The application of immuno-enzymal analysis as a method of early express diagnostics of rednoya in pond fish

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Abstract. Due to the lack of effective means of specific prevention of rubella in pond fish (CRF), it is obvious that early diagnosis is one of the most important and effective ways to combat this disease. This theoretical study is devoted to the development of an express method for the rapid detection of antibodies to rubella pathogens in pond fish (Aeromonas punctata, Pseudomonas, Rabdovirus cyprini). To date, the diagnosis of CRC (Rabdovirus cyprini) is determined by the results of the clinical picture, taking into account anatomical data, epizootological indicators and virological research results. The diagnosis of CRC (Aeromonas punctata, Pseudomonas) is determined by bacteriological results, taking into account epizootological indicators, clinical signs and pathological changes. The use of enzyme-linked immunosorbent assay (ELISA) for the determination of CRC antibodies is a comprehensive diagnostic kit that allows you to significantly reduce the time for examination (sample preparation, reaction, results recording), and also reduce the number of errors caused by the human factor. The ELISA reaction makes it possible to quickly and efficiently obtain research results. As a result of the study, indicators of the effectiveness of the application of this technique for the diagnosis of CRC were proposed. The advantage of ELISA in comparison with the bacteriological method lies in the simplicity of setting and the reduction in the time of analysis (within 1-2 hours). The proposed method for the determination of rubella in pond fish using enzyme-linked immunosorbent assay allows, within the framework of laboratory monitoring, to carry out an express diagnosis of the CRC disease. The possibility of early diagnosis of this disease, the low cost of diagnostic kits and the ability to automate most stages of the reaction, demonstrate the practical value of the presented method.

1. Introduction

Rubella is one of the most widespread and contagious diseases of pond fish (mainly carp species); it usually occurs in spring or early summer, sometimes in winter. The disease often causes massive mortality in pond aquaculture facilities. Diagnostic signs of this disease are: superficial hemorrhages of the body, dropsy, ruffled scales, ulcerative formations with a whitish or reddish rim on the skin or fins, bulging (figure 1) [1].

The diagnosis of aeromonosis caused by the bacteria Aeromonas punctata is carried out in a standard way, according to the results of the clinical picture, taking into account anatomical data, epizootological parameters and virological research results. Treatment is prescribed only by a qualified specialist ichthyopathologist for a specific body of water. If a sick fish lives in a water source supplying the entire economy, then it is practically impossible to improve it [2]; it is necessary to drain
the ponds, subject to treatment with disinfectants, and fish of all age groups are either disposed of, or, of sanitary suitability, it is possible to hand over for processing or into the distribution network [3-5].

Microbiological examination of the corpses of fish killed by the disease is carried out according to the established method: through a flexible plastic frame, with a sterile swab, a scraping is taken from 0.5 cm² of the surface of the fish body, after that, the fish is opened and 0.05-0.2 g of liver is taken and suspended. The tampon is washed with sterile saline, after which, on solid nutrient media, 50 μl is inoculated - MPA (OJSC named after I.I. Mechnikov, Russia) or Endo (HiMedia Laboratories, India), or Standard method agar (Novamed, Israel); the control of sterility is carried out according to hematology, taking blood from the tail vein of healthy fish during their lifetime [2; 6]. The virulence of the identified strains of aeromonads is assessed by their DNase activity using DNase agar with toluidine blue (Novamed, Israel) [2; 7]. The ability of strains to produce hemolysins is studied on blood agar (HiMedia Laboratories, India) (figure 2).

**Figure 1.** Characteristic signs of rubella in cyprinids.

**Figure 2.** Hemolytic activity of different strains of Aeromonas of the same individual [8].
You should always remember about the appropriateness of the chosen method to identify the causative agent of CRC [9]. For example:

- Biochemistry - slowly, not always specific;
- Typing - fast, specific, but expensive;
- Diagnostic PCR (culture required to create a target test);
- Protein method: MALDI TOF - fast, specific, expensive apparatus;
- Antibiogram (pure culture required).

