Identification and validation of a commercial cryopreservation medium for the practical preservation of Dirofilaria immitis microfilaria

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Methodology

Keywords: Aedes aegypti, Cryopreservation media, CultureSure, Dirofilaria immitis, Heartworm disease, Parasitic nematode, Microfilariae

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Abstract

Background Dirofilaria immitis is a parasitic nematode transmitted by mosquitoes and the cause of heartworm disease in dogs and dirofilariasis in humans and other mammals. The parasite is endemic worldwide. Vector stage research requires a reliable supply of D. immitis microfilariae (mf). It is believed that cryopreserved mf would retain viability and provide a powerful tool for vector stage research. However, reports on cryopreservation of D. immitis mf are limited. Therefore, this study aimed to validate commercial cryopreservation media to establish a practical, convenient, and reproducible storage procedure for D. immitis mf.

Methods Six different commercially available cryopreservation media were compared with the traditional polyvinylpyrrolidone-dimethyl sulfoxide (PVP-DMSO) preservation solution. In vitro viability of purified D. immitis mf and mf-infected total blood was analyzed using a motility assay and propidium iodide staining. In vivo infectivity of Aedes aegypti mosquitoes with cryopreserved mf was assessed using a mosquito survival test and quantifying the number of infective third stage larvae (L3) after 13 d post-infection.

Results Purified mf cryopreserved in CultureSure showed the best viability when compared to mf cryopreserved in the remaining five commercially available media and PVP-DMSO. Viability of mf in mf-infected total blood cryopreserved in CultureSure varied with the ratio of infected blood to CultureSure. Optimum results were obtained with 200 μL mf-infected blood:800 μl CultureSure. CultureSure was also the optimum medium for cryopreserving mf prior to infectivity of Ae. aegypti. The number of L3 was approximately the same for CultureSure cryopreserved mf (3×-concentrated solution) and non-cryopreserved fresh mf.

Conclusions CultureSure is an optimal commercial cryopreservation solution for the storage of D. immitis purified mf, mf-infected total blood, and mf used for in vivo mosquito experiments. Furthermore, this study describes an easy preservation method for clinical D. immitis -infected blood samples facilitating vector stage studies, as well as the education of veterinarians.

Background

The parasitic nematode *Dirofilaria immitis* is transmitted by mosquitoes causing potentially lethal heartworm disease in dogs, pulmonary dirofilariasis in humans, and dirofilariasis in various mammals [1]. Wild carnivores are also sentinels for the spread of *D. immitis*. Despite the availability of effective preventive medicines, *D. immitis* is endemic worldwide. Therefore, a better understanding of host-parasite interactions between mosquito vectors and *D. immitis* is needed to establish a truly effective control strategy.

Vector stage experiments require live *D. immitis* microfilariae (mf) obtained from infected animals [2]. Having a reliable supply of live mf on demand is very important. Cryopreservation is a powerful tool allowing pathogens to be stored in a viable state. However, there are only a few reports describing

cryopreservation of *D. immitis* mf. Initially, in 1947, Weinman and McAllister [3] demonstrated cryopreservation of mf in citrated or heparinized blood without cryoprotectant additives. Taylor in 1960 [4] and Lok *et al.* in 1983 [5] used glycerol and dimethylsulfoxide (DMSO), respectively, for cryopreservation of mf, and assessed the subsequent infectivity of mosquitoes. In the most recent study, published in 2001, Bartholomay, L. C. *et al.* [6] prepared and tested a 16% polyvinylpyrrolidone (PVP) - 6% DMSO solution (PVP-DMSO) as a cryoprotective medium.

In the previous studies, the cryopreservation media tested were originally prepared by the investigators. Recently, various commercial cryopreservatives have become available and they are commonly used, especially for mammalian cell cryopreservation. For convenience, reproducibility, and standardization purposes, we sought to identify a commercially available cryopreservation medium for effective *D. immitis* mf preservation. To this end, here, we compared six commercial cryopreservation media with the traditional PVP-DMSO preservative [6] for the cryopreservation of *D. immitis* mf. Effectiveness was evaluated by measuring *in vitro* viability and *in vivo* infectivity of mosquitoes.

