ANNOTATION

of the dissertation of Bekenova Aiganym Bekaidarovna for the degree of Doctor of Philosophy (PhD) in the educational program 8D09102 – «Sanitary and environmental safety of livestock products», on the topic «Molecular genetic differential diagnosis of opisthorchiasis and methorchiasis»

Relevance of the study. Invasive diseases caused by members of the *Opisthorchiidae* family are the 8th most common of all foodborne parasites. The most common members of the opisthorchid family are *Opisthorchis felineus (O. felineus), Clonorchis sinensis (C. sinensis), Opisthorchis viverrini (O. viverrini),* and *Metorchis spp*. The larval stages of *O. felineus* and *Metorchis bilis (M. bilis)* have similar morphology and virtually identical life cycles. However, during the marita stage, they are localized in different organs of the definitive host and cause different parasitic diseases. For example, *O. felineus* parasitizes the intrahepatic bile ducts and causes opisthorchiasis, while *M. bilis* localizes primarily in the gallbladder and causes metorchiasis, which is characterized by less pathogenicity for the host organism. In our country, the diagnosis of opisthorchiasis is established regardless of the species of the *Opisthorchiidae* pathogen infecting an animal or person. Clinical diagnosis of opisthorchiasis is difficult due to the lack of specific symptoms and syndromes characteristic of this disease. An integrated approach using various methods is necessary for an accurate diagnosis and a qualitative and quantitative assessment of the disease. The clinical manifestations of opisthorchiasis are varied and depend on both the duration and intensity of the infestation and individual characteristics of the host.

In veterinary and medical practice, coproscopic examinations and duodenal intubation are traditionally used to diagnose opisthorchiasis. These methods rely on the microscopic detection of pathogen eggs in biological material. However, the cyclical nature of helminth egg production, as well as the uneven distribution of eggs within the intestinal contents, often leads to false negative results.

Immunodiagnostics are used as an additional diagnostic method for opisthorchiasis. However, this method is only highly diagnostically valuable for acute opisthorchiasis, whereas in chronic opisthorchiasis, the immune response to opisthorchiasis is evident in only 75% of cases. Furthermore, the antigenic similarity between opisthorchiasis and metorchiasis leads to a false-positive diagnosis of opisthorchiasis in cases of mixed infestation.

The definitive host becomes infected with opisthorchiasis/metorchiasis by consuming raw or undercooked fish containing metacercariae larvae. Fish are tested for the presence of opisthorchiasis pathogens by microscopic examination of the dorsal muscles of carp. This method is labor-intensive and requires highly qualified specialists. Also, differentiating the larvae of *O. felineus* and *M. bilis* is difficult due to their similar morphological characteristics.

The use of molecular genetic methods for the diagnosis and differentiation of opisthorchiasis can solve this problem. Polymerase chain reaction (PCR) allows for the detection of *O. felineus* and *M. bilis* at any stage of the life cycle (egg,

miracidium, metacercaria, marita). The development of highly sensitive and specific PCR tests for the identification of opisthorchids is an important and pressing issue with significant veterinary and medical significance. In turn, the use of DNA from Kazakhstani isolates in the development of a PCR test for opisthorchids differentiation will improve the specificity and sensitivity of the developed method.

Research **objective**: To develop a molecular genetic method for detection and differentiation of trematodes of the *Opisthorchiidae* family.

To achieve this objective, the following tasks were defined:

- Conducting veterinary and sanitary examination of fish for the presence of pathogens of the *Opisthorchiidae* family of trematodes;
- Isolation of *Opisthorchiidae* family of trematodes metacercariae to obtain pathogen DNA;
- Conducting PCR analysis, sequencing, and bioinformatics analysis of genetic markers of *Opisthorchiidae* family of trematodes;
- Design and synthesis of primers for the detection and differential identification of pathogens;
- Determining optimal conditions, determining the sensitivity and specificity of PCR analysis for the identification and differential diagnosis of *O. felineus* and *M. bilis*;
- Developing scientific and technical documentation for the development and application of a PCR test in laboratory practice.

Research materials and methods. The study was conducted from 2018 to 2020 as part of the research project "PCR Test for Detection and Differential Diagnosis of Opisthorchiasis and Metorchiasis Agents" (AP05131132) and from 2024 to 2026 as part of the budget program "Improving Food Safety" (BR22885795).

The study was carried out at the Agricultural Biotechnology Research Platform of the S. Seifullin Kazakh Agrotechnical Research.

Object of the study. The study utilized a combination of parasitological, molecular genetic, and statistical methods.

