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Molecular evaluation of *Eimeria* spp. Infection in the Volga-Ural Saiga antelope population of the Republic of Kazakhstan

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ABSTRACT

Saiga antelope (*Saiga tatarica*) is a protected species in Kazakhstan. Little is known about the parasitofauna of these mammals. Therefore, the focus of this study was to evaluate the prevalence and species diversity of *Eimeria* spp. infection in the Volga-Ural Saiga antelope population. In June 2023, 104 Saiga antelope fecal samples collected from the district of Zhanibek, located in the province of West Kazakhstan were evaluated using microscopic and molecular techniques. Based on coprovoscopy results, *Eimeria* spp. Oocysts were present in 22 samples (21%). The four fecal samples containing the largest numbers of *Eimeria* spp. Oocysts per 10x field were selected for further genetic analysis. DNA extraction, nested PCR amplification, and sequencing were performed on 91 clones, with 80 clones forming a distinct clade and exhibiting genetic similarity to MT801034 E*imeria* sp. Voucher HY3. These clones possibly represent an *Eimeria* specific to Saiga antelopes and gazelle that has previously been morphologically described as *Eimeria elegans* (Svanbaev, 1979), underscoring the importance of further research into parasitic infections in this protected species.

1. Introduction

Although not formally included in Kazakhstan's Red Book of Rare and Endangered Species, the Saiga antelope (*Saiga tatarica*) is recognized as a protected species within the country (Ministry of Ecology and Natural Resources of the Republic of Kazakhstan, 2023). Their habitat spans ten oblasts (regions) of Kazakhstan. Three distinct populations of Saiga antelope have been identified: the Betpakdala population, residing exclusively within Kazakhstan; the Volga-Ural population, inhabiting the West Kazakhstan region and occasionally migrating into Russia; and the Ustyurt population, found in the Aktobe region of Kazakhstan and migrating to Uzbekistan during winter. The total Saiga antelope population in spring 2023 exceeded 1.9 million individuals, with over 95% of the global Saiga population inhabiting Kazakhstan (Ministry of Ecology, Geology and Natural Resources of the Republic of Kazakhstan). Notably, the Volga-Ural population boasts the largest numbers, totaling 1.13 million individuals, followed by the Betpakdala population with 745, 300 animals, and the Ustyurt population with 39,700 individuals. As per a 2023 aerial survey of Kazakhstan, Saiga antelope populations were estimated to have increased by 597,000 individuals over the previous year's estimates, indicating a 45.3% increase in population size (Ministry of Ecology, Geology and Natural Resources of the Republic of Kazakhstan). This increase in population may be due to improved conservation practices, including restoration of their natural habitat, as well as implementation of specific breeding programs (Ministry of Ecology and Natural Resources of the Republic of Kazakhstan, 2023).

Infectious diseases pose significant threats to Saiga antelope populations (Lundervold, 2001). Since 1980, there have been 11 reported instances of mass Saiga antelope mortality, with one of the most devastating occurring in 2015 due to pasteurellosis, resulting in the disposal of over 150,000 deceased animals (Lushekina, 2010; Fereidouni et al., 2019; Absatirov et al., 2013). Scientists from the Research

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Institute of Biological Safety Problems in Kazakhstan also identified theileriosis in Saiga antelopes affected by the 2015 mass mortality event, suggesting its involvement alongside *Pasteurella* infection (Orynbaev et al., 2013). Furthermore, there has been a notable decline in the diversity of Saiga antelope parasites over the past two decades. While 36 parasite species (seven protozoa, five cestodes, and 24 nematodes) were documented in the Volga-Ural Saiga antelope population during the 1990s (Bisenov et al., 1994), subsequent studies in the 2000s reported only 15 helminth species (three cestodes and 12 nematodes) (Morgan et al., 2005). More recently, only 11 parasites (nine helminth species and two protozoa species) were found in a study conducted between 2021 and 2023 (Abdybekova et al., 2023).

Literature concerning coccidia and coccidiosis in wild animals, including Saiga antelopes, is relatively scarce (Chatterjee et al., 2023). Many of the existing studies occurred more than 40 years ago and are not widely accessible (Svanbaev, 1979). In the 1950s, a new species of *Eimeria* was morphologically described in Saiga antelope and named *Eimeria elegans* (Svanbaev, 1979). Thus far, this parasite species has not been found in any other host. Additional studies focusing on coccidia infection can assist with determining overall parasite prevalence and infecting species, with a long-term goal of evaluating clinical impact on these animals. The objective of this study was to study the prevalence of coccidia infection in ground fecal samples collected from the Volga-Ural Saiga antelope population and conduct molecular analysis to determine infecting parasite species.

