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Evaluation of alternative reagents on the performance of the modified Knott's test

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Evaluation of alternative reagents on the performance of the modified Knott's test

--Manuscript Draft--

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Abstract:	The aim of the present study was to evaluate the suitability of different reagents as safe alternatives to 2% formalin in the modified Knott's test for the diagnosis of subcutaneous (Dirofilaria repens) and cardiopulmonary (D. immitis) dirofilariosis. A total of 61 blood samples from dogs naturally infected with D. immitis and D. repens were collected and analysed in two different laboratories (Lab 1, University of Parma and Lab 2, University of Napoli). For each blood sample the modified Knott's method was performed to identify and measure the mean length and width of the microfilariae (mfs) using 2% formalin (A), 2% acetic acid (B), 2% glacial acetic acid (C), 10% saponin (D) and distilled water (E). When compared to 2% formalin, there was no significant difference ($P>0.05$) among the mean length and width of either D. immitis or D. repens mfs with distilled water (E). The lengths and widths of mfs, however, were significantly reduced ($P<0.05$) when using B, C, D likely due to more pronounced parasite dehydration. Despite differences in measurements, the morphological features of the head and tail of the two species were maintained, suggesting that all the solutions tested could be a suitable alternative to formalin. All alternative reagents caused more marked haemolysis compared to formalin, improving readability of slides. The values of the mean length and the mean width of D. immitis and the mean width of D. repens mfs obtained with formalin and distilled water were statistically different ($P<0.005$) between the two laboratories. The difference in mf measurements between the two labs could be due to the use of reagents purchased from different manufacturing companies. Results suggest that distilled water could replace formalin in the modified Knott's test, as a safer reagent that allows morphology-based species differentiation of Dirofilaria spp.

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Dipartimento Medicina Veterinaria Produzioni Animali

UNIVERSITÀ DEGLI STUDI DI NAPOLI FEDERICO II

Dear Editor,

We would like thank you for accepting our manunuscript entitled "**Evaluation of alternative reagents on the performance of the modified Knott's test**" for the *Veterinary Parasitology* Journal. We appreciate the interest that you and reviewers have taken in our manuscript and the constructive criticism they have given. The manuscript has been revised according to the suggestions of the Reviewers. We have also included a point-by-point response to the reviewers, explaining the changes described in the revised manuscript.

Thank you again.
We look forward to the outcome of your assessment

Yours sincerely,

Lavinia Ciuca

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Reviewer #1:

OVERALL COMMENTS

This study aimed to evaluate the suitability of different reagents, such as acetic acid-vinegar, glacial acetic acid, saponin, and distilled water, in the execution of the modified Knott's test, instead of the usually employed 2% formalin. The study conclusion supports the use of distilled water in the above-mentioned concentration method, suggesting this reagent as a reliable and safer substitution of 2% formalin, known to be toxic and cancerogenic.

Apart some basic issues reported in the minor comments section, and the English language that must be checked by a native speaker, I recognize the importance of this finding that surely will make easier the diagnostic procedures of the filarial infections in both laboratories and veterinary facilities.

Therefore, in my opinion, the manuscript is suitable for publication in VetPar pending minor revisions.

RE: We thank the reviewer for the positive feedback on our manuscript. The manuscript has been carefully revised by a native English-speaking (LK), co-author of this manuscript, who has reviewed the revised manuscript.

MINOR COMMENTS

Highlights

First and fifth sentence: Please, add "2%" to "formalin".

RE: The manuscript has been modified accordingly.

Second and fourth sentences: Add the adjective "distilled" to "water" in order to make the sentences self-explanatory of what has been done in the study.

RE: Thank you for the suggestion. The manuscript has been modified accordingly.

Third sentence: Change "mf" in "mfs" if it has been used for the plural form "microfilariae". Check all the text and change accordingly.

RE: Done. The manuscript has been modified accordingly.

Fifth sentence: Change "purified" in "distilled". The two terms are not equivalent and, and in this case, the correct one is distilled. Check all the text and change accordingly.

RE: Done. The manuscript has been modified accordingly.

Abstract

Line 29: Remove the comma after "formalin".

RE: Done.

Line 31: Remove the comma after "D. repens".

RE: Done.

Line 32: I suggest removing "parasitology" and "in Italy" from the sentence. "Parasitology" is obvious, so there is no meaning to specify in the abstract. The same applied to the Country in which the study has been conducted. This is important to be clarified only in M&M and not in the abstract.

RE: Thank you for the comment. The manuscript has been modified accordingly.

Lines 42-45: The authors should add a short sentence explaining which is, in their opinion, the reason of the differences in the mf measures between the two laboratories and whether the main result, i.e., "... no significant difference (P>0.05) among the mean length and width of either D. immitis or D. repens mf with purified water (E)", is valid for both.

RE: The difference in mf measurements between the two labs could be due to the use of reagents purchased from different manufacturing companies. In addition, there was no significant difference (P>0.05) among the mean length and width of either *D. immitis* or *D. repens* mf with distilled water, for both laboratories (Lab 1), University of Parma and (Lab 2), University of Napoli).

Introduction

Line 35: Replace "Dirofilariasis" with "Dirofilarioses".

RE: Actually, the standard and uniform nomenclature formulated by the World Association for Advancement of Veterinary Parasitology (WAAVP) is Standardized Nomenclature of Animal Parasitic Diseases (SNOAPAD) (Kassai T). Nomenclature for parasitic diseases: cohabitation with inconsistency for how long and why? Vet Parasitol. 2006 Jun 15;138(3-4):169-78), suggest: when disease names are formed from the taxonomic name of the parasite, use solely and uniformly suffix '-osis' (in plural '-oses') of the varieties of suffixes ('-osis', '-iosis', -asis, '-iasis') currently in use for coining terms to denominate a disease or infection.

The suffix '-osis' is to be added to the stem of the name of the parasite taxon, which, in general, is formed from the nominative case of the taxa by the omission of the last one or two letters (e.g. Trypanosoma, trypanosomosis, Sarcocystis, sarcocystiosis,

Fasciola, fasciolosis, Trichostrongylidae, trichostrongylidosis, Ascaris, ascariosis, Trichinella, trichinellosis, Hypoderma, hypodermosis).

The practice of using the suffix ‘osis’ to denote parasitic disease with apparent clinical signs and the suffix ‘iasis’ for subclinical infections are to be discontinued.

Line 55: Add the references "Mendoza-Roldan et al., 2020" (Leishmania infantum and Dirofilaria immitis infections in Italy, 2009-2019: changing distribution patterns, Parasites & vectors, 13(1), 1-8) and "Brianti et al., 2021" (Dirofilaria immitis infection in the Pelagie archipelago: the southernmost hyperendemic focus in Europe. Transboundary and Emerging Diseases). Both citations strongly support the new pathogens' distribution throughout the Country and their related rapid spread.

RE: Thank you for the suggestions. The references have been added to the revised manuscript.

Lines 59-61: The underestimated risk of filarial infection is strictly related to veterinarians' point of view. Thus, the authors should specify this by adding "among the veterinary practitioners".

RE: Thank you for the suggestion. We added the information to the revised manuscript.

Lines 64- 65: Change "… is an essential part of management of disease and in the control of spread to other animals and to humans" into "… is an essential part in the disease management and in controlling the spread of pathogens to other animals and humans".

RE: Thank you for the suggestion. The manuscript has been modified accordingly.

Line 68: Remove the second "the".

RE: Done.

Line 78: Add the reference "Panarese et al., 2020" (Comparison of diagnostic tools for the detection of Dirofilaria immitis Infection in Dogs. Pathogens, 9(6), 499), in which the limitations of the main antigenic tests are well explained.

RE: Thank you for the suggestion. The reference has been added to the revised manuscript.

Line 80: After the first mention, you should replace "microfilariae" with "mfs". Check all the text and change accordingly.

RE: Done. The manuscript has been modified accordingly.

Line 95: Replace "fixation" with "concentration".

RE: Actually, the authors used the term “fixation” referring to the main activity of formalin: mfs -fixation in the modified Knott’s test.

Materials and Methods

Lines 107-111: It is not clear if these dogs were diagnosed as microfilaremic at Knott's test by the practitioners or by the respectively laboratories. Please, specify.

RE: The dogs were previously diagnosed with microfilariae with the Knott's test by the practitioners and then sent to us (both laboratories Lab 1 and Lab 2) for the species identification. All these data were added to the revised manuscript.

Lines 114-115: Change the sentence in "The blood samples were delivered by the practitioners to the laboratories within 24 hours."

RE. Done. The manuscript has been modified accordingly.

Line 125: Why distilled/bi-distilled water? Is there a specific number of blood samples that were analysed with either both, or distilled or bi-distilled water only? Is there any difference in the result between both solutions?

RE: All the samples were analysed only with distilled water. The manuscript has been modified accordingly. We did not perform the Knott test using bi-distilled water, but it is our belief that there is no difference between using distilled/bi-distilled water.

Results

Lines 158-161: Is there any dogs co-infected with both filarial species?

