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ORIGINAL PAPER

Efficacy against nematode infections and safety of afoxolaner plus milbemycin oxime chewable tablets in domestic dogs under field conditions in Europe

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Abstract Afoxolaner (AFX) plus milbemycin oxime (MO) combination chewable tablets (NexGard Spectra®, Merial) were evaluated for safety and efficacy against naturally acquired nematode infections in domestic dogs in a multi-centre, positive control, blinded field study using a randomized block design based on the order of presentation for allocation. In total, 408 dogs confirmed positive for naturally acquired infections of intestinal nematodes by pre-treatment faecal examination were studied in ten countries in Europe (Albania, Austria, Bulgaria, France, Germany, Hungary, Italy, Lithuania, Romania and Slovakia). Pre-treatment faecal examination revealed *Toxocara*, *Toxascaris*, hookworm, *Trichuris* and/or *Capillaria* nematode infections in 134, 30, 223, 155 and 14 dogs, respectively. Dogs were allocated to one of two treatment groups in a ratio of 1, AFX + MO chewables (≥2.5 mg AFX + ≥0.5 mg MO per kg body weight, according to dose bands; 207 dogs), and 1, MO plus praziquantel (PRZ) chewables (Milbemax®, Novartis; ≥0.5 mg MO + ≥5 mg PRZ per kg body weight, according to the manufacturer's instructions; 201 dogs) and treated once. For evaluation of efficacy based on reduction of faecal nematode egg counts, two faecal samples, one collected prior to

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treatment and one collected 9 to 21 days after treatment, were examined using modified McMaster techniques. For evaluation of systemic safety, dogs were examined by a veterinarian before treatment administration and at study end, and dog owners observed the health status of their dogs until the end of the study and reported any abnormal observation. For dogs treated with AFX + MO chewables, the efficacy was 99.7, 99.7, 97.2, 99.7 and 99.7 % for *Toxocara*, *Toxascaris*, hookworm, *Trichuris* and *Capillaria*, respectively; and the efficacy was 99.5, 99.4, 94.3, 99.9 and 98.0 %, respectively, for the MO + PRZ-treated dogs ($p \leq 0.002$ for all nematodes and both treatments). For *Toxocara*, hookworm and *Trichuris*, non-inferiority analysis demonstrated that the efficacy of AFX + MO chewable tablets was equal to or better than that of MO + PRZ. In spite that both treatments were ≥98 % efficacious against *Toxascaris* and *Capillaria*, a hypothesis of non-inferiority for both genera could not be established due to the low number of dogs infected with these parasites. No treatment-related adverse experiences were observed throughout the study. For both treatments, all dogs were given a systemic safety score of 'excellent' apart from one dog in each treatment group which received a score of 'acceptable'. AFX + MO combination chewables were shown to be safe and demonstrated a high level of efficacy when administered once to dogs infected with a broad range of parasitic nematodes under field conditions.

Keywords Afoxolaner · Milbemycin oxime · Dogs · Nematodes · Efficacy · Safety

Introduction

Intestinal nematode infections, particularly caused by ascarids, hookworms and/or whipworms, are a common and important diagnosis in dogs presented to veterinarians worldwide. These parasites usually cause subclinical disease, but infection may result in clinically apparent conditions, especially in puppies and young dogs but also older less well-cared-for dogs. These conditions may range from retarded growth or failure to thriving up to clinical disorders, such as vomiting, diarrhoea, anorexia and anaemia. In addition, dogs, like other companion animals, represent potential reservoirs and transmitters of several diseases including zoonotic parasitic infections. Thus, canine parasitism may have important public health implications (e.g. Bowman et al. 2010; Lee et al. 2010).

Several surveys indicated that the prevalence of intestinal nematode infection has decreased over time and attributed this decline to improved hygiene and generally better attention to canine health including veterinary care and increased use of highly effective, broad-spectrum anthelmintics. However, recently reported studies conducted throughout Europe have shown that intestinal nematode infections are still a common occurrence in dogs (e.g. Bourdeau et al. 2001; Fok et al. 2001; Lia et al. 2002; Arnold et al. 2004; Dubná et al. 2006; Pullola et al. 2006; Sager et al. 2006; Martínez-Moreno et al. 2007; Bružinskaitė et al. 2009; Claerebout et al. 2009; Kurnosova 2009; Musella et al. 2010; Tylkowska et al. 2010; Barutzki and Schaper 2011; Rinaldi et al. 2011; Becker et al. 2012; Frangipane di Regalbono et al. 2012; Napoli et al. 2012; Kirkova et al. 2013; Cardoso et al. 2014; Zanzani et al. 2014; Capári et al. 2015; Shukullari et al. 2015). However, factors like category of dogs (e.g. stray, shelter, kennel, working dog, well-cared-for pet) or environment (urban or rural) are associated with considerable variability of the prevalence of several endoparasites. In addition, most surveys were conducted in relatively small geographic areas across Europe but over a range of cultural, socioeconomic but also climatic conditions, making interpretation of results complex.

