

Systemic Activity of the Avermectins Against the Cat Flea (Siphonaptera: Pulicidae)

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ABSTRACT Ivermectin has potent systemic activity against numerous species of nematodes and arthropods, but there are some important species in these two groups, such as the cat flea, *Ctenocephalides felis* (Bouché), that appear to be refractory to it. In an effort to determine if the lack of systemic activity against *C. felis* is specific to ivermectin, or if it is a class-wide phenomenon, 20 avermectin derivatives were tested in an artificial membrane flea feeding system at concentrations of 20, 10, and 1 $\mu\text{g}/\text{ml}$. Results showed that ivermectin had LC_{90} and LC_{50} values against fleas of 19.1 and 9.9 $\mu\text{g}/\text{ml}$, respectively. Only four of the other 19 compounds evaluated possessed both LC_{90} and LC_{50} values more potent than ivermectin and even then the advantage was modest. Among those four compounds was a two-fold increase in potency relative to ivermectin when the LC_{90} values were considered (range, 9.2–10.3 $\mu\text{g}/\text{ml}$) and a two- to eight-fold increase when the LC_{50} values were examined (range, 1.23–5.26 $\mu\text{g}/\text{ml}$). Neither the possession nor the number of oleandrosyl sugars on the macrocyclic backbone were relevant for additional flea activity because among these four compounds were two disaccharides, a monosaccharide and an aglycone. Also, bond disposition between C-22 and 23 did not contribute to increase in activity because these molecules comprise members with either single or double bonds. One of these avermectin analogs was scaled-up and tested subcutaneously in a dog at >100 times the commercial ivermectin dosage and zero efficacy was observed against the flea. We conclude that even the best in vitro avermectin does not have the in vivo potential to become a commercial oral or subcutaneous flea treatment for companion animals.

KEY WORDS systemic, flea, control, avermectin, membrane feeding

THE AVERMECTIN CLASS of endectocides has potent systemic activity against numerous species of nematodes and arthropods (Egerton et al. 1979, 1980). Particularly striking, for example, are the near absolute efficacies against helminths such as immature heartworm, *Dirofilaria immitis*, in dogs at 6.0 $\mu\text{g}/\text{kg}$ (Campbell 1989) and against insects such as the larvae of the common cattle grub, *Hypoderma lineatum* (Villers), in cattle at 0.2 $\mu\text{g}/\text{kg}$ (Drummond 1984). However, despite this tremendous potency, there are other organisms within these groups that appear to be refractory to ivermectin. The cat flea, *Ctenocephalides felis* (Bouché), is a clinically relevant example. Ivermectin was orally administered weekly at 0.5 mg/kg or daily at 0.05 mg/kg and observed to be inactive against this parasite on dogs (Blair et al. 1984). Banks et al. (2000) and Shoop et al. (2001) corroborated independently those results by showing that ivermectin has weak systemic activity against the cat flea in artificial membrane flea feeding assays.

In an effort to determine if the lack of systemic activity against fleas is specific to ivermectin, or if it is a class-wide phenomenon, 20 avermectins were tested in an artificial membrane flea feeding system. The

strategically select series of avermectins tested contained representatives of most of the chemically accessible sites that have been exploited around the macrocycle. The group comprises all of the naturally occurring avermectins, as well as semisynthetic members of the biologically important aglycone, monosaccharide and disaccharide series. The commercialized compounds abamectin, ivermectin, milbemycin D, and selamectin were also included. In this article we present the relative potencies of these members of the avermectin family against fleas through testing in an artificial membrane system and show in vivo efficacy results from a dog dosed subcutaneously with one of the most potent avermectins tested.

Materials and Methods

The Greyhound. The artificial membrane flea feeding system we used is a modification of the "artificial dog" manufactured by Jay R. Georgi (FleaData, Freeville, NY). This artificial membrane system was designed to rear fleas, but it was also suggested that it could test the effects of systemic insecticides (Wade and Georgi 1988 and Pullen and Meola 1996) and it has been used to discover the novel indole terpene, nodulisporic acid A (Shoop et al. 2001). In a collaboration with Jay R. Georgi we modified the artificial dog. This new system (Fig. 1) was given the designation "Grey-

The use of cats for rearing fleas follows an approved protocol that is on file with the Merck Institutional Animal Care and Use Committee.