RE: Actually, none of the samples presented co-infection with both filarial species.

Line 167: Remove "Interestingly" What is interesting for you could not be for others…

RE: Done.

Discussion

Lines 175-195: This part sounds more like an introduction than a discussion section. I suggest moving this part later in the paragraph, after the discussion of the main results of the study. Also, the authors should shorten it a bit more, considering that this information were partially provided in the introduction.

RE: Thank you for the suggestions. We have revised the discussion from the lines 175-195 of the manuscript.

Line 205: Considering the wide range of measures of the mfs, sometimes overlapping among the filarial species, rather than "essential", it is better to replace the sentence as follows "... useful for the identification of the mfs species".**RE: Done. The manuscript has been modified accordingly.**

Line 211: Remove "subjectively" and change the sentence as follows "… was considered similar for the employed reagents by each operator".

RE: Done. The manuscript has been modified accordingly.

Line 222: Add the reference "Panarese et al., 2020" (Hyperendemic Dirofilaria immitis infection in a sheltered dog population: an expanding threat in the Mediterranean region. International Journal for Parasitology, 50(8), 555-559). Although the authors already added this reference in Table 5, it is missing in the refence list.

RE: Done. The reference has been added to the revised manuscript.

Reviewer #2:

Very interesting and well developed research. Worthy of publication for the important implications in clinical practice**.** Only 2 minor clarifications needed, the indications are in the attached document

RE: We thank the reviewer for the positive comments on our manuscript.

Dirofilaria repens the first time use the full name

RE: Done.

Formaldehyde is a basic chemical compound whereas formalin is a formulation of formaldehyde in aqueous solution. " However formaldehyde the substance contained in formalin"

RE: Thank you for the comment. The manuscript has been modified accordingly.

- Safer alternatives to 2% formalin were used in the modified Knott's test.
- The lengths and widths of mfs were reduced using glacial acetic acid and saponin.
- Knott's test using distilled water allowed differentiation of *Dirofilaria* spp.
- Distilled water could successfully replace 2% formalin in the Knott's test.

Evaluation of alternative reagents on the performance of the modified Knott's test

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Abstract

The aim of the present study was to evaluate the suitability of different reagents as safe alternatives to 2% formalin in the modified Knott's test for the diagnosis of subcutaneous (*Dirofilaria repens*) and cardiopulmonary (*D. immitis*) dirofilariosis. A total of 61 blood samples from dogs naturally infected with *D. immitis* and *D. repens* were collected and analysed in two different laboratories (Lab 1, University of Parma and Lab 2, University of Napoli). For each blood sample the modified Knott's method was performed to identify and measure the mean length and width of the microfilariae (mfs) using 2% formalin (A), 2% acetic acid (B), 2% glacial acetic acid (C), 10% saponin (D) and distilled water (E). When compared to 2% formalin, there was no significant difference ($P>0.05$) among the mean length and width of either *D. immitis* or *D. repens* mfs with distilled water (E). The lengths and widths of mfs, however, were significantly reduced ($P<0.05$) when using B, C, D likely due to more pronounced parasite dehydration. Despite differences in measurements, the morphological features of the head and tail of the two species were maintained, suggesting that all the solutions tested could be a suitable alternative to formalin. All alternative reagents caused more marked haemolysis compared to formalin, improving readability of slides. The values of the mean length and the mean width of *D. immitis* and the mean width of *D. repens* mfs obtained with formalin and distilled water were statistically different ($P<0.005$) between the two laboratories. The difference in mf measurements between the two labs could be due to the use of reagents purchased from different manufacturing companies.

Results suggest that distilled water could replace formalin in the modified Knott's test, as a safer reagent that allows morphology-based species differentiation of *Dirofilaria* spp.

Keywords: *Dirofilaria immitis*; *Dirofilaria repens*; modified Knott's test; distilled water; formalin; alternative reagents.

1. Introduction

Dirofilariosis, caused by *Dirofilaria immitis* and *Dirofilaria repens,* are two mosquito-borne diseases that are (re-) emerging and spreading in several countries (Genchi and Kramer, 2020; Mendoza-Roldan et al., 2020; Szell et al., 2020; Brianti et al., 2021; Deskne et al., 2021). Various factors have facilitated this expansion, including climate change and globalization (Genchi et al., 2009), that allowed the introduction of new competent mosquito species such as *Aedes albopictus*, together with the movement of pets to or from endemic areas. Furthermore, misdiagnosis and lack of prevention (in particular for *D. repens,* which is usually asymptomatic (Genchi et al., 2019), likely contributes to underestimation of risk for infection among the veterinary practitioners). Finally, both *D. immitis* and *D. repens* are important and emerging agents of vector-borne zoonosis, in particular *D. repens* (Simón et al., 2012).

Correct diagnosis of *Dirofilaria* spp. infection is an essential part of disease management and in controlling the spread to other animals and humans. The current American Heartworm Society guidelines (AHS, 2020) recommend testing for both circulating microfilariae (mfs) and antigens to confirm heartworm infection. The European Society of Dirofilariosis and Angiostrongylosis (ESDA, 2017) and the European Scientific Counsel Companion Animal Parasites guidelines (ESCCAP, 2019) indicate detection of circulating mfs as the best and most sensitive and specific option for diagnosis of *D. repens* infection, even when compared to molecular methods such as PCR (Ciuca et al., 2020). Moreover, the ESCCAP guidelines (ESCCAP, 2019) recommend that dogs are checked for circulating mfs before beginning annual preventive treatment in order to reduce the risk of selecting resistance against *D. immitis*. Identification of mfs is also important due to potential cross-reactivity of enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests for *D. immitis* with other filarial nematodes such as *D. repens, Angiostrongylus*

vasorum and *Spirocerca lupi* (Schnyder and Deplazes, 2012; Aroch et al., 2015; Panarese et al., 2020a).

The modified Knott's test (Knott, 1939) is an easy and inexpensive technique based on concentration, staining, detection and morphometric identification of circulating mfs of different species. The technique foresees the dilution of 1 mL of EDTA venous blood with 9 mL of 2% formalin (ESDA, 2017). However, formaldehyde, the substance contained in formalin, has been shown to be mutagenic and genotoxic in several experimental models, both *in vivo* and *in vitro* (National Toxicology Program, 2010; Bernardini et al., 2020). The toxicity of formaldehyde is thought to be due to its high water solubility and reactivity in interactions with nucleophilic groups of proteins, DNA and RNA molecules (Katsnelson et al., 2013). In 2012, the International Agency for Research on Cancer (IARC) classified formaldehyde as a human carcinogen (IARC, 2012). In addition, the ECHA (European Chemical Agency) identifies this substance as fatal if inhaled, toxic if swallowed, toxic following contact with skin, capable of causing severe skin burns and eye damage. Studies on dosimetry modelling of inhaled formaldehyde in humans have shown that more than 95% of the inhaled formaldehyde is predicted to be retained by the respiratory tract (Overton et al., 2001). Moreover, formalin requires special precautions in handling, storage and disposal and should be employed only under a chemical hood. It would therefore be of interest to find alternative fixation methods in order to increase the use of the Knott's test, especially among veterinary practitioners.

Therefore, the aim of this study was to evaluate the suitability of different reagents, i.e. acetic acid-vinegar, glacial acetic acid, saponin and distilled water, as safer alternatives to formalin to use in the modified Knott's test for the diagnosis of subcutaneous (*D. repens*) and cardiopulmonary (*D. immitis*) dirofilariosis.

2. Materials and methods

2.1 Sampling and modified Knott's tests

Between January and September 2020, blood samples from 61 dogs of different breeds, sex and age, previously found to be microfilaremic to *Dirofilaria* spp. using the modified Knott's test, by different practitioners. All the blood samples were sent to two parasitology laboratories for the species identification: Lab 1, University of Parma (19 samples) and Lab 2, University of Napoli (42 samples).

Approximately 7 ml of venous blood were collected from each dog and placed in EDTA vacutainer tubes. The blood samples were delivered by the practitioners to the laboratories within 24 hours. Moreover, all the blood samples were constantly kept refrigerated at 3-5°C and analyzed on the day of arrival in the laboratory.

All samples were analyzed with the modified Knott's test. One ml of EDTA blood was mixed with 9 ml of 2% formalin solution (A) in a 15 ml tube. The tube was gently inverted 4 times to mix the solution and centrifuged for 3 minutes at 1500 xg. The supernatant was poured off and 1-2 drops of 1% methylene blue were added (ESDA, 2017). A drop of the sediment was placed on a glass slide and covered with a coverslip. The slide was examined under the microscope at 10x to assess the presence of mfs, and at 40x to observe the morphology features.

The same procedure was applied to all the samples by replacing formalin with the following reagents: 2% acetic acid-vinegar (B), 2% glacial acetic acid (C), 10% saponin (D), distilled water (E).