Methods

Animals

Female beagles (2-4 years old) were experimentally infected with *D. immitis* SF1 strain originally isolated in Japan [7] and reared under a veterinarian's administration. *Aedes aegypti* Liverpool (LVP)-OB strain, kindly provided by Dr. R. Maeda, the Obihiro University of Agriculture and Veterinary Medicine (OUAVM), susceptible to *D. immitis* infection was maintained at 27 ˚C and >80% humidity on a diet of 5% fructose and a 12:12 h light-dark cycle.

Mf purification and cryopreservation

Cryopreservation was initially tested using purified *D. immitis* mf isolated from heparinized blood of an experimentally infected dog. The mf were purified using a PD-10 gel purification column (GE Healthcare, Amersham Place, England) equilibrated with RPMI 1640 Medium (Gibco 2120429, Thermo Fisher Scientific, Waltham, MA, USA) supplemented with 1x penicillin-streptomycin (Gibco 15140122) [8, 9]. Purified mf were centrifuged at 1,000*g* for 10 min at room temperature. The supernatant was discarded and the pelleted mf were resuspended in 1 mL of cryopreservation medium (ca. 3,000 or 15,000 mf/tube) and transferred to cryogenic tubes (Nunc 119546, Thermo Fisher Scientific). Samples were stored in freezing containers (Corning® CoolCell® LX, Corning, NY, USA) overnight at -80 ˚C before being transferred into liquid nitrogen, and stored until analysis. Cryopreservation media used in this study were as follows: PVP-DMSO (0.004 M PVP, average molecular weight of 40,000 and 6% DMSO) in phosphate buffered saline (PBS) [6, 10], CultureSure® Freezing Medium (CultureSure, FUJIFILM Wako, Osaka, Japan), Cellbanker® 1 (Cellbanker, Zenoaq Resource, Shiga, Japan), LaboBanker 1 (LaboBanker, Kurabo, Osaka,

Japan), Cos banker (Cosmo Bio, Tokyo, Japan), Bambanker® (GC Lymphotec, Tokyo, Japan), *CryoScarless*® *DMSO-Free* (CryoScarless, Bio Verde, Kyoto, Japan), and RPMI 1640 (Table 1).

In vitro motility assay

Cryopreserved mf were thawed rapidly by swirling in a water bath at 37°C. Immediately after thawing, the mf were suspended in 10 mL of RPMI 1640 medium and centrifuged at 1,500g for 10 min at room temperature. The pelleted mf were resuspended in 1 mL of RPMI 1640 medium containing 5% fetal calf serum, transferred to 12-well cell culture plates (Nunc), and incubated in 5% CO_2 in air at 37 °C for 3 d. The motility of mf was assessed microscopically on 0, 1, and 3 d post thawing (dpt) (n ≥ 3), and subjectively scored as inactive or dead (-), less active (+), moderately active (++), and highly active (+++) [11]. The results were statistically analyzed to rank the various media using a chi-square test and adjusted residuals (AR). Absolute AR values were considered significant at AR ≥ 1.96. The difference between PVP-DMSO and each cryopreservation medium was analyzed using Welch's t-test.

Cryopreservation of *D. immitis* mf-infected total blood

Fresh, heparinized blood (50-400 μL) was collected from *D. immitis*-infected dogs (ca. 25,000 mf/mL) and mixed with CultureSure up to 1 mL, and then cryopreserved as described above. Differences in motility between purified mf and mf-infected total blood were analyzed by one-way analysis of variance (ANOVA), followed by Tukey's multiple comparisons test.

Propidium iodide (PI) staining assay

To evaluate any physical damage caused by cryopreservation, cultured mf were stained with PI (2 μg/mL) (Molecular probes, Eugene, Orfegon, USA) for 10 min on 3 dpt. The stained mf were washed with PBS by centrifugation at 1500g for 10 min at 4 °C, and then subjected to fluorescence microscopy observation using the same exposure time in each experiment. Methanol-fixed mf were used as a positive control. The results were statistically compared using a chi-square test and calculated AR.

