Contemporary methods of environmental monitoring in aquaculture

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Abstract. Aquaculture is one of the promising areas of agriculture, but the intensification of the industry can negatively affect the ecosystem, which leads to the need for constant environmental monitoring, using modern methods such as genetic diagnostics using polymerase chain reaction. To expand the monitoring capabilities, the authors developed diagnostic kits based on PCR for identifying certain viruses and bacteria that are particularly dangerous for hydrobionts. Extensive use of the PCR diagnostic method for environmental monitoring will significantly reduce the duration of the study and more fully examine the population to obtain reliable information about the health of fish and the ecosystem as a whole.

1. Introduction

Russia has absolute opportunities for the development of aquaculture – 20 million hectares of lakes, 4.5 million hectares of reservoirs, 1 million hectares of reservoirs for complex purposes, more than 150 thousand hectares of ponds, therefore, the development of the country's huge water fund for fish farming is one of the most promising areas [1, 2].

The importance of the industry for the country's economy is also that it provides development of other industries, in particular, supplying livestock farms with feed products, and the chemical, pharmaceutical and cosmetic industries with valuable components. This is a complex sector of the economy, which includes various activities, ranging from forecasting the raw material base to organizing trade in fish products in the country and abroad. [3]

According to FAO estimates, to meet the projected global demand for fish, aquaculture production should more than double: from 67 million tons in 2012 to about 140 million tons in 2050. Such growth will largely ensure food security and development opportunities. It is estimated that the gap between the consumption of animal protein in the world at present and its consumption in 2050 may be reduced by about 14%. Aquaculture will also help increase income and employment, especially in developing countries where most of the industry's products are produced.

Global aquaculture production increases by 8-10% annually. The undisputed leaders in the production of artificially grown aquatic bioresources are the countries of South-East Asia and, above all, China [4]. Forecasts of global demand for fisheries products indicate a further increase in aquaculture production, which is explained by two major reasons: the lack of real opportunities to increase the volume of catch in the world's oceans and the further growth of the world's population. According to experts’ forecasts, the demand for food fish will be determined by a 60% increase in welfare and development factors, and by a 40% increase in population [5].
The global nature of aquaculture development inevitably entails an increase in the area and number of farms of various types, a higher density of aquaculture cultivation, and the use of feed resources produced outside the surrounding area. Intensification of production leads to an increase in the volume of fish production, the restoration of the number of species under threat, through overfishing, of fish populations with positive socio-economic results, but at the same time may have negative consequences for the ecosystem as a whole [6].

The industrialization of the industry leads to an increase in the consumption of mixed fodders and, as a result, the requirements of fish farms for fish flour/fat as the main components for feeding fish, so that it is inevitable to contaminate the aquatic environment with organic waste, eutrophication of water bodies and coastal zones, which in turn will provoke the restructuring of the biological environment. The introduction of chemicals used to control the state of water and, in particular, diseases, as well as the negative effect on the spread of diseases caused by artificially grown individuals can lead to the depletion of water resources. Therefore, efforts should be made to continuously monitor the impact of aquaculture on ecosystem health, as the environmental impact of the industry occurs with water pollution, fish disease and fish withdrawal from cages [7].

Thus, more intensive aquaculture activities require available tools to monitor, predict, protect and preserve the ecosystem.

Since aquaculture is a fairly new industry than traditional land-based livestock, it has greater capacity to use new means and methods of control and monitoring that further increase resource efficiency. One high-tech technique is polymerase chain reaction (PCR) based molecular analysis - direct DNA detection, with high specificity and sensitivity. The PCR method is based on the natural process of DNA replication - complementary DNA completion of the template, carried out using the enzyme DNA polymerase. The process of doubling nucleic acids can be used to produce copies of short regions of DNA specific to specific microorganisms, i.e. targeted search for specific regions is the goal of gene diagnostics to detect the agent.

2. Disease control

It is a combination of new technologies (diagnostic methods, vaccines) and wider application of monitoring and prediction methods to eliminate diseases.

