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SULFADIMETHOXINE AND RHODAMINE B AS ORAL BIOMARKERS FOR EUROPEAN BADGERS (*MELES MELES*)

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ABSTRACT: A field study was carried out on Little Island (County Waterford, Ireland) in June 2000 to evaluate the potential of a bait-marking system for use in European badgers (*Meles meles*). Two oral biomarkers, sulfadimethoxine (SDM) and rhodamine B, were incorporated into fishmeal baits and distributed by hand at main setts in five test territories for 3 consecutive days. In parallel, non-biomarked baits were distributed at a single control territory. The objectives of the study were to: (1) assess the effects of SDM and rhodamine B on palatability and thus bait acceptance, and (2) investigate the marking capacity of SDM and rhodamine B in serum and hair samples taken from badgers. Trapping was carried out in each territory for 5 consecutive days immediately after bait distribution. Analysis of data revealed that 90–100% of baits were removed in four of the test territories and from the control territory. In the fifth test territory, 61% of baits were removed. Of the badgers (n = 26) trapped in the test territories, 18 (69%) were positive when tested for both biomarkers. In contrast, the remaining eight animals and those captured in the control territory (n = 6 badgers) were negative. In the marked animals, the highest levels of SDM were recorded in serum samples taken soon after bait distribution. Thereafter, the levels declined in each badger over the course of the study. In contrast, rhodamine B was readily detectable by fluorescence microscopy of hair samples throughout the period of study. The results indicate that SDM and rhodamine B act as systemic markers in badgers and have potential future applications for monitoring of oral vaccine uptake.

Key words: Badgers, *Meles meles,* oral baits, biomarkers, sulfadimethoxine, rhodamine B, bait-marking system.

INTRODUCTION

In Ireland and Great Britain, the European badger (*Meles meles*) is the principal wildlife maintenance host for *Mycobacterium bovis,* the causative agent of bovine tuberculosis, and acts as a reservoir for transmission to cattle (Hughes et al., 1996; Krebs, 1997). Because badgers are an ecologically important and protected species, oral vaccination is a long-term disease control strategy that could help limit the spread of infection between badgers and reduce the transmission to cattle.

A crucial component of such a strategy is development of a delivery system to optimally vaccinate badgers. A recent bait delivery study in Ireland revealed that between 20–80% of badgers consumed baits in six separate study areas, ranging in size from 8.9–23 km^2 (Southey et al., 2001). Bait-delivery systems have also been successful for delivering rabies vaccine to red foxes (*Vulpes vulpes*), coyotes (*Canis latrans*), feral dogs, and raccoons (*Procyon lotor*) (Brochier et al., 1996; Fearneyhough et al., 1998; Roscoe et al., 1998; Youssef et al., 1998).

Biomarkers have played an important role in identifying individuals or the proportion of animals ingesting baits in studies with several different carnivore species (Hanlon et al., 1993; Brochier et al., 1996; Farry et al., 1998). The technique relies on using non-toxic biomarker reagents incorporated into baits to 'mark' the animals that consume baits (Mitchell, 1998). Short-term biomarkers (e.g., bromocresol green, sulfadimethoxine, clenbuterol) have been employed in studies to examine bait uptake by target species within days or weeks following bait deployment (Savarie et al., 1992; Hanlon et al., 1993; Gleixner et al., 1998). Long-term biomarkers such as iophenoxic acid and tetracycline have been used to trace and identify animals for a period of months or years after bait in-

gestion (Johnston et al., 1987; Savarie et al., 1992). Although iophenoxic acid and tetracycline have been shown to be effective biomarkers in badgers (Southey et al., 2001), iophenoxic acid detection assays are difficult to perform. Tetracycline detection also requires sectioning of teeth and bones and is often used for providing retrospective evidence of bait uptake in dead animals (Brochier et al., 1994; Hanlon et al., 1998).