2. Materials and methods

In June 2023, a total of 104 fecal samples were collected from the Volga-Ural Saiga antelope population residing in the Zhanibek District of West Kazakhstan Province. Fecal samples were collected opportunistically immediately after defecation by staff from the Kazakh Research Scientific Veterinary Institute during a field survey. At the time of sample collection, no specific animal-level data (e.g., age and sex) were recorded. The samples underwent examination utilizing the Darling method, where approximately 5–10 g of fecal matter were meticulously mixed with water in a mortar. The resulting suspension was filtered through a metal sieve into centrifuge tubes and centrifuged for 2–5 min. Subsequently, the supernatant was decanted, and a mixture comprising equal proportions of glycerol and a saturated solution of table salt was added to the sediment. The contents of the centrifuge tube were thoroughly homogenized and subjected to centrifugation once more. The upper layer of the liquid was transferred onto a slide using a loop, which was then covered with a coverslip and examined under a Carl Zeiss Primo Star upright transmitted light microscope at 10x and 40x magnification. Pictures of oocysts were taken at multiple points. Species identification of oocysts was conducted in accordance with the methodologies outlined by Heisin (1967) and Svanbaev (1979). Infection prevalence was reported as the number of samples positive for a parasite genus or species divided by the total number of samples evaluated.

Molecular analyses were carried out for *Eimeria* oocysts. Four fecal samples were selected, containing 39, 68, 75, and 101 *Eimeria* oocysts per 10x microscopic field. These samples were selected because they contained the largest numbers of *Eimeria* oocysts per 10x field during microscopic examination. Oocyst flotation was performed using 1.6 g of fecal material in a saturated saline solution following the protocol proposed by Kumar et al. (2018). Genomic DNA was extracted using a commercial kit (Wizard Genomic DNA Purification Kit, Promega, USA) according to manufacturer's instructions. Briefly, 600 µL of Nuclei Lysis Solution was added to the pellet of oocysts, homogenized, and incubated at 65 °C for 30 min. After incubation, 3 µL of RNase Solution was added, incubated for 30 min at 37 °C, followed by addition of 200 µL of Protein Precipitation Solution, vortexed, chilled on ice for 5 min, and centrifuged at 13000×*g* for 4 min. The supernatant was transferred to a fresh tube containing 600 µL of isopropanol, mixed, and centrifuged at 13000×*g* for 1 min. After centrifugation, the supernatant was removed, and 600 µL of 70% ethanol was added to the pellet and centrifuged at the conditions described above. The ethanol was aspirated, the pellet was air-dried, and the DNA rehydrated in 50 µL of DNA Rehydration Solution for 1 h at 65 °C.

2.1. Amplification of 18S rRNA fragment

The 18S rRNA fragment was amplified by nested PCR using the primers: Eimeriidae_18S_F_250 5′-GGTGATTCATATAGTAACCGAAC-3′ and Eimeriidae_18S_R_1120 5′-TCGGCATAGAGTTTATGGTAGAGAGAT-3′ for the first round of PCR, Eimeriidae_18S_F460 5′-CAATGAAAA-CAGTTTCGAGTTTCGAGGTA-3′ and Eimeriidae_18S_R_1100 5′-GGTATCTAATCTAATCGTCTTCTTCTTCAAACC-3′ for the second round of amplification. The 20 µl PCR mixture included 1 µl each of forward and reverse primer at a concentration of 10 pmol/µl, 4 µl 5X Phusion™ HF Buffer, 2 µl 2 mM dNTPs, 0.6 µl DMSO, 0.2 µl Phusion™ High-Fidelity DNA Polymerase (Thermo Scientific™, Lithuania), 5 µl DNA or 2 µl PCR mixture of 1 go-round of PCR and water to 20 µl. PCR cycling included: initial denaturation at 98 °C for 30 s, 30 cycles at 98 °C for 10 s, 55 °C for 15 s, 72 °C for 20 s; final elongation for 5 min at 72 °C. PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems). Electrophoresis was performed using 1.5% agarose gel with ethidium bromide.

