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D. E. Hill,[1,5] M. D. Samuel,[2] C. A. Nolden,[3] N. Sundar,[1] D. S. Zarlenga,[4] and J. P. Dubey[1]

[1] US Department of Agriculture, Agricultural Research Service, Animal and Natural Resources Institute, Animal Parasitic Diseases Laboratory BARC-East, Beltsville, Maryland 20705, USA
[2] US Geological Survey, Wisconsin Cooperative Wildlife Research Unit, University of Wisconsin, 1630 Linden Drive, Madison, Wisconsin 53706, USA
[3] Department of Wildlife Ecology, University of Wisconsin, 1630 Linden Drive, Madison, Wisconsin 53706, USA
[4] Bovine Functions and Genomics Laboratory, BARC-East, Beltsville, Maryland 20705, USA
[5] Corresponding author (email: dhill@anri.barc.usda.gov)

ABSTRACT: Tissues and serum from 59 raccoons (*Procyon lotor*), 42 coyotes (*Canis latrans*), and seven Striped Skunks (*Mephitis mephitis*) collected in Dane and Iowa Counties, Wisconsin, USA, between October 2005 and March 2006 were microscopically and serologically examined for the presence of *Trichinella* spp. Encapsulated larvae were found on compression slides prepared from tongue tissues from a few animals. Complete tissue digestion of tongues revealed that 19% of the raccoons, 26% of the coyotes, and none of the seven skunks tested were infected with *Trichinella* spp. Cats were subsequently experimentally infected by feeding them the raccoon tissues containing muscle larvae, and muscle larvae isolated from the collected tongues were experimentally transmitted to mice. Multiplex polymerase chain reaction analysis of the isolated muscle larvae demonstrated two distinct bands migrating at 127 base pairs (bp) and 316 bp in all samples, which together are diagnostic for *Trichinella murrelli*; the isolates were assigned Istituto Superiore di Sanita (ISS) codes ISS1656 through ISS1667, and ISS1708 through ISS1710 by the International *Trichinella* Reference Centre. These findings extend the geographic range of *T. murrelli* into Wisconsin, USA.

Key words: *Canis latrans*, carnivores, *Mephitis mephitis*, *Procyon lotor*, *Trichinella*, Wisconsin.

