

Cryopreservation and Vitality Assessment of GI Nematode Larvae in Libyan Sheep (*Ovis aries*) Using Liquid Nitrogen

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ABSTRACT

Background and Objective: Parasitic infections, particularly gastrointestinal (GI) nematodes, significantly impact livestock health and productivity. Cryopreservation offers a practical solution for long-term storage and study of parasitic larvae. This study aimed to evaluate the viability of cryopreserved GI nematode larvae from Libyan sheep (*Ovis aries*) using different cryoprotective solutions and to assess the effectiveness of vital dye exclusion for post-thaw viability determination. **Materials and Methods:** The GI nematode larvae were isolated and cryopreserved in liquid nitrogen using three solutions: distilled water, 1% ethylene glycol, and 1% acetic acid. After thawing, larval viability was assessed through survival rate calculations and confirmed via vital dye exclusion tests. Statistical analysis was conducted using ANOVA to determine significance among treatment groups, ($p \leq 0.05$). **Results:** Significant differences in larval survival were observed ($p \leq 0.01$). Distilled water yielded the highest post-thaw viability at 81.6%, while ethylene glycol showed 0% survival. Acetic acid preserved 23.3% of larvae. Vital dye exclusion assays supported these findings with statistically significant results ($p \leq 0.05$). **Conclusion:** Distilled water proved to be the most effective cryopreservation medium for GI nematode larvae. This study supports the utility of cryopreservation and rapid viability assessment in parasitology, offering valuable tools for epidemiological surveillance and disease management, particularly in regions with limited laboratory culturing options.

KEYWORDS

Gastrointestinal nematodes, culture method, cryopreservation, viability larval, sheep

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INTRODUCTION

Sheep are an important livestock species primarily raised for meat and wool production, with milk serving as a secondary product. However, they are highly susceptible to parasitic infections due to inadequate management practices and poor grazing conditions¹. These parasitic infections pose a significant challenge to global livestock production, greatly affecting animal health, growth, and productivity².



Among the various parasites, gastrointestinal nematodes are particularly harmful to sheep, highlighting the need for effective control strategies to reduce their impact³. Nematodes are one of the most common parasitic groups affecting cattle and small ruminants, resulting in serious consequences for animal health and production efficiency⁴.

Accurate diagnosis of nematode infections is essential for effective control strategies. The standard method for this involves fecal culture to isolate and identify larvae⁵. Cryopreservation has emerged as a transformative tool in biological and medical research, including the field of parasitology. By exposing organisms to ultra-low temperatures (e.g., -190°C), metabolic and enzymatic activities are halted, allowing for long-term preservation without compromising viability⁶. This technique is particularly valuable for maintaining diverse parasitic species, such as nematode larvae, in a viable state for reproduction or reinfection studies⁷.

Previous research has shown that cryopreservation is effective for nematode larvae. For example, distilled water or physiological saline has been successfully used to preserve these larvae for extended periods⁸. Interestingly, found that certain nematode larvae that infect fish remained viable after being frozen in liquid nitrogen, even though they showed no motility, as confirmed by dye exclusion tests⁹. Additionally, other studies have utilized glycerol solutions (15-25%) along with a two-step cooling process-first cooling to -70 or -25°C before storage in liquid nitrogen at -180°C-resulting in a 60.3% survival rate after six months¹⁰.

Recent advances in viability assessment have improved cryopreservation techniques. Cell survival can be evaluated using methods such as observing morphological changes, assessing membrane permeability, or conducting dye exclusion assays. In these assays, fluorescent dyes only penetrate dead or damaged cells¹¹⁻¹³. Furthermore, established a correlation between the speed of intracellular movement, ATP concentration, and cell vitality¹⁴.

This study aimed to evaluate the effects of cryopreservation in liquid nitrogen on the viability of gastrointestinal nematode larvae using various solutions. Assess the reliability of larval motility and dye exclusion tests in determining post-thaw viability.

MATERIALS AND METHODS

Study area: This study took place in Misrata, a coastal city located in northwestern Libya, from February to April, 2023. Misrata is positioned at a Latitude of 32°22'39.12"N and a Longitude of 15°05'31.26"E along the Mediterranean Coast. According to the 2012 census, the city has an estimated population of 500,000. The region is characterized by a steppe climate, which is defined by arid conditions and limited precipitation¹⁵.

