of Siberian sturgeon herpesvirus disease based on indirect enzyme-linked immunosorbent assay.

Technique. Accumulation of the virus and experimental reaction of neutralization were performed on continuous cell line SSO-2 from the pool of liver, kidneys, and spleen (5). The cells were cultured at 19 °C in the nutrient medium 199 with 10 % fetal bovine serum (FBS). Specific serum was obtained by hyperimmunization of two-year old sturgeons using the virus accumulated in continuous culture cells WSSK-1 (6). The cells were cultured at 21.5 °C in Igli MEM nutrient medium with a double set of amino acids and vitamins (2 ½ MEM), and 10 % FBS.

The used strain of SbSHV virus – SK1/0406. To provide its accumulation, daily culture of SSO-2 cells was inoculated with multiplicity of infection of about 0.03-0.10 TCID50/cell with preliminary change of growth medium for maintenance medium containing 2 % FBS and following incubation at 15 °C until obtaining 100% cytopathogenic effect (CPE).

Specific antisera to the virus were obtained by hyperimmunization of two-year old Siberian sturgeons-convalescents after experimental infection through periodic intraperitoneal injections of the virus in doses $10^{5.63}$-$10^{6.63}$ TCID50 per individual. The blood was sampled at intervals of 2 months and treated by a conventional method. The blood sera of sturgeon species-survivors of the experimental infestation were prepared as follows. Underyearlings of starlet and SBS were infected with SbSHV by placing in baths with water containing the virus at a dose of $10^{5.60}$-$10^{6.04}$ TCID50/cm^3 for 1 h with subsequent housing in flow-through tanks with temperature of water 14-16 °C and feeding with mixed feed AK-2FP. After completion of the disease course, survivors were used for obtaining the blood serum.

During isolation of IgM-like immunoglobulin from the sturgeon hyperimmune antisera to SbSHV, treatment of the blood sera included consistently conducted precipitation of lipoproteins (7), precipitation of immunoglobulins with ammonium sulfate solution at 50 % saturation, separation of immunoglobulins by gel filtration (8) on Ultrogel AcA34 ("IBF", France), and final purification of IgM-like immunoglobulin of Siberian sturgeon by ion exchange chromatography (9) on DEAE-Sepharose CL-6B ("Sigma-Aldrich", USA). Collection of fraction and registration of the protein yield from chromatographic columns were conducted using FPLC set of chromatographic equipment ("Pharmacia-LKB", Sweden). Preparation of sorbents was done according to the manufacturers' recommendations. Composition of serum protein fractions was investigated by immunoelectrophoresis according to Frimel (10). Concentration of the protein in preparations was determined as described by Lowry (11).

The reaction of neutralization of SbSHV was used to determine virus neutralizing activity of antibodies in fractions of the isolated immunoglobulin, in hyperimmune and field sera, and in the sera of fishes-survivors after experimental infection. The reaction was performed in 96-well plates ("SPL", Korea) with working dose of the virus 32 TCID50/well under the commonly adopted protocol (12).

Rabbit antiserum IgG was derived after immunization of animals with purified immunoglobulin of Siberian sturgeon. IgG was isolated from the hyperimmune sera by gel filtration (8) on a column filled with Ultrogel AcA34. Conjugation of rabbit IgG with horseradish peroxidase was carried out by a modified method of periodate oxidation (13).

Titers of antispecies antibodies in the rabbit sera and antigenic relationship of immunoglobulins derived from different sturgeon species and hybrids were determined by immunodiffusion (reaction of diffusion precipitation – RDP) of Ouchterlony. In solid-phase indirect ELISA, 96-well plate was sensibilizated with purified and concentrated viral antigen at working dilution of the solution in carbonate-bicarbonate buffer (0.05 M, pH 9.5). Residual sites of nonspecific binding of immunoglobulins were blocked by 0.5 % casein solution in Tris-HCl buffer (pH 7.6). The dilutions of tested anti-SbSHV-specific and control sera were prepared using PBS-Tween 20 (pH 7.2-7.4). Conjugate of antispecies antibodies with horseradish peroxidase was diluted in 0.5 % casein solution in Tris-HCl buffer (pH 7.6). A substrate was 0.04 M solution azinobisthiosulphonic acid (0.2 mg/cm^3) (ABTS) with 0.0001 % hydrogen peroxide solution. Optical density was measured on a microtitration plate photometer Sunrise ("Tecan", Austria) at λ= 405 nm. The sample was considered positive if optical density of the substrate solution in well was higher 2.1 times and more than that of the substrate solution in well with negative control solution.