INTRODUCTION

Sylvatic isolates of the genus *Trichinella* are widespread in the environment due to an expansive host range and worldwide geographic distribution. Wildlife serve as hosts for *Trichinella* species that can cause human disease if meats are not properly prepared. Currently, eight sibling species and three genotypes of undetermined taxonomic status have been identified in the genus *Trichinella* (Kapel, 2000; Murrell et al., 2000; Pozio and Zarlenga, 2005). Worldwide geographic distribution of these isolates has been described (Pozio et al., 1992, 1998; Zarlenga et al., 2006). Five of the sibling taxa, *Trichinella spiralis*, *Trichinella murrelli*, *Trichinella pseudospiralis*, *Trichinella nativa*, and *Trichinella T6*, occur in the continental US and have been identified in a variety of mammalian species, including domestic swine and feral swine (*Sus scrofa*), rats, black bear (*Ursus americanus*), dogs, raccoons (*Procyon lotor*), coyotes (*Canis latrans*), gray wolves (*Canis lupus*), dogs, foxes, skunks, bobcats (*Lynx rufus*), cougars (*Felis concolor*), and other carnivores (Pozio, 2007). Although *T. spiralis* is virtually absent from the US pig population (National Animal Health Monitoring System [NAHMS], unpubl.), some sylvatic isolates pose a risk for zoonotic transmission when pigs are exposed to *Trichinella*-infected wildlife in nonbiosecure pig barns or when pigs are managed in nonconfinement systems. *Trichinella murrelli* was recently recognized as a separate species (Pozio and La Rosa, 2000); to date, *T. murrelli* has been found exclusively in the Nearctic and is thought to be the predominant species circulating among sylvatic hosts in temperate North America (Zarlenga et al., 1991; Snyder et al., 1993; Pozio and La Rosa, 2000). Viable *T. murrelli* has previously been isolated from black bears, raccoons, and other carnivore hosts in Pennsylvania, Illinois, Indiana,
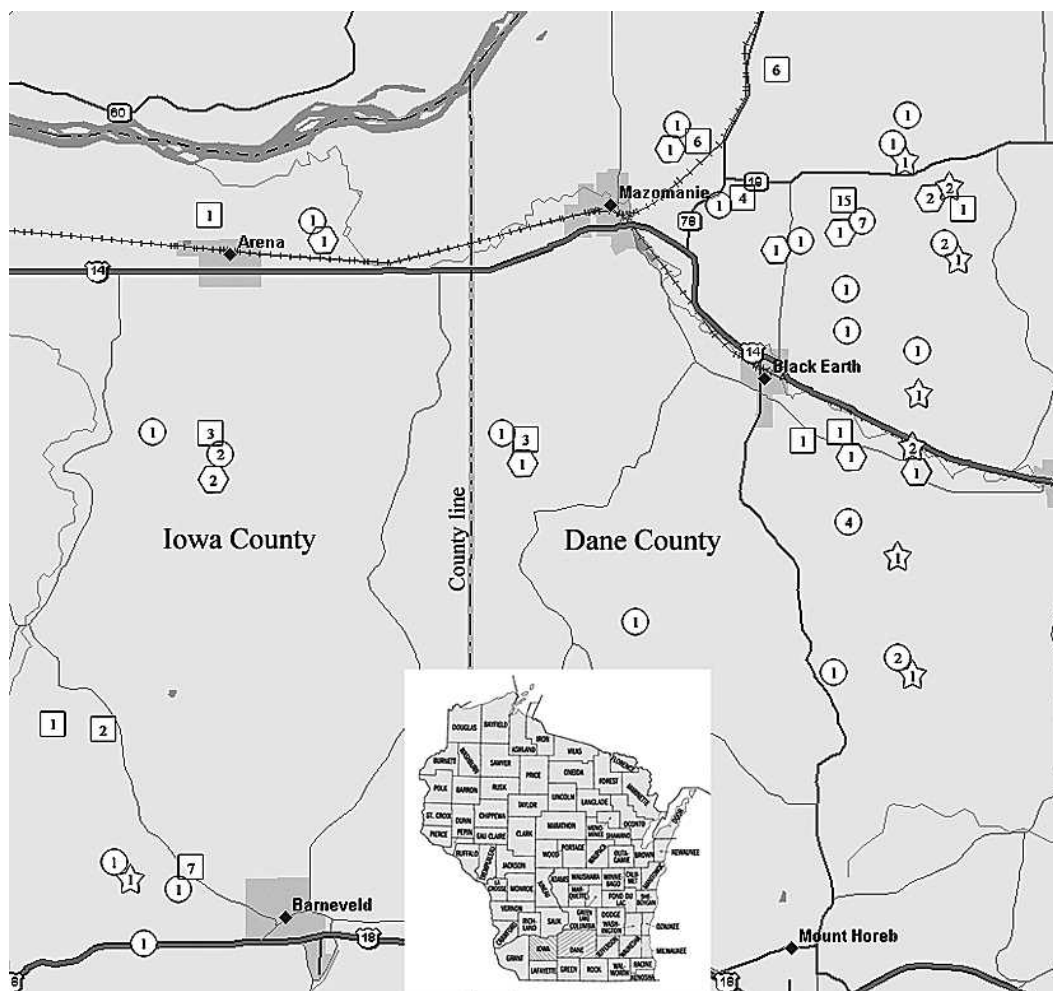
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FIGURE 1.   Wisconsin county map showing Dane and Iowa Counties in crosshatch and collection locations of scavenging mammals. Symbols refer to positive or negative tissue digest results: (stars) positive coyotes, (circles) negative coyotes, (hexagons) positive raccoon, (squares) negative raccoons. Number within symbol represents number of animals collected at the location.

