Virological research as an integral part of complex monitoring of fish diseases

E A Zavyalova

Federal State Budget Scientific Institution “Federal Scientific Centre VIEV”, Rjazanskij prospect, Moscow,109428, Russian Federation

E-mail: asdf1961@yandex.ru

Abstract. In the early 2000s, there were no Russian test systems for detecting fish viruses, the work was carried out in cell cultures and in the neutralization reaction, which is effective, but the result was obtained no earlier than 10 days, simultaneously with the release of the virus in the cell culture, which undoubtedly increased the time of diagnosis. In 2009, the addition of existing methods to polymerase chain reaction and enzyme linked immunoassay methods made it possible to detect not only open forms of the disease, but also virus carriers, which is the most important information in the organization of cross-border transport of hydrobionts. Systematic comprehensive diagnostic studies in fish farms have allowed us to get a clear idea of the epizootic situation and identify areas of trouble for viral fish diseases and take timely measures to stop the disease.

1. Introduction

The increasing demand for high-quality fish products with limited fishing opportunities, the imbalance of natural ecosystems has led to a sharp reduction in the number of hydrobionts, especially the most valuable food-related species. In recent decades, many important fish species have been at the stage of extinction and the only way to preserve salmon, whitefish, and sturgeon is to form herds in factory conditions, so there is a need to create a new direction of world fisheries - aquaculture.

Fish diseases of viral etiology are a serious factor that hinders the development of fish farming. In epizootic outbreaks, the mortality rate can reach 60-70%, which leads to large economic losses. There is no adequate therapy against viral fish diseases (except for the destruction of infected fish), so viruses often circulate in populations for years, up to a certain point without causing epizootics [1]. Spread by vertical transmission – from one generation to the next. If the virus is contained inside the eggs, it is true vertical transmission, or alternatively, on the surface of the gamete, in the ovaries, in ovarian or seminal fluid; therefore, surface disinfection of the eggs is not effective enough to prevent disease [2].

Infected individuals secrete the virus with urine, excrement and mucosal secretions from the inflamed intestine, with sexual products (ovarian and seminal fluid), through the gills, skin and fin tissue, thus forming a natural reservoir of infection in natural conditions in fresh and sea water [3].

The most effective way to combat diseases of any etiology can be considered a comprehensive diagnosis, one of the components of which is virological research. Traditional diagnostic methods for viral diseases all over the world are viral isolation in cell culture and subsequent serological identification with specific serum in the neutralization reaction, enzyme immunoassay, immunohistochemically in histological preparations, in hemagglutination reaction, or by modern
molecular methods such as PCR [4]. In the future, molecular methods may become even more important for studying the genetic profile of viruses and determining virulence [5].

For this purpose, for many years, the laboratory of ichthyopathology of the Federal State Budget Institution «Federal Scientific Centre VIEV» (FSC VIEV) has been monitoring the epizootic situation of fish diseases in various regions of the country.

The purpose of this article is a comparative analysis of the results of epizootological monitoring of viral fish diseases in Russia in 2001-2008 and 2009-2019.

2. Materials and methods

2.1. Virological studies
Samples of the liver, kidneys and spleen were taken from fish in sterile vials with the medium MEM. The samples were processed according to generally accepted methods ("Guidelines for the identification of viruses and laboratory diagnostics of viral diseases of fish" No. 13-4-2 / 1054, approved by the Department of veterinary medicine of the Ministry of agriculture of the Russian Federation 10.10.97): the Material was passed in cell cultures.

2.2. Cell culture
For virus release from pathological material and passivation of culture viruses, we used finite cell lines: RTG-2 ATCC CCL 55 (rainbow trout gonads), EPC ECACC # 93120820 (carp epithelial papilloma), CHSE-214 ATCC CRL 1681 (normal Chinook embryo), FHM ECACC # 88102401 (minnow tail stalk) [6] and OMG patent RUS 2495120 (rainbow trout gonads), SSF(viev)-1, SSF(viev)-2, SSF(viev)-3 (Siberian sturgeon fin) patent RUS 2488632, wssk-1 (white sturgeon skin) which were cultured at 15-22°C (depending on the type of virus).

2.3. Bioassay
Imported fish (carp and trout) were adapted in aquariums with a life support system for 10 days, at a temperature of 15-18°C. We formed experimental and control groups of fish (at least 10 copies into each). Fish were infected by intraperitoneal injection, and control group fish were given the same amount of saline. Observation of experimental fish was carried out for 14-30 days at a water temperature of 14-16°C. Isolation of the initial strains from infected fish was performed according to a generally accepted method.

