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RESEARCH

# Prevention of heartworm infection in dogs using a combination of moxidectin, imidacloprid and praziquantel: evidence from a randomized clinical trial

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## Abstract

The aim of this study was to evaluate the efficacy of a topical combination of moxidectin 3.5%, imidacloprid 10% and praziquantel 10% for the prevention of *Dirofilaria immitis* (Leidy, 1856) infection in dogs. For this purpose, a randomized and controlled clinical trial was conducted between August 2021 and October 2022, in the municipality of Goiana, state of Pernambuco, north-eastern Brazil, where heartworm is highly prevalent. Of the 213 dogs initially sampled (baseline), 68 (31.9%) were positive for adult antigens (SNAP 4Dx Plus, Idexx) and/or microfilariae (modified Knott's test). On day 0, 140 negative dogs were randomly included in the treatment and control groups, 70 animals each. During the study, 60 dogs (34 treated and 26 untreated) were removed for different reasons. At the end of the study (day 360 ± 2), 36 treated and 44 untreated were sampled and included in the efficacy calculation. The efficacy against the development of adults and microfilariae was 84.7%, with only one treated dog being positive for adult antigens but negative for microfilariae. On the other hand, eight untreated dogs were positive for adult antigens and/or microfilariae, resulting in a significant difference in the number of positives between groups (Chi-square test = 4.706, $df = 1$, $P = 0.0301$). Remarkably, the efficacy against the appearance of *D. immitis* microfilariae was 100% (i.e., all treated dogs negative) and three untreated dogs were positive for microfilariae. The topical combination of moxidectin 3.5%, imidacloprid 10% and praziquantel 10% significantly reduced the risk of *D. immitis* infection in treated dogs as compared with untreated dogs, in a highly endemic area in north-eastern Brazil.

**Keywords** *Dirofilaria immitis* · Heartworm · Prevention · Control · Moxidectin

## Introduction

The canine heartworm *Dirofilaria immitis* is the most widespread and clinically significant parasite among vector-transmitted helminths that affect dogs. In fact, *D. immitis* has been reported on all continents, being widely distributed in the Americas, occurring from Argentina to Canada (Simón et al. 2012; Maggi and Krämer 2019; Dantas-Torres and Otranto 2020; Dantas-Torres et al. 2023). While present in some temperate countries, *D. immitis* is more prevalent in the tropics and subtropics (Simón et al. 2012; Dantas-Torres et al. 2023). The prevalence and annual incidence may vary widely locally but may surpass 50% in highly endemic foci (Bader et al. 2020; Panarese et al. 2022). In Brazil, the prevalence of *D. immitis* infection in dogs ranges from less than 10% to more than 30% in active

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transmission-areas (Labarthe et al. 2014; Figueredo et al. 2017; Dantas-Torres et al. 2020).

Popularly known as the heartworm, *D. immitis* causes a chronic, potentially fatal cardiopulmonary disease in dogs. In addition, to its veterinary significance, *D. immitis* is a zoonotic helminth and cases of human infections have been sporadically reported worldwide (Otranto et al. 2011; Simón et al. 2012). While most human infections are sub-clinical, some patients may present overt disease (Simón et al. 2012; Saha et al. 2022). Therefore, controlling *D. immitis* infections in dogs, the primary reservoir, is also instrumental to reduce the overall risk of infection in humans.

*Dirofilaria immitis* transmission occurs when an infected female mosquito takes a blood meal on a susceptible host and deposits third-stage larvae (L3), the infective form of the parasite, at the site of the bite. The larvae then actively penetrate the animal's skin and enter the bloodstream. Mosquitoes of several species have already been incriminated as vectors of *D. immitis*, with *Aedes* spp., *Anopheles* spp. and *Culex* spp. being the most common vectors (Ledesma and Harrington 2011; Simón et al. 2012). The moult from L3 to L4 occurs 3–12 days post-infection (p.i.), while the successive moult, which produces the pre-adult (L5), occurs 50–70 days p.i. (Simón et al. 2012). The first adult worms arrive in the pulmonary artery around 70–85 days p.i. and reach sexual maturity around 120 days p.i. Females begin producing L1 (the microfilariae) 6–9 months p.i. Adults' lifespan may exceed 7 years, whereas microfilariae live in the bloodstream for approximately 2 years (Simón et al. 2012). When an uninfected female mosquito takes a blood meal on an infected dog, she ingests the circulating microfilariae which differentiate into L2 (after 8–10 days) to become L3 (the infective form), approximately 3 days later. L3s migrate to the mouthparts of the mosquito, where they reside until the next blood meal (Simón et al. 2012).