In the complex of measures to improve farms from fish diseases and prevent from development, the leading place belongs to the diagnosis and prevention of the penetration of pathogens into areas where they did not exist before. The Russian normative documentation regulates the conduct of research as follows: isolation of the agent in the sensitive culture of cells for viruses or on the nutrient medium - for bacteria, serological identification of the agent with subsequent installation of the bioprobe. Existing methods are varied: neutralization test (NT), complement fixation test (CFT), variants of enzyme-linked immunosorbent assay (ELISA), indirect immunofluorescent antibody test (IFAT), coagglutination test, western blot, etc. These techniques are actively used in diagnostic research around the world, but most of them take quite a long time and are carried out only in large research centers with specialized laboratories - such as the FSC VIEV.

For active monitoring and control of pathogens as components of medium microbiocenosis, we have developed rapid diagnostic methods and created diagnostic kits based on PCR, which are used in diagnostics not only of the most common, but also of difficult-to-cultured and non-cultured agents, as well as chronic, latent and persistent forms of infection.

Primers, as the main part of diagnostics, are used by authors, in some cases taken from literary sources [8], as well as in the international database "GeneBank." The specificity of the primers was tested by BLAST (http://www.ncbi.nlm.nih.gov/) as well as experimentally.

3. Monitoring of viral fish diseases in aquaculture and wildlife

To identify the carp spring viremia virus (SVCV), three primers were selected (N3, N4r N5r), according to the reference sequence of the N-gene of the "Fijan" strain, the conditions for RNA isolation, reverse transcription and PCR were changed and optimized, resulting in a total analysis time of not more than
4 hours. PCR uses external and internal primers complimentary to regions of the nucleoprotein gene. External primers direct the synthesis of PCR, a 418 bp product. In order to increase the sensitivity of the method, a semi-pronged PCR technique was used, which was to use the external primers N3 and N5 in the first reaction cycle and the internal primer N4 paired with the primer N3 (PCR product 388 bp) in the second reaction cycle. Standard markers - sets of DNA fragments of known length - are used to identify a specific fragment. When viewing the gel on a UV transoluminator, the presence of a fragment of a certain size in the form of a specific luminous band of greater or lesser intensity indicated the presence of cDNA obtained from viral RNA in the sample.

This technique has been successfully used to identify SVC virus isolates. During monitoring, in different regions of Russia, from 2003 to 2019, we studied biomaterial from samples of water and homogenates of fish tissues obtained in fish farms, as a result of which 22 isolates of virus were detected [9].

To identify the infectious pancreatic necrosis of salmon (IPNV), four primers to segment B (B1, B2, B3r, B4r) were selected for the "Jasper" strain in a manner that allowed the use of the nested and semi-nested PCR technique. The choice of primers was due to Segment B containing genes encoding RNA-dependent RNA-polymerase and being highly conserved regions of the virus genome. The reverse transcription reaction was carried out in the same manner as for SVCV, which produced 839 bp amplification products in the first round of PCR and 217 bp in the second round of PCR, as confirmed in all experiments.

Engineered primers and diagnostics for IPNV monitoring allowed to detect the presence of this infection in fish farms of the Russian Federation. It has been found that when examining a low virus content material, as well as to detect latent virus-bearing, it is advantageous to use a breeding PCR, i.e., sequential two PCR steps with external and internal primer pairs. While it is sufficient to use a single-stage PCR with one internal primer pair for screening in explicit epizootics. Non-culturable forms of the virus detected only in PCR were also encountered. Results obtained in PCR were correlated with the results of examination of virus-containing suspension by electron microscopy and using commercial sets ELISA Ag IPNV of "Test-line" (Czech Republic) [6].

Primers complimenting certain regions of the nucleoprotein gene: VF (direct) and VR (reverse) were used to identify VHSV in PCR. Studies conducted with these primers on the VHS virus RNA template produced a clear PCR product of expected sizes of 811 bp. The developed technique allows to detect both virus obtained from cell cultures and virus obtained from field clinical material. The effectiveness of the method is confirmed in the case of detection of virus in wild young bulltrout and lake salmon in the North-West region of Russia, when the viral cytopathogenic agent identified in the NT was isolated in cell cultures as salmon VHS virus [5]. At the same time duration of analysis by traditional method was 20 days, and in PCR - 5 hours taking into account preparation of samples.

