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8D09101 "Veterinary Medicine"
for the degree of Doctor of Philosophy (PhD)

ANNOTATION

On the topic of the dissertation: "Epizootological monitoring and molecular genetic analysis of wolf helminths in the northern and central Kazakhstan"

Астана – 2025 год

1. General characteristics of the work.

The work is devoted to a comprehensive epizootological and molecular genetic study of helminths of wolves living in the northern and central regions of Kazakhstan, in the field of veterinary parasitology.

2. Relevance of the topic:

Wild predators, such as wolves, foxes, and corsac foxes, are natural hosts for many types of parasites that pose a danger to humans and animals [1-3].

Wolves (*Canis lupus*) play an important role in natural ecosystems, such as maintaining the number of ungulates, recycling dead animals. [4,5]. However, wolves are also natural carriers and distributors of many parasitic invasions [6,7]. Among these, taeniasis, echinococcosis, toxocariasis, and trichinosis pose a serious threat to human health.

Helminthiases in Kazakhstan have their own epizootic and epidemiological features, caused by specific climatic and socio-economic conditions. The entire territory of the republic is favorable for the mass spread of various types of helminths [8].

Until now, parasitological studies in the republic have been mainly based on morphological methods, which have limited accuracy in identifying species and do not allow genotyping of parasites. This limitation hinders the development of an effective strategy for epizootiological monitoring and prevention of helminthiasis.

Currently, there are only isolated reports on studies of the parasite fauna of wild carnivores. According to available literature, parasites found in Kazakhstan belong to the families *Trichocephalida, Taeniidae, Cyclophyllidea, Toxocarida,* and *Opistarchidae*. These data were obtained based on morphological features, which have several shortcomings and limitations compared to molecular biotechnology methods [9, 10]. The use of molecular genetics and bioinformatic approaches allows not only to accurately determine the taxonomic affiliation of parasites, but also to identify intraspecific genetic variability.

Regular monitoring and prevention of helminthic invasions in the natural habitats of animals enable the control of parasite spread in ecosystems and minimise threats to agriculture and healthcare. Therefore, studies of the genetic biodiversity and epidemiology of causative agents of wolf helminthiasis that are dangerous to animals and humans are relevant for modern veterinary science, medicine and ecology in Kazakhstan [11, 12].

Current trends in molecular epizootology are influenced by global strategies of international organisations — FAO, WHO and WOAH, united within the framework of the “One Health” concept. According to updated recommendations of the World Organisation for Animal Health (WOAH, 2024), priority is given to the use of highly accurate molecular methods — PCR, sequencing and bioinformatics analysis — for epizootological surveillance, especially with respect to zoonoses transmitted from wild animals. Molecular techniques, including the sequencing of ribosomal RNA genes and mitochondrial genes (cox1, nad1, and ITS), provide a reliable basis for the accurate identification of pathogen species and genotypes. These advanced techniques not only facilitate the identification of previously unrecognised genotypes and haplotypes but also enable more efficient tracing of transmission routes and identification of infection sources. This is especially important given the high geographic variability exhibited by

Echinococcus species, which may lead to different epidemiological patterns and challenges in disease management.

In connection with the above, the dissertation employs molecular genetic methods to study the helminth fauna of *C. lupus* for epidemiological monitoring and the development of a strategy for preventing parasitosis in natural ecosystems [13, 14].

Key words: wolf (*Canis lupus*), helminthiasis, epizootological monitoring, molecular genetic analysis, parasites, PCR, sequencing, bioinformatics, genotyping, zoonoses.

3. Purpose and objectives of the research

The aim of this work is to conduct epizootological monitoring and molecular diagnostics of helminthiasis in wolves in northern and central Kazakhstan.

To achieve the goal, the following *tasks* were set:

- determination of the species composition and taxonomic affiliation of wolf parasites by morphological characteristics;
- determination of helminth infestation indicators, epizootological monitoring of wolf helminthiasis;
- molecular genetic analysis of isolated helminths for genotyping;
- bioinformatics analysis of wolf helminth DNA in comparison with reference analogues, construction of phylogenetic trees of detected parasites;
- development of methodological recommendations for molecular identification of wolf helminths.

4. Object and subject of research

The object of the study is helminths parasitising wolves (*Canis lupus*) living in the northern and central regions of Kazakhstan. The subject of the study is the epizootological features, taxonomic structure and molecular genetic characteristics of the identified helminths.