2.4. Enzyme linked immunoassay (ELISA)
Enzyme linked immunoassay (ELISA) of tissue biomaterial and viral-culture suspension was performed using kits developed in the laboratory of ichthyopathology in FSC VIEV for the identification of SVC, IPN, IHN and VHS pathogens [7,8,9].

2.5. Polymerase chain reaction (PCR)
PCR was performed using the methods and primers proposed by the OIE [10] and the author’s design [11,12].

3. Results and discussion
In the period from 2001 to 2008, research was conducted in fish farms in Moscow, Tver, Tula, Lipetsk, Vladimir, Smolensk, Murmansk regions and in the Republic of Karelia. In total, 61 fish farms of the country were surveyed, 1277 specimens of fish of different ages and species. At that time, the main object of research was carp - 38 %, while the number of sturgeon and trout - 22%. Atlantic salmon - 10%, crucian carp - 8%, as well as 22% of other fish species.

At that time, 23 virus isolates were isolated from fish of various ages and species (table 1).
The studied materials were passed through three or four passages in the cell cultures that were being transferred. In the presence of morphological changes induced in cells by the virus secreted, a neutralization reaction with typical rabbit hyperimmune antiserum was performed for identification. Further, the etiological role of the virus was confirmed in the bioassay, in some cases the virulence of viruses was confirmed, some strains were evaluated as avirulent persistent, since fish deaths were not observed, but the viruses were re-isolated. Most often, the virus is isolated from fish of the younger age groups of fingerlings, yearlings, smolts, two-year-olds and producers are less susceptible to the disease, in most cases they can be virus carriers. So in 2007, the largest number of virus strains was isolated, which was associated with the beginning of the period of mass comprehensive diagnostic studies in trout farms. Epizootic outbreaks were not observed, but virus transmission was established in trout raised in commercial production.

Table 1. Results of virological monitoring from 2001 to 2008.

| Year | Virus  | Number of cases |
|------|--------|-----------------|
| 2001 | IPNV   | 1               |
| 2002 | -      | -               |
| 2003 | SVCV   | 2               |
| 2004 | SVCV   | 1               |
| 2005 | SVCV   | 3               |
| 2006 | SVCV   | 4               |
| 2007 | SVCV   | 2               |
| 2008 | IPNV   | 2               |

As a result of identification from salmon, strains belonging to the family Birnaviridae were isolated, causing the disease infectious pancreatic necrosis (IPN) [13]. Strains obtained from carp, family Rhabdoviridae, pathogens of spring carp viremia (SVC) [14].

Since 2009, in connection with the development of diagnostics for the identification of pathogens of viral diseases, based on PCR and ELISA, the methodology of diagnostic research has undergone significant changes in favor of rapid and timely detection of pathogens, which as a result will speed up the process of taking quarantine and preventive measures.

In the test systems for the indication and identification of fish viruses by PCR, created in the laboratory of ichthyopathology of the FSC VIEV, primers proposed by the OIE and author's were used. The conditions for isolation of nucleic acids were optimized so that the analysis time does not exceed 4 hours.

A diagnostic kit of combined ELISA has been developed that allows determining the presence of pathogens of viral diseases of salmonids (IPN, IHN and VHS) in samples of biological material within 3 hours with 98% confidence, while one tablet contains from 2 samples of biomaterial in three repetitions to 24 samples without repetitions.

Over 10 years of research (2009 to 2019) laboratory methods including isolation of virus in cell culture, enzyme-linked immunosorbet assay (ELISA) and polymerase chain reaction (PCR) covered more than 40 thousand copies. fish of various species from 447 farms across the country.
Employees of the laboratory of ichthyopathology went on business trips for sampling or samples were delivered by representatives of enterprises from the republics of Karelia, Udmurtia, North Ossetia-Alania, Bashkortostan; Belgorod, Moscow, Murmansk, Leningrad, Kaluga, Tver, Irkutsk, Novgorod, Ryazan, Smolensk, Omsk, Ivanovo, Pskov, Arkhangelsk, Oryol, Yaroslavl, Voronezh regions, Stavropol, Krasnodar territory, Far Eastern Autonomous district, Khanty-Mansi Autonomous district.