Georgia, Texas, California, and New Mexico (Minchella et al., 1989; Snyder et al., 1993; Pozio and La Rosa, 2000; Pozio et al., 2001a; Pozio and Murrell, 2006). In this study, we extend the distribution area of *T. murrelli* into carnivorous mammalian hosts in Wisconsin, USA.

MATERIALS AND METHODS

During the course of an investigation of chronic wasting disease in scavenging mammals, tongues, diaphragms, hearts, brains, and blood were collected from 59 raccoons, 42 coyotes, and seven Striped Skunks (*Mephitis*

mephitis) from hunter-trapped animals in Dane and Iowa Counties, Wisconsin, USA, between October 2005 and March 2006 (Fig. 1). Animals were collected from locations near the towns of Mazomanie (northeast corner of study area, 43°13′03″N, 89°44′50″W); Mount Horeb (southeast corner of study area, 43°00′34″N, 89°41′00″W); Ridgeway (southwest corner of study area, 42°59′43″N, 89°59′23″W); and Arena (northwest corner of study area, 43°11′57″N, 89°57′12″W). Samples were packed on ice and shipped to the Animal Parasitic Diseases Laboratory, Beltsville, Maryland, USA. Upon microscopic examination of muscle tissues from each animal, coiled, encapsulated nematode larvae were noted on compression slides

from 15 raccoons, seven coyotes, and one skunk. Pepsin (1%):HCl (1%) digestion (Gamble, 1996) was performed on all tongues by first trimming the fat and connective tissue, mincing the tongue (<1 cm^2 pieces), and then mixing it with artificial digestion fluid warmed to 45 C. The mixture was stirred at 45 C for 3 hr, and the digests were allowed to settle for 20 min. The sediment containing muscle larvae (ML) was repeatedly washed with 250 ml of tap water and allowed to settle until the supernatant was clear. The settled ML were counted on a stereo microscope at ($40\times$) and then orally inoculated into two Swiss-Webster mice (500 ML each). In those instances where insufficient numbers of ML were collected, the available ML were equally divided and orally inoculated into two mice. After 35–75 days, mice were killed by cervical dislocation, skinned, eviscerated, and digested as described previously to obtain ML and to calculate the reproductive capacity index (RCI; number of ML recovered/number of ML inoculated). Muscle larvae burdens in tongues from coyote and raccoon hosts were compared using the Student's *t*-test (www.physics.csbsju.edu/stats/t-test.html).

In a concurrent study, cats were fed tissues from *Trichinella*-infected raccoons in an attempt to recover *Toxoplasma gondii* isolates also seen in these tissues. Tissues from raccoons with demonstrable *Trichinella* ML were fed to a total of five parasite-free cats from the US Department of Agriculture's cat colony (Dubey, 1995). Initially, infected muscles from three raccoons were fed to three cats. These recipient cats were killed by injection of sodium pentobarbital euthanasia solution (Schering-Plough, Union, New Jersey, USA) 17 days later, and their tongues were fixed in 10% buffered formalin. Tissues from two additional *Trichinella*-infected raccoons were fed to two more cats; these cats were bled and killed on day 30 postinoculation (PI), and their tongues and diaphragms were fixed in formalin. Paraffin-embedded histologic sections of the tissues from these five cats were examined microscopically after staining with hematoxylin and eosin (H&E). All animal work conducted at the Beltsville laboratory was approved and completed under the auspices of the Institutional Animal Care and Use Committee.