The main strategy to prevent canine heartworm infections is the monthly administration of macrocyclic lactones (e.g., ivermectin or moxidectin) to uninfected dogs at risk for infection (Bowman 2012). These drugs can eliminate L3 larvae (inoculated by vector mosquitoes) and L4 larvae (developing larvae), thus preventing the development of adult worms (Bowman 2012). For instance, moxidectin interacts with chloride channels controlled by gamma-aminobutyric acid (GABA) and glutamate. By binding to these channels, moxidectin promotes an increase in the influx of chlorine ions and hyperpolarization of the neuronal membrane, resulting in parasite paralysis and death (Bowman 2012). Besides the use in uninfected dogs, the US Food and Drug Administration (FDA) has also approved a topical formulation of moxidectin plus imidacloprid for treating microfilaria-positive dogs (Bowman and Mannella 2011; McCall et al. 2014; Bowman et al. 2015; Frangipane di Regalbono et al. 2016; Mathur et al. 2023). In this context, the aim of the present study was to evaluate the efficacy of a topical combination containing moxidectin 3.5%, imidacloprid 10% and praziquantel 10% in the prevention of *D. immitis* infection in dogs in Brazil.

## Material and methods

### Study site, design, and population

This study was a pivotal randomised, negative-controlled, GCP-compliant clinical trial conducted between August 2021 and October 2022, in the municipality of Goiana, state of Pernambuco, north-eastern Brazil. This municipality is an area on intense heartworm transmission, as confirmed by previous studies (Figueredo et al. 2017; Dantas-Torres et al. 2020). This field study was conducted in accordance with the World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for studies evaluating the efficacy of parasiticides in reducing the risk of vector-borne pathogen transmission in dogs and cats (Otranto et al. 2021) and the VICH Guideline 9 (EMA 2019).

The study population consisted of 140 privately owned dogs, which resulted negative for *D. immitis* adult antigens and microfilariae at pre-enrolment (day -7/-1). Dogs were randomly assigned to two study groups (70 dogs each): group A (dogs treated with the investigational veterinary product; Table 1) and group B (untreated controls).

The minimum sample required for this trial was calculated considering a confidence level of 95%, power of 80%, as well as the proportion of infected dogs in the treated (0.1–3%) and control (32%) groups (based on data obtained from a study carried out in the same study area; Figueredo et al. 2017). The minimum sample calculated was from 20 (assuming a theoretical efficacy of 99% and expected positivity of 0.1% in the treated group) to 33 (assuming a theoretical efficacy of 90% and expected positivity of 3% in the treated group) dogs per group. Considering potential losses, 70 dogs per group were included.

### Inclusion, exclusion, and post-inclusion removal criteria

Only dogs resulting negative in the modified Knott test for microfilariae and in the rapid test for adult worm antigens were included. Dogs resulting positive, with systemic disease, recently treated with products with known efficacy against L3 and L4 of *D. immitis*, and/or with a history of hypersensitivity to the components of the investigational veterinary product, were excluded. There was no breed or sex restriction, but females in the treatment group had to be non-pregnant.

**Table 1** Investigational veterinary product

| Commercial name | Canis Fullspot (Labyes S.A., Argentina) |
|---|---|
| Drugs | Moxidectin 3.5% + praziquantel 10% + imidacloprid 10% |
| Presentation | Pipette 0.4 ml - dogs ≤ 4 kg<br>Pipette 1.0 ml - dogs 5-10 kg<br>Pipette 2.5 ml - dogs 11-25 kg<br>Pipette 4.0 ml - dogs 26-40 kg<br>Pipette 6.0 ml - dogs 41-60 kg |
| Batch and expiry date | Pipette 0.4 ml: 0406-12 (07/2023) and 0084-11 (07/2022)<br>Pipette 1.0 ml: 0084-22 (07/2022) and 0406-11 (07/2023)<br>Pipette 2.5 ml: 0406-11 (07/2023) and 0084-32 (07/2022)<br>Pipette 4.0 ml: 0084-41 (07/2022)<br>Pipette 6.0 ml: 0558 (11/2023) and 0084-51 (07/2022) |

Post-inclusion removal criteria included hypersensitivity reaction to formulation components or use of products with known efficacy against *D. immitis* L3 and L4. Dogs that tested positive for microfilariae or adult antigens on day 120 ± 2 (4 months) were also removed, as the possibility of prior exposure (i.e., prior to study inclusion) could not be ruled out (Jacobs et al. 1994; Carmichael et al. 2017; Otranto et al. 2021). Finally, dogs from group A that were not treated on the scheduled days according to Table 2 were removed.