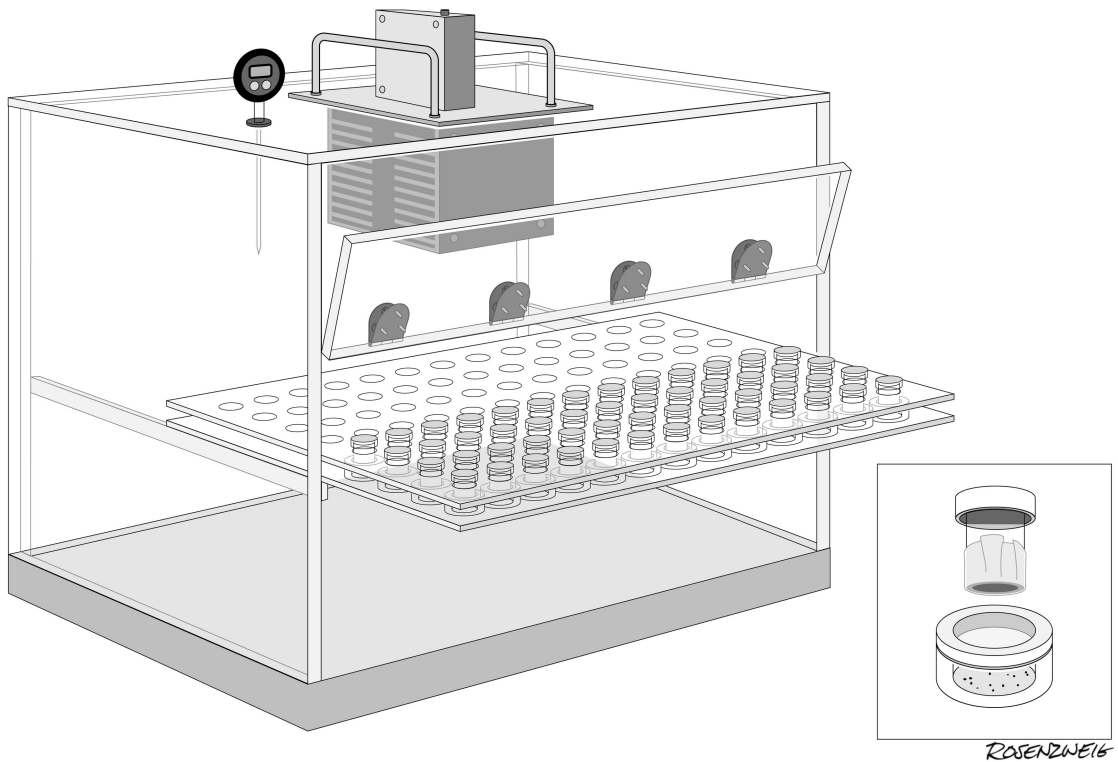


Fig. 1. Artificial membrane feeding system used to test systemic flea efficacy.

hound" because it was designed to be more efficient, easier to set up, and allowed larger numbers of compounds to be tested simultaneously. Unlike the artificial dog, which contains only 25, 5-cm cages suspended individually below a heated Plexiglas enclosure, the new system contains a removable 59 by 38-cm manifold holding 104, 2.5-cm cages. We also replaced the aluminum nondisposable feeding sleeves with plastic CVC sleeves (Costar, Cambridge, MA). The plastic sleeves were disposed after each use to minimize the likelihood of contamination by drugs.

Flea Rearing. Our flea colony was maintained on cats housed according to our Institutional Animal Care and Use Committee. Eggs collected from cats were incubated at 28°C and 85% RH in a medium consisting of eight parts sand and one part freeze dried bovine blood (California Spray Dry Company, Stockton, CA). Fleas used in this study had emerged from their puparia within 48 h.

Compound Preparation. Avermectins were tested at concentrations of 20, 10, and 1 µg/ml. Two replications of each compound at each level were tested in a side-by-side comparison using two of our feeding systems. The vehicle used was polyethylene glycol 400 and dimethyl sulfoxide (2:1). Ten microliters of vehicle per milliliter of heparinized bovine blood was used. All compounds were fermented or synthetically modified by Merck chemists except for selamectin.