A compressor method was used to conduct a parasitological study of fish for the presence of opisthorchiasis and methorchiasis pathogens in fish muscles. DNA from metacercariae was isolated using the phenol-chloroform method, with pre-treatment of the test samples with extraction and *Proteinase K*. Amplification of the extracted DNA from the metacercariae of Opisthorchiidae pathogens was performed in a 25 μl reaction mixture. PCR products were purified from residual oligonucleotides by dephosphorylation using alkaline phosphatase *SAP (shrimp alkaline phosphatase*) and endonuclease. PCR products were sequenced using the Sanger method using the BigDye Terminator kit (*Applied Biosystems, Thermo Fisher Scientific,* USA) according to the manufacturer's instructions. The same primers as for PCR were used for the reaction. The resulting sequencing products were analyzed on an *ABI 3130XL* genetic analyzer (*Applied Biosystems,* USA), and chromatogram processing and editing were performed using *Sequencing Analysis 5.2,* Patch 2 software *(Applied Biosystems,* USA). After sequencing, the products

were purified with an acetate-alcohol mixture: 28 µl of 3 M sodium acetate (pH 5.2) were added to the sample, the plate was covered with film, and kept in the dark for 20 minutes. Alignment of the resulting nucleotide sequences was performed using the Nucleotide Blast online resource. Primer design and primer mapping were performed using Ultra Edit 32 software. The specificity of the developed primers was tested using Nucleotide Blast (www.ncbi.nlm.nih.gov/blast). Conversion of the specific Reverse primer was performed using *SeqState* software. Analysis of the occurrence of homo- and heterodimers, *GC* content, and optimal annealing temperature were verified using *Oligo Analyzer* software.

Statistical analysis of the obtained data was performed using variation statistics. Infestation prevalence was expressed as a percentage as the ratio of the number of infected fish to the total number of individuals examined. Pearson's $\chi 2$ tests were used to assess the significance of differences between categorical variables.

Subject of the study. Metacercariae of the pathogens O. felineus and M. bilis, as well as the nucleotide sequences of DNA of the studied trematode species.

Scientific novelty. Primers were developed to amplify nucleotide sequences of the *ITS2* region of the nuclear ribosomal RNA gene cluster and a fragment of the mitochondrial *COX1* gene. Using these primers, a species-specific and multiplex PCR assay was developed for two important opisthorchid trematode – *O. felineus* and *M. bilis*.

Practical significance. A PCR test based on primers for the amplification of the ribosomal marker *ITS2* detects the DNA of *O. felineus* and *M. bilis* pathogens at low sample concentrations. A PCR test based on primers for the amplification of the mitochondrial gene *COX1* is well suited for species differentiation, allowing for the detection of DNA from both pathogens in a single sample. The developed PCR tests can be used to detect opisthorchiasis and methorchiasis pathogens at any life stage, including during veterinary and sanitary inspection of fish for helminths.

Scientific and technical documentation for the manufacture and use of the PCR test in laboratory practice has been developed:

- Laboratory regulations for the production of «ПЦР-тест система для детекции и дифференциальной диагностики *Opisthorchis felineus* и *Metorchis bilis»* (PCR test system for the detection and differential diagnosis of *Opisthorchis felineus* and *Metorchis bilis*);

- Methodological recommendations for the differential diagnosis of opisthorchiasis and methorchiasis.

The research results obtained as part of this dissertation are used in the educational process at the S. Seifullin Kazakh Agrotechnical Research University for lectures and laboratory and practical classes for students in the educational program 6B05102 – "Biotechnology" in the subjects "Molecular Genetic Foundations of Biotechnology" and "Molecular Genetics and Genetic Engineering".

Main provisions for defense:

- Specific primers for the identification of trematodes of the *Opisthorchiidae*

family based on the marker region of the nuclear (*ITS2*) and mitochondrial (*COX1*) genes of the pathogens;

- A species-specific PCR test based on the ribosomal cluster for the identification of *O. felineus* and *M. bilis*;

- A multiplex PCR test for the detection and differentiation of *O. felineus* and *M. bilis* based on the mitochondrial *COX1* gene.

Publications and approval. The main results of the study are reflected in 13 (thirteen) published papers, in four publications recommended by the Committee for Quality Assurance in Science and Higher Education of the Ministry of Science and Higher Education of the Republic of Kazakhstan, in articles of journals included in the Scopus database: "Advances in Animal and Veterinary Sciences" (2020), percentile 32; "Veterinary World" (2025), percentile 87 and in the proceedings of international conferences, a patent for an invention of the Republic of Kazakhstan was received

The author's personal contribution. The author was directly involved in the bulk of the work completed within the scope of this dissertation. The collection and analysis of literature data, the development of the research direction, the definition of goals and objectives, the conduct of experimental studies, the processing and interpretation of the obtained results, the preparation of scientific articles, and the writing of the dissertation text are the author's personal contributions. The author was also responsible for the formatting of the manuscript and the introduction of the obtained results into scientific circulation.

Structure and volume of the dissertation work. The work is presented on 90 pages of computer text. The dissertation consists of the following sections: introduction, literature review, materials and methods, research results, discussion of results, conclusion, references, and appendices. The dissertation contains 6 tables and 38 figures. The list of references includes 127 sources, including 102 in a foreign language.