Appropriate management practices for the control of canine nematode infections are required including the use of efficacious and safe anthelmintics. The prevalence of both nematode parasites of the gastrointestinal tract and flea and tick ectoparasites, which may co-infect dogs, at least during certain periods of the year, provides justification for their adequate and concurrent control. This implicates the development of efficacious, safe and easy-to-administer products which are acceptable for animals and their owners. During the last two decades, development of products for flea and/or tick control in dogs was based mainly on compounds for topical administration with killing, repellent and/or expellent properties and residual activity of variable length. Only recently, adulticides of the isoxazoline insecticide and acaricide class of chemicals with long residual activity for oral administration became commercially available (Shoop et al. 2014; Letendre et al. 2014). To extend the spectrum of afoxolaner, the well-known macrocyclic lactone, milbemycin oxime, which primarily targets nematode infections, was added in a fixed combination chewable product. This product, NexGard Spectra® (Merial), which has been recently authorized in Europe (European Medicines Agency 2015), is designed for oral administration to dogs suffering from, or at risk of, concurrent infestation with fleas and ticks and infection with intestinal nematodes or heartworm. The efficacy of afoxolaner plus milbemycin oxime chewables against both common ectoparasites and endoparasites and its safe use in dogs has been demonstrated in a range of well-controlled laboratory studies (European Medicines Agency 2015; Tielemans et al. 2015; Fankhauser et al. 2016; Rehbein et al. 2016a, b; Drag et al. 2016; Letendre et al. 2016). This paper reports the results of a multi-centre field study which was designed to evaluate the efficacy against naturally acquired nematode infections and the safety of afoxolaner plus milbemycin oxime chewables at the recommended commercial dose ranges in dogs in Europe.

Material and methods

The study was designed in accordance with the International Cooperation on Harmonisation of Technical Requirements for Registration of Veterinary Medicinal Products—VICH GL7, "Efficacy of Anthelmintics: General Requirements" (Vercruysse et al. 2001) and "Efficacy of Anthelmintics: Specific Recommendations for Canines" VICH GL19 (Vercruysse et al. 2002), the "World Association for the Advancement of Veterinary Parasitology (WAAVP) guidelines for evaluating the efficacy of anthelmintics for dogs and cats" (Jacobs et al. 1994) and VICH GL9, entitled *Good Clinical Practice*. An informed consent and agreement was obtained from the owners of the dogs or their designees before enrolment.

The study was performed as a double-blinded study, i.e. owners as well as all personnel involved in collecting efficacy and safety data were masked to the treatment assignment of the animals.

Study animals

Client-owned dogs of any breed and sex, with a minimum age of 2 months and harbouring naturally acquired intestinal nematode infections as confirmed by pre-treatment faecal examination, were eligible for inclusion in the study. Faecal samples of 1390 dogs from 507 clients were collected at 14 sites in ten European countries (Albania, 1 site—89 dogs; Austria/Slovakia, 1 site—112 dogs; Bulgaria, 1 site—44 dogs; France, 2 sites—33 dogs; Germany, 3 sites—426 dogs; Hungary, 1 site—160 dogs; Italy, 3 sites—286 dogs; Lithuania, 1 site—152 dogs; Romania, 1 site—88 dogs) between April and September 2012 and subjected to coproscopical examination. In total, 408 dogs diagnosed with evidence of intestinal nematode infection(s) were enrolled (Albania, 42 dogs; Austria/Slovakia, 17 dogs; Bulgaria, 42 dogs; France, 16 dogs; Germany, 47 dogs; Hungary, 56 dogs; Italy, 120 dogs; Lithuania, 37 dogs; Romania, 31 dogs). Basic demographic data of the enrolled dogs are summarized in Table 1. Each dog was assigned a unique identification number, and informed owner consent was obtained. All dogs enrolled in the study were subjected to a physical examination before treatment and considered acceptable for inclusion into the study; however, some dogs presented minor conditions at this time, e.g. external otitis, flea infestation, loose stool, monorchism, pruritus, scars, superficial wounding or vaginitis.