## Dog keeping and diagnostic procedures

Dogs included in this study remained with their owners in their respective homes. Owners were instructed to communicate to the veterinarian and/or principal investigator any need or intention to apply concomitant treatments. Dogs were also observed daily by the owners regarding their general health condition, and any adverse events documented in appropriate forms.

Dogs from both groups were clinically examined and weighed at days 0, 120 ± 2, 180 ± 2 and 360 ± 2 (Table 2). Blood samples were collected from dogs at pre-enrolment and during three subsequent visits (days 120 ± 2, 180 ± 2 and 360 ± 2), as described in Table 2. After anamnesis, the animals were physically restrained by the owner and 5 ml blood samples were collected from the cephalic, femoral, or jugular vein, as needed. In the laboratory, the EDTA-blood samples were tested for the presence of microfilariae using the Knott test (Newton and Wright 1956) and for *D. immitis* adult antigens using SNAP 4Dx Plus (Idexx Laboratories). This test also detects antibodies against *Anaplasma* spp., *Ehrlichia* spp. and *Borrelia burgdorferi*.

## Dog treatment

The dogs in group A were weighed and treated monthly with the investigational veterinary product according to the

**Table 2** Study design and procedures

| Group | Number | Preinclusion | Inclusion | Follow up | |
|---|---|---|---|---|---|
| | | | | Weighing and treatment | Physical examination and sampling |
| A (treatment) | 70 | D-7/-1: Registration, anamnesis, and sample collection | D0 ± 2: Physical examination and weighing | D0 ± 2 (TI)<br>D30 ± 2 (TII)<br>D60 ± 2 (TIII)<br>D90 ± 2 (TIV)<br>D120 ± 2 (TV)<br>D150 ± 2 (TVI)<br>D180 ± 2 (TVII)<br>D210 ± 2 (TVIII)<br>D240 ± 2 (TIX)<br>D270 ± 2 (TX)<br>D300 ± 2 (TXI)<br>D330 ± 2 (TXII)<br>D360 ± 2 (TXIII) | D120 ± 2<br>D180 ± 2<br>D360 ± 2 |
| B (control) | 70 | | | D360 ± 2 [a] | |

[a] At the end of the study (D360 ± 2), dogs in group B received 6 months of treatment with Canis Fullspot (Labyes). Abbreviators: *D* = day; *T* = treatment

manufacturer's recommendations (Table 1), with a corresponding dose of 3.5–7.0 mg/kg. The first application was made on the day the animal was included in the study (day 0), repeated monthly until the end of the study (day 360 ± 2) (Table 2). The animals in group B were not treated. At the end of the study, group B dogs received 6 months of treatment with the investigational veterinary product (Table 2).

### Efficacy calculation

Two parameters were used to verify the superiority of the investigational veterinary product compared to the absence of treatment: percent protection and efficacy. The percentage of protection was defined as the percentage of negative dogs in group A (treatment). The efficacy of the investigational veterinary product was calculated using the following formula (Otranto et al. 2021):

$$Efficacy\ (\%) = \frac{\%\ positive\ dogs\ in\ the\ control\ group - \%\ positive\ dogs\ in\ the\ treatment\ group}{\%\ positive\ dogs\ in\ the\ control\ group} \times 100$$

Both percent protection and efficacy were calculated at day 360 ± 2 for adults and microfilariae of *D. immitis*. For adults, a dog was considered positive when positive in the Knott test, SNAP 4Dx, or both. For microfilariae, a dog was considered positive when positive in the Knott test.

