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# Exploring cryopreservation alternatives for *Dirofilaria immitis* microfilariae

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ABSTRACT

Canine Heartworm Disease, caused by *Dirofilaria immitis*, primarily affects canids and felids. The earliest studies on cryopreservation were carried out at −70°C, achieving acceptable survival rates, however microfilariae (mf) showed alterations both in morphology and motility. Thereafter, liquid nitrogen was used representing an excellent tool for long-term preservation, albeit it is expensive and requires trained personnel. Accordingly, the aim of this study was to develop a protocol for cryopreservation of *D. immitis* mf at −80°C feasible to laboratories with limited specialized equipment. The cryoprotectant medium was composed by 5 % dimethyl sulfoxide, 20 % of newborn calf serum and 75 % of saline solution. At Study Day (SD) 0 whole blood from a *D. immitis* naturally infected dog was diluted with the medium at a ratio of 1:1 and stored at −80°C using a freezing container (Nalgene® Mr. Frosty® Cryo 1°C). On the SD1 and then once a month, one cryovial was thawed and examined for survival, motility, length and morphology of mf. On SD 1, the mf showed a survival rate of 99 %. By SD 120 the survival rate gradually decreased (up to 63 %) and a shift in motility patterns between the "medium" and "slow" classes, was observed. On SD 150, the survival rate exceeded 50 % and mf did not exhibit detectable morphological alterations; however, a reduction in length was observed. This study marks the first protocol where the −80°C freezer has been employed for cryopreservation of *D. immitis*, integrating the application of cryoprotectants and novel techniques for gradual temperature transition.

## 1. Introduction

Dirofilariosis is a vector-borne disease caused by nematodes of the genus *Dirofilaria*, primarily represented by *D. immitis* and *D. repens* (Dantas-Torres and Otranto., 2020). These parasites affect domestic and wild canids, felids and humans posing a zoonotic risk. Mosquitoes of the Culicidae family serve as vectors, with nearly 70 species capable of transmitting the nematodes (Simón et al., 2012). Over the past decade, *Dirofilaria* spp. infections have rapidly spread across Europe, extending from traditionally endemic regions to eastern and northeastern countries (Ionică et al., 2015; Morchón et al., 2022). *Dirofilaria immitis* and *D. repens* are now endemic in several European countries (Capelli et al., 2018) including southern Mediterranean islands (Brianti et al., 2022; Napoli et al., 2023). A plethora of factors contribute to the propagation of these agents, including climate changes, invasive mosquito species, the potential involvement of other vector species such as *Culicoides* (Napoli et al., 2022), the contribution of wild animals as reservoirs, and the increased movement of animals among territories at different endemicity, including the global relocation of dogs (Tsokana et al., 2024; Lühken et al., 2023).

The primary approach to prevent *D. immitis* infection in animals involves prophylactic use of macrocyclic lactones (ML), available in several commercial formulations for dogs, cats and ferrets. However, recent research reveals cases of ML-resistant *D. immitis* strains in Europe as well (Traversa et al., 2024), ML-resistant strains from lack-of-efficacy cases, posing concerns, especially in human filariasis caused by *Brugia malayi*, *Wuchereria bancrofti*, and *Onchocerca volvulus* (Prichard, 2021).

To enhance the studies on vectorial role, preventive measures and potential alternative therapies, cryopreservation is a valuable tool that involves freezing an organism to sustain viability, aiming to restore it to its initial state upon future thawing (Zinser et al., 2021). Since 1929, pioneering studies by Fülleborn have laid the groundwork for cryopreservation research (Fülleborn, 1929). The earliest publications employed temperatures ranging from −68° to −70°C, achieving

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acceptable survival rates reporting a survival of 660 days but resulting in several morphological and motility alterations of the cryopreserved mf (Weinman and McAllister, 1947; Taylor, 1960; Bemrick et al. 1965). Further studies have utilized liquid nitrogen reaching a final temperature of −196°C, reporting a survival rate of 30 days for *D. immitis* mf and up to 540 days for mf of other species (Bartholomay et al., 2001; Lowrie, 1983), although in a number of these studies the duration of freezing and the survival of mf over time were not reported (Ham et al., 1979; Minjas and Townson, 1980; Lok et al., 1983).