2.2. Cloning and sequencing

Round 2 PCR products were purified with AMPure XP Beads magnetic particles (Beckman Coulter) and eluted in 10 mM Tris-HCl (pH 7.4), to add adenine (A) at the 3′-end. The adenine addition reaction included: 2 µl 10X Taq Buffer with KCl, 2 µl 25 mM MgCl2, 2 µl dNTP Mix (2 mM each - dATP, dTTP, dGTP, dCTP), 0.4 µl Taq DNA Polymerase (Thermo Scientific™, EP0406), 10 µl purified PCR products from step 2 and water to 20 µl. The reaction mixture was incubated at 72 °C for 60 min. The PCR products were then cloned into the pGEM®-T vector using the pGEM®-T Easy Vector System kit (Promega, Mannheim, Germany) and transformed into *E. coli* DH5α strain according to the manufacturer's instructions. Clones containing the insert were selected using the LacZ system by blue-white screening (Gossen et al., 1992). Clones with the insert were transferred into 20 µl of 10 mM Tris-HCl (pH 7.4), incubated at 98 °C for 5 min, and centrifuged at 2000g for 15 min. The supernatant was used for amplification of the insert with M13 primers. The reaction mixture included: forward and reverse primer 1 µl each at a concentration of 10 pmol/µl, 2 µl 10X Taq Buffer with KCl, 2 µl 25 mM MgCl2, 2 µl dNTP Mix (2 mM each-dATP, dTTP, dGTP, dCTP), 0.4 µl Taq DNA Polymerase (Thermo Scientific™ EP0406), 5 µl water supernatant to 20 µl. PCR cycling included: initial denaturation of 95 °C for 3 min, 30 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, final elongation for 5 min at 72 °C. PCR was performed on a GeneAmp PCR System 9700 (Applied Biosystems). Electrophoresis was performed using 1.5% agarose gel with ethidium bromide. Positive PCR products were purified by Exonuclease I (Applied Biosystems™) and Shrimp Alkaline Phosphatase (Applied Biosystems™) as previously described (Werle et al., 1994). Sequencing was performed using second-round amplification primers and BigDye® Terminator v3 Cycle Sequencing Kit (Applied Biosystems) according to the manufacturer's instructions, followed by fragment separation on an automated genetic analyzer 3730xl DNA Analyzer (Applied Biosystems).

2.3. Analysis of 18s rRNA sequence data

The nucleotide sequences derived from forward and reverse primers were assembled into a contiguous sequence utilizing SeqMan 6.1 software (DNASTAR Inc., Madison, WI, U.S.A.). To elucidate the evolutionary relationships, the maximum likelihood method was employed along with the Kimura 2-parameter model (Kimura, 1980). The tree

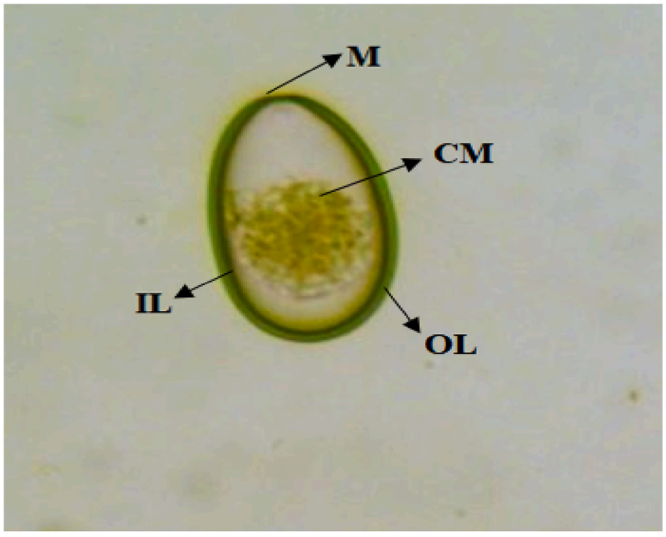

Fig. 1. Photomicroscope images showing the oocysts of *E. elegans* (40x magnification). The outer (OL) and the inner (IL) layers of the oocyst wall, cytoplasmic mass (CM), micropyle (M).

exhibiting the highest log likelihood (−4120.27) is depicted, with the percentage of trees wherein the associated taxa clustered presented adjacent to the branches. Initial trees for the heuristic search were generated automatically through the application of Neighbor-Join and BioNJ algorithms to a pairwise distance matrix estimated via the Maximum Composite Likelihood (MCL) approach. Subsequently, the topology with the optimal log likelihood value was chosen. A discrete gamma distribution was utilized to model variations in evolutionary rates among sites, with five categories (+G, parameter = 0.2858). The scale of the tree is proportional, with branch lengths measured in substitutions per site. This analysis incorporated 199 nucleotide sequences, yielding a total of 497 positions in the final dataset. The evolutionary analyses were executed using MEGA X (Kumar et al., 2018).