### Statistical analysis

To assess the homogeneity between the groups, the initial weight and age of the animals included in the treatment and control groups were compared using the Mann-Whitney U test. The percentages of male and female dogs in each group were compared using the chi-square test. The percentages of animals positive for microfilariae and adult worm antigens in the treatment and control groups were also compared using the chi-square test or Fisher's exact test. Data normality (initial weight and age) was assessed using the Lilliefors test. Differences were considered statistically significant when the *P* value was less than or equal to 0.05. The tests were carried out using BioEstat program, version 5.3 (Instituto de Desenvolvimento Sustentável Mamirauá).

## Results

During pre-inclusion, 213 dogs were sampled, of which 68 were positive in the antigen test (SNAP 4Dx, Idexx) and/or in the microfilariae test (Knott's test). This corresponds to a baseline prevalence of 31.9% (95% CI: 25.7–38.6%).

On day 0, 140 negative dogs were included, 70 in the treatment group (39 males and 31 females) and 70 in the untreated control group (38 males and 32 females); the five remaining negative dogs were not included because we reached the intended sample size. Mean ages in the treatment and control groups were 17.8 (range, 2–132 months) and 22.9 months (range, 2–108 months), respectively. The average weight in the treatment and control groups was 10.3 (range, 1.6–38.8 kg) and 11.2 kg (range, 2.2–41.4 kg), respectively. There was no significant difference between groups regarding gender (Chi-square test = 0.029, $df = 1$, $P = 0.87$) or weight (Mann-Whitney U test, Z = 0.99, $P = 0.32$), but the median age varied slightly (Mann-Whitney U Test, Z = 1.98, $P = 0.05$), being 8.5 and 12 months in the treatment and control groups, respectively.

On day 120 ± 2, four dogs (one treated and three untreated) were positive and removed from the study, as per protocol (other reasons for post-inclusion removals are described in Table 3). On day 180 ± 2, 10 dogs were positive (two treated and eight untreated) (Chi-square test = 4.129, $df = 1$, $P = 0.0422$).

During the whole study, a total of 60 dogs were removed (percentage loss of 42.9%), 34 from the treatment group and 26 from the control group. Hence, 36 treated dogs and 44 untreated ones were included in the efficacy calculation on day 360 ± 2. The efficacy of the investigational product for preventing the development of *D. immitis* adults was 84.7% (Table 4). Only one dog in the treatment group was positive adult antigen on day 360 ± 2, the same being negative for microfilariae. On the other hand, eight untreated dogs were positive for adult antigen, microfilariae, or both. There was a statistically significant difference in the number of positive dogs in the treatment and control groups (Chi-square test = 4.706, $df = 1$, $P = 0.0301$).

The efficacy of the investigational product for preventing appearance of microfilariae at day 360 ± 2 was 100% (Table 5). No treated dog was positive for microfilariae, whereas three untreated dogs were positive.

## Discussion

The topical combination of moxidectin 3.5%, imidacloprid 10% and praziquantel 10% was highly efficacious in reducing the risk of *D. immitis* infection in treated dogs as compared with untreated ones. At the end of the observational period, only one treated dog was positive for *D. immitis* adult antigens, the same being negative for microfilariae. In contrast,

