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Efficacy of afoxolaner or the combination of afoxolaner with milbemycin oxime against *Otodectes cynotis* in naturally infested dogs

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ABSTRACT

Otodectes cynotis, commonly known as "the ear mite," is a highly contagious ectoparasite and a significant cause of otitis externa in canines. The objective of the current study was to determine the efficacy of the isoxazoline afoxolaner (Nexgard®), and the combination of afoxolaner with milbemycin oxime (Nexgard Spectra®), in dogs naturally infested with *O. cynotis*. In total, 32 infested client-owned dogs from two different sites in Greece were included. The animals were randomly divided into four equal groups based on their infestation score. Group 1 served as the negative control, group 2 received one oral administration of Nexgard (Day 0), group 3 received two monthly oral administrations of Nexgard (Days 0, 30), and group 4 received two monthly oral administrations of Nexgard Spectra (Days 0, 30), according to label instructions. Otoscopic examinations for mites and observations on debris/cerumen in the ears were carried out on Days 0, 15, 30, and 45. A quantitative assessment of ear mites by ear duct flushing and live mite counts was performed on Day 45. The results demonstrated that a single oral dose of afoxolaner and two monthly doses of afoxolaner or afoxolaner with milbemycin oxime resulted in a 99.9% reduction in live mite counts compared to the untreated control group by Day 45. Additionally, treated dogs showed improved clinical symptoms, such as ear cerumen/debris decrease, while untreated dogs experienced worsening symptoms over the study duration. No adverse events were reported. Overall, these results support the use of afoxolaner-based products to treat *O. cynotis* infestation in dogs.

1. Introduction

Otodectes cynotis (Order: Acari, Sub-Order: Astigmata, Family: Psoroptidae), commonly known as "the ear mite," is an ubiquitous, non-burrowing ectoparasite and one of the leading causative agents of otitis externa in canines (Deplazes et al., 2016). It has low host-specificity and can infest different animal species such as cats, dogs, ferrets, foxes, and other wild carnivores (Deplazes et al., 2016). These mites live on the surface of the outer and inner ear canal, while sometimes they can be located on other body parts (e.g., neck, face, thorax, limbs, or tail), feeding on exudate, epithelial cells and occasionally body fluids such as blood and lymph (Deplazes et al., 2016; Powell et al., 1980). The parasite's life cycle can be completed in approximately 21 days and consists of 5 stages (eggs, larvae, protonymphs, deutonymphs, and adults), all living on the host (Deplazes et al., 2016). Adults can survive 60 days, and females adhere their eggs inside the dog's ear canal (Deplazes et al., 2016). Outside the host, the mites can live for a few days but are susceptible to different environmental conditions, such as desiccation (Deplazes et al., 2016).

Transmission occurs quickly with direct contact between animals, and puppies can get infested during suckling (Deplazes et al., 2016). Young animals are also more susceptible to the disease, while adult dogs can develop immunity (Deplazes et al., 2016; Thomson et al., 2023). Another transmission route is mechanically by using the same grooming equipment and instruments between animals (Lefkaditis et al., 2021). Cats are considered the parasite's main reservoir host in domestic environments (Thomson et al., 2023) and foxes in wild environments (Deplazes et al., 2016).

Afoxolaner (Nexgard®, Boehringer Ingelheim Animal Health, Toulouse, France) and the combination of afoxolaner + milbemycin

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(Nexgard Spectra®, Boehringer Ingelheim Animal Health, Toulouse, France) are two commercially available products in the antiparasitic category of isoxazolines (Selzer and Epe, 2021). Afoxolaner is licensed in the European Union for use against fleas, ticks, *Demodex canis,* and *Sarcoptes scabiei* in dogs after oral administration (Zhou et al., 2022). The objective of the current study was to determine the efficacy of Nexgard® and Nexgard Spectra® against naturally acquired *O. cynotis* infestations in dogs under semi-field conditions, following one administration (NexGard®) or two monthly administrations (NexGard® and NexGard Spectra®).