The immersion in liquid nitrogen represents an excellent tool for long-term preservation, albeit with higher costs and the requirement for specialized equipment and trained personnel. Accordingly, the aim of this study was to develop a protocol involving the use of a cryopreservation medium that can be stored at −80°C without liquid nitrogen. This design aims to enhance the feasibility of the technique particularly in laboratories with limited specialized equipment. This method not only aligns with ethical standards by reducing reliance on animal experimentation but also facilitates research and teaching by providing a stable and reproducible source of mf.

## 2. Materials and methods

### 2.1. Cryopreservation medium

The concentrations of cryoprotectants in the medium used in this study was: 5 % Dimethyl sulfoxide (DMSO), 20 % of newborn calf serum and 75 % of saline solution. The preparation of medium was carried out under a laminar-flow cabinet to prevent any potential contaminations.

### 2.2. Blood collection and cryopreservation

Whole blood was collected from the cephalic vein of a *D. immitis* naturally infected crossbreed male dog, housed in the Municipality of Trapani, Sicily, Italy. The blood was sampled in 6 mL tubes supplemented with lithium heparin as anticoagulant. The blood sample was kept at +4°C and transported to the laboratory within 4 hours of collection. Once at the laboratory, the sample was analyzed by Knott's test to confirm the microfilaremia (see below). The blood was quickly dispensed into 1.8 mL cryovials at a ratio of 1:1 blood to medium. In particular, each cryovial was filled with 0.9 mL of blood and 0.9 mL of cryopreservation medium. These were then placed in a Nalgene® Mr. Frosty® Cryo 1°C Freezing Containers (Termo Fisher Scientific, Waltham, MA, USA). The container was filled with isopropanol and stored into a −80°C freezer for > 24 h in accordance with the manufacturer's instructions. After 24 hours, the container was taken out, and the cryovials left at −80°C.

### 2.3. Modified Knott's test

One mL of the donor's blood was tested using a modified Knott's technique as described elsewhere (Genchi et al., 2021). The microfilariae retrieved at the Knott's test were counted and classified to species level based on morphological parameters (*i.e.*, microfilariae body length, shape of the cephalic and caudal ends) according to Magnis et al., (2013). The microfilaremia load was expressed as the number of mf per mL of blood (mf/mL).

### 2.4. Baseline

On SD 0, an aliquot of the blood sample was used to provide baseline information; briefly, microfilaremia, survival rate, motility, morphology, and length of microfilariae (see below) were evaluated both in blood with anticoagulant and in a blood sample supplemented with the cryopreservation medium but not frozen. The survival rate and motility were further evaluated at 24 h intervals (i.e., 24 h and 48 h) while the samples were stored at +4°C.

### 2.5. Thawing and evaluation procedures

One randomly selected cryovial was thawed on SD 1, 30, 60, 90, 120, 150, respectively. Samples were thawed by submerging the cryotubes into a 37 °C water bath for 2 minutes. After complete thawing, 0.9 mL of the tube content was adjusted by adding 9 mL of sterile saline solution and then centrifuged at 600 g for 3 minutes. After centrifugation, about 9 mL of the supernatant was discharged and the rinsing procedure was repeated five times. Finally, the sediment was assessed for survival rate, motility, morphology, and length of mf (see below).

Briefly, using a slide divided into four squares (1.2 ×1.2 cm), 15 μL of the sediment were placed in each square of the slide and observed at 100 magnifications under light microscopy. The survival rate was assessed by counting live and devitalized mf in the four squares of the slide, the average value was calculated and expressed as percentage. The motility was scored as fast (i.e., 3/4 coiling movements over 2 seconds) (**Video 1**), medium (i.e., 3/4 coiling movements over 5 seconds) and slow (i.e., less than 3 coiling movements over) by observing 10 mf per each field of the slide for a total of 40 mf (**Video 2**). Videos to assess the motility rate were taken using a digital camera (Axiocam Mrc Zeiss, Jena, Germany) and an imagine analysis system (Axiovision Zeiss, Jena, Germany).