Results. Obtaining specific sturgeon antisera to SbSHV. In normal sera of healthy fish before the infestation, virus-neutralizing antibodies weren't detected (detection threshold 1:8), while the blood serum of survivors after the experimental infection contained significant titers of such antibodies (114 days after the infestation – from 1:600 to 1:3000). After the first re-immunization (in 114 days after the infestation), the level of antibodies increased in 3 of 5 individuals. The second and third re-immunizations were conducted similarly in a year after the experiment; the result was rapid (within 40 and 32 days) increase in antibody titers in the blood serum of one individual up to the maximum level that had been recorded earlier during the first reimmunization, i.e. more than 3 times.

So, there were obtained five hyperimmune sera of sturgeon with titers in neutralization reaction ranging from 1:600 to 1:8600. These antisera were used to isolate immunoglobulin of Siberian sturgeon and they served as known-positive ones in development of the desired variant of ELISA.

Isolation of viral antigen for sensitization of ELISA plates. Virus-containing material of SbSHV-infected cell culture SSO-2 was obtained using the protocol including purification and concentration of the virus antigen (Fig. 1). Control antigen from non-infected cell culture was prepared similarly. The outcome was a purified and concentrated specific SbSHV antigen with the titer of infectious activity in neutralization reaction of $10^{7.35}$ TCID50/cm^3. This antigen was used to sensitize ELISA plates.

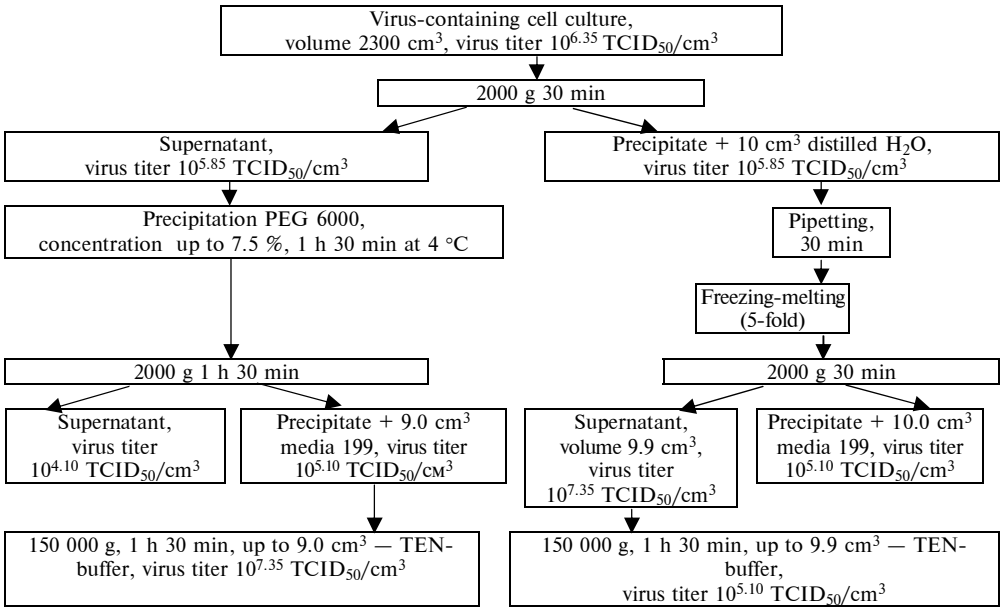

Fig. 1. Protocol of preparing SbSHV antigen for sensitization of plates (indirect ELISA)

Obtaining IgM-like immunoglobulin of Siberian sturgeon and rabbit antibodies to it. The abovementioned techniques and approaches allowed isolation of immunochemically pure immunoglobulin of Siberian sturgeon (Fig. 2) (preparation with protein content 0,638 mg/cm^3).