Ten mfs were randomly selected from the slides prepared with each of the 5 reagents body length and width were measured and the morphology of the head and tail were determined (Magnis et al., 2013). All evaluations were done by two of the authors (MG, LC), in blind. Morphometric analyses were conducted with standard diagnostic microscopes (Lab 1 used a Nikon Eclipse E200 with Zeiss Axiocam Erc 5c; Lab 2 used a Leica DM 1000 LED with

ICC50 W camera system). Moreover, an antigen test for detection of *D. immitis* (PetCheck Heartworm PF Antigen, IDEXX) was carried out on all blood samples to confirm diagnosis of *D. immitis* infection.

In order to determine the correspondence of mfs measurements taken by the two laboratories, the same histological section obtained from a naturally *D. immitis* infected dog showing mfs within the pulmonary arteries was examined by each laboratory. The microscopes to be used for the study were calibrated with a stage micrometer, measurements obtained by the two laboratories were recorded and compared.

2.2 Statistical analysis

The arithmetic mean, standard deviation (SD), standard error of the mean (SE) and ranges (min and max) of lengths and widths of *D. immitis* and *D. repens* mfs were calculated for each of the following reagents: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E). Multiple comparisons of mean ranks depending on the fulfillment of the test assumptions between the values obtained in the A, B, C, D, E solutions were performed using a parametric test, i.e. the one-way analysis of variance (ANOVA) along with post hoc testing. Before applying post-hoc test (in between the groups), the homogeneity of the variances among the groups (Levene's test) was tested. If variances were homogeneous ($P \geq 0.05$) or not homogeneous ($P < 0.05$) different tests (Bonferroni and Games-Howell, respectively), were used. For all comparisons, a level of $\alpha = 0.05$ was assumed, and the obtained *P* values were rounded to two decimal places. Moreover, Error Bars graphics were constructed in order to compare the results of measurements (length and width) of mfs of both *D. immitis* and *D. repens* using formalin and purified water in two laboratories (Napoli, Parma). Statistical analysis and graphs were performed using SPSS Statistics v.23 (IBM, Armonk, NY, USA).

3. Results

Ten out of 19 blood samples analysed by Lab 1 were positive for *D. immitis* and nine for *D. repens* mfs. Eighteen out of the 42 blood samples analysed by Lab 2 were positive for *D. immitis* and 24 for *D. repens* mfs. All *D. immitis* mf-positive samples were also positive for circulating antigens (PetCheck Heartworm PF Antigen, IDEXX). None of the samples presented co-infection with both species.

The results regarding the mean length and the mean width of *D. immitis* and *D. repens* mfs using formalin and the other solutions are shown in Tables 1-4.

Solutions B, C, D significantly underestimated the length and width of both mfs species when compared to formalin ($P<0.005$). On the other hand, there was no significant difference ($P>0.005$) between the mean length and width of mf of both *D. immitis* and *D. repens* compared to formalin (A) when using distilled water (E). However, the values of the mean length and the mean width of *D. immitis* and the mean width of *D. repens* mfs obtained with formalin and distilled water were statistically different ($P<0.005$) between the two laboratories (Figures 1, 2, 4).

Measurements obtained from the observation of the same histological section were comparable for both laboratories.

4. Discussion and conclusions

Reliable diagnosis of filarial infections in companion animals is fundamental for the prevention and control of disease and for monitoring the spread of these parasites with zoonotic potential to non-endemic areas (Genchi and Kramer, 2017; Capelli et al., 2018).

The modified Knott's test is the only available diagnostic test for *D. repens* due to the lack of a commercially available serological test. However, the discrimination between different species can be challenging in cases of mixed infections with *Dirofilaria* spp. or in cases of low parasitaemia. In these cases, as well as for discrimination between *Dirofilaria*

and *Acanthocheilonema* spp., either molecular methods (e.g. multiplex PCR) or histochemical staining are required. The use of formalin, however, is of concern and likely limits the application of the test in private practice and in diagnostic laboratories. Indeed, the final report of National Toxicology Program (NTP), lists formaldehyde in the Eleventh Report on Carcinogens (RoC), and indicates that there is sufficient evidence for the carcinogenicity of formaldehyde in humans (NTP, 2010). Moreover, a recent study has shown that exposure to formaldehyde can damage fundamental biomolecules such as DNA and proteins (Reingruber, 2018).

The results of the present study suggest that distilled water maintains the same morphological characteristics and dimensions of mfs as those observed with formalin, while the lengths and widths of mfs were significantly reduced when using the other evaluated reagents. This may be due to more pronounced dehydration. Similar results were recently reported by Evans et al. (2019) with acetic acid (vinegar) and Long et al. (2020) with glacial acetic acid. However, despite the statistically significant differences (P<0.05) of mfs length and width observed with reagents B, C and D, the morphological features of the head and tail of the two species were maintained. We can therefore affirm that all solutions could be suitable and safe alternatives to formalin for the identification of circulating mfs in clinical settings with Knott's test, but that distilled water performs best in maintaining mfs' dimensions, which are useful for the identification of the mfs species.

Hemolysis is an important factor in the modified Knott's test, as it improves the readability of the slides by removing a large fraction of red blood cells from the sample, thus making the identification and enumeration of mfs easier. Even though the present study did not evaluate the effect of the different reagents on heamolysis, the readability of slides was considered improved by each operator (Figure 6). Indeed, stronger hemolytic activity with all the alternative reagents was observed when compared to 2% formalin.

The difference in mfs measurements between the two labs cannot be fully explained. The authors performed additional Knott's tests to evaluate the influence of storage time of the blood samples (+4°C for 24h, 48h, 72h and 96h) as this was the only difference identified for sample handling between the 2 laboratories. However, differences in mfs measurements between the laboratories remained (data not shown). Moreover, the measurements recorded by Lab 2 in the present study did not agree with a previous study conducted in the same area in 2001 (Cringoli et al., 2001) (Table 5). Furthermore, the measures obtained by both laboratories were not in agreement with several previous studies (Magnis et al., 2013; Longo et al., 2020), but were in agreement with others that reported broader size ranges of both *D. immitis* and *D. repens* mfs (Sloss et al., 1994; Taylor et al., 2007; Traversa et al., 2010; Liotta et al., 2013; ESCCAP, 2019; Panarese et al., 2020b). The authors can only hypothesize that the variability among studies could be due to the influence of examination methods or to the use of reagents purchased from different manufacturing companies.

We can conclude that distilled water could successfully replace formalin in the modified Knott's test for species differentiation of *Dirofilaria* spp.

Ethical standards

The study was performed in compliance with current national laws and regulations.

Aknowledgment

This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Declarations of interest: none.

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Figure legends:

Figure 1. Comparison of modified Knott's test using formalin and distilled water between the two laboratories: Napoli (n= 18) and Parma (n=10). The circles represent the mean length of *Dirofilaria immitis* mfs.

Notes: Modified Knott's test with distilled water-Napoli: mean=309.411; Standard Deviation (SD)=9.408; Standard error of mean (SE)=2.429; Parma: mean=305.282; SD=4.429; SE=1.400; Modified Knott's test with formalin-Napoli: mean=316.568; SD=7.663; SE=1.586; Parma-mean=304.742; SD=2.259; SE=0.714.

Figure 2. Comparison of modified Knott's test using formalin and distilled water between two laboratories: Napoli (n= 18) and Parma (n=10). The circles represent the mean width of *Dirofilaria immitis* mfs. Notes: Modified Knott's test with distilled water-Napoli: mean=6.153; SD=0.299; SE=0.077; Parma: mean=5.871; SD=0.091; SE=0.029; Modified Knott's test with formalin-Napoli: mean=6.252; SD=0.254; SE=0.061; Parma-mean=6.394; SD=0.146; SE=0.046.

Figure 3. Comparison of modified Knott's tests using formalin and distilled water between two laboratories: Napoli (n= 24) and Parma (n=9). The circles represent the mean length of *Dirofilaria repens* mfs.

Notes: Modified Knott's test with distilled water-Napoli: mean=365.539; SD=6.437; SE=2.145; Parma: mean=373.808; SD=6.437; SE=2.145; Modified Knott test with formalin-Napoli: mean=366.626; SD=8.890; SE=1.895; Parma-mean=372.195; SD=6.941; SE=2.313.

Figure 4. Comparison of modified Knott's test using formalin and distilled water between two laboratories: Napoli (n= 24) and Parma (n=9). The circles represent the mean width of *Dirofilaria repens* mfs.

Notes: Modified Knott's test with distilled water-Napoli: mean=7.217; SD=0.384; SE=0.116; Parma: mean=9.059; SD=0.048; SE=0.016; Modified Knott's test with formalin-Napoli: mean=6.751; SD=0.224; SE=0.061; Parma-mean=8.915; SD=0.160; SE=0.053.

Figure 5. Morphology of *Dirofilaria immitis* and *D. repens* mfs with the five different reagents: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E), used for modified Knott's test.

Figure 6. Readability of the slides, using formalin, (A) acetic acid-vinegar, (B) saponin (C) and distilled water (D) in modified Knott's test..