5. Materials and methods:

The work was conducted between January 2019 and April 2024, and the internal organs of 81 wolves from five regions were studied. These included 3 samples from Pavlodar, 48 samples from Karaganda, 9 samples from Ulytau, and 21 samples from Kostanay regions. These samples were obtained from licensed hunting grounds that adhered to regulated limits on the removal of wolves from their natural habitat.

The delivered material was examined by methods of complete and/or incomplete helminthological dissection according to K. I. Skryabin [15, 16], as a result of which a collection of helminths was created, and taxonomic affiliation was established according to classical identifiers based on morphological characteristics of parasites [17]. In addition, muscle tissue was studied using compression diagnostics and artificial digestion methods, and coprological studies of faeces for the presence of helminths were carried out. Based on the statistical processing of the obtained data, a comprehensive analysis and epizootological monitoring of helminthiasis in wolves were carried out in the ecosystems of northern and central Kazakhstan [18].

Species identification of the detected helminths was clarified using molecular genotyping methods. For this purpose, DNA extraction methods from helminth samples were modified using commercial kits and the phenol-chloroform method; optimal reaction parameters (homogenisation, temperature conditions, volume and concentration of buffers and reagents) for extracting genetic material for each species were selected. The biodiversity of the detected parasites was analysed using primers for the marker regions of ITS1, ITS2, cox1, and *18s rRNA*, specific for each species. At the next stage of the research, the parameters for setting up PCR analysis for each marker gene were optimized. Then, sequencing of the obtained amplicons for the marker region of the gene and species identification was performed.

Next, bioinformatic analysis of the nucleotide sequences was performed by determining identity with known reference analogues using the Basic Local Alignment Search Tool (BLASTtn) programs. Phylogenetic trees of the identified species were then constructed in MEGA11 software [19], using ClustalW multiple alignment and maximum likelihood mathematical modelling.

6. Scientific novelty of the work:

For the first time in the conditions of northern and central Kazakhstan, a comprehensive study of the helminth fauna of wolves was conducted using molecular genetic methods, which enabled the clarification of species affiliation, genetic diversity, and phylogenetic relationships of parasites. During the study, for the first time in the republic, the circulation of *Trichinella nativa* and *Dirofilaria repens* in wolves was confirmed with the registration of the corresponding nucleotide sequences in the GenBank database. Five unique haplotypes of *Echinococcus granulosus*, previously not described in the studied ecosystems, were identified. The obtained data significantly supplement the information on the taxonomic structure of the parasite fauna of wild predators of Kazakhstan and demonstrate the importance of molecular approaches for epizootological monitoring and prevention of zoonotic infections.

7. Main provisions submitted for defense:

1. Taxonomic affiliation of helminths in wolves based on morphological features;
2. Helminth infestation, epizootological monitoring of helminthiasis in wolves in northern and central Kazakhstan;
3. Molecular genetic identification of wolf helminths;
4. Bioinformatics analysis of DNA of helminth species.

8. Practical significance

The distribution and epizootic state of wolf helminthiasis in the northern and central regions of the country were studied. The main methods for isolating genomic DNA of parasites using phenol-chloroform isoamyl and commercial kits (Gene Jet genomic DNA purification kit, Biolabmix DNA extraction kit, Monarch DNA purification kit) were developed. Species- and genus-specific primers for marker regions of the genome for genotyping helminths were selected.

Based on the developed protocols, methodological recommendations for genetic identification of helminth species are proposed: *Echinococcus granulosus, Taenia hydatigena, Mesocestoides* spp.*, Toxascaris leonina, Trichinella nativa, Alaria alata* and *Dirofilaria repens*.

The obtained results serve as a basis for developing effective strategies for monitoring and managing helminthiasis in the wild wolf population in the ecosystems of northern and central Kazakhstan. The results are important for veterinary medicine, sanitary epidemiology, wildlife conservation, and public health. The data can be used to develop national programs for the prevention and control of echinococcosis.

9. Main research results, conclusions

Conclusion 1: Taxonomic analysis and epizootological monitoring of the parasite load on the wolf population in the studied ecosystems showed a high overall level of helminthic invasion - 72.4%. The degree of infestation varied significantly depending on the region. The highest parasite load was noted in wolves living in steppe and semi-desert ecosystems, where natural conditions contribute to the circulation of helminths. Thus, in the territory of central Kazakhstan in the Karaganda region, the level of infestation was 42%, in the Ulytau district, it was 12.3%. In the territory of northern Kazakhstan in the Kostanay region, 21%, the minimum indicators were recorded in the Pavlodar region, 4%, which is probably due to the limited sample size. Due to the fact that no limits were set on the removal of wolves in the North Kazakhstan region, there are no data provided for this region. The findings highlight the influence of environmental factors on the spread of helminthiasis among wild predators.