In the present study we evaluated the potential usefulness of sulfadimethoxine (SDM) and rhodamine B as biomarkers for live badgers. Sulfadimethoxine is a broad-spectrum anti-microbial agent that has been previously evaluated as a serum marker for rabies vaccine bait consumption by raccoons (Hanlon et al., 1993) and dogs (Matter et al., 1998). The duration of marking with SDM is short-term and normally persists in the bloodstream for up to 7 days following ingestion (Savarie et al., 1992). However, SDM has the advantage that it can be easily detected and qualitatively measured using fresh blood samples and a commercially available rapid card test (Matter et al., 1998; Youssef et al., 1998). Alternatively, it can be quantitatively measured in an enzyme-linked immunosorbent assay (ELISA).

Rhodamine B is an intense fluorescent dye detectable in hair samples with the unaided eye at high concentrations and under ultraviolet (UV) light at low concentrations (Savarie et al., 1992). It has been used as a topical marker of bait consumption in animals such as dogs and coyotes (Perry et al., 1988; Farry et al., 1998). Perry et al. (1988) reported that dogs were stained bright red on the tongue, oral mucosa, mouth, nose, and other parts of the body after contact with baits containing rhodamine B. Systemic marking by rhodamine B has been observed in coyotes (Johns and Pan, 1981) and mountain beavers (*Aplodontia rufa*) (Lindsey, 1983). In these animals, systemic marking is characterized by UV-fluorescent deposits in the hair and claws, which persists for several months following rhodamine B consumption.

We conducted a field experiment in which badgers were exposed to baits containing SDM and rhodamine B. The aims of the study were to assess the palatability and acceptance of bait-delivered biomarkers by badgers and to investigate test systems for measuring SDM and rhodamine B in serum and hair samples, respectively.

MATERIALS AND METHODS

Badgers used in this study were cage-trapped in June 2000 on Little Island (52°15′N, 7°15′W), a riverine island (area 1.18 km^2) that lies 3 km east of Waterford, Ireland. There are six badger social groups in six territories (called A–F) on the island, which were mapped by feeding a peanut, treacle (molasses), and colored plastic pellet mixture as described previously (Kruuk, 1989; Delahay et al., 2000). Each territory has one main sett except for territory B which has three. The badger population density is 37 badgers/km^2, estimated by capture-recapture and direct enumeration. To our knowledge, this is one of the highest recorded densities of badgers. The only other wildlife species on the island likely to consume baits are tame red foxes, though they are accustomed to being hand-fed.

Each Du Pont bait (Bait-Tek Inc., Beaumont, Texas, USA) is a rigid 5 × 3 × 2 cm, 35–40 g, parallelepiped composed of fishmeal and fish oil aggregated by a synthetic polymer (Hanlon et al., 1989; Brochier et al., 1994). Sulfadimethoxine sodium and rhodamine B were purchased (Sigma, Dorset, UK). The cavities of the Du Pont baits were partially filled with melted chocolate and allowed to set. The SDM (80 mg/ml) and rhodamine B (93.3 mg/ml) were added to melted gelatin (final concentration of 10%) and kept liquid at 50 C. Approximately 3 ml of the mixture was pipetted into the remainder of each bait cavity. Minimum dose of SDM was 280 mg/bait and of rhodamine B was 240 mg/bait. After the gelatine mixture solidified, baits were coated with melted chocolate and stored at 4 C until deployment.

Ten Du Pont baits containing SDM and rhodamine B were placed under stones (>1 kg) around active entrances and on paths leading to each of seven main setts in territories B–F. Likewise, ten baits without biomarkers were distributed at the single main sett in territory A. Bait removal from the bait deployment sites was recorded each day for 3 consecutive days, and missing baits were replaced daily. Cage