Specific primers selected for the nucleoprotein gene and the nest PCR technique were used to detect IHNV [1]. The reverse transcription reaction was performed at 42°C - 45 minutes, then the resulting cDNA was heated at 95°C - 5 minutes and used for setting up a polymerase chain reaction. In the first round, a product with a length of 786 b.p. was obtained, in the second - 323 b.p. with the help of the developed method, 80 field isolates were investigated and a virus was detected in one of the famous fish hatcheries of the Krasnodar territory. The results of the polymerase chain reaction correlated with the results obtained in cell culture and serological methods. [7]

4. Monitoring of agents of fish bacterial diseases

Currently, the diagnosis of bacterial infections in laboratories is based on the detection of microbes in nutrient media, followed by the study of their cultural and biochemical properties. This method is long, requiring financial costs, and the result of the analysis often depends on factors such as the experience and competence of the person conducting the work, the specificity of the reagents used and the feed media. Therefore, the use of operational modern methods will undoubtedly be relevant and in demand in diagnostics. We have developed oligonucleotide primers for genotyping by PCR and based on them
created diagnostic kits for differential diagnosis of some bacterial diseases of fish, for example, yersiniosis of salmon fish in samples of biological material in a short time with reliability of 100%.

Among the infectious diseases of rainbow trout grown in fresh water, yersiniosis – ERM (Enteric Red Month, "intestinal redness"), caused by the bacterium *Yersinia ruckeri*, a member of the *Enterobacteriaceae* family, is a serious danger. Yersiniosis is recognized as an enzootic infection in a number of regions that are actively engaged in trout farming – in North America, Europe, Australia, South Africa, France, and Germany. Since 2010, yersiniosis has been detected in the Russian Federation, where it causes severe damage to fish farms due to mass death and damage to the product type.

Polymerase chain reaction is a direct method of detecting DNA, it has a high specificity and sensitivity.

Three genes (glnA, gurB, recA) were selected as primers targets. After gene analysis, areas specific only to *Y.ruckeri* were selected: primers glnA-1 and YrgA-2 for the gene flanking 154 bp; for gurB gene the primers of YrgB-1 and YrgB-2 flanking site 225 bp; for the recA gene, YrrA-1 and YrrA-2 flanking the 294 bp. The specificity assessment of the designed primers confirmed the homology with the nucleotide sequence of recA, glnA and gurB genes and the absence of significant homology with the nucleotide sequences of *Enterobacteriaceae* and other species. When analyzing the selected primers, the absence of self-complementary sites within the primers and complementarity to each other was found, in order to avoid the occurrence of secondary structures and "dimers" of primers that can block the reaction or lead to non-specific amplification. The difference in the size of the amplified areas allowed the study to be carried out in a single tube, which reduces material costs and increases the "capacity" of the thermocycler when examining a large number of samples [10]. The diagnostic kit has been tested as a positive result for a number of years, which allowed to detect more than 28 outbreaks of yersiniosis in the period 2016-2018 in the North-West region of Russia [11].

This method can be used in scientific research to obtain new knowledge about the spread and patterns of circulation of species of *Yersinia ruckeri* in aquaculture and natural areas, and is of practical interest in the use of this diagnostic kit in the environmental monitoring system, which will allow to control the habitat and incidence of yersiniosis, preserve the health of wild and cultivated fish.

5. Conclusion

Currently, the molecular genetic method of PCR is increasingly used to monitor human and animal diseases, but in ichthyopathology and eco-control it is not a common technique. Compared to traditional methods, the application of the PCR method and diagnostics, which include all the necessary components, has certain advantages: high sensitivity, short execution time, possibility of early analysis and diagnosis, it does not require deep knowledge from the operator performing routine research. This will make it possible to make the method more practical and to more fully examine the number of fish in aquaculture with a view to environmental monitoring of infectious agents or, if necessary, to determine the causes of the disease.

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