Faecal examination

To qualify animals for the study and for evaluation of efficacy based on reduction of faecal nematode egg counts following treatment, fresh faecal samples were obtained before treatment and nine to 21 days post-treatment (enrolled animals only) for examination. The faecal samples were examined first grossly for the presence of parasites or parasite stages and subsequently processed using modified McMaster techniques to establish faecal nematode egg per gram (EPG) counts. Medium used for floatation (saturated sodium chloride solution with a specific gravity of 1.2, six sites; zinc sulphate solution adjusted to a specific gravity of 1.3, eight sites) and sensitivity of counting techniques (1 egg ≡ 15 EPG, two sites; 1 egg ≡ 20 EPG, one site; 1 egg ≡ 25 EPG, four sites; 1 egg ≡ 50 EPG, seven sites) varied between sites. Oocysts of *Cystisospora* species and sporocysts of *Sarcocystis* species, when present, were handled similarly, i.e. oocysts per gram (OPG) or sporocysts per gram (SPG) counts, respectively, were recorded. For cestodes, only the presence of either proglottids or eggs was recorded. Parasites were identified based on the morphology of the faecal forms; thus, there was no identification to the species level.

Evaluation of systemic safety

The dogs were physically examined by a veterinarian upon enrolment (before treatment) and at the end of the study when the dogs were presented for the post-treatment faecal examination. Dog owners/designees were instructed to report any adverse reactions as soon as possible after the reaction was noticed until end of the study. At study end, the systemic safety was evaluated based on the occurrence of adverse events throughout the study either reported by the owner or observed by the veterinarian (Table 2).

Allocation of dogs to treatment groups and treatment

A unique allocation sequence was provided for each study site. Blocks of two dogs each were formed based on order of presentation for allocation. Within blocks, dogs were assigned to one of two treatment groups according to the allocation sequence in a ratio of 1, afoxolaner plus milbemycin oxime chewables (NexGard Spectra®, Merial; 207 dogs) and 1, milbemycin oxime plus praziquantel (Milbemax®, Novartis; 201 dogs). Dogs were treated once on day 0 following determination of dose based on body weight measured on the day of treatment administration. NexGard Spectra® chewables for oral administration was used in the five commercial market sizes for dogs weighing 2.0 to 3.5, 3.6 to 7.5, 7.6 to 15.0, 15.1 to 30.0 or 30.1 to 60.0 kg, respectively, thus providing 2.5–5 mg AFX + 0.5–1 mg MO per kilogram body weight.

Milbemax®, available in two sizes (S and M chewables, providing ≥0.5 mg milbemycin oxime + ≥5 mg praziquantel per kg body weight), was administered orally according to the manufacturer's instructions.

All dogs remained with their owners, were kept in their usual environments and received their usual food and water.

Table 1 Basic demographics of the dogs ($n = 408$) which were enrolled in the study

Breeds	Pure breed dogs: 196 (Old German Shepherd, 23; Scent Hound, 18; German Shepherd, 17; Anglo-Français Tricolore, 12, Russo-European Laika, 10; Beagle, 7; Hounds, 7; American Staffordshire, 6; Blue Gascony Griffon, 5; West Siberian Laika, 5; Border Collie, 4; French Bulldog, 4; German Basset, 4; Jagdterrier, 4; Pekingese, 4; Pitbull, 4; Spitz, 4; Atlas Shepherd Dog, 3; Boxer, 3; Caucasian Shepherd, 3; Foxhound, 3; Mioritic Sheepdog, 3; Pointer, 3; Rottweiler, 3; Vizsla, 3; Dachshund, 2; Dalmatian, 2; Golden Retriever, 2; Miniature Pinscher, 2; Setter, 2; Bavarian Scent Hound, 1; Blue Gascony Basset, 1; Bukovina Shepherd, 1; Bulldog, 1; Central Asia Shepherd, 1; Cocker, 1; Deutsch Drahthaar, 1; English Setter, 1; Fox Terrier, 1; Husky, 1; Irish Setter, 1; Jack Russell Terrier, 1; Lithuanian Hound, 1; Malinois, 1; Maremma Sheepdog, 1; Miniature Spitz, 1; Pomeranian, 1; Poodle, 1; Ratonero, 1; Saint Bernard, 1; Sharpei, 1; Siberian Husky, 1; Tawny Britany Griffon, 1; Tibet Terrier, 1)
	Crossbred dogs, 39
	Mixed breed dogs (mongrels), 173
Gender/neutering status	Male, 198; male castrated, 19; female, 147; female spayed, 44
Age (range)	approximately 2 months to 15 years
Weight (range)	2.0 to 74.0 kg

Data analysis

Data from all study sites were combined for analysis, including data from dogs of complete and incomplete blocks.

The primary efficacy endpoint was the faecal egg count (EPG) for each identified nematode parasite from the individual dog. Percent efficacy was computed comparing the post-treatment (days 9 to 21) sample with the pre-treatment (days −14 to 0) sample for each treated group separately. Only those dogs that tested positive for the nematode in the pre-treatment sample were included in the efficacy evaluation and analysis. The faecal egg counts of each animal for each nematode of interest were transformed to the natural logarithm of (count [EPG] +1) for the calculation of geometric means. The percent efficacy of each treatment group was calculated using the formula $100 \times [(B - E) / B]$, where B is the pre-treatment geometric mean of the treatment group (baseline) and E is the post-treatment geometric mean of the treatment group (end visit).