Table 1. Comparison of mean length of *Dirofilaria immitis* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	28	312.34	300.20	335.22	8.48	A vs. B=0.0000
						A vs. C=0.0000
B	27	285.12	271.65	312.83	11.09	A vs. D=0.0000
						A vs. E=0.0789
C	24	290.09	274.89	326.22	9. 78	B vs. C=0.0620
D	25	277.91	258.35	297.89	9.38	B vs. D=0.0660
E	25	307.75	287.99	325.87	7.95	B vs. E=0.0000
						C vs. D=0.0000
						C vs. E=0.0000
						D vs. E=0.0000

Table 2. Comparison of mean width of *Dirofilaria immitis* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	28	6.30	5.70	6.60	0.22	A vs. B=0.0000
B	27	5.84	5.40	6.90	0.35	A vs. C=0.0000
C	24	5.84	5.27	6.34	0.23	A vs. D=0.0000
D	25	5.70	5.40	6.63	0.25	A vs. E=0.0983
E	25	6.25	5.70	7.13	0.39	B vs. C=1.0000
						B vs. D=0.5060
						B vs. E=0.0400
						C vs. D=0.0231
						C vs. E=0.0010
						D vs. E=0.0000

Table 3. The comparison of mean length of *Dirofilaria repens* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	31	368.24	347.45	386.97	8.64	A vs. B=0.0000
B	24	344.17	326.09	353.65	6.63	A vs. C=0.0000
C	32	339.21	321.80	354.55	8.56	A vs. D=0.0000
D	21	331.84	307.82	351.35	13.63	A vs. E=1.0000
E	23	368.77	352.48	387.54	10.11	B vs. C=0.0121
						B vs. D=0.0060
						B vs. E=0.0000
						C vs. D=0.0204
						C vs. E=0.0000
						D vs. E=0.0000

Table 4. The comparison of mean width of *Dirofilaria repens* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	20	7.72	6.40	9.18	1.12	A vs. B=0.0000
B	20	5.96	5.05	6.55	0.33	A vs. C=0.0010
C	21	6.48	5.70	7.85	0.48	A vs. D=0.0000
D	20	5.94	5.29	6.62	0.29	A vs. E=0.0869
E	20	8.04	6.48	9.13	0.98	B vs. C=0.0200
						B vs. D=1.0000
						B vs. E=0.0000
						C vs. D=0.0010
						C vs. E=0.0000
						D vs. E=0.0000

399 Table 5. Comparison of mean length and width of *Dirofilaria immitis* and *D. repens* between
400 the results obtained by the authors from the present study (Lab 1, Parma and Lab 2, Napoli)
401 and other authors from previous studies, using modified Knott test with 2% formalin.
402

***Dirofilaria immitis* mfs**		***Dirofilaria repens* mfs**		**Authors**
Mean length µm	Mean width µm	Mean length µm	Mean width µm	
304.74	6.39	372.20	8.92	Laboratory 1(Parma)
315.31	6.27	365.05	6.75	Laboratory 2 (Napoli)
311.30	5.69	366.20	6.40	Cringoli et al., 2001
311.96		364.53		Panarese et al., 2020 Site 1
294.97		354.93		Panarese et al., 2020 Site 2
316				Evans et al., 2019
295 to 325				AHS, 2020
301.77	6.30	369.44	8.87	Magnis et al., 2013
259.2 ± 28.3		322.7 ± 21.1		Liotta et al., 2013
306.2	5.5			Long et al., 2020
260-340	5-7.5			Taylor et al., 2007

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Evaluation of alternative reagents on the performance of the modified Knott's test

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Abstract

The aim of the present study was to evaluate the suitability of different reagents as safe alternatives to 2% formalin in the modified Knott's test for the diagnosis of subcutaneous (*Dirofilaria repens*) and cardiopulmonary (*D. immitis*) dirofilariosis. A total of 61 blood samples from dogs naturally infected with *D. immitis* and *D. repens* were collected and analysed in two different laboratories (Lab 1, University of Parma and Lab 2, University of Napoli). For each blood sample the modified Knott's method was performed to identify and measure the mean length and width of the microfilariae (mfs) using 2% formalin (A), 2% acetic acid (B), 2% glacial acetic acid (C), 10% saponin (D) and distilled water (E). When compared to 2% formalin, there was no significant difference ($P>0.05$) among the mean length and width of either *D. immitis* or *D. repens* mfs with distilled water (E). The lengths and widths of mfs, however, were significantly reduced ($P<0.05$) when using B, C, D likely due to more pronounced parasite dehydration. Despite differences in measurements, the morphological features of the head and tail of the two species were maintained, suggesting that all the solutions tested could be a suitable alternative to formalin. All alternative reagents caused more marked haemolysis compared to formalin, improving readability of slides. The values of the mean length and the mean width of *D. immitis* and the mean width of *D. repens* mfs obtained with formalin and distilled water were statistically different ($P<0.005$) between the two laboratories. The difference in mf measurements between the two labs could be due to the use of reagents purchased from different manufacturing companies.

Results suggest that distilled water could replace formalin in the modified Knott's test, as a safer reagent that allows morphology-based species differentiation of *Dirofilaria* spp.

Keywords: *Dirofilaria immitis*; *Dirofilaria repens*; modified Knott's test; distilled water; formalin; alternative reagents.

1. Introduction

Dirofilariosis, caused by *Dirofilaria immitis* and *Dirofilaria repens,* are two mosquito-borne diseases that are (re-) emerging and spreading in several countries (Genchi and Kramer, 2020; Mendoza-Roldan et al., 2020; Szell et al., 2020; Brianti et al., 2021; Deskne et al., 2021). Various factors have facilitated this expansion, including climate change and globalization (Genchi et al., 2009), that allowed the introduction of new competent mosquito species such as *Aedes albopictus*, together with the movement of pets to or from endemic areas. Furthermore, misdiagnosis and lack of prevention (in particular for *D. repens,* which is usually asymptomatic (Genchi et al., 2019), likely contributes to underestimation of risk for infection among the veterinary practitioners). Finally, both *D. immitis* and *D. repens* are important and emerging agents of vector-borne zoonosis, in particular *D. repens* (Simón et al., 2012).

Correct diagnosis of *Dirofilaria* spp. infection is an essential part of disease management and in controlling the spread to other animals and humans. The current American Heartworm Society guidelines (AHS, 2020) recommend testing for both circulating microfilariae (mfs) and antigens to confirm heartworm infection. The European Society of Dirofilariosis and Angiostrongylosis (ESDA, 2017) and the European Scientific Counsel Companion Animal Parasites guidelines (ESCCAP, 2019) indicate detection of circulating mfs as the best and most sensitive and specific option for diagnosis of *D. repens* infection, even when compared to molecular methods such as PCR (Ciuca et al., 2020). Moreover, the ESCCAP guidelines (ESCCAP, 2019) recommend that dogs are checked for circulating mfs before beginning annual preventive treatment in order to reduce the risk of selecting resistance against *D. immitis*. Identification of mfs is also important due to potential cross-reactivity of enzyme-linked immunosorbent assay (ELISA) and immunochromatographic tests for *D. immitis* with other filarial nematodes such as *D. repens, Angiostrongylus*

vasorum and *Spirocerca lupi* (Schnyder and Deplazes, 2012; Aroch et al., 2015; Panarese et al., 2020a).

The modified Knott's test (Knott, 1939) is an easy and inexpensive technique based on concentration, staining, detection and morphometric identification of circulating mfs of different species. The technique foresees the dilution of 1 mL of EDTA venous blood with 9 mL of 2% formalin (ESDA, 2017). However, formaldehyde, the substance contained in formalin, has been shown to be mutagenic and genotoxic in several experimental models, both *in vivo* and *in vitro* (National Toxicology Program, 2010; Bernardini et al., 2020). The toxicity of formaldehyde is thought to be due to its high water solubility and reactivity in interactions with nucleophilic groups of proteins, DNA and RNA molecules (Katsnelson et al., 2013). In 2012, the International Agency for Research on Cancer (IARC) classified formaldehyde as a human carcinogen (IARC, 2012). In addition, the ECHA (European Chemical Agency) identifies this substance as fatal if inhaled, toxic if swallowed, toxic following contact with skin, capable of causing severe skin burns and eye damage. Studies on dosimetry modelling of inhaled formaldehyde in humans have shown that more than 95% of the inhaled formaldehyde is predicted to be retained by the respiratory tract (Overton et al., 2001). Moreover, formalin requires special precautions in handling, storage and disposal and should be employed only under a chemical hood. It would therefore be of interest to find alternative fixation methods in order to increase the use of the Knott's test, especially among veterinary practitioners.

Therefore, the aim of this study was to evaluate the suitability of different reagents, i.e. acetic acid-vinegar, glacial acetic acid, saponin and distilled water, as safer alternatives to formalin to use in the modified Knott's test for the diagnosis of subcutaneous (*D. repens*) and cardiopulmonary (*D. immitis*) dirofilariosis.