Conclusion 2: It has been established that the helminth fauna is represented by four classes of parasites (*Trematoda, Cestoda, Nematoda* and *Acanthocephala*), belonging to three types: *Platyhelminthes, Nemathelminths* and *Acanthocephala*. The species composition of helminths includes *Alaria alata* (trematodes), *Echinococcus granulosus*, *Taenia hydatigena, Mesocestoides* spp. (cestodes), *Toxascaris leonina, Trichinella nativa*, *Dirofilaria repens* (nematodes) and *Oncicola* spp. (acanthocephals). The most common were cestodes, the prevalence of which reached 54.3%, and taeniids, 50%, with an average prevalence of 31.1 specimens, including *E. granulosus*, 4.3%, with a prevalence of 20.5 specimens, which indicates their leading role in the formation of the parasite load in wolves.

Conclusion 3: It was found that individuals aged 1 to 4 years were the most vulnerable to helminthic invasion, with an infection rate of 88%. Young wolves aged 3 months to 1 year demonstrated a significantly lower rate of infection – only 20%. At the same time, males (70%) were more prone to infection than females (30%), which is explained by the dominant social status of the former in the pack. The study showed that wolves living in steppe and semi-desert regions were more prone to infection with helminths. Among these regions, the semi-desert Karaganda region had the highest prevalence of helminthic infections, indicating that the environment in this region is particularly favorable for the spread and circulation of helminths.

Finding 4: Molecular genetic analysis was performed on 81 muscle tissue samples from wolves to clarify the species of *Trichinella* spp., of which 17 (21%) tested positive for *Trichinella*. Species identification was performed using multiplex PCR with amplification of the *ESV*, *ITS1*, and *ITS2* genes [20], as well as PCR of the *5s rDNA* and ITS1 gene regions [21] followed by sequencing. Based on the obtained sequences, a phylogenetic tree was constructed, which confirms the circulation of *T. nativa* (Genbank accession number: OP829905, OP829904) among wolves in Kazakhstan.

Conclusion 5: Of the 81 analyzed samples, 8 wolves (9.6%) from Kostanay (4 individuals), Ulytau (2 individuals) and Karaganda (2 individuals) regions were found to be infected with *Toxascaris leonina*, which was confirmed by the results of molecular identification (GenBank accession numbers: OR647588, OR647594). The conducted ribosomal DNA analysis using *NC5/NC2* primers [22] not only reliably confirmed the species affiliation of the parasite but also identified three clearly formed phylogenetic clades, including a separate clade of *T. leonina* found in wolves. These data indicate a stable circulation of this nematode parasite among wild predator populations of the region and also indicate potential risks for domestic animals and humans. It is noteworthy that *Toxocara canis*, which is widespread among carnivores, was not detected in this study, which may reflect specific regional features of the distribution of nematodes in the northern and central regions of Kazakhstan.

Conclusion 6: Among the studied wolves infected with echinococcosis, two individuals were identified from Karaganda and Kostanay regions, from which a total of seven samples of the pathogen were isolated. The level of echinococcosis infection was 4.3%, which confirms the presence of stable natural foci in these regions. To assess the genetic diversity of *Echinococcus granulosus*, mitochondrial markers *cox1* and *nad1* [23] were used, with parameters adapted to the characteristics of the biomaterial. Sequencing of amplified fragments enabled the identification of five different haplotypes (GenBank: OM319836, OM319830, OM319844, OM640352, OM640353, OM640354), indicating high intraspecific variability of the parasite. The obtained data are important for understanding the genetic structure of *E. granulosus* populations in Kazakhstan, and also serve as a basis for further epidemiological monitoring and the development of effective strategies for the control of echinococcosis in wild and domestic animals. The results fill significant gaps in knowledge about the spread of this pathogen among key reservoir species - in particular, wolves.

Conclusion 7: For the first time, the presence of *Dirofilaria repens* in the wolf heart was confirmed using molecular genetic methods, providing important evidence of the circulation of this parasite among wild predators in Kazakhstan. Species identification was performed based on the amplification of the *SSU* rRNA region using species-specific primers, and the resulting nucleotide sequence was deposited in the GenBank database under the number MT877205.