To compare the faecal egg counts of the two time points for the nematode genera for each treatment group, the van Elteren rank test was used with sites treated as blocks. The Freq procedure in SAS® Version 9 was used employing the Cochran-Mantel-Haenszel 2 option with score = modridit.

Non-inferiority analysis was used to compare the efficacy of afoxolaner plus milbemycin oxime chewables with milbemycin oxime plus praziquantel: the null hypothesis that afoxolaner plus milbemycin oxime chewables are worse than milbemycin oxime plus praziquantel versus the alternative hypothesis that afoxolaner plus milbemycin oxime chewables are equal to or better than milbemycin oxime plus praziquantel was tested. The expected faecal egg counts per gram of the two treated groups post-treatment for each nematode represented were compared using the Mixed procedure in SAS® Version 9. The non-inferiority threshold was 2 EPG. The log-faecal egg counts were the dependent variable, treatment group was the fixed effect, and site was the random effect. A two-sided 95 % confidence interval was computed on the afoxolaner plus milbemycin oxime chewables − milbemycin oxime plus praziquantel difference. If the upper limit (the 97.5 % confidence limit) was at or below 0.69 (log(2)), then the null hypothesis was rejected and the data supported that afoxolaner plus milbemycin oxime chewables are equal to or better than milbemycin oxime plus praziquantel.

Table 2 Categories for assessment of systemic safety

Score	Safety	Guidance criteria
0	'Excellent'	No adverse event noted
1	'Acceptable'	Mild and acceptable adverse event(s) or health abnormality(ies)
2	'Poor'	Serious and unacceptable adverse event(s) or health abnormality(ies)

Systemic safety scores for the dogs of the two treatments were summarized descriptively.

Results

Infection rate of endoparasite infections in the dogs examined for enrolment

Of the 1390 dogs that were examined for potential enrolment in the study, 425 (30.6 %) had evidence of infection with endoparasites (nematodes, cestodes and/or protozoans), as determined by standard coproscopic examination (gross examination plus examination by modified McMaster techniques). Total infection rate and range of counts of endoparasite forms detected, if applicable, are summarized in Table 3. Identification of the faecal forms revealed eggs of nematodes (*Toxocara*, *Toxascaris*, hookworm, *Trichuris* and *Capillaria*), *Dipylidium* and taeniid proglottids and/or eggs, as well as *Cystoisospora* oocysts and *Sarcocystis* sporocysts. Overall, hookworms were the most prevalent parasites detected, followed by *Trichuris* and *Toxocara* nematodes. Of the dogs examined, 20.9 % showed infection with a single parasite and 9.7 % with multiple parasites (up to four parasites concurrently were observed) (Table 4). Table 5 summarizes the percent infection rates of endoparasite infections and ranges of counts of faecal forms for the dogs examined in the respective countries.

Evaluation of efficacy

Of the 408 (29.4 %) dogs which had evidence of infection with intestinal nematodes and were enrolled in the study, allocated and treated once on day 0, four dogs did not complete the study (two dogs with *Toxocara* infections and one dog each positive for hookworm or *Trichuris* infection; three dogs treated with afoxolaner plus milbemycin oxime chewables and one dog treated with milbemycin oxime plus praziquantel) for reasons unrelated to the study. Thus, analysis was based on data from 404 dogs of which 132, 30, 222, 154 and 14 dogs had pre-treatment (baseline) evidence of *Toxocara*, *Toxascaris*, hookworm, *Trichuris* and *Capillaria* nematodes, respectively.

Dosing of the animals according to the commercial dose bands of the two treatments provided actual milbemycin oxime dose rates of 0.72 ± 0.14 mg per kilogram body weight for the dogs which received afoxolaner plus milbemycin oxime chewables and 0.87 ± 0.39 mg per kilogram body weight for the dogs which received milbemycin oxime plus praziquantel.

For dogs treated with afoxolaner plus milbemycin oxime chewables, the efficacy in terms of reduction of faecal egg counts from baseline was 99.7, 99.7, 97.2, 99.7 and 99.7 % for *Toxocara*, *Toxascaris*, hookworm, *Trichuris* and *Capillaria*, respectively, and the efficacy was 99.5, 99.4, 94.3, 99.9 and 98.0 %, respectively, for the milbemycin oxime plus praziquantel-treated dogs ($p \leq 0.002$ for all nematodes and both treatments) (Table 5). For *Toxocara*, hookworm and *Trichuris* nematodes, the null hypothesis was rejected, and the data supported that afoxolaner plus milbemycin oxime chewables were equal to or better than milbemycin oxime plus praziquantel at the (one-sided) 2.5 % significance level; however, because insufficient numbers of animals were infected with both *Toxascaris* and *Capillaria* nematodes, the upper confidence limit was not less than the non-inferiority threshold in spite of the two treatments having similar efficacies of ≥98 % (Table 6).