2. Materials and methods

2.1 Sampling and modified Knott's tests

Between January and September 2020, blood samples from 61 dogs of different breeds, sex and age, previously found to be microfilaremic to *Dirofilaria* spp. using the modified Knott's test, by different practitioners. All the blood samples were sent to two parasitology laboratories for the species identification: Lab 1, University of Parma (19 samples) and Lab 2, University of Napoli (42 samples).

Approximately 7 ml of venous blood were collected from each dog and placed in EDTA vacutainer tubes. The blood samples were delivered by the practitioners to the laboratories within 24 hours. Moreover, all the blood samples were constantly kept refrigerated at 3-5°C and analyzed on the day of arrival in the laboratory.

All samples were analyzed with the modified Knott's test. One ml of EDTA blood was mixed with 9 ml of 2% formalin solution (A) in a 15 ml tube. The tube was gently inverted 4 times to mix the solution and centrifuged for 3 minutes at 1500 xg. The supernatant was poured off and 1-2 drops of 1% methylene blue were added (ESDA, 2017). A drop of the sediment was placed on a glass slide and covered with a coverslip. The slide was examined under the microscope at 10x to assess the presence of mfs, and at 40x to observe the morphology features.

The same procedure was applied to all the samples by replacing formalin with the following reagents: 2% acetic acid-vinegar (B), 2% glacial acetic acid (C), 10% saponin (D), distilled water (E).

Ten mfs were randomly selected from the slides prepared with each of the 5 reagents body length and width were measured and the morphology of the head and tail were determined (Magnis et al., 2013). All evaluations were done by two of the authors (MG, LC), in blind. Morphometric analyses were conducted with standard diagnostic microscopes (Lab 1 used a Nikon Eclipse E200 with Zeiss Axiocam Erc 5c; Lab 2 used a Leica DM 1000 LED with

ICC50 W camera system). Moreover, an antigen test for detection of *D. immitis* (PetCheck Heartworm PF Antigen, IDEXX) was carried out on all blood samples to confirm diagnosis of *D. immitis* infection.

In order to determine the correspondence of mfs measurements taken by the two laboratories, the same histological section obtained from a naturally *D. immitis* infected dog showing mfs within the pulmonary arteries was examined by each laboratory. The microscopes to be used for the study were calibrated with a stage micrometer, measurements obtained by the two laboratories were recorded and compared.

2.2 Statistical analysis

The arithmetic mean, standard deviation (SD), standard error of the mean (SE) and ranges (min and max) of lengths and widths of *D. immitis* and *D. repens* mfs were calculated for each of the following reagents: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E). Multiple comparisons of mean ranks depending on the fulfillment of the test assumptions between the values obtained in the A, B, C, D, E solutions were performed using a parametric test, i.e. the one-way analysis of variance (ANOVA) along with post hoc testing. Before applying post-hoc test (in between the groups), the homogeneity of the variances among the groups (Levene's test) was tested. If variances were homogeneous ($P \geq 0.05$) or not homogeneous ($P < 0.05$) different tests (Bonferroni and Games-Howell, respectively), were used. For all comparisons, a level of $\alpha = 0.05$ was assumed, and the obtained *P* values were rounded to two decimal places. Moreover, Error Bars graphics were constructed in order to compare the results of measurements (length and width) of mfs of both *D. immitis* and *D. repens* using formalin and purified water in two laboratories (Napoli, Parma). Statistical analysis and graphs were performed using SPSS Statistics v.23 (IBM, Armonk, NY, USA).

3. Results

Ten out of 19 blood samples analysed by Lab 1 were positive for *D. immitis* and nine for *D. repens* mfs. Eighteen out of the 42 blood samples analysed by Lab 2 were positive for *D. immitis* and 24 for *D. repens* mfs. All *D. immitis* mf-positive samples were also positive for circulating antigens (PetCheck Heartworm PF Antigen, IDEXX). None of the samples presented co-infection with both species.

The results regarding the mean length and the mean width of *D. immitis* and *D. repens* mfs using formalin and the other solutions are shown in Tables 1-4.

Solutions B, C, D significantly underestimated the length and width of both mfs species when compared to formalin ($P<0.005$). On the other hand, there was no significant difference ($P>0.005$) between the mean length and width of mf of both *D. immitis* and *D. repens* compared to formalin (A) when using distilled water (E). However, the values of the mean length and the mean width of *D. immitis* and the mean width of *D. repens* mfs obtained with formalin and distilled water were statistically different ($P<0.005$) between the two laboratories (Figures 1, 2, 4).

Measurements obtained from the observation of the same histological section were comparable for both laboratories.

4. Discussion and conclusions

Reliable diagnosis of filarial infections in companion animals is fundamental for the prevention and control of disease and for monitoring the spread of these parasites with zoonotic potential to non-endemic areas (Genchi and Kramer, 2017; Capelli et al., 2018).

The modified Knott's test is the only available diagnostic test for *D. repens* due to the lack of a commercially available serological test. However, the discrimination between different species can be challenging in cases of mixed infections with *Dirofilaria* spp. or in cases of low parasitaemia. In these cases, as well as for discrimination between *Dirofilaria*

and *Acanthocheilonema* spp., either molecular methods (e.g. multiplex PCR) or histochemical staining are required. The use of formalin, however, is of concern and likely limits the application of the test in private practice and in diagnostic laboratories. Indeed, the final report of National Toxicology Program (NTP), lists formaldehyde in the Eleventh Report on Carcinogens (RoC), and indicates that there is sufficient evidence for the carcinogenicity of formaldehyde in humans (NTP, 2010). Moreover, a recent study has shown that exposure to formaldehyde can damage fundamental biomolecules such as DNA and proteins (Reingruber, 2018).

The results of the present study suggest that distilled water maintains the same morphological characteristics and dimensions of mfs as those observed with formalin, while the lengths and widths of mfs were significantly reduced when using the other evaluated reagents. This may be due to more pronounced dehydration. Similar results were recently reported by Evans et al. (2019) with acetic acid (vinegar) and Long et al. (2020) with glacial acetic acid. However, despite the statistically significant differences (P<0.05) of mfs length and width observed with reagents B, C and D, the morphological features of the head and tail of the two species were maintained. We can therefore affirm that all solutions could be suitable and safe alternatives to formalin for the identification of circulating mfs in clinical settings with Knott's test, but that distilled water performs best in maintaining mfs' dimensions, which are useful for the identification of the mfs species.

Hemolysis is an important factor in the modified Knott's test, as it improves the readability of the slides by removing a large fraction of red blood cells from the sample, thus making the identification and enumeration of mfs easier. Even though the present study did not evaluate the effect of the different reagents on heamolysis, the readability of slides was considered improved by each operator (Figure 6). Indeed, stronger hemolytic activity with all the alternative reagents was observed when compared to 2% formalin.

The difference in mfs measurements between the two labs cannot be fully explained. The authors performed additional Knott's tests to evaluate the influence of storage time of the blood samples (+4°C for 24h, 48h, 72h and 96h) as this was the only difference identified for sample handling between the 2 laboratories. However, differences in mfs measurements between the laboratories remained (data not shown). Moreover, the measurements recorded by Lab 2 in the present study did not agree with a previous study conducted in the same area in 2001 (Cringoli et al., 2001) (Table 5). Furthermore, the measures obtained by both laboratories were not in agreement with several previous studies (Magnis et al., 2013; Longo et al., 2020), but were in agreement with others that reported broader size ranges of both *D. immitis* and *D. repens* mfs (Sloss et al., 1994; Taylor et al., 2007; Traversa et al., 2010; Liotta et al., 2013; ESCCAP, 2019; Panarese et al., 2020b). The authors can only hypothesize that the variability among studies could be due to the influence of examination methods or to the use of reagents purchased from different manufacturing companies.

We can conclude that distilled water could successfully replace formalin in the modified Knott's test for species differentiation of *Dirofilaria* spp.

Ethical standards

The study was performed in compliance with current national laws and regulations.

Aknowledgment

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Declarations of interest: none.

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Figure legends:

Figure 1. Comparison of modified Knott's test using formalin and distilled water between the two laboratories: Napoli (n= 18) and Parma (n=10). The circles represent the mean length of *Dirofilaria immitis* mfs.

Notes: Modified Knott's test with distilled water-Napoli: mean=309.411; Standard Deviation (SD)=9.408; Standard error of mean (SE)=2.429; Parma: mean=305.282; SD=4.429; SE=1.400; Modified Knott's test with formalin-Napoli: mean=316.568; SD=7.663; SE=1.586; Parma-mean=304.742; SD=2.259; SE=0.714.

Figure 2. Comparison of modified Knott's test using formalin and distilled water between two laboratories: Napoli (n= 18) and Parma (n=10). The circles represent the mean width of *Dirofilaria immitis* mfs. Notes: Modified Knott's test with distilled water-Napoli: mean=6.153; SD=0.299; SE=0.077; Parma: mean=5.871; SD=0.091; SE=0.029; Modified Knott's test with formalin-Napoli: mean=6.252; SD=0.254; SE=0.061; Parma-mean=6.394; SD=0.146; SE=0.046.