The morphology and length of microfilariae were assessed on the microfilariae isolated through a modified Knott's test performed with 0.9 mL of the content of cryotube soon after the thawing. All the microfilariae of the Knott's test were morphological evaluated, while a sample of 30 mf was examined to determine the total length of mf and measures were expressed as average, maximum, and minimum (± standard deviation).

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### 2.6. Statistical analysis

The differences in survival rate at the different study days were investigated statistically by Yates corrected chi-square test for trend. Differences in the measure of the mf at baseline and at SD 150 were compared using an unpaired T-*test.* Statistical analyses were performed using GraphPad Prism version 9.0 for Windows (GraphPad Software, www.graphpad.com) and the level of significance was set at $p < 0.05$.

## 3. Results

Upon the Knott's test the microfilariae in the blood sample were morphologically classified as *D. immitis* with a high microfilaremia load (*i.e.*, 15,000 mf/mL). The results of the survival rate, motility, morphology, and length carried out on the blood sample supplemented with anticoagulant and with the cryopreservation medium are reported in Tables 1 and 2, respectively. No significant differences were observed among microfilariae parameters recorded either in the blood sample with anticoagulant or supplemented with the cryopreservation medium.

**Table 1**
Data on survival, motility, and length of microfilariae of *Dirofilaria immitis* extracted from the blood sample, and neither exposed to the cryopreservation medium nor frozen. Survival and motility rate were further evaluated at 24 h, and 48 h post extraction.

| Study Day | Survival (%) | Motility (%) | | | Lenght |
|---|---|---|---|---|---|
| | | Fast | Medium | Slow | Min-max length (mean ± standard deviation) |
| SD0 | 99.0 | 18.9 | 72.2 | 8.9 | 300.1–331.8 *μm* (316.7 ± 11.1 *μm*) |
| + 24 h | 94.6 | 14.4 | 71.1 | 14.4 | - |
| + 48 h | 92.9 | 11.1 | 68.9 | 20.0 | - |

**Table 2**
Data on survival, motility, and length of microfilariae of *Dirofilaria immitis* extracted from the blood sample exposed to the cryopreservation medium but not frozen. Survival and motility rate were further evaluated at 24 h, and 48 h post extraction.

| Study Day | Survival (%) | Motility (%) | | | Lenght |
|---|---|---|---|---|---|
| | | Fast | Medium | Slow | Min-max length (mean ± standard deviation) |
| SD0 | 99.0 | 12.2 | 77.8 | 10.0 | 295.1–331.8 μm (315.0 ± 5.9 μm) |
| + 24 h | 93.4 | 10.0 | 73.3 | 16.7 | - |
| + 48 h | 90.0 | 8.9 | 73.3 | 17.8 | - |

The survival and motility rates recorded at the different timepoints are summarized in Table 3. On SD 1, the mf exhibited a survival rate of 99 %. The observed movements were complete and the predominant motility category was medium (77.8 %) followed by fast (12.2 %) and slow (10 %). On SD 30, a decrease in the survival rate (83.5 %) was observed, and a more even distribution of motility categories. "Medium" remained dominant motility category (42.5 %), followed by "fast" (32.5 %) and "slow" (25 %). The trend progressed on SD 60, with a further reduction in survival rate (81.2 %) and a shift in motility distribution. On SD 90 and SD 120, there was a progressive decline in both survival rate (74.1 % and 63 %, respectively) and motility across all categories. On SD 150 a significant decrease in the survival rate (51.4 %) and a pronounced shift in motility was recorded. Overall, the data indicates a correlation between the duration of cryopreservation and a gradual decrease in both survival rate and motility of mf over the study period. The decrease in the survival rate was progressive and not linear (Fig. 1) and the differences observed among the different timepoints were statistically significant ($\chi^2 = 325.901$; $p < 0.0001$).

Across the study period, any morphological alterations or signs of vacuolar degeneration was observed; despite a progressive reduction in length of mf has been observed from SD 1 to SD 150 (Table 4, Fig. 2). The unpaired T-*test* showed that difference between the measures of the mf at the base line and in the other SDs was statistically significant ($t = 6.8150$, $df = 52$, $p < 0.0001$).