traps (n = 79) were distributed in the six territories (approximately 10 per main sett) and were set on day 3 of bait deployment. Cages were pre-baited daily with peanuts and trapping was carried out for 5 days. All of the badgers trapped over the course of the study were anesthetized with 0.1 mg/kg ketamine hydrochloride (Vetalar®, Pharmacia and Upjohn Ltd., Crawley, UK) and 0.1 mg/kg medetomidine hydrochloride (Domitor®, Orion Corporation, Espoo, Finland), administered by intramuscular injection. Once anesthetized, the animals were weighed, tattooed, ear-tagged, and checked for ectoparasites (fleas, ticks, lice) and other signs of disease or injury (bite wounds, trap wounds). Sex, approximate age by tooth wear, and body length were also recorded. Five milliliters of blood were collected by venipuncture from each badger. Guard hairs, including the follicle, were removed from the shoulder region of each animal with tweezers. Anesthetized badger's mouth, anus, hair, and claws were examined using a UV lamp (Spectraline, Sigma, Dorset, UK) under a cloth hood to detect topical marking by rhodamine B. Following sampling, animals were returned to the cage and allowed to recover. After at least 1 hr of visual monitoring badgers were released near the setts.

Blood samples were centrifuged and the serum removed and stored at −20 C prior to analysis. An SDM rapid card test (EZ-Screen Sulfadimethoxine, Meditox Diagnostics Inc., Burlington, North Carolina, USA) was used to screen sera collected from five badgers trapped on day 1 for the presence of SDM as an indication of bait ingestion. For quantitative analysis, sera collected from badgers during capture were analysed using an ELISA kit (General Sulfa One-Step ELISA, International Diagnostic Systems Corp., St. Joseph, Michigan, USA). The SDM standard was diluted to produce a standard curve ranging from 0.005–0.04 ppm. Prior to analysis, sera were heat-inactivated for 15–30 sec in a microwave set on high power and diluted as required to within standard range. Serum SDM values were calculated using the SDM standard curve and then multiplying by the appropriate dilution factor. Values <0.005 ppm were considered negative for SDM.

Guard hair samples from each badger were examined by fluorescence microscopy. Hairs were washed by immersion in methanol for 5 min and allowed to air dry. These were then placed length-wise onto a glass microscope slide and covered with a cover slip and viewed at 10× magnification using a fluorescence microscope (Olympus BX60, Olympus Optical Co. Gmbh, Hamburg, Germany) fitted with a BX-FLA reflected light fluorescence attachment connected to a U-RFL-T mercury lamp burner. Hair samples were recorded positive for systemic rhodamine B marking when a brilliant orange fluorescence was observed in the root and/or along the hair shaft (Fisher et al., 1999).

Data obtained on weights of badgers and serum SDM levels were analyzed by the unpaired students t-test and analysis of variance (ANOVA).

RESULTS

Following delivery of baits, badgers were trapped over 5 consecutive days. Thirty-two badgers (19 females, 13 males) were captured, 26 from the test territories and six from the control territory. Including recaptures, animals were trapped a total of 56 times. Individual badgers were captured in the test territories a median of two times (range 1–4) and badgers within the control territory were captured a median of two times (range 1–3). As judged by extent of tooth-wear, the ages of animals ranged from cubs (<6 mo old) to mature adult (>3 yr old). Animals appeared healthy on gross examination, though many had high numbers of ectoparasites and several had bite wounds. There was a difference (P = 0.04) in mean body weights of adult badgers which were captured in the test territories (7.97 ± 0.80 kg, n = 22) compared to those captured in the control territory (7.23 ± 0.41 kg, n = 6). Four badger cubs were captured in the test territories (mean body weight 3.83 ± 0.85 kg) while none was caught in the control territory.

With the exception of the remains of five incompletely eaten baits found in territories B, E, and F, baits were removed without trace. The cumulative total of baits removed was greatest (90–100%) in both test (C–F) and control territories (Table 1). In territory B, containing three main setts, the cumulative bait removal was determined to be 61% (Table 1). This relatively lower removal rate was attributed to poor bait uptake at two of the main setts.

Of 26 badgers trapped in test territories, 18 (69%) were positive for serum SDM.

TABLE 1. Cumulative total of baits removed by European badgers from main setts in territories A–F over 3 days of bait distribution.

Territory	Number of main setts baited	Cumulative number baits taken (%)
A[a]	1	27 (90)
B[b]	3	55 (61)
C	1	30 (100)
D	1	30 (100)
E	1	29.5 (98)
F	1	28 (93)

[a] The main sett in territory A was fed with non-biomarked baits.

[b] The main setts in territories B–F were fed with baits containing sulfadimethoxine and rhodamine B.