2. Materials and methods

2.1. Ethical standards and approval

This study was conducted with the approval of the Ethics Committee of the Aristotle University of Thessaloniki (188132/2022) and following the VICH GL9 (June 2000) guideline on Good Clinical Practice (The European Agency for the Evaluation of Medicinal Products, 2000). The experiments were also carried out in conformity with nationally applicable regulations and guidelines, and the dogs were handled with care and consideration for their welfare. Before participating in the research, dog owners consented to all procedures and completed an informed consent form. Dogs were kept in individual enclosures throughout the study, providing environmental enrichment and aural and visual interaction among them, but not direct contact. The kennels were considered adequate according to the EU Directive 2010–63 specifying kennel dimensions.

2.2. Study design and animal inclusion

The study was conducted from August to October 2022. It was an efficacy study with negative controls, employing a parallel-group design with blinding, randomization, and a single-center approach. The experimental unit was the individual dog. In total, 32 client-owned dogs were included, selected from two sites in Greece (east and west areas of the country), 16 from site 1 in Kavala (40°57'49" N, 24°27'35" E), and 16 from site 2 in Ioannina (39°42'48" N, 20°49'37" E). The 32 dogs belonged to different breeds (mostly mixed-breed) and were between 1 and 9 years old at the time of inclusion. Four males and 28 females were included (females were neutered before the study start to ensure they were not lactating or pregnant). There was no restriction on body weight; the dogs weighed between 14.1 kg and 30.1 kg. None of the dogs had received an ectoparasite treatment at least three months prior to the start of the study. The two study kennels were property of the respective dog owners and eeach dog was individually housed in a numbered enclosure and displayed a good temperament deemed suitable for the study. Water was offered in individual bowls refilled ad libitum, and commercial standard dry food was given in individual plates once daily according to the dogs' body requirements. Considering Day 0 (D0) as the day when the dogs received their first treatment, the dogs were screened by an initial otoscopic examination on Day −8 (D-8). All 32 dogs displayed high naturally acquired *O. cynotis* infestations in both ears and were assessed for inflammation, pruritus scores, and live mite counts (in a semi-quantitative manner as described in Section 2.4 below) as ranking criteria. The dogs were then acclimatised to the study's environment for eight days before the treatment day. Clinical examinations were performed by the same veterinarian on all dogs on D-8 and D0 for enrolment and inclusion purposes, while general health observations were carried out daily. After physical examination at D0 for the study initiation, all dogs were healthy except for the *O. cynotis* infestations. The dogs were weighed on a calibrated and verified electronic scale on Days −8, 0, 30 (D30), and 45 (D45). The 32 dogs were randomly allocated into four groups of eight dogs each based on mite infestation scores to ensure similar infestation levels across all groups, and every group consisted of four dogs from each site. The groups were the following:

- Group 1: Dogs (n = 8) remained untreated (negative control),
- Group 2:Dogs (n = 8) were treated with Nexgard as a single oral dose on Day 0,
- Group 3:Dogs (n = 8) were treated with Nexgard as an oral dose once on Days 0 and 30,
- Group 4:Dogs (n = 8) were treated with Nexgard Spectra as an oral dose once on Days 0 and 30.

All dogs remained the property of their respective owners at the end of the study, and the owners' veterinarians were responsible for treatment of the dogs.

2.3. Treatment administration

On D0, dogs in groups 2, 3, and 4 were treated with their respective investigational veterinary products (IVPs), and on D30, dogs in groups 3 and 4 were treated with the second dose of their respective IVP. Dogs in group 1 remained untreated. Administration of the afoxolaner formulation was the responsibility of non-blinded personnel. All other people involved in the study were blinded to the group allocation. Individual doses on D0 were determined based on each animal's weight on D0 and doses on D30 using each animal's weight on D30. For afoxolaner, combinations of whole chewable tablets were provided to ensure a dose rate as close as possible to the dose rate of 2.7 mg/kg, the minimum label dose. Similarly, the combination of afoxolaner with milbemycin oxime tablets was provided to ensure a dose rate as close as possible to 2.5 mg/kg afoxolaner and 0.5 mg/kg milbemycin oxime, the minimum label dose.