Preliminary purification of selamectin proceeded as follows. Six ampules of Revolution (240 mg each)

were diluted in minimal volumes of CH₂Cl₂ and loaded onto a pad of silica gel (4 inches high). Gradient elution [hexanes (500 ml), then 11 EtOAc/hexanes (500 ml) followed by 95/5 MeOH/2 M NH₄OH (1 liter) yielded partially purified selamectin. The fractions containing partially purified selamectin were pooled, concentrated under reduced pressure, and further purified by repeating the protocol described above. Final purification to homogeneity was accomplished by preparative TLC (1,000 µm plates) using 97/3 MeOH/2 M NH₄OH as eluant.

Drug Testing. Twenty-five adult fleas were placed into each of the individual cages, the cages were placed in the removable manifold, and feeding sleeves with untreated bovine blood were added. The manifold was slid into the Plexiglas feeder and fleas were incubated at 28°C and 85% RH, and blood was maintained at 40°C. Untreated blood was fed to fleas for the first 48 h to acclimate them. Blood sleeves were removed and new sleeves with fresh blood were added every 24 h for both the 48-h prefeed and the subsequent 48-h drug exposure periods. Four cages in each box were used as vehicle treated controls. At the completion of the feeding cycles, cages were opened and live and dead fleas were separated using a flea separator (Wade and Georgi 1988). Mortality was assessed and efficacy determined using Abbott's equation (1925). LD₉₀ and LD₅₀ values were calculated using linear regression.

Dog Study. Two beagles (10 and 11.5 kg) were prebled and one was chosen at random to be dosed with the avermectin monosaccharide 4',4a-bis-(O-methoxyethoxymethyl)-22,23-dihydro Avm B₁ at 1 mg/kg subcutaneously. At 24 h postinoculation, blood samples were taken from each dog, after which both dogs were infested with 100 unfed adult fleas. The blood samples of the treated and control dogs were evaluated *in vitro* in the artificial membrane feeding system. Seventy-two hours after dosing, *in vivo* efficacy was also determined on the dogs by an 8-min comb-count (Gregory et al. 1995).

Results

Artificial Membrane Feeding. Compounds evaluated in the artificial membrane flea feeding system had representative bond or atom changes at virtually all of the chemically accessible sites of the avermectin/milbemycin pharmacophore including C-4a, C-5, C-10, C-11, C-13, C-22,23, C-25, C-4', and C-4''.

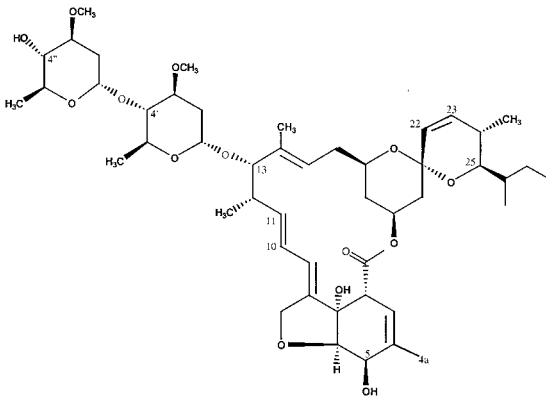


Table 1 shows that ivermectin (compound 17) had 88, 64, and 4% efficacy at the 20, 10, and 1 $\mu\text{g}/\text{ml}$ concentrations, respectively; and LC_{50} and LC_{90} values of 9.9 and 19.1 $\mu\text{g}/\text{ml}$, respectively, were calculated from the dose-response curve. The most closely related structural analog to ivermectin was avermectin B₂ (compound 11). Avermectin B₂ differs from ivermectin only in the presence of an axial hydroxy at C-22 and their efficacies are strikingly similar. Avermectin B₂ had 86, 61, and 0% efficacy at the 20, 10, and 1 $\mu\text{g}/\text{ml}$ concentrations, respectively; and LC_{50} and LC_{90} values of 10.5 and 19.4 $\mu\text{g}/\text{ml}$, respectively.