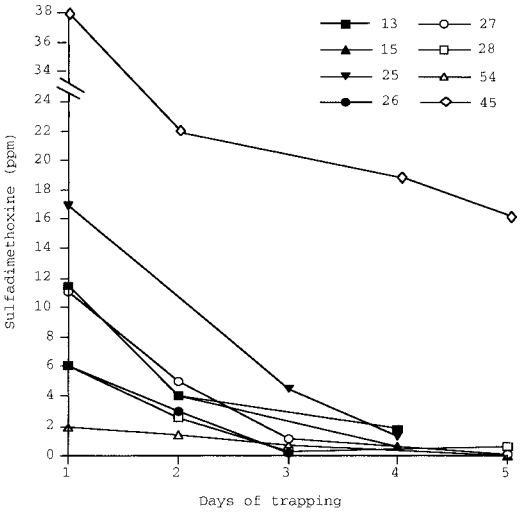

FIGURE 1. Change in serum sulfadimethoxine concentration in eight European badgers trapped at least three times following the consumption of one or more biomarked baits on Little Island in June 2000. Numbers in legend are badger identification numbers.

The serum SDM levels from 16 badgers captured on day 1 ranged from 0.2–38.0 ppm (mean = 11.0 ± 9.4 ppm). There was no significant difference in SDM levels detected in males (9.8 ± 3.6 ppm, $n = 5$), females (11.1 ± 14.1 ppm, $n = 7$), or cubs (12.4 ± 5.0 ppm, $n = 4$). No SDM was detected in the sera of the six badgers ($n = 6$) caught in the control territory.

Figure 1 shows the change in serum SDM levels in eight badgers sampled on at least three occasions. Highest concentrations of SDM were recorded on day 1 of trapping. There was a decline in SDM levels over 3 days with the half-life estimated as lying between day 2 and day 3. In seven of the eight animals, serum SDM levels approached the limits of detection by day 4 or 5 of trapping. However, badger No. 45 (female) displayed relatively high serum SDM (38 ppm) levels on day 1 (Fig. 1), which decreased to 22 ppm on day 2 and remained relatively constant for the rest of the trapping period.

Rhodamine B fluorescent marking was observed under UV illumination on seven of thirty-two badgers examined, either in the corner of the mouth, hair, or in the feces. The claws or guard hairs did not fluoresce under the UV lamp. In three of the test territories, bright red urine was observed on the grass close to where biomarked-baits were deployed. In all SDM-positive badgers ($n = 18$) follicles of guard hairs fluoresced bright orange under a fluorescence microscope (Fig. 2). In contrast, a much fainter coloration was observed in follicles of hair samples taken from badgers that did not show evidence of bait consumption. Fluorescence was readily observed in hairs removed on day 1 and there did not appear to be any diminution of fluorescence when hairs were removed from the same animal on day 5. There did not appear to be a strong positive correlation in intensity of fluorescence

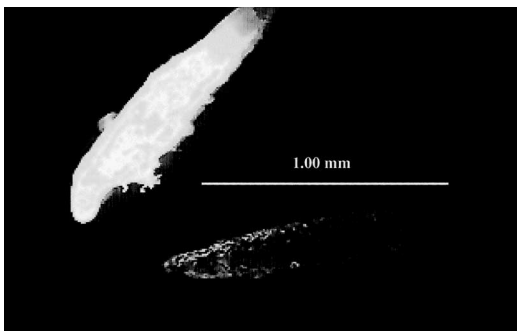

FIGURE 2. Rhodamine B fluorescence in a hair follicle of a European badger trapped after consuming biomarked bait. A control hair follicle is shown below for comparison. Bar = 1 mm.

and levels of serum SDM in any individual animal.