Non-nested, multiplex polymerase chain reaction (PCR) was carried out on genomic DNA isolated from both the original isolate and from the mouse-amplified sample using a DNeasy Tissue Kit, following the manufacturer's instructions (Qiagen Inc., Valencia, California, USA) and as described by Zarlenga et al. (1999). Amplified products were separated on a 2% NuSieve agarose gel, which was subsequently stained with ethidium bromide and photographed. Identification of the *Trichinella* ML collected from the original host was verified by multiplex PCR testing at the International *Trichinella* Reference Centre (ITRC; Pozio et al., 2001b; www.iss.it/site/Trichinella/index.asp), after which Istituto Superiore di Sanita (ISS) numbers were assigned (ISS1656 through ISS1667, and ISS1708 through ISS1710). Each serum sample collected during the course of the experiment was tested in duplicate by enzyme linked immunosorbent assay (ELISA) for the presence of anti-*Trichinella* antibodies using a commercial ELISA kit (SafePath Laboratories, Carlsbad, California, USA), which uses a *T. spiralis* excretory/secretory (E/S) antigen. Sera were tested at a 1:200 dilution as recommended by the manufacturer, except that horseradish peroxidase conjugated (hrp) antidog antibody was used as the second antibody in assays of coyote sera, and hrp-antiraccoon antibody was used in assays of raccoon and skunk sera as described by Cheadle et al. (2001). Positive and negative control canine sera were included on each ELISA plate since no known positive coyote, raccoon, or skunk sera were available. The ELISA values were reported as the mean of duplicate wells and were considered positive if the optical density (OD) exceeded 0.300 after subtraction of the negative control well.

RESULTS

Tissue digest results revealed that 11 of 59 (19%) raccoons, and 11 of 42 (26%) coyotes were infected with *Trichinella* sp. (Table 1). None of the seven skunks tested was infected with *Trichinella* based on digest results. The number of *Trichinella* ML isolated from coyote tongues was significantly lower (range 0.1 to 141 larvae per gram (lpg), mean 34.3, standard deviation [SD] 42.8) than those isolated from raccoon tongues (range 5 to 481 lpg, mean 280.3, SD 162; *t*-test, $P<0.05$).

Multiplex PCR analysis in this laboratory and confirmatory results by the ITRC clearly demonstrated two distinct bands migrating at 127 base pairs (bp) and 316 bp (data not shown), which together are diagnostic for *T. murrelli*. The possibility of a mixed infection containing other

TABLE 1. *Trichinella* spp. tissue digest and serologic results for scavenging mammals sampled in Wisconsin, USA.

Species	County	Total no. collected	No. tissue positive	Larvae/gram of tissue; min–max	OD range in ELISA-positive samples, min–max[a]	OD range in ELISA-negative samples, min–max
Procyon lotor	Dane	44	9	200–481	0.376–1.425, $n=8$	0.016–0.247, $n=36$
	Iowa	15	2	5, 351	0.310, 0.919, $n=2$	0.012–0.031, $n=13$
Canis latrans	Dane	32	9	0.1–141	0.319–3.017, $n=16$	0.013–0.236, $n=16$
	Iowa	10	2	13, 34	0.312–2.39, $n=5$	0.043–0.086, $n=5$
Mephitis mephitis	Dane & Iowa	7	0	0	0.539, $n=1$	0.025–0.097, $n=6$

[a] ELISA cutoff >0.300.

sylvatic species of *Trichinella* (127 bp) and *T. murrelli* (127 bp and 316 bp) could not be ruled out because individual larvae were not tested.

Of the 11 coyotes that tested positive by tissue digestion and from which sera were available (nine), seven were also positive by ELISA. Of the 11 raccoons that were positive by tissue digestion, six of nine available sera were positive by ELISA. Additionally, 15 coyotes, four raccoons, and one skunk that were negative by tissue digestion were ELISA positive (Table 1).

All (44) Swiss-Webster mice became infected after oral inoculation with ML collected from raccoon and coyote tongue tissue. The RCI values of the *T. murrelli* isolates from raccoons and coyotes ranged from 3.0 to 15.

An unencapsulated *Trichinella* larva was found in the tongue of one of the three cats killed 17 days PI, and there were multifocal areas of mononuclear cell infiltrations in the tongue (Fig. 2A). Encapsulated larvae were found in the tongue and diaphragm of the two cats killed 30 day PI (Fig. 2B).