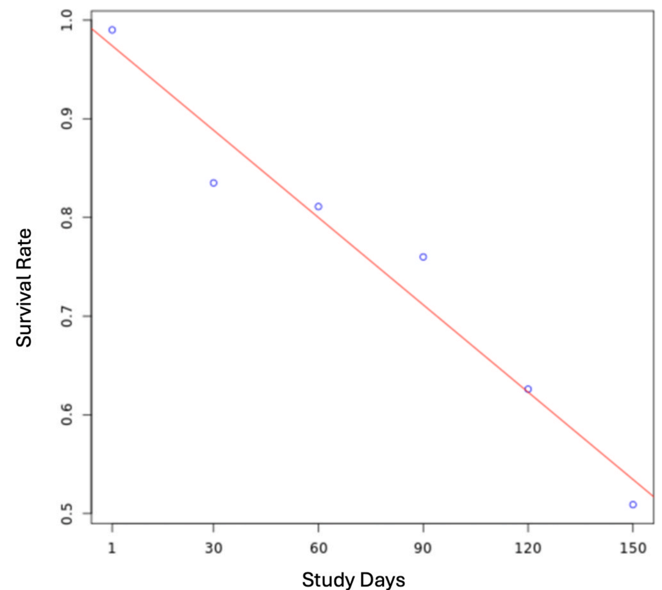


**Fig. 1.** Scattered plot with regression line describing the reduction of the survival rate of *Dirofilaria immitis* microfilariae added with the cryopreservation medium and stored at −80°, from SD 1 to SD 150.

## 4. Discussion

This study provides the first protocol for cryopreservation of *D. immitis* mf where −80°C freezer has been complemented with contemporary cryoprotectants and novel techniques for gradual temperature transition. The findings herein reported demonstrate that the cryopreserved mf can survive at least up to 150 days at −80°C with a survival rate exceeding 50 % and no obviously detectable morphological alteration.

In 1929, Fülleborn carried out pioneering work on storing *D. immitis* mf at low temperatures, maintaining viability for 4–7 weeks at 0°C. Later on, Weinman and McAllister (1947) explored prolonged storage of human pathogenic protozoa and nematodes such as *Wuchereria bancrofti*, *D. immitis*, and *Litomosoides carinii*. They found that *D. immitis* mf still showed 96 % and 83 % motility at −15°C and −70°C, respectively, with a survival of 69 days at −70°C.

In 1960, a significant study on mf of various parasites, including *D. immitis*, introduced the use of a cryopreservation medium (Taylor, 1960). Microfilariae stored at −79°C in a glycerol and serum mixture, as well as at room temperature in oxygenated blood, showed 50–80 % survival and activity at both temperatures. The duration of freezing was not stated however, those stored at room temperature did not display morphological alterations been vacuolar degeneration the main issue observed in the cryopreserved one. Moreover, a better rate of recovery in mosquitoes for those stored at room temperature was reported in the study. Bemrick and collaborators (1965) carried out a study on mf of *D. immitis* stored at −68°C without the use of any cryopreservation medium reporting a survival of mf up to 660 days and a successful development into L3 larvae in mosquitoes; however, a lower rate of recovery compared to the unfrozen control was observed (Fülleborn, 1929; Weinman and McAllister, 1947; Taylor, 1960; Bemrick et al. 1965).

Since 1965, studies on the cryopreservation of microfilariae have utilized liquid nitrogen, reaching a final temperature of −196°C. However, the duration of freezing and the survival of cryopreserved

**Table 3**
Data on survival and motility, from SD 1 to SD 150 of mf of *Dirofilaria immitis* added with the cryopreservation medium and stored frozen at −80°.

| Study Day | Survival Rate (%) | Motility (%) | | |
|---|---|---|---|---|
| | | Fast | Medium | Slow |
| SD 1 | 99.0 | 12.2 | 77.8 | 10 |
| SD 30 | 83.5 | 32.5 | 42.5 | 25.0 |
| SD 60 | 81.2 | 22.5 | 50.0 | 25.0 |
| SD 90 | 74.1 | 25.0 | 50.0 | 22.5 |
| SD 120 | 63.0 | 12.5 | 55.0 | 32.5 |
| SD 150 | 51.4 | 7.5 | 32.5 | 60.0 |