The LC_{90} 's of four of the other 18 avermectin compounds tested indicate that they were more potent than ivermectin. These four compounds consist of an aglycone (compound 3), a monosaccharide (compound 6), and two disaccharides (compounds 10 and 12) with LC_{90} values of 9.2, 10.3, 10.1, and 10.2 $\mu\text{g}/\text{ml}$, respectively. It is important to note that, with respect to LC_{90} values, these four compounds enjoyed only a two-fold advantage over ivermectin. These data also indicate that neither the possession nor number of

sugars on the macrocyclic backbone were important for flea activity. Likewise, bond disposition between C-22 and 23 contributed nothing to increase in activity because two of these molecules (compounds 3 and 6) are saturated and possess a single bond between C-22 and 23 and two (compounds 10 and 12) are unsaturated and have a double bond.

Two of these four compounds (compounds 10 and 12) also showed a two-fold advantage when the LC_{50} values were compared with ivermectin. However, compounds 3 and 6 showed slightly more potent LC_{50} values of five- and eight-fold increases, respectively, over ivermectin, reflecting a different slope in their dose-response curves.

Two molecules, selamectin (compound 7) and the naturally occurring avermectin A₂ (compound 9), possessed LC_{90} values similar to ivermectin, but the slope of their response curve was steeper than that of ivermectin and their LC_{50} values were the lowest among the 20 compounds evaluated. Both of these molecules differ from most members of the avermectin B subfamily at C-5 in that they possess a ketoxime (=NOH) in the case of selamectin and a methoxy (OCH₃) for avermectin A₂. However, possession of a ketoxime or methoxy alone cannot be responsible for the increase in activity because neither compound 20 nor compound 8, each of which possess one of the substituents, showed activity superior to ivermectin.

Flea Efficacy Study with Dogs. There was zero efficacy observed against fleas *in vitro* when the blood taken from the dog treated with compound 6 was fed to fleas in the artificial membrane feeding system. *In vivo* results also showed zero efficacy when fleas placed on the dog 24 h after dosing were evaluated relative to a control dog 48 h later. The 1 mg/kg dose of compound 6 given to the treated dog was >100 times the commercial ivermectin dosage of 6.0 $\mu\text{g}/\text{kg}$. This dose was given subcutaneously to maximize bioavailability of the drug.

Discussion

To date, no avermectin/milbemycin derivative has been shown to have useful oral or subcutaneous activity in dogs against fleas and the systematic examination of this chemical family herein supports this. As was noted previously, ivermectin has poor oral activity against the cat flea both *in vivo* (Blair et al. 1984) and *in vitro* (Banks et al. 2000, Shoop et al. 2001). The data contained in this article indicate that despite exploiting most of the chemically accessible sites using a variety of substituents, little gain in potency over ivermectin was observed. This point was made dramatically when compound 6, one of the most potent of the group, was tested in the dog at >100 times the commercial ivermectin dosage and no efficacy was observed against the flea.

The range of potencies observed in this study against fleas was minimal when compared with the range of activities found against other parasites for which the avermectins have had commercial success. For example, Michael et al. (2001) observed an in-

Table 1. Efficacy of avermectin analogs fed to fleas in an artificial membrane feeding system

Compound no.	Subfamily	Chemical name	Mean efficacy, %			LC ₉₀ ^a	LC ₅₀ ^a
			20 ^a	10 ^a	1 ^a		
1	Milbemycin	13-deoxy-22,23-dihydro Avm B ₁ (milbemycin D)	87	48	46	24.05	5.78
2	Aglycone	13-deoxy-13- <i>epi</i> -fluoro Avm B ₁	94	46	0	19.11	11.03
3		13-deoxy-13- <i>epi</i> -fluoro-22,23-dihydro Avm B ₁	97	94	45	9.24	1.89
4		13-O-methoxymethyl Avm B ₁	10	0	16	>20.0	>20.0
5		13- <i>epi</i> -O-methoxymethyl Avm B ₁	67	35	0	26.07	14.78
6	Monosaccharide	4',4a-bis(O-methoxyethoxymethyl)-22,23-dihydro Avm B ₁	93	89	49	10.34	1.23
7		25-cyclohexyl-25-des-2-butyl-22,23-dihydro-5-ketoximino Avm B ₁ monosaccharide (selamectin)	89	74	63	21.03	<1.0
8	Disaccharide	Avermectin A ₁	52	8	0	35.61	21.17
9		Avermectin A ₂	86	69	64	25.19	<1.0
10		Avermectin B ₁ (abamectin)	94	89	23	10.08	4.67
11		Avermectin B ₂	86	61	0	19.41	10.52
12		4"-methyl-5-ketoximino Avm B ₁	90	89	15	10.19	5.26
13		4a-hydroxy Avm B ₁	94	58	15	18.57	8.95
14		4a-hydroxy-22,23-dihydro Avm B ₁	76	65	0	21.21	11.11
15		4"- <i>epi</i> -formylamino-4"-desoxy-22,23-dihydro Avm B ₁	82	62	24	21.45	8.33
16		4"-cyanomethyl Avm B ₁	64	50	19	29.52	12.67
17		22,23-dihydro Avm B ₁ (ivermectin)	88	64	4	19.07	9.91
18		4"- <i>epi</i> -methoxyacetylamino-4"-desoxy-22,23-dihydro Avm B ₁	75	33	32	28.94	11.82
19		10-methoxy-10,11-dihydro Avm B ₁	77	53	0	21.96	11.98
20		4"- <i>epi</i> -acetylamino-4"-desoxy-22,23-dihydro-5-ketoximino Avm B ₁	77	72	6	20.73	9.90