Thus, the purpose of developing a method for determining rubella in pond fish species using enzyme-linked immunosorbent assay (ELISA) is to carry out rapid identification using a complex diagnostic kit, with the determination of not only the viral pathogen of the disease, but also bacterial pathogens within the framework of laboratory control over the spread of CRC.

2. Materials and methods

Our proposed ELISA methodology, with the help of special biochemical reactions, helps to determine the presence or absence of antibodies and their amount and to identify specific antibodies to the pathogens of rubella in pond fish (Aeromonas punctata, Pseudomonas, Rabdovirus cyprini).

The most important immunological processes in the body include the formation of antibodies (immunoglobulins - specific blood proteins). To antigens that can induce the body's immune response, i.e. specific immunological reactions include: polysaccharides, proteins, nucleic acids, incl. in the form of various biological components and structures (cells, tissues, viruses, etc.).

Functional groups or residues of antigenic determinants or epitopes that confer antigenic specificity can be found on the surface of the antigen molecule; the valence of an antigen depends on the number of epitopes on the surface of a complex molecule. So, in protein molecules, it can vary from 5 to 20; if the antigenic determinant is formed by a set of amino acid residues, then often, a single amino acid substitution in the structure of the antigenic determinant is quite sufficient for changes in antigenic specificity.

The function of antibodies is to protect the body from the penetration of foreign substances and their subsequent removal from the body, for which specific immune complexes are formed with the corresponding antigens. At the heart of immunochemical methods of analysis is the study of the ability of antibodies to form strong highly specific immunocomplexes with various substances and in the required quantities.

The body is able to recognize any foreign antigen due to specific blood cells of B-lymphocytes (antibodies), on the surface of which there are up to 100 thousand receptors of the same specificity. Meeting with a complementary receptor, the antigen selects the corresponding B-lymphocyte, which transforms into a plasma cell, each of which, dividing repeatedly, creates clones of cells, secreting antibodies that are homogeneous in their structure, therefore, such types of immune response and antibodies are polyclonal [2]

It is fundamentally important that polyclonal antibodies are heterogeneous both in their physicochemical properties and in the structure of the active center; those, if the antigen is polyvalent, then antibodies in the blood serum are formed against each individual antigenic determinant, thus the complication of antibodies occurs [10].

The advantages of ELISA over other methods for the detection of antigens and antibodies:

- Early diagnosis of infection and uniformity in mass research;
- Speed and high sensitivity, up to 90%;
- Convenience of diagnostics and low cost of diagnostic kits;
- The minimum volume of the studied biomaterial;
- The ability to automate most stages of the reaction;
Stability of all ingredients required for ELISA during storage (a year or more);
Ease of tracking the dynamics of the development of the process of an infectious disease.

Based on this, preferably within the framework of preventive measures, incl. to determine rubella in pond fish species, it is precisely the methodology of enzyme-linked immunosorbent assay (ELISA) that will significantly reduce the analysis time to 1-2 hours (sample preparation, reaction, registration of reaction results), make an accurate diagnosis and determine CRC (Aeromonas punctata, Pseudomonas, Rabdovirus cyprini).

3. Results
As a test material, to detect antigens to Aeromonas punctata, Pseudomonas, Rabdovirus cyprini, biopsy samples of internal organs (liver, kidney, spleen), ulcerative scrapings (from the surface of the body of sick fish), exudate of cell cultures and / or virus-containing supernatant are used. The biomaterial is homogenized on PBS (pH 7.2-7.4) until a homogeneous mass is obtained. The resulting 10% suspension is used for further research by pouring it into a sterile container [11].

When setting up the reaction, prepare a phosphate-saline buffer containing tween-80 for washing the plates, for which, the contents of the vial with FSB-T * 25 are stirred and diluted with distilled water to 700 ml.; in case of precipitation, the concentrate is heated until the salts are completely dissolved. Storage is carried out in a bottle at 4 ° C for up to 5 days.