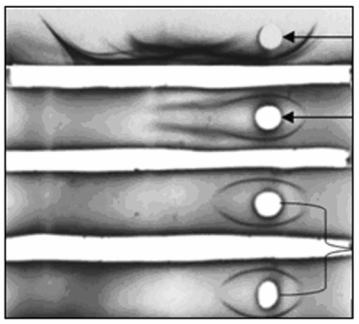

Fig. 2. Immunoelectrophoregram of Siberian sturgeon Ig isolated from hyperimmune serum to SbSHV.

In grooves – antispecies rabbit serum to the blood serum of Siberian sturgeon.

The next step after hyperimmunization of rabbits against Siberian sturgeon immunoglobulin it was obtaining antispecies sera with the activity in diffuse precipitation reaction 1:64-1:256. Preparation of antispecies antibodies was prepared using the antisera with maximum activity; the result was isolation of rabbit IgG (preparation with protein concentration 17 mg/cm^3).

To assess applicability of the developed ELISA method for detection of antibodies to SbSHV in sturgeon species and hybrids, it was investigated antigenic relationship of immunoglobulins of closely related sturgeon species. The reaction of diffusion precipitation with antispecies rabbit IgG against Ig of Siberian sturgeon revealed a close antigenic relationship of immunoglobulins of at least four sturgeon species and hybrids – Siberian sturgeon, sterlet, beluga ½ Lena sturgeon, beluga ½ sterlet (bester), sterlet ½ beluga ½ sturgeon (SBS). Therefore, antispecies rabbit IgG can be used to detect antibodies to SbSHV in different sturgeon species and hybrids.

The resulting rabbit IgG against immunoglobulin of Siberian sturgeon was used to prepare peroxidase conjugates.

Indirect variant of ELISA. Optimal dilutions of reagents for performing indirect ELISA were determined by chessboard titration. The resulting working dilution of the specific antigen for sensitization of plates amounted 1:800, antispecies immunoperoxidase conjugate – 1:1000. The identified antibody titers in the sera of investigated sturgeon fish ranged from 1:90 to 1:590490.

To compare the results of neutralization reaction and indirect variant of ELISA, hyperimmune sera of Siberian sturgeon and the blood sera of Siberian sturgeon obtained from surveyed fish farms were titrated by both methods. In regression analysis, the data of both methods exhibited close correlation ($R^2 = 0.8413$) in a linear relationship described by the empirical formula: $y = 1.2789 x + 2.6992$ (Fig. 3).

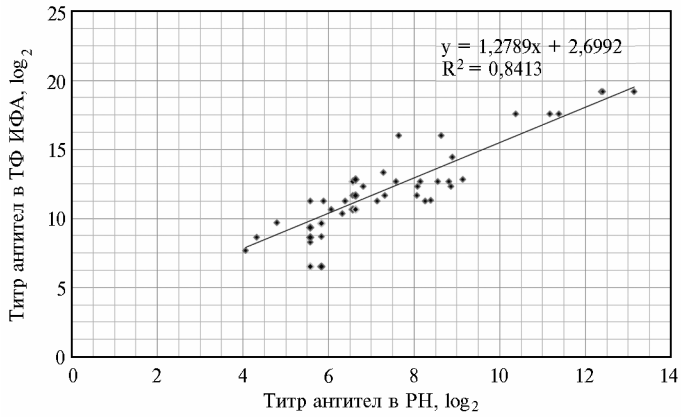

Fig. 3. Correlation between SbSHV antibody titers in neutralization reaction (NR) **and indirect ELISA** (ELISA) (description see in "Technique").

The presence of such relationship indicates that the developed indirect variant of ELISA provides reliable data about specific immune status of sturgeon fish in retrospective diagnosis and surveillance of Siberian sturgeon herpes virus disease.

Thus, the developed method based on indirect variant of ELISA allows rapid and specific detection of antibodies to Siberian sturgeon herpes virus (SbSHV) disease in Siberian sturgeon and related sturgeon species and hybrids. The method can be used in retrospective diagnosis of Siberian sturgeon herpesvirus disease as conducted in conditions of scientific institutions and specialized diagnostic laboratories.

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