^a µg/ml.

crease in range of potency >10,000-fold against *Haemonchus contortus* when 14 different avermectins were evaluated. In addition, they noted clear structure/activity patterns among the avermectins, which could guide a medicinal chemistry effort toward an optimized compound. We observed only a two-fold advantage in potency against fleas when the LC₉₀ was considered and an eight-fold advantage when the LC₅₀ was examined. Moreover, unlike the avermectins against *H. contortus*, we were unable to identify any clear structure/activity trends that would guide a chemistry program for control of the cat flea. In this context, it appears unlikely that an avermectin derivative could be successfully developed for orally or subcutaneously administered flea control when ivermectin has little or no such activity and careful evaluation of its chemical class revealed such a narrow range of potency between it and the best analog.

Banks et al. (2000) reported the results of their evaluation of avermectin derivatives in an artificial membrane flea feeding apparatus. They set as their threshold for significance a minimum of 30% mortality after a 24-h feeding period on blood containing a 0.25 µg/ml concentration of various avermectins and concluded that only monosaccharide C-5 oximes derivatives met the criterion. Specifically, the compound they found most interesting was the analog designated compound 7 in our test. Our data indicate that the monosaccharide derivatives did perform among the best compounds tested, but were not unique in this capacity. We used more traditional readouts for potency such as LC₅₀ and LC₉₀ values. Irrespective of which of these values one might consider more important it would be difficult to argue that the monosaccharide compound 7 was substantively more potent

than the milbemycin compound 1, the aglycone compound 3, or the disaccharide compound 9.

Identification of an avermectin with extended duration of flea activity following oral or subcutaneous administration would be a remarkable achievement in flea control. To better appreciate the daunting task of flea control through oral administration, let us accept that the ivermectin LC₉₀ of 19 µg/ml identified in this study is the blood concentration necessary in the dog to eliminate 90% of the fleas at the end of a 24-h period. Also consider that an ivermectin 100 µg/kg oral dose will produce a blood C_{max} of 40 ng/ml in dogs (Pivnichny et al. 1983). One can then calculate the amount of ivermectin the dog has to be given orally to achieve the requisite LC₉₀ concentration in the blood at the end of a 24-h period. To attain that blood concentration it would require a 47.5 mg/kg oral dose. It is now clear why the 0.5 mg/kg oral dose of ivermectin given by Blair et al. (1984) did not produce any efficacy in the dog and is likely the same reason why our 1 mg/kg subcutaneous dose of compound 6 failed.

Sustained flea activity following oral administration would depend on many factors, including absorption, metabolism, potency, and half-life. Herein we have dealt only with inherent potency. For example, if one was aiming to identify a compound with month-long flea activity following oral administration, then half-life of the molecule in relation to potency in the dog would be critical factors. Ivermectin in dogs has a known half-life of 1.6 d (Kojima et al. 1987). After accounting for 17 half-lives through the course of a 28-d month, more than 3 kg of ivermectin per kilogram of dog bodyweight would have to be given orally to maintain the LC₉₀ concentration in the blood at the end of the month (assuming linear pharmacokinetics).

This absurd figure illustrates how distant ivermectin is to becoming a monthly, systemic flea control product in dogs and how far a new avermectin/milbemycin analog would have to advance.

