Determining a Diagnostic Dose for Imidacloprid Susceptibility Testing of Field-Collected Isolates of Cat Fleas (Siphonaptera: Pulicidae)

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ABSTRACT The susceptibility of four laboratory strains of cat fleas, *Ctenocephalides felis* (Bouché), to imidacloprid was determined by three different laboratories, by using a standardized bioassay protocol. The probit lines generated by the different laboratories were very similar, with LC_{50} values ranging from 0.32 to 0.81 ppm. Based on these data, a diagnostic dose (DD) of 3 ppm imidacloprid in larval rearing media was provisionally identified for detecting shifts in tolerance, possibly as a consequence of incipient imidacloprid resistance. None of the larvae from the susceptible laboratory strains survived the DD. Eighteen field-collected isolates were evaluated for their susceptibility to imidacloprid and to validate a DD of 3 ppm. Probit lines from 18 field-collected isolates were very similar, with LC_{50} values ranging from 0.14 to 1.52 ppm. When exposed to the DD, between 3 and 10% of the exposed larvae emerged as adults from only three of the 18 isolates. All other field isolates gave 100% mortality at the DD. Under the criteria established (>5% survivorship at 3 ppm), two isolates would be established on mammalian hosts and more extensive tests conducted to exclude or confirm the presence of resistance. The DD of 3 ppm is robust enough to eliminate most of the susceptible isolates collected until today, yet low enough to identify possible isolates for further testing.

KEY WORDS cat fleas, *Ctenocephalides felis*, insecticide resistance, monitoring, imidacloprid

ADVANCES IN TOPICAL AND SYSTEMIC therapy for cat flea control have revolutionized clinical practices (Gortel 1997). Strategies to delay the development of insecticide resistance and conserve these new active ingredients that have proved so valuable in veterinary practice for controlling cat fleas are needed. Monitoring the susceptibility of field-collected populations of fleas is an important first step in that process. Moyses and Gfeller (2001) proposed a method of topically applying insecticides to adult fleas and provided baseline information for several strains. Even though the technique is extremely sensitive, large populations of adult fleas (140–850 individuals) are needed. A larval bioassay was developed to monitor imidacloprid susceptibility that eliminated the need to maintain field strains on laboratory hosts or artificial feeding systems (Rust et al. 2002). Advantages of the larval bioassay are that it does not require the laboratory maintenance of field-collected cat flea isolates and that as few as 40 eggs are used.

Insecticide resistance in cat fleas, *Ctenocephalides felis* (Bouché), has been reported for a number of organophosphate and pyrethroid insecticides as reviewed by Bossard et al. (1998) and Krämer and Mencke (2001). Bardt and Schein (1996) reported that a field-collected strain ("Cottontail") exhibited resistance to hexachlorocyclohexane, carbamates, phosphoric acid esters, rotenone, synergized pyrethrin, pyrethroids, and lufenuron. It showed some decreased susceptibility to fipronil and no change in sensitivity to imidacloprid and most of the insect growth regulators, except lufenuron.

To date, no resistance has been reported to imidacloprid in cat fleas. However, imidacloprid resistance

This study was conducted according to the Guide for the Care and Use of Laboratory Animals promulgated by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council, 1996, and protocols adopted by each institution.

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Bayer Corporation Flea Susceptibility Monitoring Project: Flea Egg Collection Questionnaire

To be completed by Veterinarian or Veterinary Technician **IMPORTANT: PLEASE COMPLETE THIS ENTIRE FORM**

Date_____ Initials of Person Completing
Questionnaire:_____

Clinic Information:
Veterinary Hospital/Clinic
Name:_____
City:_____
State:_____
Phone:_____
Fax:_____

Client Information:

Client's
Name:_____

Household Information:

How many **other** pets are in the household?
Cats_____ Dogs_____
Others
(Specify)_____

Have these other pets been treated for flea infestation in the last year?
1-2 mos. 2-6mos. 6-12 mos. Never
Unknown

Were any premise treatments used in this household? Y N Unknown

Method of household treatment
and product :_____
Unknown_____

Is this a recurring problem or first time problem with fleas on pets in this home?
Recurring_____
First time_____

Pet Collection/Treatment Information:

Pet's Name:_____ Dog___ Cat___
Breed_____ Age_____ Sex____

Level of Flea Infestation: Low(0-20) Medium(20-50)
High(>50) Unknown

How long was animal caged for the flea egg collection?_____

How many hours per day does pet spend indoors?_____Don't Know_____

When was this pet last treated for fleas?: 1-2 mos 2-6 mos
6-12 mos Unknown

List product last used:_____
Unknown_____

Was Treatment Successful? Yes No

Fig. 1. Questionnaire sent to each participating clinic to accompany each egg collection.