3. Results

Among the 104 fecal samples examined, *Eimeria* spp. Oocysts were found in 22 samples (21%), with one to 101 oocysts per microscopic field at 10x magnification. Among these samples, 19 contained *Eimeria* oocysts morphologically identified as *Eimeria elegans* (Fig. 1). The oocysts of *Eimeria elegans* were ellipsoidal in shape and differently colored (green, yellow-green, lilac) with a smooth, double-layered wall. A small micropyle was observed at the narrow end of the oocyst. It should be noted that lilac-colored oocysts prevailed in our studies.

While the remaining three samples exhibited oocysts differing in morphological characteristics from E. elegans. The oocysts of *Eimeria* spp. Had an ellipsoidal, oval shape with a smooth, yellowish-green, brown, double-layered wall. A clearly defined micropyle covered by a cap was observed at one pole of the oocyst. Mature oocysts contained 4 oval spores (Fig. 2 a, b).

A total of 91 clones of 18s rRNA were sequenced from four DNA samples, with sample 1 yielding 29 clones, sample 2 yielding 18 clones, sample 3 yielding 22 clones, and sample 4 yielding 22 clones. Among these, 80 clones formed a distinct clade and exhibited the highest genetic similarity to MT801034 *Eimeria* sp. Voucher HY3 (Fig. 3). Nevertheless, the identity percentage (98.2–99.2%) of 80 *Eimeria* sp. Clones with MT801034 *Eimeria* sp. Voucher HY3 suggests that they belong to different species. Considering the morphological similarity of the oocysts detected in 19 samples to those of *E. elegans*, and the absence of the 18S rRNA sequence of this *Eimeria* species in the NCBI database, we can conclude that these 80 clones might belong to *E. elegans*.This clade encompassed all clones from samples 1 and 2, 21 of 22 clones from sample 3, and 12 of 22 clones from sample 4. One clone from sample 3 clustered with AF345998 *Eimeria faurei* (Svanbaev, 1979), while two clones from sample 4 were most genetically similar to LC507797 *Eimeria arloingi* (Marotel, 1905). The remaining eight clones from sample 4 clustered with *Eimeria ahsata* (Musaev and Mamedova, 1981), *Eimeria christenseni* (Levine et al., 1962), *Eimeria hirci* (Chevalier, 1966), *Eimeria crandallis* (Chevalier, 1966), and *Eimeria weybridgensis* (Norton et al., 1974).

4. Discussion

Since 1980, 11 cases of mass mortality in Saiga antelopes from various infectious diseases have been recorded in Kazakhstan (Lushekina, 2010). To this day, the cause of these outbreaks remains unknown. Therefore, Saiga antelopes need to be investigated for parasitic infections, including eimeriosis, babesiosis, and theileriosis, which can make infected animals susceptible to secondary infections or cause morbidity and mortality on their own. While *Eimeria* spp. Have previously been found in Saiga antelope, little is known about how these parasites are genetically related to *Eimeria* found in other species and in other geographic locations.

This study is the first to molecularly evaluate *Eimeria* spp. infecting Saiga antelope from the Volga-Ural population. The parasite *E. elegans* is host-specific to Saigas and was initially identified in the 1950s in Saiga antelopes from the Betpakdala and Volga-Ural populations (Svanbaev, 1979). It's noteworthy that eight non- *E. elegans* clones clustered with *E. faurei*, *E. arloingi*, *E. ahsata*, *E. christenseni*, *E. hirci*, *E. crandallis*, and *E. weybridgensis*, which are known to parasitize sheep and goats (El-Alfy et al., 2020; Battelli and Poglayen, 1980; Bawm et al., 2020). Among them, *E. faurei* and *E. arloingi* were discovered in the Betpakdala Saiga population in the 1960s by Svanbaev (1979). *E. arloingi* oocysts have been detected in sheep on farms in the Kaztalov and Zhanibek districts of