In vivo third stage larva (L3) development assay

Mf cryopreserved with commercial CultureSure, LaboBanker media, and traditional PVP-DMSO were used to determine the infectivity of mosquitoes *in vivo*. Thawed mf was fed to female *Ae. aegypti* on 7 d post-emergence for 2 h by artificial membrane feeding using Parafilm (Bemis Flexible Packaging, USA). Blood feeding experiments were completed within 4 h post-thawing. The concentration of thawed mf was

adjusted to 18 mf/μL using heparinized blood collected from a healthy dog. Non-cryopreserved fresh blood (6 mf/μL) was collected from an infected dog and used as the control. Fully fed mosquitoes were selected under CO_2 anesthesia and maintained for 13 d. Mosquito survival was monitored every day. At 13 d post-infection (dpi), all surviving female mosquitoes were dissected under a stereomicroscope to investigate the number of L3. Mosquito survival was analyzed by chi-square test, and the L3 count was analyzed by Welch's t-test.

Statistical analyses

All statistical analyses were performed using GraphPad Prism 7 (GraphPad, San Diego, CA, USA). A significant difference was defined by $p < 0.05$ and $p < 0.10$ was considered a marginal difference.

Results And Discussion

In vitro viability of purified mf

The viability of cryopreserved mf was compared between traditional PVP-DMSO and several commercially available cryopreservation media. Microscopic observation revealed that mf cryopreserved with CultureSure exhibited the highest motility (AR = 8.5 on 0 dpt, 10.51 on 1 dpt, and 12.19 on 3 dpt) (Fig. 1a). Mf cryopreserved with LaboBanker exhibited the second highest motility (AR = 7.67 on 0 dpt, 9.88 on 1 dpt, and 6.55 on 3 dpt), and Mf in CryoScarless exhibited the lowest motility (AR = -24.03 on 0 dpt, -20.06 on 1 dpt, and -10.12 on 3 dpt). When mf motility was compared based on moderately active and highly active levels, mf in CultureSure, LaboBanker, and Cellbanker media all exhibited significantly higher motility on 0, 1, and 3 dpt than mf preserved in traditional PVP-DMSO media. Mf in CryoScarless medium showed significantly lower motility compared to PVP-DMSO on 0 and 1 dpt.

The viability of mf on 3 dpt was quantified based on the ability of PI to penetrate dead or dying cells but not living cells. CultureSure-cryopreserved mf showed significantly lower PI values compared to all of the other cryopreservation media (AR = 5.7) (Fig. 1b). The mf cryopreserved in PVP-DMSO (AR = 3.59), LaboBanker (AR = 2.87), and Bambanker (AR = 2.53) showed significantly lower PI positive rates (Fig. 1b). These results are supported by fluorescent imaging of mf PI staining in each medium (Fig. 1c). Translating our results, to do a parallel with other published studies, the total motile parasite ratio of CultureSure-cryopreserved mf after thawing (97.2%, Fig. 1A) was considered equivalent or slightly better than the reported for cryopreserved mf using 5% DMSO medium (78.8% - 91.6%) [5] or PVP-DMSO (>95%) [6]. From these experiments, it is clear that CultureSure is the optimal cryopreservation medium tested for the preservation of D. immitis mf.

Parasite concentration levels were a factor influencing motility in the various media. In general, motility of mf cryopreserved at a high concentration (ca. 15,000 mf/mL) was significantly greater than mf cryopreserved at a low concentration (ca. 3,000 mf/mL). This was true for mf cryopreserved in PVP-

DMSO on 0 to 3 dpt, in CultureSure on 1 and 3 dpt, and in LaboBanker on 0 and 3 dpt (Fig. 1d). These results suggest that cryopreservation of mf should be carried out at the higher mf concentration to retain better viability.

In vitro viability of mf in mf-infected total blood

Cryopreservation of D. immitis mf-infected total blood is especially important in clinical settings. The motility of mf in mf-infected total blood was compared over a range of blood volumes in CultureSure (1 mL total volume) and with purified mf (ca. 25,000 mf/mL) (Fig. 2a). The results were comparable to purified mf cryopreservation, in most cases. Significant differences were observed between purified mf and mf-infected total blood volumes of 400 μL on 0 dpt and 300 μL on 3 dpt. In addition, a significant difference was seen for mf-infected volumes of 200 μL and 400 μL on 0 dpt. Analysis of PI staining of mf on 3 dpt, demonstrated that mf-infected total blood at a volume of 200 μL showed significantly lower PI positive values (AR = 2.36, Fig. 2b), whereas the 300 μL sample showed the highest PI staining values (AR = 1.98, Fig. 2b). These findings were supported by fluorescent imaging (Fig. 2c). Altogether, these results suggest that cryopreservation is optimal with 200 μL (ca. 25 mf/μL) of infected blood brought to a total volume of 1 mL with CultureSure.