has been confirmed in other insect pests of plants (Nauen and Denholm 2005), highlighting the importance of establishing a proactive flea monitoring program (Schroeder et al. 2003). The objective of this study was to establish a diagnostic dose (DD) of imidacloprid to test against field-collected strains and to eliminate the need to establish such isolates on laboratory hosts. Using a larval bioassay (Rust et al. 2002), four laboratory strains of *C. felis* were each tested by three different laboratories to ensure the consistency of results and to identify a provisional DD for monitoring purposes. Each laboratory then tested six field-collected isolates to confirm the relevance of the DD to contemporary flea populations. The potential use of this monitoring procedure in both research and clinical practice is discussed.

Materials and Methods

Laboratory Strains. Four laboratory strains of cat flea, *Ctenocephalides felis* (Bouché) (UCR, University of California, Riverside; KSU, Kansas State University; AUB, Auburn University; and MON, Bayer Animal Health Laboratories in Monheim, Germany) were maintained on separate cats with a standard rearing procedure adopted by each laboratory (Rust et al. 2002). None of the laboratory strains are known to have been exposed to insecticides and probably represent susceptible populations. Research laboratories at the University of California, Riverside; Auburn University, Auburn, AL; and Kansas State University, Manhattan, KS, reared and maintained each of the four laboratory flea strains on separate cats.

Field-Collected Isolates. Veterinary clinics throughout the United States were recruited to collect and ship flea eggs to one of the designated research laboratories. Each clinic was provided a kit and instructions on how to collect and ship cat flea eggs from infested pets. The shipping kit consisted of a Styrofoam cooler (928 by 23 by 25 cm), ice pack (454 g), two sheets of standard newsprint (4.6 by 6.1 cm), ≈300 g of polyester fiber quilt batting, two cotton gauze pads (7.6 by 7.6 cm), one roll of 5.1-cm packing tape, one glass tube containing flea rearing media, and a questionnaire. The questionnaire requested information concerning the pet, treatment history, and other pets in the household (Fig. 1).

Table 1. Probit analyses of the four susceptible laboratory strains tested at the three different research laboratories

Strain	Laboratory	*n*	Slope ± SE	LC_{50} (95% CI)	LC_{95} (95% CI)
UCR	KSU	339	4.57 ± 0.85	0.59 (0.48–0.71)	1.35 (1.06–2.07)
	AUB	397	9.43 ± 2.21	0.32 (0.28–0.35)	0.47 (0.41–0.65)
	UCR	561	3.70 ± 0.58	0.36 (0.24–0.44)	0.99 (0.76–1.78)
MON	KSU	349	4.07 ± 0.85	0.64 (0.46–0.79)	1.61 (1.24–2.73)
	AUB	357	7.58 ± 1.83	0.35 (0.29–0.41)	0.58 (0.49–0.86)
	UCR	364	5.21 ± 0.97	0.39 (0.27–0.48)	0.80 (0.62–1.58)
KSU	KSU	1,208	4.34 ± 0.62	0.73 (0.57–0.87)	1.75 (1.40–2.71)
	AUB	452	6.37 ± 1.24	0.45 (0.38–0.51)	0.81 (0.69–1.14)
	UCR	595	2.78 ± 0.33	0.46 (0.29–0.61)	1.77 (1.22–3.90)
AUB	KSU	1,301	6.01 ± 1.34	0.81 (0.61–0.93)	1.51 (1.26–2.36)
	AUB	429	5.28 ± 0.95	0.47 (0.39–0.55)	0.97 (0.80–1.38)
	UCR	274	4.98 ± 1.13	0.70 (0.51–0.82)	1.50 (1.21–2.37)

To collect flea eggs, the blank newsprint was placed on a table or floor and a stainless steel grate was set on the paper. An animal cage with an open grating floor and pet infested with fleas was put over the paper and grate. Food, water, and litter pan, especially for cats, were provided as needed. After 4–24 h, the pet was gently brushed or combed to dislodge the eggs, and the newsprint was examined for their presence. The debris and eggs were gently brushed to the center of the paper and the number of eggs was counted with the hand lens when possible. The debris and eggs were poured onto the sieve and funnel, and the eggs were collected into the glass tube. The tube was covered with a piece of Whatman filter paper and sealed with the white tape.