The chew(s) was (were) administered orally, and treated animals were observed for a few minutes to ensure that part of the dose was not lost or refused. Specific health observations were conducted prior to each oral administration and at 1 h (h) (± 15 min), 3 h (± 15 min), 6 h (± 15 min), and 24 h (± 30 min) after. Protective clothing and disposable gloves were changed between groups for all study procedures where animals were handled, such as otoscopic assessments or body weight measurements, to avoid cross-contamination.

2.4. Ear flushing and assessment of ear mite counts

Otoscopic assessments were conducted on D0, D15, D30, and D45. These assessments were semi-quantitative, and the presence or absence of visible live ear mites was recorded according to the parameters below: Negative (0 live mites); low infestation (1 - 4 live mites); medium infestation (5 - 10 live mites) and high infestation (> 10 live mites). During the otoscopic examinations, observations were made on the absence or presence of debris/cerumen (slight, moderate and severe) in the ears.

At the end of the study (D45), after otoscopic examination, a quantitative assessment of ear mites by ear duct flushing, mite collection, and live mite count was performed. In order to facilitate the removal of the content, the ear ducts were filled with paraffin oil and massaged lightly externally until sufficient dissolution of the ear duct contents had occurred. The paraffin oil was then removed with a pipette and collected into a suitably labeled container. After completion of the flushing procedure, both ears were otoscopically examined, and if persistent cerumen deposits and/or mites were observed, the flushing procedure was repeated until both ear ducts were judged clean.

All collected materials were transported to the Laboratory of Parasitology and Parasitic Diseases, School of Veterinary Medicine, Aristotle University of Thessaloniki, Greece, for further examination. The contents from each container were transferred to a Petri dish for counting under a stereo microscope at a magnification of 120x (Olympus, Research Stereomicroscope System SZH10). All living mites (larvae, nymphs, and adults) were identified based on morphological keys (Sweatman, 1958) and counted, and each dog's total ear mite count was calculated by adding the mites from its two ears. For viability, a

correlation between the otoscopic examination performed before the ear duct flushing procedure and the ear mite count was made. If signs of viability (live mites) were seen at the otoscopic examination, entire and normal-looking ear mites (excluding desiccated or fragmented mites) observed under the microscope were recorded as live. In order to avoid bias or unmasking, otoscopy and microscopy were carried out by experienced, blinded personnel in a random order.

2.5. Efficacy assessment and statistical analyses

SAS Version 9.4 was used for all the statistical analyses, the level of significance of the formal tests was set at 5%, and all tests were two-sided. The statistical unit was the individual dog. The primary efficacy criterion was the number of live mites collected from the treated groups on D45 compared to that of the negative control group. To calculate the efficacies, the total number of live mite counts were used, considering both ears (right and left) and including adults, nymphs, and larvae. These counts were assessed during ear flushing on D45. Because minor and even zero mite counts could be recorded, it could be expected that the mite counts did not follow a normal distribution. Therefore, percentage reduction calculations were based on geometric means of the mite (count + 1) data rather than arithmetic means. One (1) was subsequently subtracted from the result to obtain a meaningful value for the geometric mean of each group. The log counts (count + 1) of live mites of the treated groups (groups 2, 3, and 4) were compared to the log counts (count + 1) of the control group (group 1), using a mixed analysis of variance model to confirm the acaricidal efficacy results. Fixed factors in the model were the group (1 and 2) and sex (male and female). The block to which each animal was assigned for randomisation was included as random effect. As the mite counts did not follow a normal distribution, the Wilcoxon Mann-Whitney test was also used for group comparisons instead. Furthermore, efficacy calculations based on arithmetic means were also reported. The percentage efficacy for the IVP groups against mites was calculated as follows:

Efficacy (%) against mites = 100 x (Mc – Mt) / Mc, where:
Mc = Geometric or arithmetic mean number of live mites in dogs in the negative control group (group 1).
Mt = Geometric or arithmetic mean number of live mites in dogs in the IVP groups (group 2, 3, or 4).