Vector infectivity and survival of cryopreserved mf

Infectivity of mosquito vectors with D. immitis mf cryopreserved with CultureSure or LaboBanker was compared with traditional PVP-DMSO cryopreservation. Non-cryopreserved, fresh mf were used as a positive control. The survival rate of mosquitoes fed LaboBanker cryopreserved mf was significantly higher compared to mosquitoes fed CultureSure or PVP-DMSO cryopreserved mf on 13 dpi (Fig. 3a). The number of L3 in mosquitoes fed CultureSure cryopreserved mf tended to be higher than that of the L3 for mf cryopreserved in PVP-DMSO and fresh mf (Fig. 3b). Although different mosquito colonies and mf infectious inocula were used, the mf infectivity reported here was similar to the one previously reported for PVP-DMSO cryopreserved mf [6]. The ratios of mean L3 numbers in individual mosquitoes/feeding inoculum concentration were 0.85 and 0.77 for PVP-DMSO cryopreserved mf in our study and a previous study, respectively [6]. Remarkably, this ratio, determined for CultureSure was 1.03. The number of L3 of mosquitoes fed LaboBanker cryopreserved mf was significantly lower compared to the other treatments. These results indicate that CultureSure is an optimal medium for D. immitis mf cryopreservation. Lok et al. demonstrated that 6.0% - 20.0% of mf cryopreserved in 5% DMSO medium reached the L3 stage when inoculated to mosquitoes using the enema method [5]. The estimated average blood volume ingested by Ae. aegypti per blood meal was 2.3 - 2.6 μl [12, 13]. In our study, assuming a blood intake of 2.5 μl (18 mf/μl) per mosquito, the L3 development ratio was calculated as 41.8% (18.8 L3/45 mf), suggesting CultureSure is better than 5% DMSO medium for the cryopreservation of D. immits mf.

Importance of DMSO in cryopreservation media

DMSO appears to be an important component in cryopreservation media for D. immitis mf. In the present study, most of the commercial cryopreservation media examined yielded a positive performance, except for CryoScarless, which was the only medium that did not contain DMSO. DMSO is known to inhibit ice crystal formation during the freezing process and it is commonly used for mammalian cell cryopreservation [14]. Selection of a DMSO-containing medium seems critical for successful cryopreservation of D. immitis mf.

Justification and practical applications for using cryopreserved mf

Preparation of D. immitis-infected animals and fresh mf is a bottleneck for in vivo mosquito vector experiments. Here, we demonstrated that cryopreserved mf could be used for in vivo mosquito experiments instead of fresh mf, thereby eliminating the need to maintain infected animals. The concentration of cryopreserved mf fed to mosquitoes in mf-infected blood was adjusted to 3-fold higher levels than non-cryopreserved, fresh mf. Under these conditions, the number of L3 for the mosquitoes was almost equivalent to that of fresh mf.

Standardization of D. immitis mf cryopreservation method is essential for the study of drug-resistant D. immitis. Macrocyclic lactones have been widely used to prevent heartworm infections for the past three decades [15]. Since the emergence of macrocyclic lactone resistance in D. immitis in 2005 [16], numerous field and laboratory-induced isolates have been studied [17-19]. These isolates are essential for further studies to completely understand drug-resistance in D. immitis. However, it is quite expensive and time-consuming to maintain each of these isolates by continuous alternate passages in mosquitoes and dogs. Although cryopreservation methods are not without limitations, such as the need for continuous and relatively frequent replenishment of liquid nitrogen levels, they offer unique advantages over continuous biological maintenance. In addition and importantly, cryopreservation allows the reduction of animal use (fewer dogs are needed), respecting the ethics in animal experimentation, and makes the continuous maintenance of an insectary for mosquito production expendable.