Evaluation of systemic safety

No treatment-related adverse experiences were observed throughout the study. For both treatments, all dogs that

Table 3 Overall infection rate and range of counts of parasite stages in the faeces of 1390 dogs from ten countries (Albania, Austria, Bulgaria, France, Germany, Hungary, Italy, Lithuania, Romania and Slovakia) examined for enrolment in the study, as determined by gross faecal examination for expelled parasites and modified McMaster techniques

Parasite stage	Infection rate		Range of counts
	Number of positive dogs (pre-treatment)	%	
Toxocara eggs	134	9.64	20–10,225 EPG
Toxascaris eggs	30	2.16	15–3700 EPG
Hookworm eggs	223	16.04	20–4230 EPG
Trichuris eggs	155	11.15	25–4450 EPG
Capillaria eggs	19	1.37	50–4800 EPG
Dipylidium eggs/proglottids	3	0.21	Not recorded
Taeniid eggs/proglottids	10	0.72	Not recorded
Cystoisospora oocysts	11	0.79	50–1100 OPG
Sarcocystis sporocysts	2	0.14	50–150 SPG

EPG eggs per gram of faeces, *OPG* oocysts per gram of faeces, *SPG* sporocysts per gram of faeces

Table 4 Occurrence of single and mixed endoparasite infections of 1390 dogs from ten countries (Albania, Austria, Bulgaria, France, Germany, Hungary, Italy, Lithuania, Romania and Slovakia) examined for enrolment in the study, as determined by gross faecal examination for expelled parasites and modified McMaster techniques

	Infection rate, total (%)
Single endoparasite infections	290 (20.86 %)
Toxocara	73 (5.25 %)
Toxascaris	15 (1.08 %)
Hookworm	117 (8.42 %)
Trichuris	70 (5.04 %)
Capillaria	5 (0.36 %)
Dipylidium	1 (0.07 %)
Taeniid	3 (0.22 %)
Cystoisospora	4 (0.29 %)
Sarcocystis	2 (0.14 %)
Mixed endoparasite infections	135 (9.71 %)
Toxocara + *Toxascaris*	5 (0.36 %)
Toxocara + hookworm	22 (1.51 %)
Toxocara + *Trichuris*	7 (0.50 %)
Toxocara + *Capillaria*	1 (0.07 %)
Toxocara + taeniid	1 (0.07 %)
Toxocara + *Cystoisospora*	3 (0.22 %)
Toxascaris + hookworm	3 (0.22 %)
Toxascaris + *Trichuris*	4 (0.29 %)
Hookworm + *Trichuris*	53 (3.81 %)
Hookworm + *Capillaria*	4 (0.29 %)
Hookworm + *Dipylidium*	1 (0.07 %)
Hookworm + taeniid	1 (0.07 %)
Hookworm + *Cystoisospora*	1 (0.07 %)
Trichuris + *Capillaria*	1 (0.07 %)
Trichuris + *Dipylidium*	1 (0.07 %)
Trichuris + *Cystoisospora*	1 (0.07 %)
Capillaria + taeniid	2 (0.14 %)
Toxocara + *Toxascaris* + hookworm	1 (0.07 %)
Toxocara + *Toxascaris* + *Trichuris*	1 (0.07 %)
Toxocara + hookworm + *Trichuris*	13 (0.94 %)
Toxocara + hookworm + *Capillaria*	2 (0.14 %)
Hookworm + *Trichuris* + *Capillaria*	1 (0.07 %)
Hookworm + *Trichuris* + taeniid	1 (0.07 %)
Toxocara + hookworm + *Cystoisospora*	1 (0.07 %)
Toxocara + *Capillaria* + *Cystoisospora*	1 (0.07 %)
Toxocara + *Toxascaris* + hookworm + *Trichuris*	1 (0.07 %)
Toxocara + hookworm + *Capillaria* + taeniid	1 (0.07 %)
Toxocara + *Trichuris* + *Capillaria* + taeniid	1 (0.07 %)

completed the study received a systemic safety score of 'excellent' apart from one dog for each treatment which received a score of 'acceptable'. These two dogs, which belonged to the same owner and were treated the same day, were reported exhibiting a period of diarrhoea 6 to 9 days after treatment.