3. Results

3.1. Otoscopic assessment

All dogs in groups 1 (negative control), 2 (Nexgard as a single oral dose on D0), 3 (Nexgard as an oral dose once on D0 and D30), and 4 (Nexgard Spectra as an oral dose once on D0 and D30) had visible mite infestations in both ears (low to high infestation) and debris/cerumen (slight to severe) at the start of the study (D-8 and D0).

Dogs in group 1 remained infested in both ears and with debris/cerumen for the duration of the study (D-8 until D45). All dogs in the treated groups 2, 3, and 4 improved throughout the study regarding mite counts and the presence of debris/cerumen. No visible mites were recorded for dogs in group 2 by D45, except for one dog, with a low infestation in the left ear (D45). In contrast, all dogs from groups 3 and 4 had no visible mites by D45.

3.2. Mite counts and efficacy data

No product losses occurred, no signs of emesis in any of the dogs were observed, and all animals received the full dose. Moreover, no adverse events occurred during the study. The geometric mean (minimum and maximum) mite counts at the end of the study (D45) for groups 1, 2, 3, and 4 were 144.4 (72.0–225.0), 0.1 (0.0–2.0), 0.1 (0.0–1.0), and 0.2 (0.0–1.0), respectively. Based on geometric means, all treatments in groups 2, 3, and 4 resulted in 99.9% efficacy (Table 1). All IVPs thus showed adequate efficacy ($\geq$ 90%), and the differences in terms of total live *O. cynotis* mite counts at the end of the study (D45) were statistically significant compared to the negative control group 1. Specifically, using the Wilcoxon-Mann-Whitney test, the p-valuesbetween the negative control group (Group 1) and the treated groups (Groups 2, 3, and 4) were highly significant: $p = 0.0006$ for Group 1 vs. Group 2, $p = 0.0006$ for Group 1 vs. Group 3, and $p = 0.0007$ for Group 1 vs. Group 4. According to arithmetic means, group 3 showed the highest efficacy with 99.9% compared to 99.8% for groups 2 and 4 (Table 1).

Regarding the presence of debris/cerumen inside the ears, all dogs in the treated groups (2, 3, and 4) had progressively lower debris/cerumen scores from D0 until D45 and had a comparatively better clinical picture than the untreated dogs in group 1 (Fig. 1).

4. Discussion

The current study demonstrated the efficacy of the isoxazoline afoxolaner and the combination of afoxolaner with milbemycin oxime against *O. cynotis* in naturally infested dogs. Afoxolaner at a dose rate of 2.7 mg/kg administered orally once (D0) or twice monthly (D0 and D30) and the combination of afoxolaner with milbemycin oxime at a respective dose rate of 2.5 mg/kg and 0.5 mg/kg after two monthly oral administrations (D0 and D30), all reduced the number of ear mites by 99.9% compared to negative controls by D45. The high efficacy in reducing mite counts in the treated groups were accompanied by decreased otitis externa symptoms like ear cerumen/debris, while clinical signs in dogs from the negative control group progressively worsened. Similarly, treating *O. cynotis* infestations in dogs has also been correlated with alleviating or resolving otocariosis symptoms in other studies (Panarese et al., 2021).