Sample collection: Fecal samples were collected from various sheep farms in Misrata between September, 2022 and May, 2023. A total of 87 fresh sheep dung samples were randomly collected during the early morning hours using sterile sampling bags. Each sample was labeled with essential metadata, including a unique sample number, the collection location, and the date. Immediately after collection, the samples were transported to the Research Unit at the Department of Zoology, Faculty of Science, Misurata University, for further processing and analysis.

Sample examination: In the laboratory, immediately processed all fecal samples. Each sample was accurately weighed using a digital analytical balance, and a standardized 20 g aliquot was taken for further analysis. These aliquots underwent coproculture examination according to established protocols to promote larval development and identification. This standardized approach ensured consistency in the processing of all specimens.

Experiment of cryopreservation of nematode larvae in liquid nitrogen: After collection, live larvae were thoroughly washed with distilled water through three consecutive rinses to ensure complete removal of debris. The purified larval suspension was then divided into three experimental groups: one group was suspended in distilled water (6 replicates, 10 larvae per replicate), the second group was treated with ethylene glycol (6 replicates, 10 larvae per replicate), and the third group was treated with a 5% acetic acid solution (6 replicates, 10 larvae per replicate). All groups were transferred to 1.8 mL cryogenic vials and stored in the vapor phase of liquid nitrogen at -196°C for 30 days.

After cryopreservation, the samples underwent a standardized thawing protocol, which involved gradually warming them to room temperature ($22\pm 2^{\circ}\text{C}$) over the course of 30 min. To assess viability, conducted the following evaluations: Direct observation of motility under phase-contrast microscopy (magnification $400\times$) and vital staining with a 0.1% iodine solution⁸. Survival rates were calculated based on the proportion of motile larvae and the patterns of dye exclusion. Statistical analysis was performed on triplicate measurements.

Experiment of larvae vital measurement: Exclusion dyes, such as eosin aqueous dye, are fluorescent compounds that are excluded from living cells but accumulate in dying cells with compromised membranes^{12,13}. In this study, 50 live and active larvae were placed in distilled water to measure their vitality by observing their movement. The larvae's activity was monitored at the beginning and then every half hour for a total duration of two and a half to three hours. Over time, the movement of the larvae gradually slowed down until it was completely lost.

Additionally, the dye exclusion technique was used to assess the vitality of the larvae. Another group of 50 live and active larvae was placed in a 1% aqueous eosin dye solution. The healthy larvae remained vital and did not allow the dye to penetrate their membranes. Their response was monitored for the first 10 min and then every half hour. The rate of dye penetration into the larvae was recorded as occurring between four to four and a half hours.

Statistical analysis: The statistical analysis employed in this study included a One-way ANOVA test to determine the most effective method for cryopreservation of larvae, as well as survival analysis using the Kaplan-Meier test to assess larval vitality¹². The analyses were conducted using the SPSS software, and differences were considered significant at a p-value of less than 0.05.

RESULTS

The results of the cryopreservation experiment involving larvae in liquid nitrogen are as follows: Three physiological solutions were used in the experiment, with six repetitions for each solution. Each repetition included 10 larvae, resulting in a total of 60 larvae per solution, and the duration of the experiment was one month.

The findings indicated that the survival rate of larvae in distilled water was 42 out of 60, which accounts for 81.6%. In contrast, the survival rate in a 1% acetic acid solution was only 14 out of 60 larvae, translating to 23.3%. For ethylene glycol, the survival rate was 0%, as illustrated in Table 1.