Table 2. Results of virological monitoring from 2009 to 2019.

| Year | Number of enterprises surveyed, indicating the type of activity | Number of fish | Virus agent | Number of cases of virus detection |
|------|---------------------------------------------------------------|---------------|-------------|-----------------------------------|
|      |                                                                |               |             | Cell culture | ELISA | PCR |
| 2009 | 20, of which:                                                 | 700           | IPNV        | 1           | 2     | 2   |
|      | Ca – 6; Stb – 4,                                               |               |             |             |       |     |
|      | Trc – 10                                                      |               |             |             |       |     |
| 2010 | 20, of which:                                                 | 1200          | IPNV/SSHV   | 1           | N/d   | 1   |
|      | Ca – 6; St – 4,                                                |               |             |             | 1     |     |
|      | Tr – 10                                                       |               |             |             |       |     |
| 2011 | 37, of which:                                                 | 1500          | IPNV/INHV   | 2           | 2     | 2   |
|      | Ca – 5; St – 5,                                                |               |             |             | 1     |     |
|      | Tr – 27                                                       |               |             |             |       |     |
| 2012 | 33, of which:                                                 | 2122          | IPNV/VHSV   | 1           | 1     | 1   |
|      | Ca – 4; St – 5,                                                |               |             |             | 1     |     |
|      | Tr – 23, Sa – 1                                               |               |             |             |       |     |
| 2013 | 40, of which:                                                 | 1680          | IPNV/VHSV/ | 1           | 2     | 2   |
|      | Ca – 3; St – 3,                                                |               | KHV         |             | 2     |     |
|      | Tr – 22, Sa – 2                                               |               |             |             |       |     |
| 2014 | 32, of which:                                                 | 2991          | Not detected| -           | -     | -   |
|      | Ca – 2; St – 2,                                                |               |             |             |       |     |
|      | Tr – 27, Sa – 1                                               |               |             |             |       |     |
| 2015 | 49, of which:                                                 | 2891          | ISAV        | 1           | N/d   | 1   |
|      | Ca – 5; St – 5,                                                |               |             |             |       |     |
|      | Tr – 37, Sa – 2                                               |               |             |             |       |     |
| 2016 | 42, of which:                                                 | 3836          | IPNV (Armenia) | 1       | 1     | 1   |
|      | Ca – 1; St – 2,                                                |               | IPNV (Finland) | 1       | 1     | 1   |
|      | Tr – 37, Sa – 2                                               |               | KHV         |             | 1     |     |
| 2017 | 49, of which:                                                 | 5967          | Not detected| -           | -     | -   |
|      | Ca – 1; St – 2,                                                |               |             |             |       |     |
|      | Tr – 37, Sa – 2                                               |               |             |             |       |     |
| 2018 | 57, of which:                                                 | 8954          | Not detected| -           | -     | -   |
|      | Ca – 5; St – 5,                                                |               |             |             |       |     |
|      | Tr – 45, Sa – 2                                               |               |             |             |       |     |
| 2019 | 68, of which:                                                 | 9188          | Not detected| -           | -     | -   |
|      | Ca – 5; St – 5,                                                |               |             |             |       |     |
|      | Tr – 45, Sa – 2                                               |               |             |             |       |     |
| Total:|                                                               | 447           | 41 029      | 18          | 22    | 26  |

a Carp enterprise.
b Sturgeon enterprise.
c Trout enterprise.
d Not do.
e Salmon enterprise.
Table 2 shows that in the period 2009-2013, cases of viral diseases were registered: infectious pancreatic necrosis (IPN), infectious hematopoietic tissue necrosis (IHN) and viral hemorrhagic septicemia of salmon (VHS), as well as sturgeon herpesvirus disease (SSHV) and koi herpesvirus disease (KHV). Their number is small, 1-3 cases, in a number of facts it is a virus carrying, since in a parallel study the virus was confirmed by immunological and molecular methods, but it did not cause cytopathogenic action in cell cultures, and the development of epizootics was not observed at enterprises. This period coincides with the rapid development of trout production in the North-West region, which also follows from the table, the number of trout farms that provided fish for research has increased 3 times in 5 years. The reasons for the spread of fish diseases were, most often, violations of the rules of transportation, in connection with the development of the industry and the shortage of fish planting material, the young were transported from poor to prosperous farms, as well as the presence of natural foci of pathogens in natural reservoirs.

Later, starting in 2014, owners of large fish farms come to understand that mass therapy in fish is very difficult, due to the inability to prepare large amounts of medicinal feed on site and the lack of treatment as such for viral infections, which in the event of an outbreak of the disease can lead to large economic losses and to the imposition of quarantine. Thus, some enterprises pay more attention to the health of imported fish stocking material, for this purpose, in cooperation with the laboratory of ichtiopathology in FSC VIEV, conduct a quality assessment of juveniles before delivery to the farm, through a comprehensive survey of fish – including virological, bacteriological and parasitological laboratory and diagnostic studies, with sampling on the site of its cultivation. According to the results of the study, infected fish are not allowed for interregional transportation, which gives a positive result, the number of cases of detection of pathogens of viral diseases is steadily decreasing.