In addition, during the study of the ribosomal gene of trematodes found in wolves, the species *Alaria alata* was identified, and its DNA sequence was also deposited in GenBank (accession number: ON358106). These results expand the understanding of the spectrum of helminths infecting wild carnivores of the region and emphasise the importance of molecular diagnostics for the accurate detection of pathogens with potential zoonotic danger.

Conclusion 8: Bioinformatic analysis of nucleotide sequences obtained during molecular genetic studies of wolf helminths confirmed the species affiliation of parasites and their phylogenetic relationships with known reference strains. Phylogenetic trees constructed based on ribosomal regions showed that all positive *Trichinella* samples form a single clade with *Trichinella nativa* circulating among wolves in Kazakhstan. Analysis of nematode sequences revealed three distinct phylogenetic clades, including a separate clade of *Toxascaris leonina*. Sequencing of mitochondrial regions allowed us to identify five unique haplotypes of *E. granulosus*, reflecting the high genetic diversity of the pathogen in the studied ecosystems. The phylogenetic identity of the *D. repens* and *A. alata* species with analogues from neighboring regions of Eurasia was also confirmed, which emphasizes the relevance of using bioinformatic methods for epidemiological monitoring of natural foci of parasitic diseases.

10. Relationship with research and development and government programs

The study was carried out with the financial support of the Committee of Sciences of the Ministry of Education and Science of the Republic of Kazakhstan within the framework of the project AP08052252 for 2020-2022 "Creation of a method for determining the species identity of parasites most common among wild carnivores using molecular biotechnology methods"; Project leader: PhD, Associate Professor V.S. Kiyan.

11. Reliability and validity of the results obtained

The use of a set of modern and classical research methods, including morphological identification of helminths, epizootological analysis methods, and molecular genetic approaches such as PCR, sequencing, and bioinformatics analysis, confirms the reliability and validity of the obtained results. All methods were carried out in compliance with reproducibility standards, with multiple repetitions of experiments and control of negative and positive samples. To confirm the species affiliation of parasites, validated primers and international databases (NCBI GenBank) were used, where the nucleotide sequences obtained during the study were also entered. Phylogenetic trees were constructed using generally accepted software (MEGA11), which made it possible to reliably determine the evolutionary relationships of the identified parasites with reference strains. In addition, the data obtained are consistent with the results of previous international and domestic studies, which further confirms the scientific validity of the conclusions.

The obtained results were confirmed by publications in journals with a high impact factor and in 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 the proceedings of international scientific conferences near and far abroad.

12. Information on publications on the main results:

1. Uakhit R, Smagulova A, Lider L, Leontyev S and Kiyan V (2024) Epizootiological monitoring of wolf helminths in Northern and Central Kazakhstan, Veterinary World, 17(7): 1648–1654. DOI: 10.14202/vetworld.2024.1648-1654. **Percentile: 82%; Q2**
2. Uakhit, R., Mayer-Scholl, A., Shin, C., Smagulova, A., Lider, L., Leontyev, S. & Kiyan, V. (2023) Genetic identification of Trichinella species found in wild carnivores from the territory of Kazakhstan. *Front Vet Sci*. 10: 1266561. doi: https://doi.org/10.3389/fvets.2023.1266561. **Percentile: 92%; Q1**
3. Uakhit R, Smagulova A, Lider L, Shevtsov A, Berber AA, Berber AP, Bauer C and Kiyan V (2024) Molecular identification of Baylisascaris melis (Gedoelst, 1920) from the Eurasian badger (Meles meles) and ascarids from other wild carnivores in Kazakhstan. *Front. Vet. Sci*. 11:1452237. doi: https://doi.org/10.3389/fvets.2024.1452237 **Percentile: 92%; Q1**
4. Uakhit, R., Smagulova, A., Syzdykova, A., Abdrakhmanov, S. & Kiyan, V. (2022) Genetic diversity of Echinococcus spp. in wild carnivorous animals in Kazakhstan. *Vet World*. 15(6): 1489-1496. doi: https://doi.org/10.14202/vetworld.2022.1489-1496. **Percentile: 82%; Q1**
5. Uakhit, R.S., Lider, L.A., Smagulova, A.M., Leontyev, S.V., Abdrakhmanov, S.K., & Kiyan, V.S. (2021). First molecular identification of Dirofilaria repens found in

a wolf heart in Kazakhstan. *Adv. Anim. Vet. Sci.* 9(9): 1342-1346. https://doi.org/10.17582/journal.aavs/2021/9.9.1342.1346. **Percentile: 47%; Q3**

