Veterinary Parasitology 192 (2013) 353–358

Contents lists available at SciVerse ScienceDirect

Veterinary Parasitology

journal homepage: www.elsevier.com/locate/vetpar

The efficacy of eprinomectin extended-release injection against *Hypoderma* spp. (Diptera: Oestridae) in cattle

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ARTICLE INFO

Keywords:
Eprinomectin
Extended-release injection
Efficacy
Hypoderma bovis
Hypoderma lineatum
Cattle

ABSTRACT

The efficacy of eprinomectin in an extended-release injection (ERI) formulation was determined in cattle harboring naturally acquired infestations of first- or second- and third-stage larvae of *Hypoderma* spp. in three studies conducted according to the same protocol in the USA (two studies) and Germany (one study). Thirty cattle sourced from herds with a history of *Hypoderma* infestation were included in each study. Cattle were formed into replicates of three animals each on the basis of pre-treatment anti-*Hypoderma* antibody titers. Within replicates each animal was randomly allocated to one of the following treatments: ERI vehicle (control) at 1 mL/50 kg bodyweight, administered once on Day 0; Eprinomectin 5% ERI at 1 mL/50 kg bodyweight (1.0 mg eprinomectin/kg), administered once on Day 0 (when larvae were expected to be first instars); or Eprinomectin 5% ERI at 1 mL/50 kg bodyweight (1.0 mg eprinomectin/kg), administered once when larvae were second or third instars (study dependent, Day 73, 119, or 140). Treatments were administered by subcutaneous injection in front of the shoulder. In all studies, emerging and/or expressed *Hypoderma* larvae were recovered, speciated, and counted and viability was determined. Eprinomectin LAI treatment was 100% ($p < 0.05$) efficacious against first- and second- or third-stage larvae of *Hypoderma bovis* (two studies) and *Hypoderma lineatum* (one study). All animals accepted the treatment well. No adverse reaction to treatments was observed in any animal in any study.

1. Introduction

Warble flies are relatively host-specific species of myiasis-producing dipterans whose larvae dwell as subcutaneous parasites and may produce boil-like swellings (warbles) under the skin of mammals. *Hypoderma bovis* (Linnaeus, 1758) and *Hypoderma lineatum* (De Villers, 1789) (Diptera: Oestridae: Hypodermatinae) are commonly referred to as 'ox warbles', warble flies, cattle grubs or northern hemisphere grubs and are parasites of cattle, rarely affecting horses, sheep, or man. They occur throughout the Holarctic region (Zumpt, 1965).

The biology of *H. bovis* and *H. lineatum* is similar in many respects. The adult flies, which do not feed and live only for a short time, occur in late spring and summer, particularly from May to August/early September. *H. lineatum* tends to appear about one month before *H. bovis*. The female flies affix their eggs directly to cattle hairs (mainly legs, more rarely on lower parts of the body). The first stage larvae hatch from the eggs within a week and penetrate the host's skin. The larvae of *H. bovis* and *H. lineatum* reach the skin of the animal's back after migration from the sites of oviposition via the epidural fat in the spinal canal (*H. bovis*) or the submucosal connective tissue of the esophagus

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0304-4017
http://dx.doi.org/10.1016/j.vetpar.2012.11.042

(*H. lineatum*) by burrowing between the sheets of connective tissue. Several months elapse before the larvae reach their final site in the region of the lumbar and posterior thoracic vertebrae. With the onset of the following spring, the larvae arrive under the skin of the back, where swellings begin to develop as a host reaction to the larvae. The infestation is in the 'warble' stage, when the skin over each swelling becomes perforated, allowing the larva to breathe. Warbles of *H. lineatum* appear in the backs of animals from January up to April/May, while those of *H. bovis* appear between March and July. Within a period of one to two months, the larva in the warble molts twice and grows rapidly. The mature larva emerges through the warble pore and falls to the ground to pupate for one to three months. The entire life cycle is approximately one year in duration and thus results in only a single generation per year.

Hypoderma spp. are important economic pests of cattle, and losses arise from a number of causes. Although the principal impact from these flies is associated with the parasitism of the larval stages, losses also are attributed to the imagos. The disturbance of grazing cattle caused by ovipositing flies, especially *H. bovis*, may result in an avoidance behavior which is known as 'gadding'. It has been associated with interruption of grazing, altered reproduction patterns, self-injury, poor weight gain, and reduced milk yield. The most significant losses, however, are associated with the *Hypoderma* larvae: warbles and damage to other tissues during migration of the larvae may require excessive trimming of discolored flesh in slaughtered cattle, and the perforation of the skin by the larvae, as well as the scars of healed warbles, cause condemnation or greatly reduce the value of the hides (Kettle, 1984; Wall and Shearer, 2001; Catts and Mullen, 2002; Colwell et al., 2006).