Intra-auricular ear mange treatments can prove ineffective in cases of ectopic infestations, where some mites live outside the ear canal (Deplazes et al., 2016), and thus, systemic treatments provide a better solution (Deplazes et al., 2016). Additionally, formulations administered directly in the ear canal typically require repeated applications due to their short residual activity and because they may not be effective against eggs, larvae, or nymphs, leading to reinfestations. Consequently, it is recommended to implement efficient, year-round parasite control programs as advised by the European Scientific Council of Companion Animal Parasites (ESSCAP, 2023). Besides, some dogs do not tolerate the in-ear application of compounds, and aural obstruction can interfere with compound absorption. Topical systemic treatment options include macrocyclic lactones such as moxidectin (Arther et al., 2015; Krieger et al., 2005), and selamectin (Shanks et al., 2000; Six et al., 2000). However, in recent years, dog owners and clinical veterinarians have come to prefer systemically and more easily administered antiparasitic compounds with a prolonged residual action (Selzer and Epe, 2021). Towards this goal, certain isoxazolines like afoxolaner, fluralaner, and sarolaner have been developed, providing good results in treating experimentally and naturally induced *O. cynotis* infestations in dogs (Carithers et al., 2016; Six et al., 2016; Taenzler et al., 2017).

Considering that the duration of the study (45 days) corresponds to two *O. cynotis* mite generations (the life cycle is typically 21 days), the treatment regimens not only affect the mite population already present but also break the *O. cynotis* life cycle, protecting the dogs from reinfestation. These high efficacies were on par with or even superior to the reported efficacies of other commonly used topical compounds used for treating canine otocariosis, such as moxidectin (85.7% (Krieger et al., 2005), 82% (Arther et al., 2015)) and selamectin (88.2% (Krieger et al., 2005), 74% (Arther et al., 2015), 90% (Six et al., 2000), 100% (Shanks et al., 2000)), although each study employed different observation periods and methodologies. Therefore, afoxolaner can be safely administered for treating a plethora of ectoparasites in dogs, such as fleas, different tick species, *Demodex canis*, *Sarcoptes scabiei,* and *O. cynotis* (Zhou et al., 2022).

Table 1
Percentage efficacy of Nexgard® and Nexgard Spectra® using geometric and arithmetic means of the total live *Otodectes cynotis* mite counts at the end of the study (D45). The minimum and maximum mite counts per group are inside the parentheses.

	Group 1	Group 2		Group 3		Group 4	
	Mean	Mean	Percentage efficacy	Mean	Percentage efficacy	Mean	Percentage efficacy
Geometric	144.4 (72.0-225.0)	0.1 (0.0-2.0)	99.9	0.1 (0.0-1.0)	99.9	0.2 (0.0-1.0)	99.9
Arithmetic	153.9	0.3	99.8	0.1	99.9	0.3	99.8

*Group 1: Dogs remained untreated (negative control)
Group 2: Dogs were treated with Nexgard® as a single oral dose on Day 0
Group 3: Dogs were treated with Nexgard® as an oral dose once on Days 0 and 30
Group 4: Dogs were treated with Nexgard Spectra® as an oral dose once on Days 0 and 30