The Styrofoam cooler was packed in several layers of materials to insulate the flea eggs. First, a 2-cm layer of polyester fiber batting was placed in the cooler and a frozen ice pack was placed on top. Approximately 4 cm of batting was placed on top of the ice pack and covered with two sheets of newsprint. The glass tube with flea eggs was placed on the newsprint and covered with polyester batting. Two gauze pads moistened with water were placed on top of the batting, and the container was sealed and taped. The Sytrofoam container was shipped overnight to one of the three laboratories.

The flea eggs were placed in additional UCR larval rearing medium and held at 80% RH and 26 ± 2°C (Rust et al. 2002). Larval medium was passed through a 16-mesh screen at day 12 to remove the cocoons. Adults that emerged between day 16 and 18 were lightly anesthetized with CO_2 and ≈30 adult males and females were placed on each cat. Two cats were used as hosts for each field-collected isolate.

Larval Bioassays. Each laboratory determined the activity of imidacloprid against the susceptible laboratory strains and field-collected isolates of larval cat fleas according to the protocol reported by Rust et al. (2002). Larval rearing medium was treated with technical imidacloprid to provide the following concentrations: 30, 15, 10, 5, 3, 1, 0.5, 0.1, and 0.05 ppm. Treated medium was placed into glass petri dishes (5 cm in diameter by 1.5 cm). To determine the number of flea eggs that hatched, 20 eggs were cemented to the upper inner surface of the petri dish. A thin streak of glue (UHUStic, Saunders, Winthrop, ME) was applied to the glass with a moistened paint brush. Eggs were carefully placed in the petri dish lid and rolled on to the tacky surface with a fine camel's-hair brush (size 0000). Once the glue dried, the eggs remained attached to the petri dish lid. As the eggs hatched, the larvae fell into the medium. The glass petri dishes and flea eggs were placed in incubators in each laboratory that were maintained at 26 ± 2°C and 80% RH. A minimum of three replicates was tested for each concentration.

The medium and cocoons were passed through a 16-mesh screen at day 12. The cocoons were placed in a plastic snap cap vial (2.5 cm in diameter by 4.5 cm), and a disk of Whatman filter paper (5.5 cm in diameter) was placed over the top and secured with a snap cap lid rim. The vials and cocoons were returned to a chamber maintained at 26 ± 2°C and 80% RH. The number of adults that emerged or developed in the cocoons was counted at day 28. The adult emergence data were analyzed by probit analysis (Robertson and Preisler 1992) by using the POLO program (LeOra Software, Menlo Park, CA).

Results

The four laboratory strains UCR, MON, KSU, and AUB gave very similar LC_{50} and LC_{95} values within each laboratory, and results for each strain between laboratories also were consistent (Table 1). For example, the LC_{50} values ranged from 0.59 to 0.81 ppm in the KSU laboratory, from 0.32 to 0.47 ppm in the Auburn laboratory, and from 0.36 to 0.70 ppm in the UCR laboratory. Within a strain, the greatest difference between laboratories was for the MON strain between the KSU and AUB laboratories, resulting in a 1.94-fold difference at LC_{50}. The slopes were parallel in most cases, and LC_{95} values also were comparable within and between laboratories. The LC_{95} values ranged from 0.47 to 1.77 for all strains and laboratories. No flea larvae survived exposure to 3.0 ppm. The average (±SD) LC_{50} and LC_{95} values for all strains and laboratory tests were 0.52 ± 0.169 and 1.16 ± 0.447 ppm, respectively.

Flea strains were collected from six different states beginning in June 2000 (Table 2). Interestingly, most strains were collected in late summer and early fall during September and October. Eight of the 18 strains

Table 2. Field-collected isolates of cat fleas tested for susceptibility to imidacloprid

Strain	Locality	Collected	Host	Treatment history[a]	Last treated
B01	Columbia, MO	26 Sept. 2000	Cat	Pet store drops	1–2 mo
B02	Ponchatoula, LA	27 Sept. 2000	Dog	Advantage	1–2 mo
B03	Virginia Beach, VA	11 Oct. 2000	Dog	Adams spray	6–12 mo
B05	Ponchatoula, LA	23 Oct. 2000	Dog	Flea collar/dip	2–6 mo
B07	Columbia, MO	25 Oct. 2000	Cat	Pet store drops	1–2 mo
B08	Kirksville, MO	30 Oct. 2000	Dog	Advantage	6–12 mo
D01	Milton, NH	5 Sept. 2000	Dog	Advantage	6–12 mo
D02	Columbia, MO	18 Sept. 2000	Cat	Advantage	6–12 mo
D03	Ponchatoula, LA	2 Oct. 2000	Dog	Advantage	1–2 mo
D04	Kirksville, MO	3 Oct. 2000	Dog	Yard spray	1–2 mo
D05	Virginia Beach, VA	5 Oct. 2000	Cat	Hartz	6–12 mo
D06	Boonville, MO	10 Oct. 2000	Cat	Flea dip	1–2 mo
R01	Gainesville, FL	7 June 2000	Dog	None	
R02	Gainesville, FL	8 June 2000	Dog	Hartz top spot	1–2 mo
R04	Gainesville, FL	22 June 2000	Dog	Adams flea shampoo, Frontline spray	1–2 mo
R06	Riverside, CA	27 Sept. 2000	Cat	Flea collar	6–12 mo
R07	Mountain Grove, MO	2 Oct. 2000	Cat	Hartz flea spray	1–2 mo
R08	Virginia Beach, VA	18 Oct. 2000	Cat	None	