**Table 4**
Data on length (mean length ± sd and minimum and maximum length) of microfilariae of *Dirofilaria immitis* cryopreserved at −80° and thawed at different Study Day (i.e., SD1 to SD150).

| Study Day | Mean lenght μm (± sd) | min-Max length μm |
|---|---|---|
| SD 1 | 317.1 (±5.9) | 300.1–330.1 |
| SD 30 | 298.7 (±12.1) | 265.8–325.8 |
| SD 60 | 299.1 (±10.3) | 281.1–316.4 |
| SD 90 | 298.4 (±15.9) | 267.8–329.3 |
| SD 120 | 298.4 (±9.5) | 281.8 – 316.7 |
| SD 150 | 294.5 (±10.9) | 272.9–318.6 |

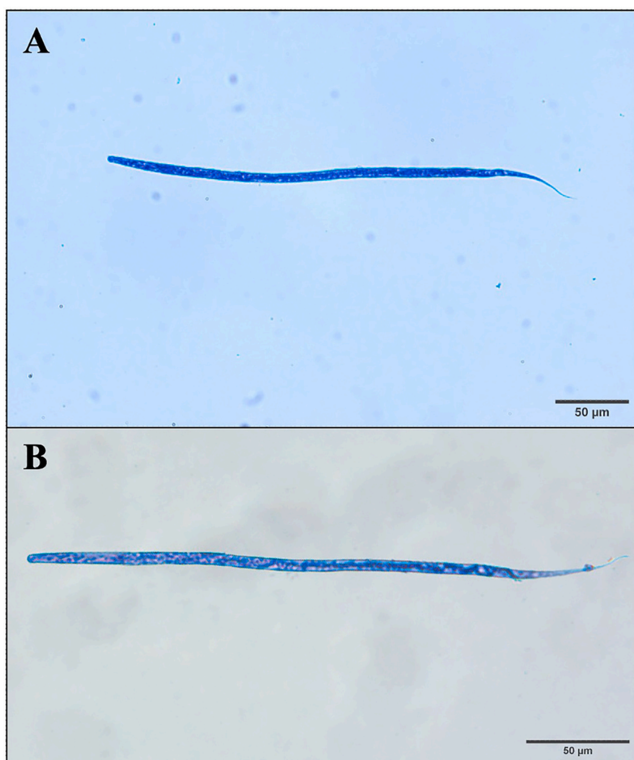


**Fig. 2.** Morphology of *Dirofilaria immitis* mf using Knott's test preformed at the different study days (SD). SD0329 $\mu m$ (A). SD150, 294 $\mu m$ (B). (20x, scale bar 50 $\mu m$).

microorganisms over time are not reported in several studies (Ham et al., 1979; Minjas and Townson, 1980; Lok et al., 1983). Microfilariae species other than *D. immitis* such as *D. corynodes*, *B. malayi*, and *W. bancrofti,* yield a survival length in liquid nitrogen ranging from 4 to 540 days (Lowrie, 1983). Bartholomay and colleagues (2001) reported a storage period of *D. immitis* for 30 days at −196° testing a 16 % polyvinylpyrrolidone (PVP) - 6 % DMSO solution (PVP-DMSO) as a cryoprotective medium. Shirozu and colleagues (2020) validated several commercial cryopreservation media for *D. immitis*, one year later Zinser and colleagues (2021) completed the life cycle with cryopreserved microfilariae of *D. immitis* up to the definitive host.

In the current study, at the baseline evaluation (SD 0), *D. immitis* mf supplemented with cryopreservation medium showed no alteration in survival compared to those not exposed to the medium. Over the next 48 hours, survival was nearly identical with a slight decrease in motility in those preserved in the medium. However, the morphology and average length of the mf did not exhibit significant variations between the whole blood sample and the one supplemented with the cryopreservation medium. These findings were obtained employing a standardized methodology as previously elucidated, with the specific aim of assessing the impact of the cryopreservation medium on mf without freezing.