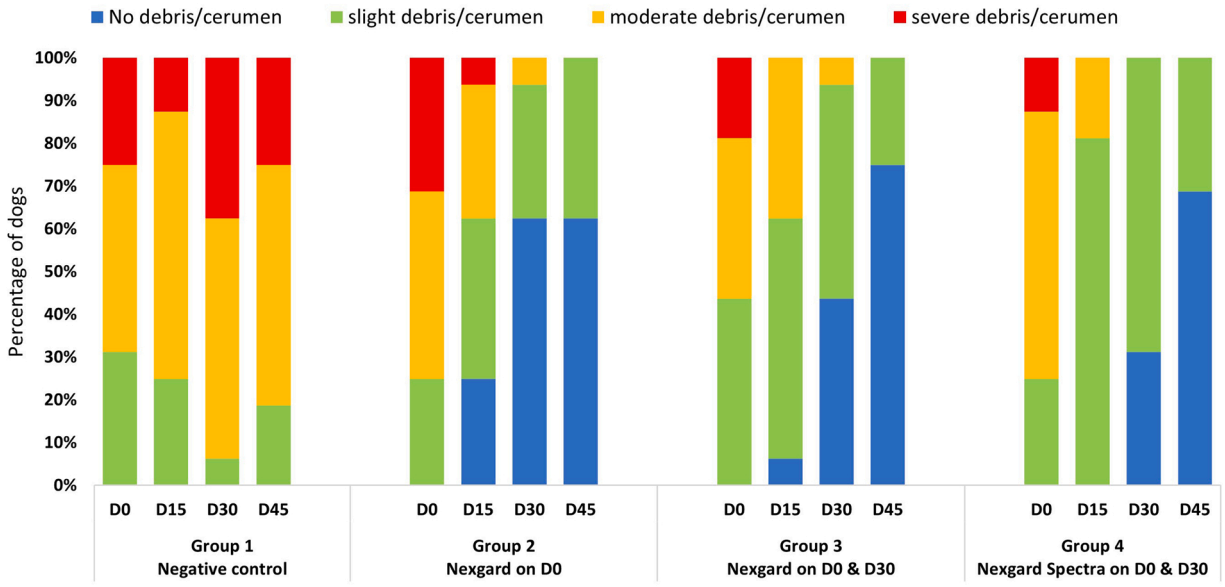

Fig. 1. Percentages of dogs with presence or absence of cerumen/debris and their respective scores observed during otoscopic examinations on Days 0, 15, 30, and 45 of the study. Group 1: Dogs remained untreated (negative control). Group 2: Dogs were treated with Nexgard® as a single oral dose on Day 0. Group 3: Dogs were treated with Nexgard® as an oral dose once on Days 0 and 30 Group. 4: Dogs were treated with Nexgard Spectra® as an oral dose once on Days 0 and 30.

Concerning infestation levels, in the current study, the arithmetic mean number of live mites in dogs in the negative control group (group 1) at the end of the study (D45) was high (153.9) like the arithmetic mean in a previous study with induced infestations in dogs (109.9) (Carithers et al., 2016). These high mite counts in the present study provide additional evidence of the strong efficacy of the isoxazoline afoxolaner against *O. cynotis*, even against high infestation levels.

5. Conclusions

After a single dose or two monthly doses, both afoxolaner (Nexgard®) and the combination of afoxolaner with milbemycin oxime (Nexgard Spectra®) displayed high efficacies (99.9%) against *O. cynotis* in naturally infested dogs. During the 45 days of the study, treatment(s) with afoxolaner or afoxolaner with milbemycin oxime improved symptoms of otitis externa, such as ear cerumen/debris, while there were no adverse events reported, and the treatments were well tolerated by the dogs.

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CRediT authorship contribution statement

Georgios Sioutas: Conceptualization, Validation, Formal analysis, Investigation, Resources, Data curation, Writing – original draft preparation, Writing – review and editing, Visualization. **Elias Papadopoulos:** Conceptualization, Validation, Formal analysis, Investigation, Resources, Writing – review and editing, Visualization, Supervision, Project Administration, Funding acquisition. **Maxime Madder:** Validation, Formal analysis, Resources, Data curation Writing – review and editing, Visualization, Supervision, Project Administration. **Frederic Beugnet:** Conceptualization, Validation, Formal analysis, Writing – review and editing, Supervision, Project administration, Funding acquisition. **Eric Tielemans:** Conceptualization, Validation, Formal analysis, Writing – review and editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

F.B. and E.T. are employees of Boehringer Ingelheim Animal Health. The other authors declare no competing interests.

Data Availability

The data presented in this study are available in the main text.

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study.

Animal welfare statement

This animal study protocol was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Aristotle University of Thessaloniki (188132/2022).

Informed Consent Statement

Written informed consent has been obtained by the dog owners to publish this paper.

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