[a] Advantage (imidacloprid); Frontline (fipronil).

were collected from cats and 10 from dogs. Only two of the 18 field-collected isolates did not have a treatment history within the previous year. Imidacloprid (Advantage) was reportedly used on five of the pets. A variety of other products was used for which only a few descriptions were specific enough to identify the active ingredient.

The 18 field-collected isolates (each tested in one laboratory only) gave LC_{50} and LC_{95} values that ranged from 0.14 to 1.52 and from 0.92 to 5.55 ppm, respectively (Table 3). The probit lines of all the field-collected strains overlapped extensively (Fig. 2). Although many calculated probit lines encompassed the proposed DD of 3 ppm, only three isolates produced adults emerging from larval media treated with this concentration (Table 4).

Discussion

Imidacloprid is a neonicotinoid insecticide that acts on the insect central nervous system as an agonist of the postsynaptic nicotinic acetylcholine receptors (Bai et al. 1991, Liu and Casida 1993). When applied as a spot treatment on the pelage of cats or dogs (Advantage), imidacloprid provides nearly 100% flea control for 4 wk (Jacobs et al. 1997, Dryden et al. 1999). Since its introduction into the United States in 1996, there have been no published reports of documented cases of fleas developing resistance to imidacloprid. Developing a reliable and cost-effective bioassay methodology is the first phase of an extensive survey to monitor the sensitivity of field-collected isolates of cat fleas to imidacloprid. This is a proactive approach to conserving this important chemistry as an effective therapeutic agent to control cat fleas.

The development of a sensitivity monitoring program requires accurate information on the baseline response of susceptible individuals, and on the consistency of this response between sites and over time. If more than one laboratory is to be involved in the program, ensuring standardization of techniques and the repeatability of results between laboratories is also

Table 3. Probit analyses of the field-collected isolates tested at the three research laboratories

Laboratory	Strain	n	Slope ± SE	LC_{50} (95% CI)[a]	LC_{95} (95% CI)[a]
AUB	B01	383	3.56 ± 0.46	0.73 (0.57–0.90)	2.13 (1.61–3.51)
	B02	686	3.91 ± 0.55	1.26 (0.99–1.52)	3.33 (2.50–6.09)
	B03	311	4.06 ± 0.84	0.97 (0.74–1.18)	2.48 (1.89–4.36)
	B05	400	2.45 ± 0.41	0.74 (0.53–0.94)	3.47 (2.32–7.69)
	B07	381	3.89 ± 1.06	1.52 (1.01–1.89)	4.12 (2.88–11.60)
	B08	669	4.20 ± 0.71	1.10 (0.80–1.31)	2.70 (2.09–5.02)
KSU	D01	810	5.04 ± 0.67	0.97	2.05
	D02	487	7.48 ± 1.47	0.56 (0.45–0.66)	0.93 (0.78–1.34)
	D03	512	4.22 ± 0.77	1.10 (0.85–1.33)	2.70 (2.14–4.13)
	D04	997	3.24 ± 0.38	0.55 (0.43–0.66)	1.77 (1.40–2.52)
	D05	555	4.17 ± 1.45	0.97 (0.65–1.30)	2.41 (1.72–5.13)
	D06	453	5.79 ± 1.09	0.77 (0.63–0.89)	1.48 (1.23–2.06)
UCR	R01	741	3.28 ± 0.42	0.57 (0.35–0.74)	1.80 (1.29–3.77)
	R02	344	3.37 ± 0.64	0.99 (0.54–1.39)	3.05 (1.99–12.27)
	R04	582	3.35 ± 0.41	0.54 (0.42–0.65)	1.68 (1.30–2.59)
	R06	728	1.04 ± 0.33	0.14	5.55
	R07	325	2.99 ± 0.52	0.61 (0.38–0.82)	2.16 (1.46–5.15)
	R08	446	3.86 ± 0.61	0.51 (0.35–0.65)	1.36 (1.00–2.71)

[a] Only a "g" statistic of <0.5 was used to calculate confidence intervals (Robertson and Preisler 1992).