DISCUSSION

As part of the development of an antituberculosis vaccination program, we evaluated the potential of an oral baiting system for delivering an oral vaccine to badgers. The Du Pont baits chosen for this work have proved successful in rabies vaccine campaigns in Western Europe (Brochier et al., 1994) and the USA (Hanlon et al., 1989; Robbins et al., 1998; Roscoe et al., 1998). We previously tested these baits on mainland populations of badgers using tetracycline and iophenoxic acid as biomarkers (Southey et al., 2001). Both reagents were effective in monitoring bait uptake; however, the cost of measuring iophenoxic acid and the requirement of postmortem analysis for tetracycline deposits in badgers demonstrated their limited usefulness in large scale vaccination programs. In the current study we tested two biomarkers that can be conveniently and rapidly assayed in hair and blood samples. We also used an island high density population of badgers, which facilitated daily recapture and sampling of animals to perform assays for longevity of biomarker in the host.

Deployment of bait at main setts in each territory was designed to optimize the uptake by the highest proportion of badgers. Cumulative bait removal was >90% in four of the test territories (C–F) and in the control territory. In the fifth test territory only 61% of baits was removed. We can speculate that in the latter case, habitat and uptake by non-target species may have contributed to the relatively low uptake. Our strategy of placing baits under heavy stones was designed to minimize bait contact by non-target species. However, foxes were regularly seen and trapped during the study and may have removed some of these baits. It has been suggested from similar studies with foxes that the probability of encountering a bait depends on the number of baits exposed each night, the spatial distribution of baits, the fox density, and their territorial use in relation to bait distribution (Fleming, 1997). It is likely that badgers encountering baits also would be influenced by similar factors.

Sulfadimethoxine was a useful short-term marker of bait consumption by badgers, being detectable from 1–5 days after bait ingestion. In addition, the rapid card test for SDM was useful as a potential field test by generating an immediate assessment of bait consumption in five badgers tested. There was a strong concordance in the test territories between the number of baits removed and the proportion of animals which were biomarked. The SDM ELISA was modified to facilitate quantification of SDM levels in badger sera. A wide range of SDM levels (0.2–38 ppm) was recorded in the sample of badgers captured on day 1. When SDM levels were measured in recaptured animals, there was no apparent difference in the rate of SDM decline in badger sera, irrespective of day 1 serum SDM levels. This may indicate that SDM is metabolized at a similar rate among badgers. It was estimated from the data that 50% of the SDM was metabolized between days 2 and 3. However, further work is required to assess the metabolism of different initial SDM concentrations as a function of age and health status of the animal.

If the day 1 serum SDM concentrations approximate the relative number of baits taken by each badger then it appears that there was considerable variability in the numbers of baits eaten. High levels of SDM were measured in all of the captured cubs and this may be due to a concentrating effect because of their small size relative to adults. Alternatively, it may reflect behavioral differences in cubs that do not wander far from the main setts and are therefore more likely to encounter and eat baits.

Rhodamine B fluorescence of hair was evident in all badgers that were positive for SDM. Fluorescence was visible on day 1 after the final bait delivery and was still

present in animals captured on day 5. There did not appear to be any change in fluorescence in any individual over the course of the study. In addition, there did not appear to be a strong correlation between the degree of fluorescence and the levels of SDM detected in any particular animal. However, it was noted that female badger No. 45, with the highest SDM levels on day 1, also displayed the most intense fluorescence. Rhodamine B staining of hair has been reported to persist up to 175 days in coyotes (Johns and Pan, 1981) and for several weeks in mountain beavers and pocket gophers (*Thomomys mazama*) (Lindsey, 1983). Johns and Pan (1981) also found that rhodamine B is only incorporated into actively growing hair.

Both biomarkers were suitable and reliable in badgers. An advantage of SDM is that it can be rapidly assayed using a commercially available card test. As part of oral vaccination campaigns this would be valuable in allowing strategic distribution of baits. However, the short duration and rapid elimination of SDM from the host limits its usefulness in monitoring uptake over a longer period of time. Conversely, detection of rhodamine B may be more cumbersome because it relies on fluorescence microscopy; however there would be a greater persistence of marking than with SDM. This would be ideal in large-scale field studies where exposure of badger populations to oral vaccine-laden baits could be investigated several weeks or months after bait distribution. Future studies will be required to investigate the longevity of fluorescence in hair samples and how this relates to multiple bait uptake, moulting, and badger health status.

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