Figure 3. Comparison of modified Knott's tests using formalin and distilled water between two laboratories: Napoli (n= 24) and Parma (n=9). The circles represent the mean length of *Dirofilaria repens* mfs.

Notes: Modified Knott's test with distilled water-Napoli: mean=365.539; SD=6.437; SE=2.145; Parma: mean=373.808; SD=6.437; SE=2.145; Modified Knott test with formalin-Napoli: mean=366.626; SD=8.890; SE=1.895; Parma-mean=372.195; SD=6.941; SE=2.313.

Figure 4. Comparison of modified Knott's test using formalin and distilled water between two laboratories: Napoli (n= 24) and Parma (n=9). The circles represent the mean width of *Dirofilaria repens* mfs.

Notes: Modified Knott's test with distilled water-Napoli: mean=7.217; SD=0.384; SE=0.116; Parma: mean=9.059; SD=0.048; SE=0.016; Modified Knott's test with formalin-Napoli: mean=6.751; SD=0.224; SE=0.061; Parma-mean=8.915; SD=0.160; SE=0.053.

Figure 5. Morphology of *Dirofilaria immitis* and *D. repens* mfs with the five different reagents: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E), used for modified Knott's test.

Figure 6. Readability of the slides, using formalin, (A) acetic acid-vinegar, (B) saponin (C) and distilled water (D) in modified Knott's test..

Table 1. Comparison of mean length of *Dirofilaria immitis* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	28	312.34	300.20	335.22	8.48	A vs. B=0.0000
B	27	285.12	271.65	312.83	11.09	A vs. C=0.0000
C	24	290.09	274.89	326.22	9. 78	A vs. D=0.0000
D	25	277.91	258.35	297.89	9.38	A vs. E=0.0789
E	25	307.75	287.99	325.87	7.95	B vs. C=0.0620
						B vs. D=0.0660
						B vs. E=0.0000
						C vs. D=0.0000
						C vs. E=0.0000
						D vs. E=0.0000

Table 2. Comparison of mean width of *Dirofilaria immitis* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	28	6.30	5.70	6.60	0.22	A vs. B=0.0000
B	27	5.84	5.40	6.90	0.35	A vs. C=0.0000
C	24	5.84	5.27	6.34	0.23	A vs. D=0.0000
D	25	5.70	5.40	6.63	0.25	A vs. E=0.0983
E	25	6.25	5.70	7.13	0.39	B vs. C=1.0000
						B vs. D=0.5060
						B vs. E=0.0400
						C vs. D=0.0231
						C vs. E=0.0010
						D vs. E=0.0000

Table 3. The comparison of mean length of *Dirofilaria repens* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	31	368.24	347.45	386.97	8.64	A vs. B=0.0000
B	24	344.17	326.09	353.65	6.63	A vs. C=0.0000
C	32	339.21	321.80	354.55	8.56	A vs. D=0.0000
D	21	331.84	307.82	351.35	13.63	A vs. E=1.0000
E	23	368.77	352.48	387.54	10.11	B vs. C=0.0121
						B vs. D=0.0060
						B vs. E=0.0000
						C vs. D=0.0204
						C vs. E=0.0000
						D vs. E=0.0000

Table 4. The comparison of mean width of *Dirofilaria repens* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	20	7.72	6.40	9.18	1.12	A vs. B=0.0000
B	20	5.96	5.05	6.55	0.33	A vs. C=0.0010
C	21	6.48	5.70	7.85	0.48	A vs. D=0.0000
D	20	5.94	5.29	6.62	0.29	A vs. E=0.0869
E	20	8.04	6.48	9.13	0.98	B vs. C=0.0200
						B vs. D=1.0000
						B vs. E=0.0000
						C vs. D=0.0010
						C vs. E=0.0000
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399 Table 5. Comparison of mean length and width of *Dirofilaria immitis* and *D. repens* between
400 the results obtained by the authors from the present study (Lab 1, Parma and Lab 2, Napoli)
401 and other authors from previous studies, using modified Knott test with 2% formalin.
402

***Dirofilaria immitis* mfs**		***Dirofilaria repens* mfs**		**Authors**
Mean length µm	Mean width µm	Mean length µm	Mean width µm	
304.74	6.39	372.20	8.92	Laboratory 1(Parma)
315.31	6.27	365.05	6.75	Laboratory 2 (Napoli)
311.30	5.69	366.20	6.40	Cringoli et al., 2001
311.96		364.53		Panarese et al., 2020 Site 1
294.97		354.93		Panarese et al., 2020 Site 2
316				Evans et al., 2019
295 to 325				AHS, 2020
301.77	6.30	369.44	8.87	Magnis et al., 2013
259.2 ± 28.3		322.7 ± 21.1		Liotta et al., 2013
306.2	5.5			Long et al., 2020
260-340	5-7.5			Taylor et al., 2007

Table

Table 1. Comparison of mean length of *Dirofilaria immitis* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
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						C vs. E=0.0000
						D vs. E=0.0000

Table

Table 2. Comparison of mean width of *Dirofilaria immitis* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. Samples	Mean	Min.	Max.	SD	*P* value (post hoc analysis)
A	28	6.30	5.70	6.60	0.22	A vs. B=0.0000
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Table 3. The comparison of mean length of *Dirofilaria repens* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	**No. Samples**	**Mean**	**Min.**	**Max.**	**SD**	***P* value (post hoc analysis)**
A	31	368.24	347.45	386.97	8.64	A vs. B=0.0000
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						D vs. E=0.0000

Table 4. The comparison of mean width of *Dirofilaria repens* using different solutions for the modified Knott's test: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E).

Solution	No. samples	Mean	Min.	Max.	SD	P-value (post hoc analysis
A	20	7.72	6.40	9.18	1.12	A vs. B=0.0000
B	20	5.96	5.05	6.55	0.33	A vs. C=0.0010
C	21	6.48	5.70	7.85	0.48	A vs. D=0.0000
D	20	5.94	5.29	6.62	0.29	A vs. E=0.0869
E	20	8.04	6.48	9.13	0.98	B vs. C=0.0200
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Table

Table 5. Comparison of mean length and width of *Dirofilaria immitis* and *D. repens* between the results obtained by the authors from the present study (Lab 1, Parma and Lab 2, Napoli) and other authors from previous studies, using modified Knott test with 2% formalin

***Dirofilaria immitis* mfs**		***Dirofilaria repens* mfs**		**Authors**
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Figure

Figure 1. Comparison of modified Knott's tests using formalin and distilled water between two laboratories: Napoli (n= 18) and Parma (n=10). The circles represent the mean of length of *D. immitis* mfs.

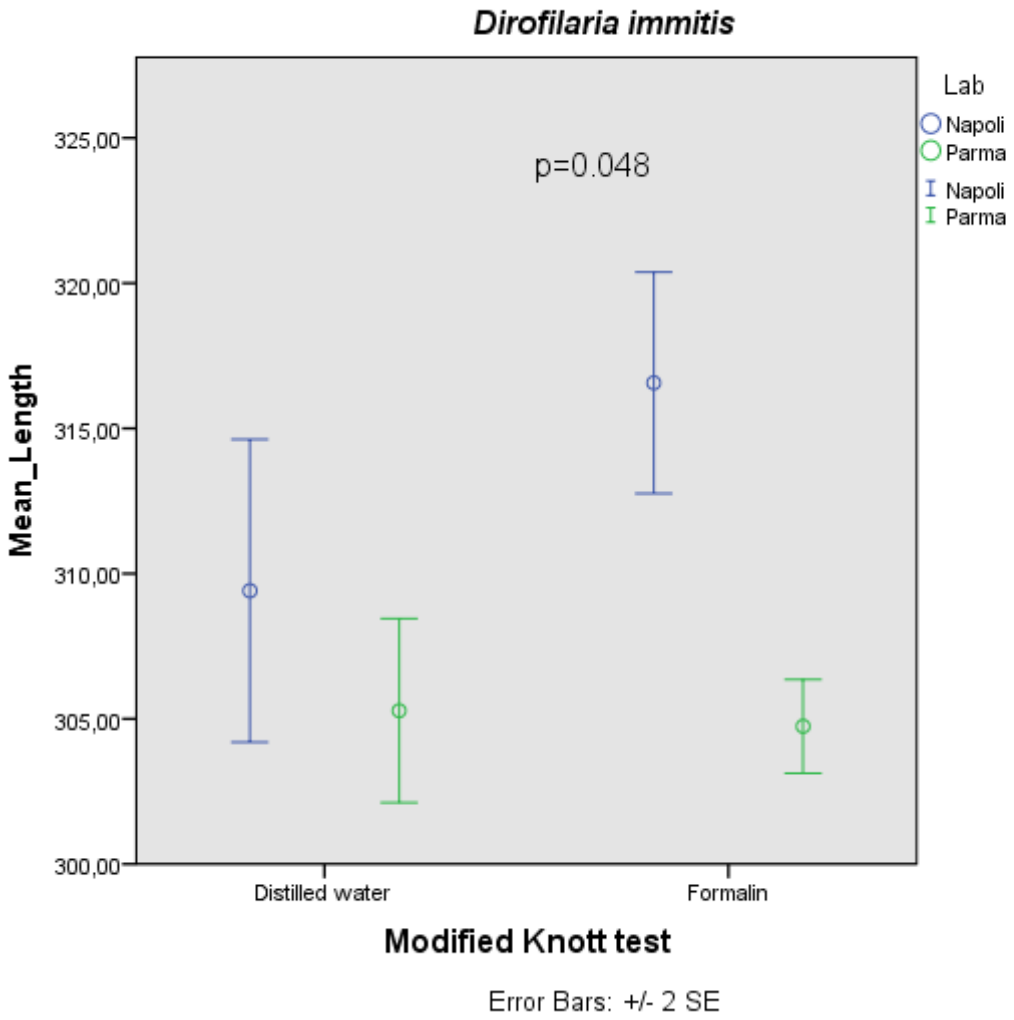

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Figure

Fig 2. Comparison of modified Knott's tests using formalin and distilled water between two laboratories: Napoli (n= 18) and Parma (n=10). The circles represent the mean of width of *D. immitis* mfs.