**Table 3** Post-inclusion removal

| Code | Group | Reason for removal |
|---|---|---|
| GO-003 | Treatment | Pregnancy |
| GO-017 | Control | Death |
| GO-018 | Treatment | Fractious animal |
| GO-023 | Treatment | Fractious animal |
| GO-026 | Treatment | Address change |
| GO-027 | Control | Owner decision |
| GO-029 | Treatment | Address change |
| GO-030 | Control | Concomitant treatment |
| GO-031 | Control | Death |
| GO-032 | Treatment | Pregnancy |
| GO-047 | Control | Death |
| GO-050 | Treatment | Address change |
| GO-056 | Treatment | Owner decision |
| GO-057 | Treatment | Owner decision |
| GO-062 | Treatment | Pregnancy |
| GO-065 | Control | Address change |
| GO-066 | Control | Address change |
| GO-067 | Treatment | Owner decision |
| GO-068 | Treatment | Owner decision |
| GO-070 | Control | Positive for *Dirofilaria* at D120 ± 2 |
| GO-071 | Control | Owner decision |
| GO-072 | Treatment | Owner decision |
| GO-075 | Control | Death |
| GO-080 | Treatment | Death |
| GO-081 | Control | Death |
| GO-082 | Control | Positive for *Dirofilaria* at D120 ± 2 |
| GO-083 | Treatment | Address change |
| GO-086 | Treatment | Address change |
| GO-090 | Control | Address change |
| GO-102 | Treatment | Death |
| GO-109 | Control | Death |
| GO-115 | Treatment | Positive for *Dirofilaria* at D120 ± 2 |
| GO-119 | Treatment | Death |
| GO-120 | Control | Address change |
| GO-123 | Treatment | Pregnancy |
| GO-131 | Control | Death |
| GO-132 | Treatment | Address change |
| GO-133 | Control | Address change |
| GO-136 | Control | Address change |
| GO-151 | Treatment | Pregnancy |
| GO-153 | Control | Fractious animal |
| GO-155 | Control | Death |
| GO-156 | Treatment | Death |
| GO-161 | Treatment | Pregnancy |
| GO-162 | Control | Death |
| GO-167 | Control | Death |
| GO-171 | Treatment | Death |
| GO-179 | Treatment | Death |
| GO-180 | Treatment | Death |
| GO-181 | Treatment | Death |
| GO-182 | Control | Death |
| GO-184 | Control | Address change |
| GO-186 | Control | Positive for *Dirofilaria* at D120 ± 2 |
| GO-189 | Treatment | Owner decision |
| GO-190 | Treatment | Address change |
| GO-191 | Treatment | Death |
| GO-193 | Treatment | Death |
| GO-194 | Control | Disappearance |
| GO-203 | Treatment | Fractious animal |
| GO-210 | Treatment | Death |

**Table 4** Efficacy and protection in preventing the development of heartworm adults [a]

| Day 360 ± 2 | Positive | Total (*n*) | Positivity (%) | Efficacy (%) | Protection (%) |
|---|---|---|---|---|---|
| Treatment | 1 | 36 | 2.8 | 84.7 | 97.2 |
| Control | 8 | 44 | 18.2 | - | - |

[a]For this calculation, the results of the test for antigens (SNAP 4Dx, Idexx) and for microfilariae (Knott test) were considered, considering that both confirm the presence of adults (i.e., for microfilariae to be present, adults must be present)

**Table 5** Efficacy and protection in preventing the circulation of microfilariae [a]

| Day 360 ± 2 | Positive | Total (*n*) | Positivity (%) | Efficacy (%) | Protection (%) |
|---|---|---|---|---|---|
| Treatment | 0 | 36 | 0.0 | 100.0 | 100.0 |
| Control | 3 | 44 | 6.8 | - | - |

[a]For this calculation, only the results of the test for microfilariae (Knott test) are considered

eight untreated dogs were positive for *D. immitis* adult antigens, three of which were also positive for microfilariae. These results confirm the efficacy of this moxidectin-containing product in reducing the risk of *D. immitis* infection in dogs from a coastal area in north-eastern Brazil, where this parasite is historically highly prevalent (Labarthe et al. 2003, 2014; Figueredo et al. 2017). The baseline prevalence of *D. immitis* infection detected in this study (i.e., 31.9%) is in line with that reported previous studies conducted in the very same area. Indeed, based on adult antigen detection only, a cross-section study conducted during early 2015 reported a prevalence of 32% (Figueredo et al. 2017). A subsequent study conducted from late 2015 to early 2016 reported an even higher prevalence (39.8%), again, based on adult antigen detection only (Dantas-Torres et al. 2020). Altogether, these studies confirm the study site as a hotspot of intense

heartworm transmission in Brazil, making it an interesting location for conducting clinical trials on the prevention of *D. immitis* as well as other vector-borne pathogens [e.g., *Ehrlichia canis*, *Babesia vogeli*, and *Leishmania infantum*], which are highly prevalence in the same geographical area (Dantas-Torres et al. 2020).