As for vitality parameters recorded in frozen mf, both the survival and the motility gradually decreased over the course of the study period. Specifically, the survival rate remained very high (< 80 %) in the first two months of the study (SD1-SD60) and then decreased gradually up to 51 % on SD 150. Similarly, on SD1, SD30, and SD60, the most representative motility class was "medium". However, by SD90, a shift was observed from the "medium" to the "slow" class, and the study ended with 60 % of the observed mf ranked in the “slow” mobility class. The primary limitation of this evaluation is its inherent subjectivity, as it is operator-dependent. However, all evaluation procedures were conducted by the same operator, following objective criteria for the classification of mf movements. In this regard, further studies should consider the use of a machine-aided evaluation system such as that provided by "The Worminator" to limit the potential biases in the assessment of motility classes (Storey et al., 2014).

Regarding the results obtained on measurements, it was observed that there was a tendency for the mf to decrease in length overtime and the decrease is linked to cryopreservation. Baseline results, indeed, suggest that this shrinkage of the mf is likely due to exposure to low temperatures rather than the effect of cryoprotectants as mf supplemented with the medium but not frozen did not show any length variations. The decrease in the length of cryopreserved mf has been observed in previous studies highlighting concerns in the field of education, length, indeed, is one of the key identification features and students may not properly recognize the species of cryopreserved mf (Long et al., 2020).

In previous studies carried out at −70°C as freezing temperature, the main reported issues were related to variations in motility and morphology of the microfilariae. Particularly, vacuolar degeneration was noted (Taylor, 1960). Additionally, in mosquitoes infected with cryopreserved blood a low recovery of L3 compared to the unfrozen control was observed. (Bemrick et al., 1965). Our findings align with the studies conducted by Taylor (1960) and Bemrick and collaborators (1965), though in those studies there was some limitations concerning the alteration in the exterior morphology and vacuolar degeneration. In our experiment, no alterations in the morphology of the mf were observed. This difference could be attributed to the use of DMSO as a cryoprotectant and to the progressive degree of freezing (1 C°/min) provided by the freezing container. The main physical stresses associated with cell cryopreservation are cryogenic damage and ice crystal formation. As a matter of fact, sudden temperature reduction leads to damage in temperature-sensitive structures, affecting their functions, for example, by altering membrane permeability or damaging intracellular organelles). The freezing container Nalgene® Mr. Frosty® Cryo 1°C employed in this study allows a progressive temperature reduction that follows a very precise timing (cooling rate of 1 °C/min), likely contributing to the reduction of cryogenic damage and consequentially maximizing the survival of frozen mf. Additional studies with a larger sample size and for an extended time should be carried out to corroborate these results and to subsequent assess the development of cryopreserved mf into L3 within the mosquito vector.

## 5. Conclusion

In conclusion, this study provides a simple and feasible protocol for cryopreservation of *D. immitis* mf suitable for a wide range of laboratories without the need of specialized equipment. This protocol combines the use of a cryopreservation medium with the gradual control of temperature decrease without the use of liquid nitrogen. This combination, results in a survival rate greater than 50 % over 5 months, and neither morphological alterations nor signs of cryogenic damage of the preserved mf. This facilitates studies on *D. immitis* and has practical applications in teaching field.

## Funding

This research received no external funding.

## Ethical statement

All experimental procedures were carried out in accordance with the European legislation regarding the protection of animals used for scientific purposes (European Directive 2010/63), as recognized and adopted by the Italian law (D.Lgs. 26/2014).

## CRediT authorship contribution statement

**Emanuele Brianti:** Writing – original draft, Validation, Supervision, Conceptualization. **Francesco Origgi:** Writing – review & editing, Validation, Supervision. **Gabriella Gaglio:** Writing – review & editing, Validation, Supervision. **Alexandre Lemaire:** Methodology, Formal analysis, Data curation. **Ettore Napoli:** Writing – review & editing, Validation, Formal analysis. **Jennifer Varet:** Methodology, Formal analysis, Data curation. **Emanuela Sturiale:** Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Giovanni De Benedetto:** Writing – review & editing, Methodology, Formal analysis, Data curation.

## Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper

## Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.vetpar.2024.110355.

## Data Availability

Raw data and derived data supporting the findings of this study are available from the corresponding author Giovanni De Benedetto on request.

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