DISCUSSION

Trichinella murrelli has been identified in a number of carnivorous mammals in North America. Snyder et al. (1993) identified *T. murrelli* as likely the most common sylvatic genotype circulating in the US. This was later supported by results from Pozio and La Rosa (2000); however, fewer than two dozen isolates

had been deposited with the ITRC before the current study. The host and geographic range of *T. murrelli* has only partially been described (Pence et al., 2001; Pozio et al., 2001b), and it is of importance because *T. murrelli* can infect wild game destined for human consumption in the US, as well as aberrant host species such as horses, which are exported from the US for human consumption.

Typically, *T. spiralis* is maintained in the peridomestic environment and involves rats, pigs, and humans when infected pigs enter the food chain. Sylvatic genotypes, including *T. murrelli*, are maintained only in a sylvatic cycle involving scavenging carnivorous mammals and occasionally aberrant hosts (Pozio, 2000), and they become a zoonotic concern when humans insert themselves into the sylvatic food chain through the consumption of game meats or other susceptible animals.

An outbreak of trichinellosis in humans occurred in France as a result of the consumption of horse meat; two of 325 infected individuals died (Ancelle et al., 1988). Epidemiologic investigations concluded that the infection originated from a horse carcass imported from Connecticut, USA. This resulted in an import ban on horse meat from the US to the European Union. Although parasites were never recovered from the original source, a biopsy performed on a chronically ill patient nearly 6 yr after the outbreak determined that the etiologic agent was indeed *T. murrelli* (Dupouy-Camet et al.,
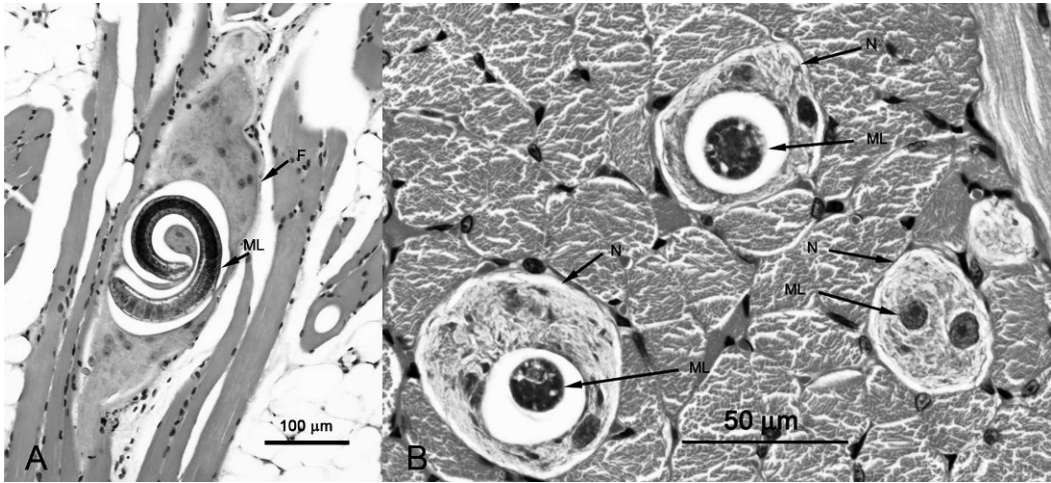
FIGURE 2.   A. Unencapsulated *Trichinella* muscle larvae in cat tongue muscle fiber, day 17 postinfection. ML, muscle larvae; F, parasitized muscle fiber. H&E stained, 40×. B. Mature, well-defined nurse cell containing *Trichinella* muscle larvae in cat tongue, day 30 postinfection. ML, muscle larvae; N, nurse cell. H&E stained, 10×.

2001). Since *T. murrelli* has only been found in North America, this was confirmatory evidence for the source of the infected horse meat and identified *T. murrelli* as a serious human pathogen for consumers of fresh game and horse meats. Sylvatic isolates of *Trichinella* have frequently been cited as a concern for the pork industry based on the potential threat of crossover of sylvatic species into the domestic pig cycle. However, consumption of domestic pork poses little risk for human exposure to *T. murrelli* because *T. murrelli* demonstrates moderate to low infectivity in pigs (Kapel and Gamble, 2000). This was recently supported by Zarlenga et al. (2006), who concluded that pigs are refractory to the crown species of *Trichinella*.