Discussion

By examination of faecal samples of domestic dogs from ten countries in Europe using conventional coproscopic techniques, it was determined that almost one third of the dogs were shedding endoparasite stages. The spectrum of parasites identified in terms of their faecal forms comprised mainly nematode parasites of the gastrointestinal tract: infections with hookworms (Ancylostomatidae), whipworms (*Trichuris*) and *Toxocara* ascarids were detected most frequently while infections with cestodes and coccidians were observed rarely. All nematodes are common canine parasites throughout Europe, and their occurrence in dogs in the different countries has been documented previously including several recently conducted coproscopical surveys (e.g. Bourdeau et al. 2001; Fok et al. 2001; Lia et al. 2002; Arnold et al. 2004; Negrea 2005; Szabová et al. 2007; Bružinskaitė et al. 2009; Musella et al. 2010; Barutzki and Schaper 2011; Rinaldi et al. 2011; Becker et al. 2012; Napoli et al. 2012; Kirkova et al. 2013; Capári et al. 2015; Shukullari et al. 2015).

The examination of the dogs for potential enrolment in the study did not aim to establish prevalence data for intestinal parasitism in dogs. Given that the results, based on a single faecal examination with modified McMaster techniques, capable of detecting 15 to 50 eggs per gram, most probably underestimate the true prevalence, this data should be considered the minimum rate of infection for these parasites. The overall rate of intestinal parasite infection of the dogs varied between countries. However, the observed infection rate for the different nematodes does not only confirm the particular importance of *Toxocara*, hookworm and *Trichuris* infections but is also representative of the level of intestinal parasitism of dogs in Europe. Different from most surveys conducted in Europe (e.g. Fok et al. 2001; Lia et al. 2002; Arnold et al. 2004; Dubná et al. 2006; Pullola et al. 2006; Sager et al. 2006, Bružinskaitė et al. 2009, Claerebout et al. 2009; Kurnosova 2009; Tylkowska et al. 2010; Barutzki and Schaper 2011; Becker et al. 2012; Napoli et al. 2012; Zanzani et al. 2014), hookworm and whipworm infections were more frequently diagnosed than *Toxocara* in the dogs examined for the present study. However, in other studies from Europe, predominance of hookworms or *Trichuris* in canine faecal examinations has been found (e.g. Bourdeau et al. 2001; Martínez-Moreno et al. 2007; Musella et al. 2010; Frangipane di Regalbono et al. 2012; Kirkova et al. 2013; Cardoso et al. 2014; Capári et al. 2015). In our study, this order of infection rate with a relatively high rate of hookworm and *Trichuris* co-infections likely reflect, at least in part, the age structure of the dogs considered. Almost 77 % of the dogs were 1 year of age

Table 5 Percent infection rate and (range of counts) of endoparasite infections in the dogs examined in Albania, Austria, Bulgaria, France, Germany, Hungary, Italy, Lithuania, Romania and Slovakia as determined by gross faecal examination for expelled parasites and modified McMaster techniques

Parasite	Percent infection rate and (range of counts)[a] of endoparasite infections									
	Albania (89 dogs)	Austria (77 dogs)	Bulgaria (44 dogs)	France (33 dogs)	Germany (426 dogs)	Hungary (160 dogs)	Italy (286 dogs)	Lithuania (152 dogs)	Romania (88 dogs)	Slovakia (35 dogs)
Toxocara	39.3 (50–2800)	1.3 (300)	15.9 (100–1750)	0	4.7 (25–10,225)	10.6 (25–775)	12.6 (60–765)	9.2 (20–2400)	4.5 (200–1900)	0
Toxascaris	0	0	0	0	1.4 (25–2300)	0.6 (100)	7.3 (15–500)	0	0	5.7 (600–3700)
Hookworm	14.6 (100–700)	1.3 (100)	75.0 (100–3900)	33.3 (50–475)	2.8 (25–375)	11.9 (25–475)	30.8 (50–4230)	17.1 (20–2160)	13.6 (50–150)	22.9 (50–800)
Trichuris	15.7 (50–2050)	0	47.7 (100–2400)	18.2 (25–500)	2.8 (50–325)	26.9 (25–1900)	11.2 (30–2490)	0	23.9 (50–4450)	17.1 (50–2850)
Capillaria	0	0	9.1 (200–1100)	3.0 (550)	1.4 (50–275)	1.3 (50–200)	0	3.9 (80–4800)	0	0
Dipylidium[b]	0	0	2.3	0	0	0	0	0.7	1.1	0
Taeniid[b]	0	0	4.5	0	1.6	0.6	0	0	0	0
Cystoisospora	0	0	0	0	1.2 (100–775)	0	0	0.7 (80)	3.4 (50–1100)	5.7 (300–600)
Sarcocystis	0	0	0	0	0	0	0	0	2.3 (50–150)	2.9 (300)
Overall nematode infection rate	47.2 %	2.6 %	97.7 %	48.5 %	12.0 %	35.0 %	42.0 %	24.3 %	36.4 %	42.9 %