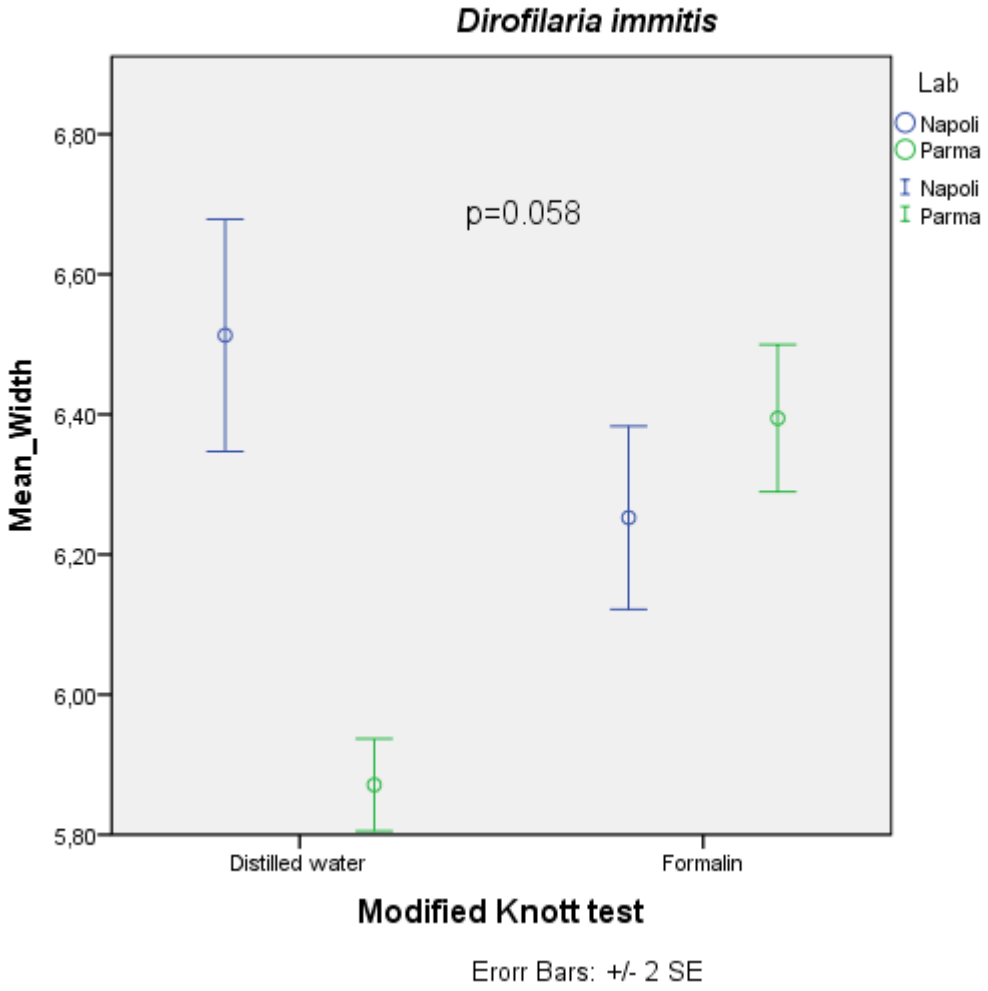

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Figure

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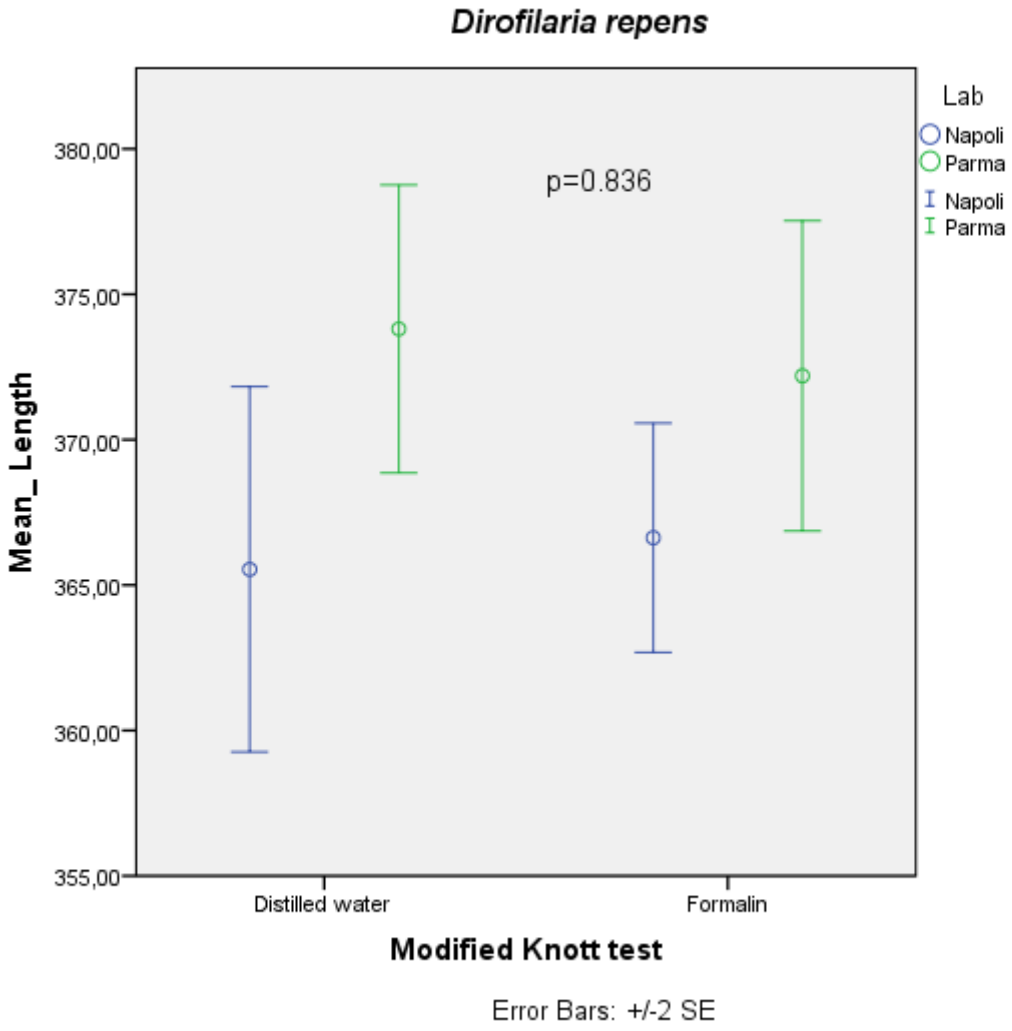
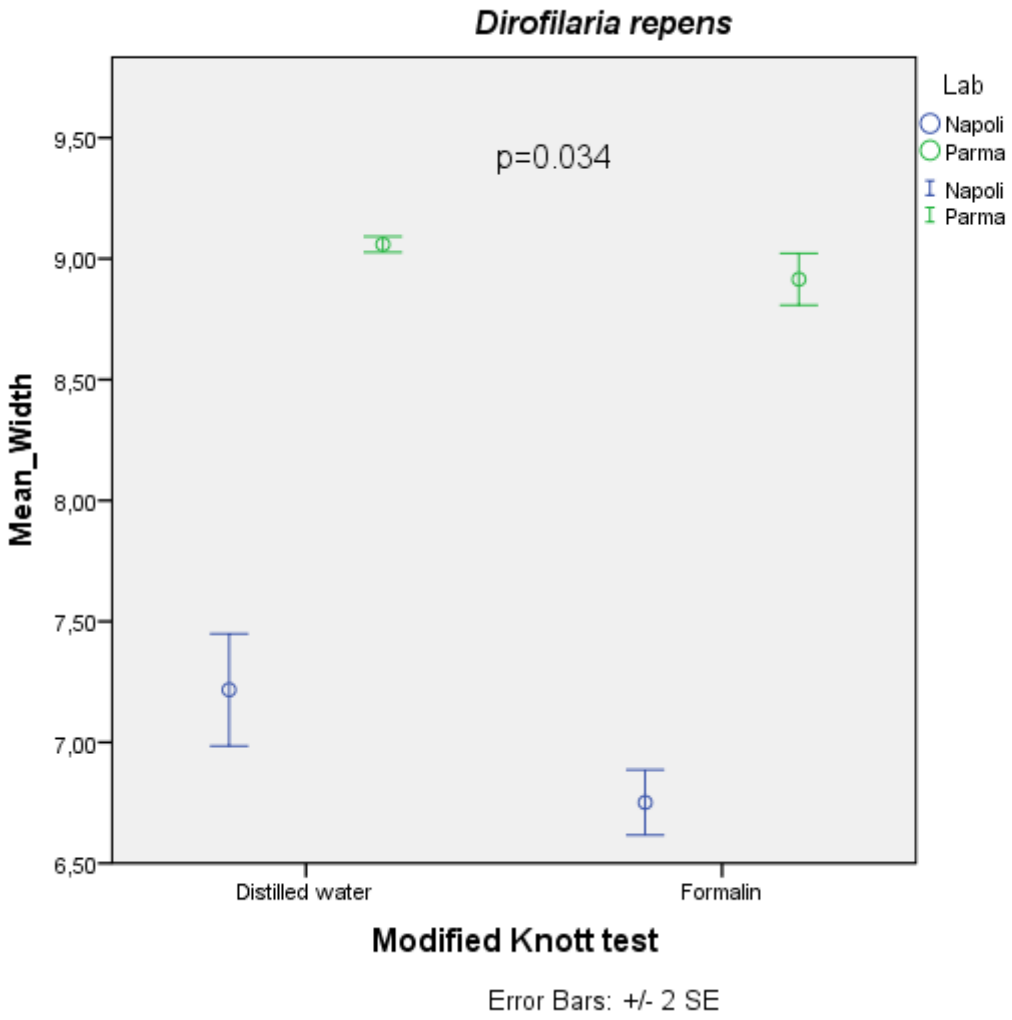