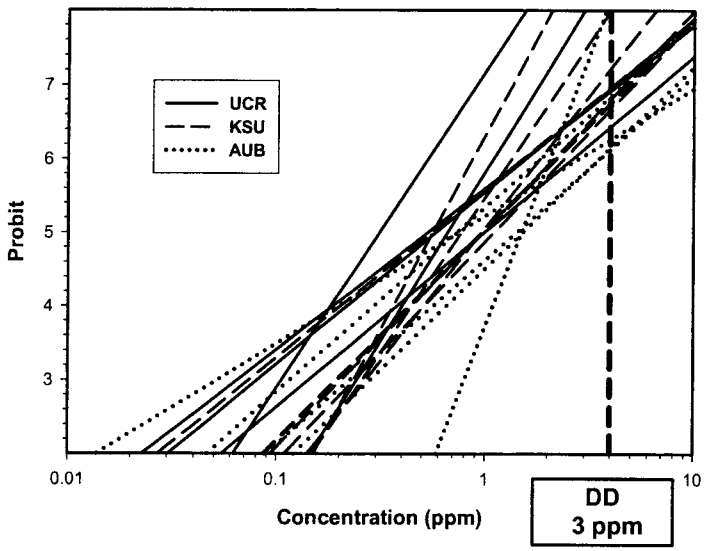

Fig. 2. Probit lines from field-collected isolates tested at UCR, KSU, and AUB.

a prerequisite for effective implementation and diagnosis of any resistance that may exist. The current project was fortunate in having access to a number of strains with a long history of laboratory culture and no known history of exposure to imidacloprid. Testing of these strains in three laboratories gave extremely consistent results, fostering confidence in the accuracy and reliability of the larval bioassay method (Table 1). A range of field isolates with contrasting treatment histories responded similarly to the laboratory ones, implying that the latter remain representative of contemporary field populations (Table 3).

In addition to the appraisal and refinement of bioassays, much attention has been paid to the statistical design of monitoring programs (Roush and Miller 1986, Sawicki et al. 1989, Halliday and Burnham 1990). Use of full probit lines has numerous advantages in toxicological research, but several disadvantages for routine monitoring compared with a single dose or concentration optimized to distinguish between susceptible and putatively resistant individuals. As well as being time-consuming and labor-intensive to obtain, probit parameters such as LC_{50} and LC_{95} values are very insensitive to slight changes in susceptibility that may nonetheless be of clinical significance (Sawicki et al. 1989, Denholm 1990, Halliday and Burnham 1990). Single doses represent a more efficient use of resources and have become widely used, for example, when tracking temporal changes in the susceptibility to insecticides of important agricultural pests (Sawicki et al. 1989, Forrester et al. 1993). However, such doses must be chosen with care to minimize the likelihood of "false positives" while maximizing the prospect of detecting resistance at the earliest stage possible in its development.

Table 4. Percentages of individual fleas of field-collected isolates that survived the DD of 3 ppm

Strain	*n*	% survival at 3 ppm
B02	49	10.2
B07	46	8.7
R02	41	2.4

The DD resulting from this study (3 ppm) reflects such a compromise. None of the laboratory strains showed any survival when exposed to 3 ppm imidacloprid, and the majority of individuals from field-collected isolates also were killed at this concentration. Isolates showing low levels of survival at 3 ppm, which have subsequently been shown to be extremes of the normal range of susceptibility rather than cases of resistance (unpublished data), demonstrate the need for some caution with interpretation of results. Thus, we have adopted the criterion that >5% survival at 3 ppm in subsequent surveys will trigger additional testing of insects reared from the original collection or resampled from the same locality.

Flea eggs were easily collected by veterinary personnel and shipped to laboratories for bioassays. Even though fleas are also a problem during spring in warmer climates, most of the isolates were collected in September and October. The reason for greater numbers in the fall is not known. Establishing a DD for the larval assay now permits us to determine whether strains are susceptible with as few as 40 eggs and also eliminates the need to have a host in the laboratory for each strain. Adult fleas in the control vials are available to place on a host in the event that larvae exposed to the DD develop into adults. A single laboratory could assay as many as 12 strains per day. With some training, veterinary personnel could conduct the tests if they were provided with treated larval rearing media and had a chamber to hold the fleas at ≈26°C and 75% RH.

The development of the larval bioassay and a DD will permit the widespread evaluation of field populations of cat fleas. This program will permit the early

detection of any reduced susceptibility and serve as the foundation of developing alternative pest management strategies.

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