[a] Eggs per gram (*Toxocara*, *Toxascaris*, hookworm, *Trichuris*, *Capillaria*), oocysts per gram (*Cystoisospora*) or sporocysts per gram (*Sarcocystis*)

[b] Presence of proglottids/eggs

and older, but *Toxocara* prevalence is inversely related to dog age while *Trichuris* infections are more prevalent in older dogs (e.g. Visco et al. 1977; Little et al. 2009). Interestingly, the results of the pre-treatment coproscopic examination revealed that approximately one third of the dogs enrolled harboured mixed infections of up to four nematodes. These findings are indicative of the common occurrence of multi-parasitism in dogs and that indicate the value of using a product with an appropriately broad spectrum. Although the number of eggs shed with the faeces was rather moderate in the majority of dogs with excessive counts recorded in relatively few cases only, these counts provide an indication of the substantial dissemination of nematode eggs into the environment through untreated, patently infected dogs.

This multi-centre clinical field study was conducted to confirm the efficacy and systemic safety of NexGard Spectra® under conditions of practical use in Europe. With enrolment of dogs representing a broad range of breeds of different sizes and age groups from northern (Lithuania), western (France), central (Germany, Austria, Slovakia, Hungary) and southern Europe (Italy, Albania, Romania, Bulgaria), results of this study demonstrated that afoxolaner plus milbemycin oxime chewables are an efficacious and safe treatment against naturally acquired infections of the most common nematodes of dogs. Following a single administration, the efficacy, in terms of reduction of faecal egg shedding, was consistently >97 % against all nematodes demonstrated, i.e. *Toxocara*, *Toxascaris*, hookworm, *Trichuris* and *Capillaria* infections. Results of this field study thus confirm the results of controlled studies conducted under laboratory conditions which included dogs with either induced or naturally acquired infections of *Ancylostoma caninum*, *Toxocara canis*, *Toxascaris leonina* or *Trichuris vulpis* from widely disjunct geographies (Fankhauser et al. 2016; Rehbein et al. 2016a).

In Europe, there are two species of hookworms parasitizing dogs and wild canids: *A. caninum* and *Uncinaria stenocephala*. For examination of faecal samples in this field study, no attempt was made to differentiate the eggs of the two species. In principle, the majority of eggs of the two species, which are typical strongyle in appearance, can be fairly readily identified based on their size (Georgi and McCulloch 1989). However, because of using McMaster counting slides, eggs frequently do not lay flat under the top plate which hampers differentiation.

In comparison to the climatic conditions with the temperatures allowing for the development and survival of the free-living larval stages, *U. stenocephala* is a parasite that is adapted to more temperate or cooler climates while *A. caninum* is found usually in southern temperate and subtropical regions (Georgi and Georgi 1992). Thus, it is believed that *A. caninum* occurs predominantly in southern Europe and only sporadically in northern Europe while *U. stenocephala* infection is more prevalent in central and northern Europe. However, the endemic areas of the two species overlap and

Table 6 Pre- and post-treatment faecal nematode egg counts, percentage efficacy and results of data analysis

Nematode	Treatment[a]	N[b]	Analysis of faecal egg counts (van Elteren rank test)				Non-inferiority analysis	
			Geometric mean (EPG)[c]		% Efficacy[d]	*p* value[c]	Upper 97.5 % CI[e]	Threshold[f]
			Pre-treatment	Post-treatment				
Toxocara	1	60	258.8	0.7	99.7	<0.0001	0.44	0.69
	2	72	203.2	1.0	99.5	<0.0001	NA	NA
Toxascaris	1	19	134.7	0.4	99.7	<0.0001	0.78	0.69
	2	11	75.4	0.4	99.4	0.0002	NA	NA
Hookworm	1	111	212.8	6.0	97.2	<0.0001	0.30	0.69
	2	111	152.5	8.7	94.3	<0.0001	NA	NA
Trichuris	1	85	142.8	0.4	99.7	<0.0001	0.42	0.69
	2	69	192.0	0.3	99.9	<0.0001	NA	NA
Capillaria	1	6	249.4	0.7	99.7	0.0079	1.47	0.69
	2	8	209.1	4.1	98.0	0.0020	NA	NA

NA not applicable

[a] Treatment 1 = afoxolaner + milbemycin oxime, treatment 2 = milbemycin oxime + praziquantel