In 2015, an outbreak of infectious salmon anemia (ISA) in mariculture was detected in the Murmansk region, when there was a mass death of Atlantic salmon with an average weight of 1.5-2.0 kg, previously imported from Norway without preliminary studies. To isolate the virus, we used transferable CHSE-214 cell lines. After the appearance of focal CPE caused by ISAV growth in CHSE-214 cells, the virus was identified in PCR according to OIE protocols (Manual of Diagnostic Tests for Aquatic Animals, OIE, 2009). After receiving the results of the study, together with the Committee for veterinary medicine of the Murmansk region, it was decided to pass all the remaining fish for sale in the commodity network, which was completed as soon as possible.

Checking fish before importation is widely used abroad and is reflected in the lists of requirements of many countries importing hydrobionts, for example, India and Japan. In 2016, a study of rainbow trout samples delivered to the laboratory from Armenia for testing before planned import to the Republic of Karelia identified a virus-pathogen of infectious necrosis of salmon hematopoietic tissue (IHN); and in the study of fish delivered from Finland - a virus-pathogen of infectious necrosis of the pancreas of salmon (IPN). In both cases of detection of salmon viruses by the Ministry of agriculture of the Republic of Karelia, the import of planting material from the tested enterprises was prohibited, which prevented infection.

4. Conclusion
For laboratory diagnostics and identification of pathogens of viral diseases of fish all over the world, including in Russia, such methods as virus isolation in cell culture and subsequent serological identification with specific serum in the neutralization reaction and/or enzyme immunoassay, as well as molecular methods of various PCR variants are used.

Each of these methods has a different diagnostic value – some of them require significant time and equipment, while others have low sensitivity. Therefore, only the use of several methods at the same time can ensure the sensitivity and specificity of laboratory diagnostics.

References
[1] Van Regenmortel M H V et al. 2000 Virus Taxonomy. Seventh Report of the International Committee on Taxonomy of Viruses (San Diego: Academic Press)
[2] Bullock G L, Rucker R R, Amend D and Wolf K 1976 Infectious pancreatic necrosis: transmission with iodine-treated and nontreated eggs of brook trout (Salvelinus fontinalis) Jour. Of the Fish. Res. Board of Can. 33 1197-8

[3] Reno P W 1999 Infectious pancreatic necrosis and associated aquatic birnaviruses Fish Diseases And Disorders 3 1-55

[4] OIE 2006 Aquatic code

[5] Evensen O, Skjelstad B, Rimstad E, Brun E and Johansen L H 2003 IPN in salmonids. A review FHF 3 118

[6] Zavyalova E A, Pichugina T D, Dyakonov L P and Borisova M N 2005 Fish cell cultures for virological research and diagnostics of viral diseases of fish Cell cult. 20 53-7

[7] Zavyalova E A and Droshnev A E 2017 Enzyme-linked immunoassorbent assay in the diagnosis of viral haemorrhagic septicaemia of salmonids (VHS) Vet. And Fed. 3 43-4

[8] Zavyalova E A, Karpova M A and Droshnev A E 2017 Solid-phase enzyme immunoassay in the diagnosis of viral diseases of salmon fish Cur. Iss. of vet. Biol. 2 (34) 25-9

[9] Karpova M A and Zavyalova E A 2018 Obtaining components of the ELISA diagnostic kit for IPNV identification in monitoring studies Vet. and fed. 7 39-41

[10] OIE 2019 Diagnostic manual for Aquatic animal diseases

[11] Zavyalova E A, Kandrina N Yu, Lomakina N F and Gulukin M I 2015 Indication and identification of some particularly dangerous fish viruses by PCR Fish. And fishery 3 21-5

[12] Zavyalova E A, Kandrina N Yu, Lomakina N F and Gulukin M I 2011 Polymerase chain reaction in the diagnosis of viral diseases of fish Vet. med. 95 59-60

[13] Pichugina T D, Zavyalova E A, Borisova M N, Dyakonov L P, Nadtochey G A and Shulak A F 2005 Isolation of infectious pancreatic necrosis virus in salmon Vet. 1 31-2

[14] Pichugina T D and Zavyalova E A 2005 Impact of viral infections on the development of aquaculture in Russia vol. 2 (Kharkiv: Vet. med.-85) pp 906-11