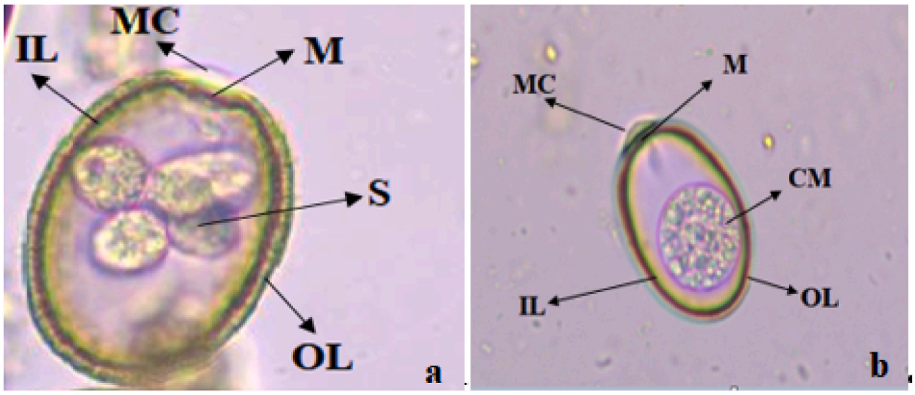

Fig. 2. Eimeria spp. Oocyst at 40x magnification. The outer (OL) and the inner (IL) layers of the oocyst wall, micropyle (M), micropyle cap (MC), spores (S).

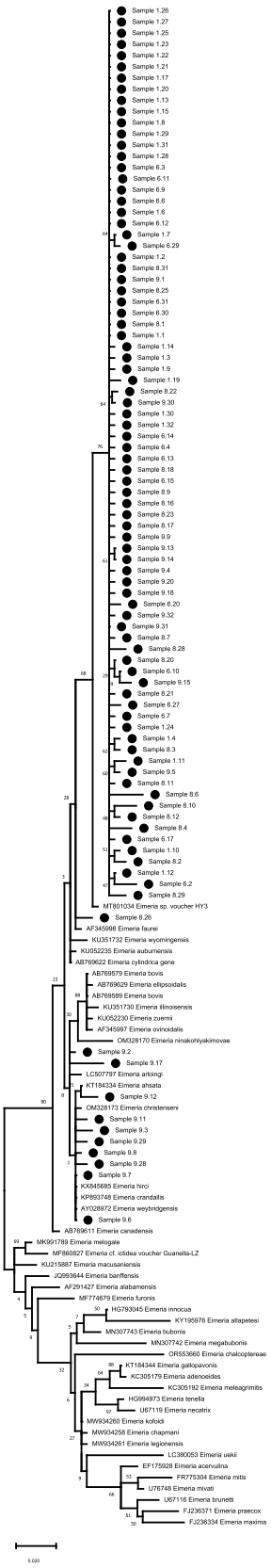

Fig. 3. Phylogenetic tree generated by the Maximum Composite Likelihood (MCL) method using partial sequences of the 18S rRNA of the *Eimeria* species that infect the *Saiga tatarica*.

the West Kazakhstan region (Final report of the grant project AP09260294, 2023).

The 80 clones forming a distinct clade and exhibiting genetic similarity to MT801034 *Eimeria* sp. Voucher HY3 likely represent a specific species (previously described as *Eimeria elegans*) unique to Saiga antelopes. This species type has not been previously cataloged in international gene banks due to lack of prior molecular investigations. The 18S rRNA gene is highly conserved and is widely used for the molecular detection of *Eimeria* spp. The most genetically similar 18S rRNA sequence (*Eimeria* sp. Voucher HY3), previously identified was collected from a forest musk deer (*Moschus berezovskii* (Flerov, 1929)), being kept in captivity in Hanyuan Province, China. This should be considered an initial step in exploring the parasite diversity of Saiga antelopes and possibly other rare wildlife species using more recently available molecular tools (Ren et al., 2021).

CRediT authorship contribution statement

Erzhan Zh Kidiraliyev: Writing – original draft, Methodology, Investigation, Data curation. **Ainur A. Zhaksylykova:** Investigation, Data curation. **Eleonora A. Kydyrkhanova:** Investigation, Data curation. **Kaissar Zh Kushaliyev:** Investigation, Data curation, Conceptualization. **Alexandr Shevtsov:** Investigation. **Ryskeldina Anara:** Investigation. **Zhibek P. Sembaeva:** Investigation. **M.A. Arzybaev:** Investigation. **Christine M. Budke:** Writing – review & editing. **Aida M. Abdybekova:** Writing – original draft, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

We, authors of this article (the list of authors below) declare that there are no conflicts of interest regarding the research presented in this article titled “Molecular evaluation of *Eimeria* spp. infection in the Volga-Ural Saiga antelope population of the Republic of Kazakhstan”. The work was carried out under grant No. AP09260294 from the Science Committee of the Ministry of Science and Higher Education of the Republic of Kazakhstan “Complex methods of diagnostics of helminthiasis (coenurosis, monieziasis and echinococcosis) of saigas of the Uralsk population, development of an algorithm for preventive measures".

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