13. Testing the results of the dissertation:

1. Uakhit R.S., Zhumalin A.Kh., Syzdykova A.S., Smagulova A.M., Migushin A.A., Leontyev S.V. Helminth fauna of wild carnivores of the steppe zone of Kazakhstan. Proceedings of the international scientific and theoretical conference "Seifullin Readings - 17: "Modern Agricultural Science: Digital Transformation", dedicated to the 30th anniversary of Independence of the Republic of Kazakhstan. - 2021. - Vol. 1, Part 1 - P. 316-318.
2. Uakhit R.S., Lider L.A., Kan M.Advantages of the staining method for studying the morphological characteristics of cestodes. Proceedings of the international scientific and practical conference "Seifullin Readings - 18(2): "Science of the XXI century - the era of transformation", 2022.- T.I, Ч.II.-P.205-207.
3. Uakhit R.S., Lider L.A., Kiyan V.S. Molecular identification of cryptic cysticercosis: Taenia krabbei in Kazakhstan wild carnivores. European Biotechnology Congress, 2021. Special issue. – P. 32 – 35. DOI: https://doi.org/10.2478/ebtj-2021-0031.
4. Rabiga Uakhit, Vladimir Kiyan. Molecular genetic methods in species identification of wolf helminth fauna. 3rd International Scientific and Practical Internet Conference “Integration of education, science and business in modern environment: summer debates”.
5. Rabiga Uakhit, Vladimir Kiyan, Lyudmila Lider. Identification of species taenia (Cestoda: Taeniidae) isolates from wolf. II International Scientific and Practical Conference "Issues of Practice and Science". London, UK, 2021, -pp. 195-198.
6. Uakhit R.S., Kiyan V.S., Smagulova A.M. Genetic identification of helminthiases in wild carnivores. Proceedings of the International Scientific Conference "Zoological Research in Kazakhstan in the 21st Century: Results, Problems and Prospects" dedicated to the 90th anniversary of the RSE "Institute of Zoology" of the KN MNVO RK. Almaty, 2023. - P. 101-107.
7. Rabiga Uakhit, Lyudmila Lider, Sergey Leontyev, and Vladimir Kiyan. Molecular identification of *Echinococcus* spp. in Kazakhstan wolves. International scientific conference "XXIV Satpayev readings" dedicated to the 125th anniversary of academician Kanysh Satpayev, Pavlodar, 2024, - P. 340-342.
8. Uakhit R.S., Kiyan V.S., Lider L.A., Smagulova A.M. Genetic diversity of echinococcosis in the Republic of Kazakhstan // Abstracts of the VII All-Russian Congress on Medical Microbiology, Clinical Mycology and Immunology (XXVI Kashkin Readings). - V. 25, No. 2. - St. Petersburg, 2023. - P. 123.
9. Uakhit R.S., Mannapova N.E., Jazina K.T. Preliminary study of echinococcus species distribution in Kazakhstan // Proceedings of the international scientific and practical conference “Seifullin Readings – 19”, Astana, 2023. Vol. 1, part 2, p. 88-90.
10. The obtained scientific results were used in the development of methodological recommendations on methods of molecular identification of wolf helminths. The methodological recommendations were approved at a meeting of the Academic Council of the National Centre for Biotechnology, Minutes No. 20 dated October 16, 2024. "Methodological recommendations on methods of molecular

identification of wolf helminths" / Prepar. Uakhit R.S., Kiyan V.S. / National Center for Biotechnology, Ministry of Health of the Republic of Kazakhstan. - Astana, 2024. - 27 p.

14. Description of the doctoral student's contribution

The doctoral student personally prepared and conducted laboratory research of biological material, including helminthological dissection and morphological identification of parasites. Protocols for DNA extraction from various types of helminths were modified, and optimal conditions for PCR amplification and sequencing were selected. The doctoral student participated in the development of primers and setting up reactions to marker regions of the parasite genome, and also conducted a bioinformatics analysis of nucleotide sequences and the construction of phylogenetic trees.

All stages of the work - from setting tasks, conducting epizootological monitoring to interpreting the results and writing scientific articles - were carried out under scientific supervision, with the active personal participation of the doctoral student, which indicates her high research independence and qualifications in the field of veterinary parasitology and molecular diagnostics.

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