The prevalence of *Hypoderma* spp. has decreased markedly following the use of systemically acting macrocyclic lactones in national eradication programs which have been undertaken in many countries. Nevertheless, the prevalence of *Hypoderma* infestation in cattle can be considerable in certain areas, and *Hypoderma* spp. have a great capacity for population (re)generation if eradication or control measures are incomplete or if re-introduction (importation of infested cattle or fly immigration) occurs (O'Brien, 1998; Scholl, 1998; Colwell, 2002; Colebrook and Wall, 2004).

Eprinomectin, a second-generation avermectin, is a member of the macrocyclic lactone class of parasiticides. Its endectocidal activity in a 0.5% formulation consisting of natural oils for pour-on administration at 0.5 mg eprinomectin/kg bodyweight has been documented in numerous laboratory and field studies world-wide (e.g., Shoop et al., 1996; Barth et al., 1997; Gogolewski et al., 1997a,b; Holste et al., 1997, 1998; Pitt et al., 1997; Williams et al., 1997; Yazwinski et al., 1997; Batty et al., 1999; Epe et al., 1999; Cramer et al., 2000; Dorny et al., 2000; Campbell et al., 2001; Davey and George, 2002; Rehbein et al., 2005).

In order to extend the persistency of activity of eprinomectin against endoparasites, an injectable formulation has been developed which releases the active over time in concentrations that provide effective prevention of nematode infections in cattle for up to 150 days (Soll et al., 2013). In this extended-release injection (ERI) formulation, eprinomectin is released from a matrix formed with poly(D,L-lactide-*co*-glycolic)acid (PLGA). PLGA is known as a safe and effective biodegradable material which has been used as a drug delivery system for extended-release applications of various pharmaceutical compounds including ivermectin (Lewis, 1990; Miller et al., 1999; Clark et al., 2004; Winzenburg et al., 2004).

The objective of the present studies was to confirm the efficacy of Eprinomectin ERI against naturally acquired infestations of first or second and third larval stages of *Hypoderma* spp. in cattle.

2. Materials and methods

A total of three controlled studies were conducted according to the same protocol, two in the USA and one in Germany. The studies were designed and conducted to comply with the regulatory requirements of both the FDA/CVM and the European Medicines Agency/Committee for Medicinal Products for Veterinary Use, and according to relevant guidelines for good clinical practices (GCPs) and for establishing the efficacy of ectoparasiticides against myiasis-causing parasites (Holdsworth et al., 2006).

The studies were performed as blinded studies, i.e., all personnel involved in collecting data were masked to the treatment assignment of the animals.

2.1. Experimental animals

A total of 90 (1 male, 49 male castrate and 40 female) healthy, ruminating Angus, Angus cross, Hereford, Hereford/Angus cross, Hereford/Red Angus cross, Hereford cross, Red Angus, Rotbunte, Schwarzbunte, Shorthorn cross or Simmental cross cattle were included in three studies, conducted in the USA (Studies 1 and 3) or in Germany (Study 2). The cattle were approximately 5–25 months old and weighed 150–482 kg prior to treatment (Day −1 or Day 0). The animal descriptions and details are presented in Table 1. The animals were sourced from herds with *Hypoderma* infestation history in Wyoming (Study 1) or Wisconsin (Study 3), USA and in the Rhine Palatinate, Germany (Study 2). Animals had not been treated previously with an avermectin or milbemycin product and had previous exposure to *Hypoderma* spp. as confirmed by the presence of anti-*Hypoderma* antibodies prior to treatment.

Animals were handled with due regard for their welfare and in compliance with Merial Institutional Animal Care and Use Committee (IACUC) approvals, local regulations and requirements of any local IACUC.

2.2. Experimental design

A randomized block design based on pre-treatment anti-*Hypoderma* antibody levels was used with the individual animal being the experimental unit in each study. Ten replicates of three cattle each were formed sequentially based on decreasing pre-treatment anti-*Hypoderma* antibody test (Studies 1 and 3: ELISA test as described

Table 1
Animal description and details.

Study[a]	Treatment[b]/animals per treatment		Breed	Sex	~Age (months)	Pre-treatment bodyweight (kg)
1	Control EpERI L1[c] EpERI L2/L3[d]	$n = 10$ $n = 10$ $n = 10$	Angus cross, Hereford	27 male castrate, 3 female	8	150–254
2	Control EpERI L1 EpERI L2/L3	$n = 10$ $n = 10$ $n = 10$	Angus cross, Rotbunte, Schwarzbunte	1 male, 29 female	6–25	190–482
3	Control EpERI L1 EpERI L2/L3	$n = 10$ $n = 10$ $n = 10$[e]	Angus, Hereford, Hereford cross, Hereford/Angus cross, Hereford/Red Angus cross, Red Angus, Shorthorn cross, Simmental cross	22 male castrate, 8 female	5–9	200–390