Solutions are prepared immediately before use; to 0.05 ml of the concentrated solution add, stirring thoroughly, 15 ml of a solution for diluting conjugates No. 1,2,3 (RK). Immediately before use, the TMB solution is taken into a plastic bath, in an amount of 12 ml.; Remains of TMB solution from the bath must not be poured back into the bottle with the original TMB solution. If necessary, it can be stored during the entire shelf life of the kit, at 4 ° C.

Before the start of the analysis, the wells of the plate are washed once with a special washing solution, in each of which, 300 μl of solution is added, after filling all the wells in this way, after five minutes, the solution and residual moisture from the wells are carefully removed by tapping the inverted plate on the filter paper.

The introduction of the material into the plate is followed by thorough mixing by pipetting for 5-7 seconds. The plate is incubated for 60 minutes at room temperature (21-25 °C). Wells on the plate are sensitized as follows: immunoglobulins to IPN (1, 4, 7, 10 rows), IHN (2, 5, 8, 11 rows), VHS (3, 6, 9, 12 rows):

- In the first wells of each row of the plate sensitized to IPN, IHN and VHS - add 100 μl of specific positive antigens (IPN K +, IHN K +, VHS K +);
- In the second wells of each row - add 100 μl of negative antigen;
- In all other wells - 100 μl of the test samples (preferably 2-3 replicates).

Solutions of conjugates No. 1,2,3 in working dilution are prepared 5-10 minutes before the end of incubation; at its end, the plate is washed four times with FSB-T from unbound antigens, then carefully removing moisture on filter paper.

100 μl of TMB solution is added to all used wells of the plate, using a plastic bath for this, and the plate itself is incubated at room temperature, protected from light, for 25-30 minutes.

The reaction is terminated by adding 100 μl of stop reagent to all wells.

After adding the stop reagent, the reaction results should be taken into account after 2-3 minutes, by measuring the optical density (OD) in each well on a spectrophotometer with a vertical light beam, at a wavelength of 450 nm, or visually.

Results should only be considered when the following conditions are met:

- OD value in a well with negative control - no more than 0.30 p.u.
- OD value in the well with positive control - not less than 0.62 p.u.
The reaction is evaluated by the formula:

\[
\text{PERCENTAGEOFREACTIVITY} = \left( \frac{OD - ODC + ODC}{ODC + OD} \right) \times 100\%
\]  

Where \( OD \) is the optical density of the sample
\( ODC + \) optical density of the positive control
\( ODC - \) is the optical density of the negative control

The limits of the threshold values of reactivity (limits of the effectiveness of the method): at a value of \( % \geq 22\% \) - the reaction is considered positive (high efficiency); \( % \) value \( <10\% \) - negative reaction (not effective); range from 10\% to 22\% - questionable reaction results (low efficiency).

4. Discussion

Rubella in fish is a fairly common and contagious disease that, according to various researchers, is caused either by a virus or bacteria. There is objective evidence for both hypotheses. It is believed that under the term "rubella", with similar symptoms, there are several diseases. In this connection, the All-Union Institute of Experimental Veterinary Medicine (FGBNU FNTs "VIEV" RAS) proposed to distinguish three independent diseases caused by various bacteria: aeromonosis (Aeromonas punctata), pseudomonosis (genus Pseudomonas), and spring viral disease (VVD) caused by the Rabdovirus virus cyprini [12].

To determine the pathogenicity of the causative agent of carp aeromonosis, white mice can be used as a bioassay. However, along with this, when determining the virulence of the isolated aeromonads, it is mandatory to set up a bioassay on susceptible fish.

The diagnosis of pseudomonosis caused by bacteria of the genus Pseudomonas is determined by bacteriological results, taking into account epizootological indicators, clinical signs and pathological changes. The procedure for bacteriological studies is the same as for aeromonosis. Pseudomonoses are differentiated from aeromonosis, associative infections and complications, pseudomonas of chronic toxicosis. Confirmation of the pathogenicity of pseudomonads in a bioassay on susceptible fish is mandatory [7; 8].

You should also remember about the virulence of bacteria Aeromonas punctata, Pseudomonas. According to the literature, these bacteria have developed resistance to most chemotherapeutic agents. From this it follows that it will take great efforts to control the disease, and not to treat it, which in most cases is risky and expensive [1].