Cryopreservation of D. immitis mf has practical applications for veterinary science education [20]. Currently, diagnosis of D. immitis infection is usually performed with a commercial antigen detection kit; however, false negatives are common [21]. Adequate skills need to be developed to ensure mf detection in clinical settings. The supply of D. immitis mf available for veterinary education curricula is limited due to the difficulty of maintaining live animals infected with D. immitis mf. We believe our results validate the use of a commercial cryopreservation medium for D. immitis mf, overcoming the current issues.

Conclusions

In summary, we determined that CultureSure is an optimal commercial cryopreservation solution for the storage of D. immitis mf. CultureSure was successful in cryopreserving purified mf, mf-infected total blood, and mf for in vivo mosquito vector experiments. This study will facilitate not only the study of the vector stage of D. immitis but also the study of macrocyclic lactone resistance in heartworms and the consequent education of veterinarians.

Abbreviations

ANOVA: analysis of variance; AR: adjusted residual; DMSO: dimethyl sulfoxide; Dpi: days post-infection; Dpt: days post-thawing; L3: third stage larvae; LVP: Liverpool; Mf: microfilariae; OUAVM: Obihiro University of Agriculture and Veterinary Medicine; PBS: phosphate buffered saline; PI: propidium iodide; PVP: polyvinylpyrrolidone

Declarations

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Declarations

None.

Ethics approval and consent to participate

This study was carried out in strict accordance with the recommendations in the Guide for Laboratory Animals of OUAVM. The protocol was approved by the committee of the Animal Experiments of the OUAVM (permit number 19-115).

Consent for publication

Not applicable.

Availability of data and materials

Data supporting the conclusions of this manuscript are provided within the article.

Competing interests

The authors state that they have no competing interests.

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Authors' contributions

TS and SF designed the experiments. TS, AS and SF carried out the experiments. TS analyzed the data. TS and SF wrote the manuscript, which was edited by AS. SF supervised the study.

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Table

Table 1. Information about the composition in commercial cryopreservation media

Product name	DMSO	Bovine serum	Manufacturer
Cellbanker 1	10%	<80%	Zenoaq Resource
LaboBanker 1	10%	containing	Kurabo
CultureSure Freezing Medium	10%	free (containing BSA)	Fujifilm
Bambanker	10%	free (containing bovine serum-derived components)	GC Lymphotec
Cos banker	containing	free	Cosmo Bio
CryoScarless DMSO-Free	free	free	Bio Verde