Notes: Modified Knott's test with distilled water-Napoli: mean=365.539; SD=6.437; SE=2.145; Parma: mean=373.808; SD=6.437; SE=2.145; Modified Knott test with formalin-Napoli:mean=366.626; SD=8.890; SE=1.895; Parma-mean=372.195; SD=6.941; SE=2.313.

Figure

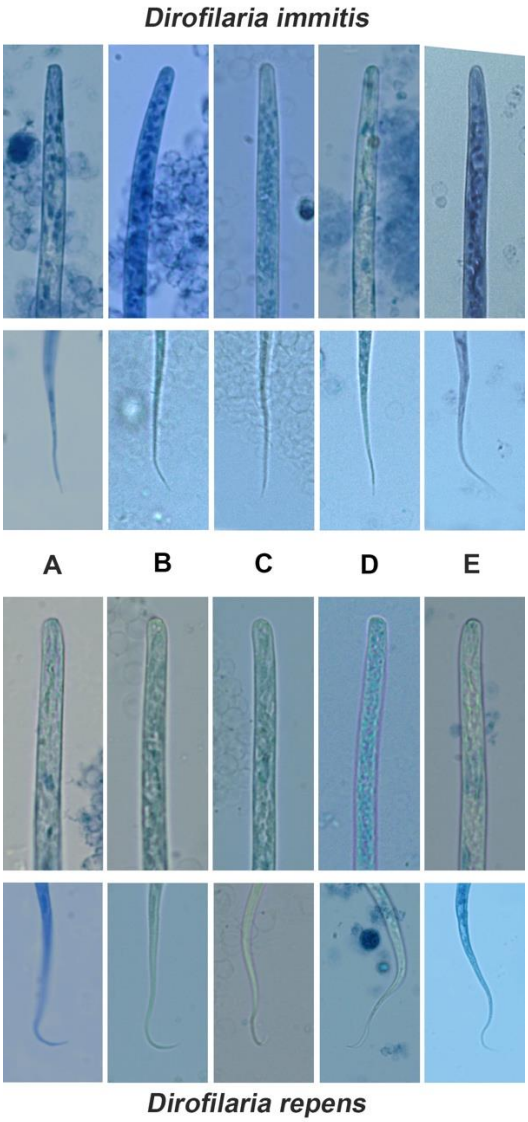
Fig 4. Comparison of modified Knott's tests using formalin and distilled water between two laboratorires: Napoli (n= 24) and Parma (n=9). The circles represent the width of *D. repens* mfs.



Notes: Modified Knott's test with distilled water-Napoli: mean=7.217; SD=0.384; SE=0.116; Parma: mean=9.059; SD=0.048; SE=0.016; Modified Knott's test with formalin-Napoli: mean=6.751; SD=0.224; SE=0.061; Parma-mean=8.915; SD=0.160; SE=0.053.

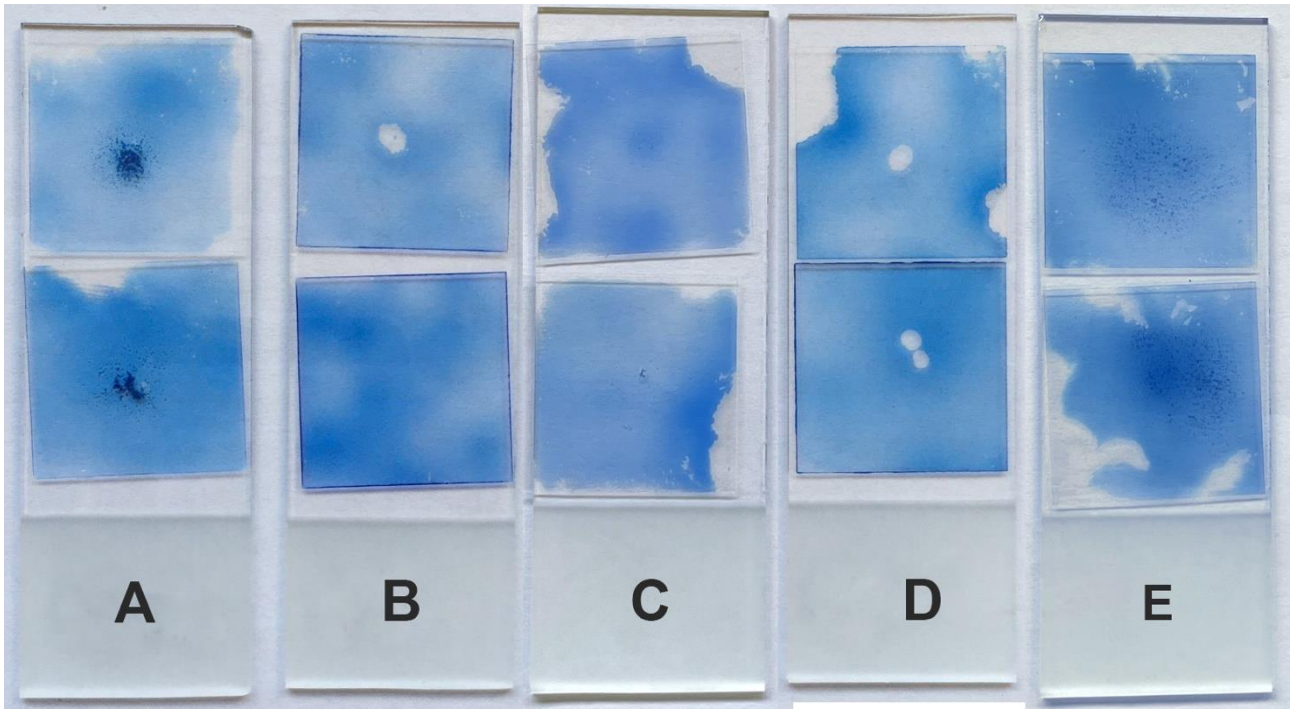
Figure

Figure 5. Morphology of *Dirofilaria immitis* and *D. repens* mfs with the five different reagents: Formalin (A), Acetic acid-vinegar (B), Glacial acetic acid (C), Saponin (D), Distilled water (E), used for modified Knott's test.



Figure

Figure 6. Readability of the slides, using formalin (A), acetic acid-vinegar (B), saponin (C) and distilled water (E) in modified Knott's test.



Declarations of interest: none.

Author Contributions

Marco Genchi: Project administration; Conceptualization; Formal analysis; Investigation; Methodology; Writing - original draft; Writing - review & editing. **Lavinia Ciuca**: Formal analysis; Investigation; Methodology; Software; Writing - original draft. **Alice Vismarra:** Data curation; Formal analysis; Methodology; **Elena Ciccone:** Data curation; Formal analysis; **Giuseppe Cringoli:** Supervision; Writing - review & editing. **Laura Kramer:** Supervision; Writing - review & editing. **Laura Rinaldi:** Conceptualization; Formal analysis; Validation; Writing - original draft; Writing - review & editing.