The diagnosis of the spring viral disease (VVD) caused by the Rabdovirus cyprini virus is made according to the clinical picture, taking into account anatomical parameters, epizootic data and virological studies: i.e. - isolation of the virus, its serological identification and confirmation of virulence in a bioassay, as well as the use of the IMP method and PCR [13]. Such studies should be carried out according to the “Methodological guidelines for laboratory diagnosis of viral fish diseases” [6]. The final diagnosis is based on the results of virological studies for the isolation and serological identification of the virus. VVB is differentiated from pseudomonosis and aeromonosis, guided by the provisions of the above document; identification of the isolated virus is carried out using hyperimmune serum in a neutralization reaction. When working with epizootic material, the formulation of the reaction is combined with the isolation of the virus.

The main method for identifying CRC (Aeromonaspunctata, Pseudomonas, Rabdovirus cyprini) today is the clinical and anatomical analysis of pathological changes. The clinical and morphological picture in aeromonosis of pond fish differs from IHC and carp pseudomonosis in a more diverse symptom complex and pathological dynamics of the development of the process, namely: a gradual change or combination of acute septic, subacute ascites-ulcerative and chronic ulcerative forms - this stage of manifestation and development of the disease - an important differential diagnostic sign of aeromonosis, which distinguishes it from pseudomonosis and IHC [14].

With CRC, the disease is associated with a high mortality rate of 80\%-100\%. Such a rapid course of the disease may be the result of mutations leading to the emergence of a new property that allows
bacteria to adapt to new environmental conditions. The incubation period lasts from 7 to 30 days, depending on the temperature of the water [15]. It is allowed to carry out the identification of bacterial species using chemical methods of analysis. Thus, when analyzed by the MALDI-TOF-MS (matrix laser desorption / mass spectrometry) system, isolates are used; they are prepared for mass spectrometry in accordance with the standard protocol recommended by Bruker [16].

Within the framework of epizootological and environmental studies, abroad, the following methods of identification of CRC pathogens are mainly used today [10]: Aeromonas hydrophila - identified with the gel diffusion method, the method of direct fluorescent antibodies, the method of indirect fluorescent antibodies, immunoblotted sodium dodecyl sulfate (SDS) gel - polyacrylamide gel electrophoresis (PAGE) - these methods, however, are of limited value, as various serological types of A. hydrophila are abundant in fish farms [11].

Along with the above-described methods, foreign experts also note a great help for fish farms in the early diagnosis of the bacterial pathogen by the PCR method before the appearance of a clinical picture, which already allows organizing surgical intervention and treatment [17].

Thus, it seems that the capabilities of enzyme-linked immunosorbent assay (ELISA), as a type of immuno-chemical analysis based on the specific immunological reaction of an antigen with the formation of an immune complex.

5. Conclusion

Due to the lack of effective means of preventing rubella in pond fish at the present stage, it is obvious that early diagnosis is one of the most important and effective ways to combat this disease, which is why this work is devoted to the development of a methodology for the rapid detection of antibodies to rubella pathogens in pond fish (Aeromonas punctata, Pseudomonas, Rabdovirus cyprini).

The proposed enzyme-linked immunosorbent assay (ELISA) method for the determination of rubella in pond fish, both theoretically and technologically, makes it possible to quickly and efficiently diagnose rubella in pond fish species within the framework of laboratory activities.

The advantage of ELISA in the diagnosis of CRC in comparison with the bacteriological method lies in the simplicity of setting and a significant reduction in the time of the analysis - 1-2 hours, instead of 4 to 10 days (operational efficiency of the method).

The effectiveness of this technique for the diagnosis of CPR is proposed to be evaluated based on the percentage of optical density obtained with the control ELISA: > 22% - high efficiency; <10% - not effective; from 10% to 22% - low efficiency.

At the same time, the possibility of early diagnosis of this disease, the low cost of diagnostic kits and the ability to automate most stages of the reaction, demonstrate the practical value of the presented express-method for establishing the diagnosis of CRC.

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