Table 1: Larval survival rates following cryopreservation in liquid nitrogen using different solutions

Solutions	Total number of larvae	Damaged larvae	Live larvae don't move	Live larvae moving
Distilled water	60	11	7	42
Ethylene glycol	60	60	0	0
Acetic acid 1(%)	60	46	14	0

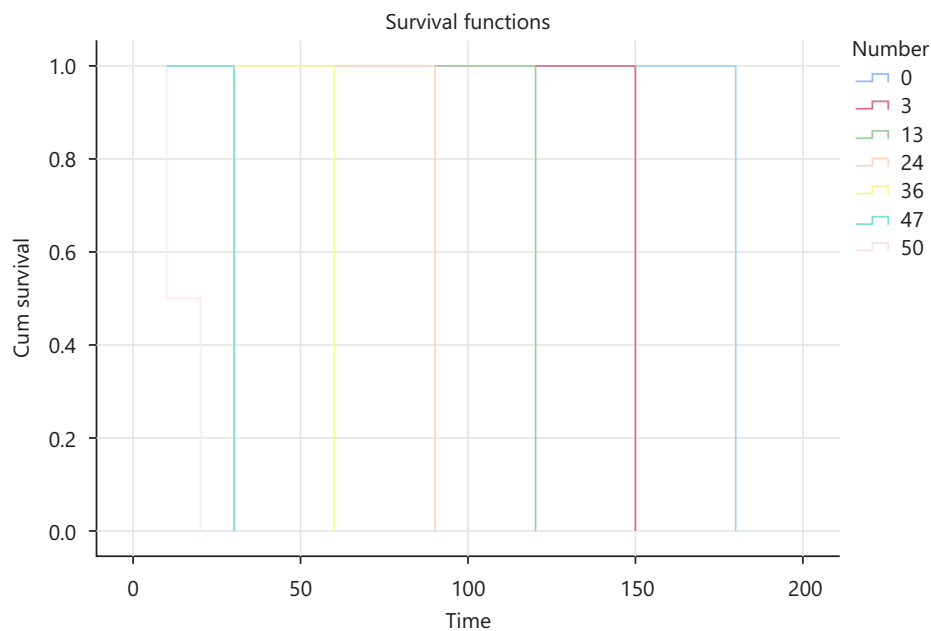


Fig. 1: Measurement of larval viability based on their movement

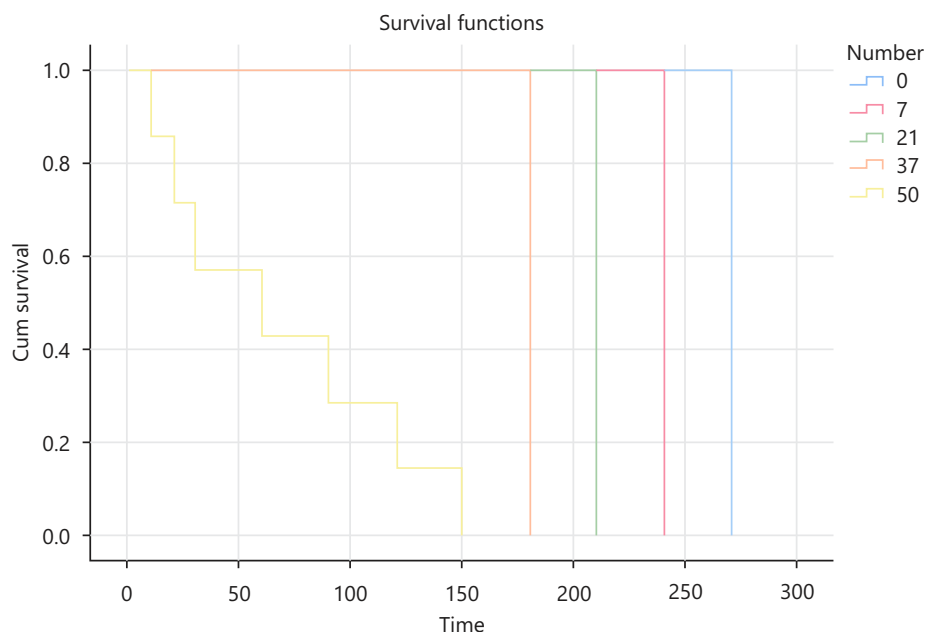


Fig. 2: Measurement of larval vitality through the exclusion of dye

Statistical analysis confirmed a significant difference in the survival rates, with a $p \leq 0.01$, as shown in Table 2 placed in a 1% aqueous eosin dye solution. The healthy larvae remained vital and did not allow the dye to penetrate their membranes. Their response was monitored for the first 10 min and then every half hour. Larval survival post-cryopreservation, confirming distilled water as the most effective medium. Ethylene glycol was completely ineffective. The results were statistically significant.