Figures
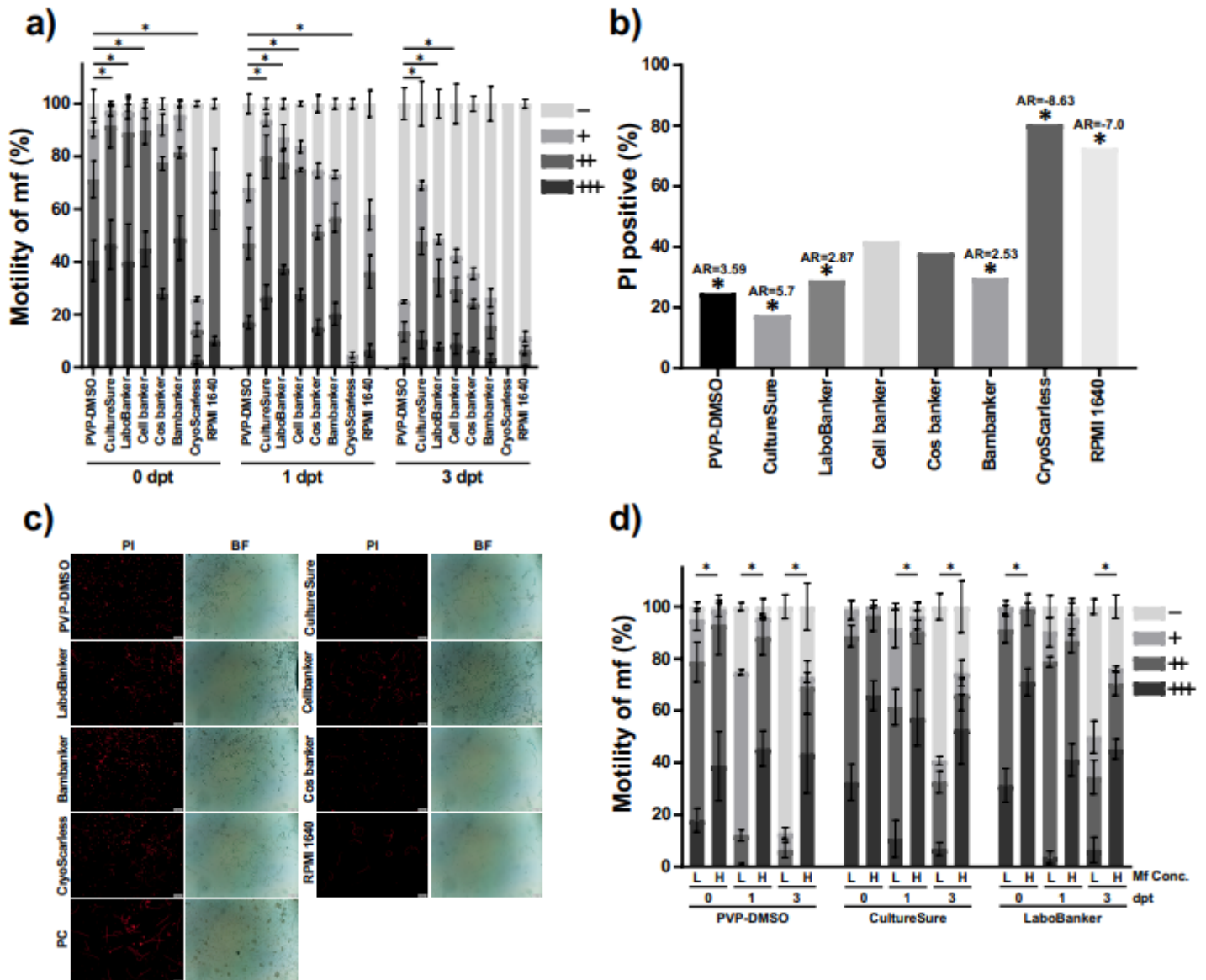
Figure 1

Validation of commercial cryopreservation media for the preservation of D. immitis microfilariae (mf) in vitro. a) Comparison of mf motility in vitro for various cryopreservation media. Motility of mf (ca. 3,000 mf/mL) was analyzed on 0, 1, and 3 d post-thawing (dpt) and scored at four levels as follows: inactive or dead (-), less active (+), moderately active (++), and highly active (+++). An asterisk indicates a significant difference ($p < 0.05$) using Welch's t-test between the PVP-DMSO control and the other cryopreservation media. Error bars indicate ± standard deviation (SD). b) Percent of mf showing positive propidium iodide (PI) staining 3 dpt, indicating dead or dying mf. An asterisk indicates a significant difference based on chi-square test and adjusted residuals (AR). An absolute value of AR ≥ 1.96 was considered significant ($p < 0.05$). c) Fluorescent images of PI staining of mf 3 dpt (exposure, 200 ms; gain, 0.5). PI image (left) and bright field (BF) image (right). PC, positive control for PI staining fixed by methanol. Scale bar = 100 μm. d) Comparison of mf motility at high (H; ca. 15,000 mf/mL) and low (L; 3,000 mf/mL) concentrations during cryopreservation. Motility was scored at four levels as described in Panel A. An asterisk indicates a significant difference ($p < 0.05$) by Welch's t-test. Error bars indicate ± SD.
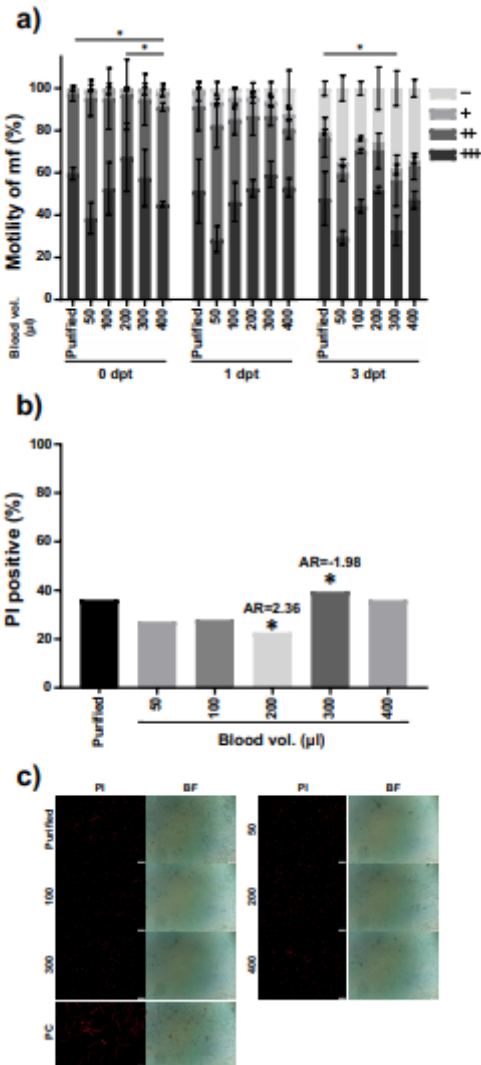
Figure 2