Mice became infected after oral inoculation of isolated ML from raccoons and coyotes. The RCI in Swiss-Webster mice seen here was similar to that reported previously by Pozio and La Rosa (2000), who also reported a 3–16 times higher RCI value in wild mice (29.5–159.8) than in Swiss mice (1.2–9.5), perhaps reflecting the sylvatic host adaptation of this *Trichinella* species.

This first experimental infection of cats with *T. murrelli* was also accomplished by feeding infected raccoon tissues to parasite-free cats. Nurse cell development was complete, and encapsulated larvae were seen in cats by day 30 PI, which is early in the range reported for this species of *Trichinella* (24–70 days PI; Pozio and La Rosa, 2000). It is likely that the nonencapsulated *Trichinella* larvae seen in the cat killed at day 17 had not yet begun nurse cell development. These data demonstrate that animals that frequent both the sylvatic and the peridomestic environment, such as dogs (Dubey et al., 2006) and cats, can become infected and increase the risk of transmission to domesticated animals. Recent studies of poorly managed pig farms have demonstrated that nonconfined pigs will readily consume carcasses of dead pigs as well as other wild and peridomestic animals in their environment (Hill, unpubl. data).

Results of the multiplex PCR indicated that all of the isolates were *T. murrelli*; however, because the single band (127 bp) amplified in multiplex PCR from *T. nativa* and *Trichinella* T6 (both of which occur in the US) is identical to one

of the bands amplified from *T. murrelli*, it is possible but unlikely that some of the animals also harbored a mixed infection containing one of these sylvatic genotypes. Mixed infections of *Trichinella* genotypes are known to occur in nature, but at relatively low rates (Pozio et al., 1995); mixed infections could not be detected here because individual larvae were not tested.

Serologic results from the ELISA identified 77% of the tissue positive coyote samples, and 67% of the tissue positive raccoon samples. The serologic test used in this study utilized *T. spiralis* excretory/secretory (ES) antigen to detect serum antibodies. Previous studies in this laboratory have established its usefulness for detection of anti-*Trichinella* antibodies in canids (Dubey et al., 2006). In addition, Moller et al. (2005) evaluated this test using serum and tissue fluids from canids and found that the test performed well with sensitivity of 99–100%. The sensitivities of the ELISA and the digestion assay are reduced in animals with low worm burdens; worm burdens were low in most animals in the current study. However, in studies published to date, the ELISA was more sensitive than the digestion assay at detecting infection in experimentally infected animals with low worm burdens (Gamble, 1998; Gamble et al., 2004). Fifteen coyotes, four raccoons, and one skunk that were parasite negative by tissue digestion were ELISA positive. It is likely that the volume of tissue digested from the tongues and the low to moderate larval burdens found in the tissues (0.1 to 141 lpg in coyotes and 5 to 481 lpg in raccoons) was responsible for the lack of detection of ML in these serologically positive animals. Additionally, the digestion method used here may not have been sufficiently sensitive to detect ML in lightly infected animals. The theoretical sensitivity of the digestion method utilized in this study is ~1–3 lpg using a minimum of 5 g of tissue (Gamble, 1996, 1998; Gamble et al., 1996), and serologic detection of antibodies to *Trichinella* was found to be more sensitive than the digestion method in pigs (Gamble, 1998). As such, serologic results suggest a much higher rate of *Trichinella* infection in both coyotes (62%) and raccoons (25%), indicating widespread infection with *T. murrelli* in scavenging mammals and a possible risk to consumers of game meats collected in this geographic area. The possibility of transmission through consumption of normally herbivorous host species should also be taken into consideration given the previously documented occurrence of *T. murrelli* in horse meat.

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