In the current study, the results of the viability larval experiment were assessed based on two main factors: The movement of the larvae and the exclusion of dye. The vitality of the larvae, indicated by their movement, was observed to last approximately two and a half to three hours, as shown in Fig. 1.

Meanwhile, the penetration of dye into the larvae was noted to occur throughout approximately four to four and a half hours, as illustrated in Fig. 2.

Table 2: Average survival rate of larvae after cryopreservation in liquid nitrogen

	Distilled water	Ethylene glycol	Acetic acid 1(%)
Mean±S.E	8.2±1.2	0.00±0.000	2.3±0.6
Significance	**		

Significance levels: ** $p \leq 0.05$ and indicates the highest mean value among treatments

Table 3: Average larval vitality estimated by movement and dye exclusion (Kaplan-Meier survival analysis)

Larvae viability by estimation	Movement	Exclusion of the dye
Mean±S.E	82.5±20.8	125.4±26.3
Significance	*	

Significance levels: * $p \leq 0.05$ and indicates the highest mean value among treatments

Survival analysis conducted using the Kaplan-Meier test revealed a significant difference in these parameters ($p \leq 0.05$), as presented in Table 3. A higher exclusion rate over time indicates increasing mortality.

DISCUSSION

The prevalence of gastrointestinal parasites in sheep is a significant constraint to sheep production worldwide, leading to substantial economic losses and negative impacts on the sheep industry¹⁶.

In this study, the results of cryopreservation indicated that the best survival rates for larvae stored in liquid nitrogen occurred when they were stored in distilled water. This finding aligns with⁸, who noted that distilled water is one of the most commonly used solutions for the cryopreservation of nematode larvae over extended periods, resulting in longer survival times. Larvae stored in a 1% acetic acid solution exhibited cases of merging and tearing, which can be attributed to the properties of acetic acid. Mazur¹⁷ confirmed that a reduction in the amount of water inside the cells enhances their ability to survive cryopreservation. In our study, larvae stored in an ethylene glycol solution showed merging of all larvae placed in it, contrasting with the findings of Irdani *et al.*¹⁸, who reported good survival rates for larvae in similar conditions.

Nematodes that infect plants sustain damage after being cooled to -140°C , likely due to differences in osmotic pressure between the interior of the larvae and the surrounding solution. This variation may also stem from differences among the larvae and the types of hosts they infect.

To measure larval vitality, we used the dye exclusion technique, which involved observing larval movement and the exclusion of dye until the larva was completely pierced. The results suggest that temperature plays a critical role, as larvae can be stored at 4°C without immediate loss of vitality. Various environmental factors such as temperature, humidity, and pH can adversely affect larval vitality and cause permanent cessation of movement. The protective leather sheath that encases the larvae helps them withstand harsh environmental conditions¹⁹. Initially, the larvae exclude the dye, evidencing their vitality, but this ability diminishes over time as indicated by the dye's eventual penetration, as confirmed by^{12,13}. Exclusion dyes are fluorescent compounds that are expelled from living cells and accumulate in dying cells that have compromised membranes.

CONCLUSION

This study demonstrates that distilled water is the most effective cryopreservation medium for gastrointestinal (GI) nematode larvae from Libyan sheep, achieving an 81.6% post-thaw viability rate. In contrast, ethylene glycol resulted in complete larval mortality, while acetic acid preserved only 23.3% of larvae. The vital dye exclusion assay further validated these results, confirming its reliability for rapid viability assessment.

SIGNIFICANCE STATEMENT

This study discovered the effectiveness of distilled water as a low-cost and efficient cryoprotective medium for gastrointestinal (GI) nematode larvae, achieving 81.6% post-thaw viability. This finding can be beneficial for enhancing long-term parasite preservation, especially in regions with limited laboratory infrastructure. It provides a practical tool for parasitologists to conduct further research on nematode biology, drug resistance, and vaccine development. Additionally, it supports disease surveillance through improved sample storage and transport for epidemiological studies. The technique also aids in livestock health management by facilitating diagnostic training and anthelmintic testing. This study will help researchers to uncover the critical areas of parasite cryobiology that many researchers were not able to explore. Thus, a new theory on larval survival mechanisms may be arrived at.

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