In conclusion, modification of an existing artificial membrane flea feeding system enabled us to determine the relative potencies of avermectin analogs representing all four naturally occurring avermectin sub-families as well as many of the chemically most accessible sites simultaneously, reliably, and efficiently. By testing these series, we found the monosaccharide (compound 6) to possess superior LC₉₀ and LC₅₀ values to ivermectin and evaluated its likely candidacy for systemic flea efficacy in dogs. The lack of any activity obtained from the flea efficacy study with dogs, however, suggests that even the best in vitro avermectin tested in our study does not appear to have the in vivo potential to become a commercial oral or subcutaneous flea treatment for companion animals.

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References Cited

- Abbott, W. S. 1925. A method for computing the effectiveness of an insecticide. *J. Econ. Entomol.* 18: 265–267.
- Banks, B. J., B. F. Bishop, N. A. Evans, S. P. Gibson, A. C. Goudie, K.A.F. Gration, M. S., Pacey, D. A. Perry, and M. J. Witty. 2000. Avermectins and flea control: structure-activity relationships and the selection of selamectin for development as an endectocide for companion animals. *Bioorg. Med. Chem.* 8: 2017–2025.
- Blair, L. S., J. R. Egerton, and D. E. Ewanciw. 1984. *Ctenocephalides felis*: failure of ivermectin given per os in miglyol vehicle to control experimental flea infestations of dogs in a double blind controlled trial, p. 14. *In* Proceedings, American Association of Veterinary Parasitologists, 15–17 July 1984, New Orleans, LA.
- Campbell, W. C. 1989. Use of ivermectin in dogs and cats, pp. 246–250. *In* W. C. Campbell [ed.], ivermectin and abamectin. Springer, New York.
- Drummond, R. O. 1984. Control of larvae of the common cattle grub (Diptera: Oestridae) with animal systemic insecticides. *J. Econ. Entomol.* 77: 402–406.
- Egerton, J. R., D. A. Ostlind, L. S. Blair, C. H. Eary, D. Suhayda, S. Cifelli, R. F. Riek, and W. C. Campbell. 1979. Avermectins, new family of potent anthelmintic agents: efficacy of the B_{1a} component. *Antimicrob. Agents Chemother.* 15: 372–378.
- Egerton, J. R., J. Birnbaum, L. S. Blair, J. C. Chabala, J. Conroy, M. H. Fisher, H. Mrozik, D. A. Ostlind, C. A. Wilkins, and W. C. Campbell. 1980. 22,23-Dihydroavermectin B_{1a}, a new broad-spectrum antiparasitic agent. *Br. Vet. J.* 136: 88–97.
- Gregory, L. M., M. Zakson, R. G. Endris, and W. L. Shoop. 1995. A further comparison of the thumb-counting and comb-counting techniques used to determine *Ctenocephalides felis* infestation levels on dogs. *Vet. Parasitol.* 56: 349–352.
- Kojima, K., K. Yamamoto, and Y. Nakanishi. 1987. Determination of 22,23-dihydroavermectin B_{1a} in dog plasma using solid-phase extraction and high-performance liquid chromatography. *J. Chromatogr.* 413: 326–331.
- Michael, B., P. T. Meinke, and W. Shoop. 2001. Comparison of ivermectin, doramectin, selamectin and 11 intermediates in a nematode larval development assay. *J. Parasitol.* (in press).
- Pivnichny, J. V., J.-S.K. Shim, L. A. Zimmerman. 1983. Direct determination of avermectins in plasma at nanogram levels by high-performance liquid chromatography. *J. Pharm. Sci.* 72: 1447–1450.
- Pullen, S. R., and R. W. Meola. 1996. Use of an artificial membrane system for feeding the cat flea (*Ctenocephalides felis*). *Lab. Anim.* May 1996: 39–40.
- Shoop, W. L., L. M. Gregory, M. Zakson-Aiken, B. F. Michael, H. W. Haines, J. G. Ondeyka, P. T. Meinke, and D. M. Schmatz. 2001. Systemic efficacy of nodulisporic acid against fleas on dogs. *J. Parasitol.* (in press).
- Wade, S. E., and J. R. Georgi. 1988. Survival and reproduction of artificially fed cat fleas, *Ctenocephalides felis* Bouché (Siphonaptera: Pulicidae). *J. Med. Entomol.* 25: 186–190.

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