[b] Number of dogs analyzed per treatment

[c] Geometric mean faecal egg count, EPG = eggs per gram of faeces, pre-treatment (days –14 to 0, baseline) and post-treatment (days 9 to 21, end visit)

[d] Percent efficacy = 100 x [(B – E) / B], where B and E are the geometric mean pre-treatment (baseline) and post-treatment (end visit) faecal egg counts, respectively. Geometric means were calculated by averaging the log-counts, taking the anti-logarithm and then subtracting 1

[c] (Two-sided) probability comparing post-treatment with pre-treatment for each treatment within each nematode

[e] The upper 97.5 % confidence limit on the difference of treatment 1 – treatment 2 was computed on the log-counts in the Mixed procedure. If the upper limit was less than the threshold, then for that nematode, treatment 1 was significantly not inferior to treatment 2 at the 5 % significance level and under the hypothesis that the two treatments had comparable faecal egg counts if the ratio of the means is less than 2

[f] Threshold = logarithm of 2

with increasing travelling of dogs to and importation of dogs from southern Europe and climate tending to more favourable conditions in central and northern Europe, *A. caninum* may spread northwards. Based on faecal examination, dogs with hookworm infections were diagnosed in all ten countries which enrolled animals for this study. In all of these countries, the abundance of canine hookworms was demonstrated previously through identification of adult parasites at postmortem or by thorough egg differentiation. These studies demonstrated the prevalence of both species of hookworm in dogs in Albania, Bulgaria, France, Hungary, Italy, Lithuania and Romania (e.g. Panebianco and Sciutteri 1955; Kazlauskas and Prūsaitė 1976; Bourdeau and Chermette 1985; Fok et al. 1988; Martini and Poglayen 1990; Fok 1992; Franc et al. 1997; Georgieva et al. 1999; Mureşan et al. 2002; Xhaxhiu et al. 2011) while for Austria, Germany and Slovakia, there are records of canine *U. stenocephala* infections only (Gräfner and Danailov 1964; Mituch 1968; Supperer and Hinaidy 1986; Dubinský et al. 1995). The significant, >94 % reduction of hookworm egg counts following treatment with both products thus suggests a substantial effect on the infection with both species of hookworm through reduction of parasite burden and/or affecting the fecundity of female worms. Therapeutic response to treatment with milbemycin oxime in case of *U. stenocephala* infection in dogs has been variable. However, the findings of the present study are supported by controlled studies in naturally infected dogs treated with afoxolaner plus milbemycin oxime chewables (Rehbein et al. 2016a) and results obtained in another field study which tested a milbemycin oxime combination product at a minimum dose of 0.75 mg/kg body weight (Hayes et al. 2015).

Interestingly, *Capillaria* eggs were detected in the faeces of dogs from five countries with infection rates ranging from 1.3 to 9.1 %. Unless faeces are contaminated by urine or eggs are accidentally ingested during grooming, *Capillaria* eggs found in the faeces of dogs are unlikely to be derived from bladder worms (*C. plica*). *Capillaria* eggs recovered from faeces may originate from capillarids residing in the respiratory tract (*Eucoleus aerophilus*, *E. boehmi*), or the eggs may have passed through the alimentary tract following ingestion of contaminated food or coprophagia (Georgi and Georgi 1992). There was no detailed microscopic examination of the morphology of the eggs to distinguish the eggs of *E. aerophilus* or *E. boehmi* from those of other capillarids which is possible based on size and egg shell wall surface pattern (Conboy et al. 2013). However, the detection of *Capillaria* eggs in the dogs' faeces may support the hypothesis that the capillarid lungworm infections are spreading in

Europe in both dogs and cats (Traversa et al. 2009, 2010; Di Cesare et al. 2011). Coincidentally, cases of canine *E. boehmi* nasal infections have recently been reported from Europe (Di Cesare et al. 2012; Magi et al. 2012; Macchioni et al. 2013), and there is also a discussion on intestinal *Aonchotheca putorii* potentially parasitizing dogs in areas of the occurrence of this capillarid in wildlife (Macchioni et al. 2013). Noteworthy, treatment with both products tested in this study resulted in ≥98 % reductions of shedding of *Capillaria* eggs. The number of treated dogs was small; therefore, more extensive studies, including identification of the capillarid nematodes, are necessary to confirm the apparent effectiveness. According to a recently published report, milbemycin oxime at a dose of 2 mg/kg body weight appeared to be efficacious against *E. boehmi* infection in a dog based on the cessation of faecal egg shedding (Conboy et al. 2013).

In conclusion, the results of this multi-centre study demonstrated that NexGard Spectra®, when administered once orally to dogs infected with nematodes, were safe and highly effective for the treatment of a broad range of important nematode parasites in dogs under field conditions in Europe.

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Compliance with ethical standards

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