Viability of cryopreserved D. immitis microfilariae (mf)-infected total blood. a) In vitro motility was scored over a range of blood volumes (x-axis) in CultureSure (1 mL total volume) and compared to purified mf at 0, 1, and 3 d post-thawing (dpt). Scoring was at four levels as follows: inactive or dead (-), less active (+), moderately active (++), and highly active (+++). An asterisk indicates a significant difference (p < 0.05) by one-way ANOVA, followed by Tukey's multiple comparisons test. Error bars indicate ± SD. b) Percent positive PI staining of mf-infected total blood on 3 dpt. An asterisk indicates a significant difference by chi-square test and adjusted residual (AR). AR ≥ 1.96 was considered significant (p < 0.05). C) Fluorescent images of propidium iodide (PI) staining of mf-infected total blood on 3 dpt (exposure, 100 ms; gain, 0.5). PI image (left) and bright field (BF) image (right). PC, positive control. Scale bar = 100 μm.
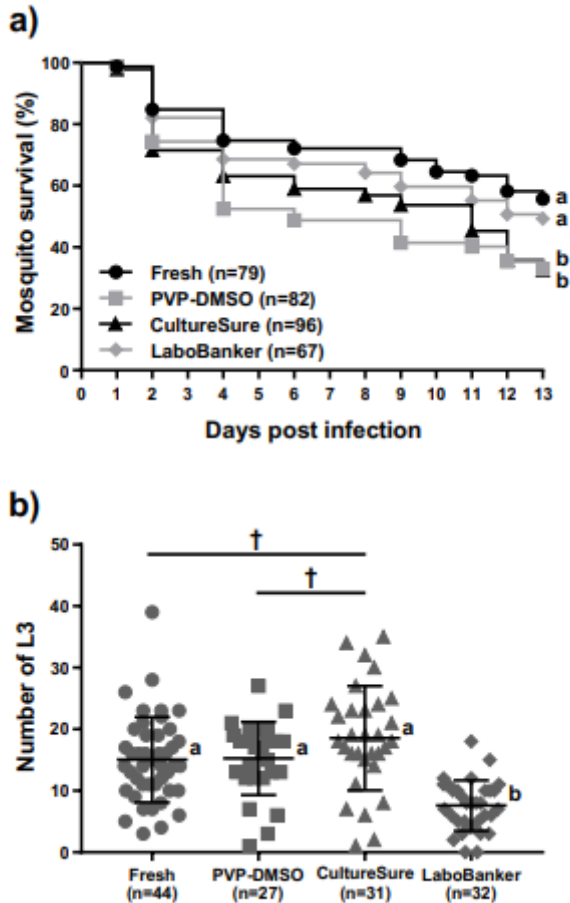
Figure 3

Analysis of in vivo infectivity of Ae. aegypti mosquitoes with cryopreserved D. immitis mf. a) Survival of mosquitoes infected with mf cryopreserved in PVP-DMSO, CultureSure, and LaboBanker, and non-cryopreserved control (Fresh) over 13 d post-infection (dpi). The survival rate on 13 dpi was statistically compared using a chi-square test. Different letters (a, b) indicate significant differences (p < 0.05). b) The number of third stage larvae (L3) in the mosquitoes on 13 dpi. Each plot shows the L3 of individual mosquitoes. Error bars = mean ± SD. The L3 were statistically compared using Welch's t-test. Significant differences (p < 0.05) were observed between different letters (a, b). †, Marginal differences.