Moxidectin is highly efficacious against *D. immitis* and is available in veterinary products for oral, topical, and injectable administration (Myers et al. 2022; Savadelis et al. 2022; Mitchell et al. 2023). The efficacy of moxidectin-containing depends on the several factors, including formulation, dosages, and treatment times post-experimental inoculation (Savadelis et al. 2022). For instance, efficacy of oral moxidectin against macrocyclic lactone-susceptible *D. immitis* ranged from 47.8% to 100%, depending on dosage and treatment time post-experimental inoculation (Savadelis et al. 2022). The efficacy of oral moxidectin against macrocyclic lactone-resistant strains (JYD-34, ZoeLA, and ZoeMO) was much lower (19–53.2%) and required extremely higher dosages monthly for three consecutive months to reach higher efficacies (98.9% for 24 µg/kg and 100% for 40 or 60 µg/kg). Injectable and topical products containing moxidectin also presented high efficacy against macrocyclic lactone-resistant strains of *D. immitis* (Kryda et al. 2020; McTier et al. 2021).

While pivotal, laboratory studies cannot always mimic the field conditions. Laboratory studies have well defined inoculation days and treatment times post-experimental inoculation (Savadelis et al. 2022). So, dogs are exposed to the parasites under defined conditions in terms of number of L3 inoculated and timing of L3 inoculation. Under field conditions, dogs can be infected multiple times, with variable number of L3, and at any timepoint during the study. Therefore, field studies are fundamental to assess the efficacy of a given product under natural transmission conditions, particularly in highly endemic foci. Another advantage of field studies is that they do not require the euthanasia of dogs at the end of the study, which should also be considered from an ethical perspective.

This is the first randomized, negative-controlled clinical trial, conducted according to the good clinical practice (GCP) guidelines, to assess the efficacy of a moxidectin-containing topical product against *D. immitis* under field conditions in Brazil. A previous study conducted in Rio de Janeiro assessed the efficacy of a topical product containing moxidectin (2.5–6.25 mg moxidectin/kg) and imidacloprid, as compared with topical selamectin (6–12 mg/kg) (Labarthe et al. 2015). None of 14 dogs from the moxidectin + imidacloprid treated group presented *D. immitis* adult antigen or microfilariae, whereas four out of 15 selamectin-treated dogs were infected at the end of the study (day 300 ± 5) (Labarthe et al. 2015). Altogether, these studies confirm the efficacy of topical products containing moxidectin to reduce the risk of *D. immitis* infection in dogs in transmission hotspots in Brazil.

Besides its preventive efficacy, different formulations of moxidectin have also been successfully used in combination with doxycycline as slow kill therapy for treating existing *D. immitis* infections in dogs (Jacobson and DiGangi 2021; Alberigi et al. 2020; Dantas-Torres et al. 2023). For example, the administration of topical imidacloprid/moxidectin monthly coupled with oral doxycycline (10 mg/kg, BID, for 4 weeks) showed to be an efficacious treatment option in remote areas where animals cannot be monitored after adulticide treatments (Brianti et al. 2023). Further research is needed to assess the efficacy of the above combination (i.e., topical formulation of moxidectin plus doxycycline), as a slow kill therapy for treating dogs in Brazil and other Latin American countries, where the first line adulticide (melarsomine) is unavailable (Dantas-Torres et al. 2023).

According to the results of clinical trial, the investigational veterinary product provided significant protection against *D. immitis* in treated dogs, when compared to untreated dogs. The high efficacy (100%) against microfilariae stands out, which denotes the usefulness of the investigational product in controlling the transmission of *D. immitis*, considering that the presence of circulating microfilariae is a sine qua non condition for transmission to occur through the bite of mosquito vectors. Therefore, the veterinary product investigated herein represents an important tool for reducing the risk of transmission of *D. immitis* among dogs and possibly to humans, helping to control this zoonotic parasite in Brazil and other endemic areas.

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**Author contribution** Study design and coordination: FDT. Data acquisition: LAF, KGSS, RLNL, LCSP, LGS and LLNB. Data analysis: FDT, DO. Manuscript writing: FDT. Manuscript revision: LAF, KGSS, RLNL, LCSP, LGS, LLNB and DO. All authors read and approved the final manuscript.

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**Data availability** All data supporting the conclusion of this study are included in the manuscript.

## Declarations

**Ethics approval** This project was approved by the Animal Use Ethics Committee (CEUA) of the Aggeu Magalhães Institute (Fiocruz-PE), under protocol number 154/2019.

**Consent to participate** Dog owners signed an informed consent form allowing the participation of their dogs in the study.

**Consent for publication** Not applicable.

**Competing interests** This project was sponsored by Labyes. We declare the sponsor did not play any role in study design, conduction, data acquisition, interpretation, and manuscript preparation.

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