[a] Source of animals: Study 1 – Wyoming, USA; Study 2 – Rhine Palatinate, Germany; Study 3 – Wisconsin, USA.
[b] Control = ERI vehicle-treated; EpERI = Eprinomectin ERI.
[c] Treatment during the first larval stage of development.
[d] Treatment during the second/third larval stages of development.
[e] One animal not treated (died of a lung hemorrhage on Study Day 42, prior to treatment).

by Colwell et al. (1997); Study 2: Hypodermosis ELISA, Vétoquinol, Lure, France) results. Within replicates, animals were randomly allocated to treatment: one to the control (vehicle-treated) group, one to the Eprinomectin ERI group to be treated when the *Hypoderma* spp. larvae were expected to be first-stage larvae, and one to the Eprinomectin ERI group to be treated when the *Hypoderma* spp. larvae were in the second/third stage, for a total of 10 animals per group each.

Cattle were held indoors and were individually stanchioned (Study 2) or were held in indoor/outdoor (Study 3) or outdoor (Study 1) pens by treatment group. Animals were fed as per local practice and were provided water ad libitum. The studies were initiated either in November (Study 1) or in December (Studies 2 and 3).

Both vehicle (formulation consisting of the excipients of the Eprinomectin ERI) and Eprinomectin 5% (w/v) ERI (eprinomectin in a PLGA-based formulation; dosed at 1.0 mg eprinomectin/kg), were administered at 1 mL/50 kg (1 mL/110 lb) bodyweight by subcutaneous injection in front of the shoulder using commercial disposable syringes and needles. Two groups of cattle were treated on a single occasion with either ERI-vehicle or Eprinomectin ERI when the *Hypoderma* spp. larvae were expected to be first-stage larvae and no warbles were visible (Day 0: all studies). A third group was treated with Eprinomectin ERI once when warbles were visible and the *Hypoderma* spp. larvae were in the second/third stage (Day 73: Study 1; Day 119: Study 2; Day 140: Study 3).

Animals were weighed prior to treatments (Days 0 and 73: Study 1; Days 0 and 119: Study 2; Days −1 and 140: Study 3) for dose calculation.

All cattle were observed hourly for 4 h post-treatment and thereafter once daily throughout the studies for health problems or adverse drug events.

Cattle were inspected for warbles starting on study Day 38 (Study 1) or Day 42 (Studies 2 and 3) at intervals no greater than two weeks until warbles were detected in a minimum of six cattle in the group to be treated when the *Hypoderma* spp. larvae were in the second/third stage. Thereafter, cattle were inspected, and adequately mature *Hypoderma* spp. larvae were collected, viability was determined, and larvae were identified to species, when possible. Cattle were examined until study termination, i.e., until larvae were no longer emerging.

2.3. Statistical methods

The counts of live *Hypoderma* spp. larvae were transformed to the natural logarithm of (count + 1) for calculation of geometric means. Eprinomectin ERI treatment groups were compared separately to the ERI vehicle-treated (control) group using the Wilcoxon rank sum test. A two-sided test was used at the significance level of 0.05. Efficacy was calculated as $100[(C - T)/C]$, where C is the geometric mean for the vehicle-treated (control) group and T is the geometric mean for the Eprinomectin ERI-treated group.

Table 2
Hypoderma larval counts and therapeutic efficacy of Eprinomectin ERI against natural infestations of first-stage and second or third-stage larvae of *Hypoderma* spp.

Study[a]	Treatment[b]	Live *Hypoderma* larval counts[c]		Efficacy[d]	Probability[e]
		Number of cattle infested	GM[f] (range)		
Hypoderma lineatum larvae					
	Control	8/10	4.9 (0–26)	–	–
1	EpERI L1[g]	0/10	0	100%	<0.05
	EpERI L2/L3[h]	0/10	0	100%	<0.05
Hypoderma bovis larvae					
	Control	10/10	12.3 (1–52)	–	–
2	EpERI L1	0/10	0	100%	<0.01
	EpERI L2/L3	0/10	0	100%	<0.01
	Control	7/10	1.8 (0–9)	–	–
3	EpERI L1	0/10	0	100%	<0.05
	EpERI L2/L3	0/9[i]	0	100%	<0.05

[a] Source of animals: Study 1 – Wyoming, USA; Study 2 – Rhine Palatinate, Germany; Study 3 – Wisconsin, USA.
[b] Control = ERI vehicle-treated; EpERI = Eprinomectin ERI.
[c] Number of larvae collected after treatment of cattle with second/third stage *Hypoderma* larvae until study termination (Study 1: Day 108, Study 2: Day 189 and Study 3: Day 182).
[d] Efficacy = 100 × (GM control − GM Eprinomectin ERI/GM control).
[e] Probability from the Wilcoxon rank sum test.
[f] Geometric mean live larval counts (based on transformation to the natural logarithm of [count + 1]).
[g] Treatment during the first larval stage of development.
[h] Treatment during the second/third larval stages of development.
[i] One animal died of a lung hemorrhage on Study Day 42, prior to treatment.

3. Results

All larvae collected in Study 1 were identified as *H. lineatum*, while all larvae collected in Studies 2 and 3 were identified as *H. bovis*. At the time of treatment of animals with second/third stage *Hypoderma* larvae, the following warble numbers were counted: Study 1, Day 73: 1–34 warbles in 9/10 animals treated, and 2–33 warbles on 8/10 control animals; Study 2, Day 119: 2–37 warbles in 9/10 animals treated, and 4–32 warbles on 10/10 control animals; Study 3, Day 140: 1–12 warbles in 6/9 animals treated, and 1–9 warbles on 6/10 control animals.

Following treatment of groups apparently infected with second/third-stage *Hypoderma* larvae, the number of warbles regressed to zero, within two (Studies 1 and 3) or six weeks (Study 2). *Hypoderma* spp. larvae did not emerge from any of the cattle treated when the larvae were either expected to be first-stage larvae or when the *Hypoderma* spp. larvae were in the second/third stage. Each study was continued until larvae were no longer emerging from the vehicle-treated (control) animals (Study Days 108, 189, or 182 in Studies 1, 2, and 3, respectively.). By the conclusion of each study, 97 live *H. lineatum* larvae and 175 and 30 live *H. bovis* larvae were collected from the vehicle-treated (control) animals in Studies 1, 2 and 3, respectively. No live *Hypoderma* larvae were collected from any treated animal (Table 2).

Cattle treated with Eprinomectin ERI when *Hypoderma* spp. were expected to be at the first or second/third stages had significantly ($p < 0.05$) fewer *H. lineatum* or *H. bovis* larvae emerging and/or expressed than the vehicle-treated (control) animals. The efficacy of Eprinomectin ERI against the larval stages of *H. lineatum* or *H. bovis* was 100% (Table 2).

All animals were reported as normal during hourly observations for 4 h post-treatment, indicating that the treatment (either Eprinomectin ERI or ERI vehicle) was well accepted. There were no drug related health problems or adverse drug events observed at any time during the studies.

4. Discussion

The results presented herein clearly demonstrate that eprinomectin in a ERI formulation is 100% efficacious in the treatment of cattle naturally infested with *H. bovis* and *H. lineatum* larvae at all stages of development. Eprinomectin ERI can, therefore, be used as "prophylactic" treatment for *Hypoderma* spp. infestations, i.e., when there is no external evidence of their presence and before damage to flesh and skin occurs, and as "therapeutic" treatment, e.g., when warbles are already present. Similar results were reported by Holste et al. (1997) who used a 0.5% eprinomectin pour-on treatment at 0.5 mg eprinomectin/kg bodyweight in cattle.

Serodiagnosis of hypodermosis was used to select study animals as it is the only way to detect infested animals well before the appearance of the warbles on the animals; however, there is no correlation between titer level and intensity of infestation (Sinclair et al., 1984; Boulard, 1985; Colwell and Baron, 1990). Although exclusively anti-*Hypoderma* antibody positive animals were enrolled in the current studies, detectable warbles ultimately developed in only 81.4% of the controls and the groups of animals scheduled for late-treatment, where larvae were allowed to develop to the second/third stage prior to treatment. This finding is consistent with the established predictive value of available ELISA's (Colwell and Baron, 1990; Pruett and Kunz, 1996; Colwell, 2001; Colwell and Jacobsen, 2002),

but it may also reflect to a certain degree the high larval mortality in *Hypoderma* spp. infestations as they develop.

In studies using the intraruminal ivermectin sustained-release bolus designed to deliver ivermectin for approximately 130 days, protection from *Hypoderma* spp. infestation has been demonstrated in cattle for the whole ivermectin release period during the season of warble fly activity (Freedom of Information Summary, IVOMEC®[1] SR Bolus for Cattle; http://www.fda.gov/cvm/FOI/140-988.pdf).

In conclusion, based on the efficacy profile of Eprinomectin ERI which provides effective control of nematode infections in cattle for up to 150 days (Rehbein et al., 2013; Soll et al., 2013) and the exquisite sensitivity of *Hypoderma* spp. to the avermectins in general (Boulard, 1999; Vercruysse and Rew, 2002), it can be assumed that Eprinomectin ERI treatment during the period when the adult flies are ovipositing on cattle will also provide protection to cattle from *Hypoderma* spp. larval infestation.

Conflict of interest

The work reported herein was funded by Merial Limited, GA, USA.

All authors were/are current employees (S.R. and J.H.) or contractors (L.S. and J.L.) of Merial and assisted with the study design, conduct, data analysis and review of the manuscript.

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