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SEROLOGIC EVIDENCE OF INFECTION WITH GRANULOCYTTIC EHRLICHIAE IN BLACK BEARS IN PENNSYLVANIA

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ABSTRACT: Serum samples from 381 black bears (*Ursus americanus*) killed in Pennsylvania (USA) on 24 November 1997 were analyzed for antibodies reactive to the agent of human granulocytic ehrlichiosis (HGE; *Ehrlichia* sp.) by indirect immunofluorescence assay. Antibody reactivity to HGE antigen was detected in 21% (81/381) of the samples collected. Reactive samples were reported from 56% (14/25) of the counties where bear samples were collected. Endpoint antibody titer ranged from 1:8 to 1:16,192, with a geometric mean titer of 1:582. There was no significant difference in antibody prevalence between male and female bears ($P < 0.01$). However, adult bears were significantly more likely to have reactive antibodies than juvenile bears ($P < 0.01$). Attempts to amplify and detect granulocytic ehrlichial DNA from corresponding bear blood clots ($n = 181$) through nested polymerase chain reaction assays were unsuccessful. Further studies are needed for identification of the pathogen-responsible for induction of HGE-reactive. This is the first description of antibodies reactive to the HGE agent in black bears and suggests these mammals are infected with the agent of HGE or an antigenically related ehrlichial species.

Key words: Black bear, *Borrelia burgdorferi*, *Ehrlichia* sp., human granulocytic ehrlichiosis, indirect immunofluorescence assay, *Ixodes scapularis*, polymerase chain reaction, *Ursus americanus*.

INTRODUCTION

Human granulocytic ehrlichiosis (HGE) was first described in 1994 in Minnesota and Wisconsin. The etiologic agent of HGE is an ehrlichia closely related to or conspecific with *Ehrlichia phagocytophila* and *E. equi* (Bakken et al., 1994; Chen et al., 1994), veterinary pathogens causing tick-borne fever and equine granulocytic ehrlichiosis (EGE), respectively (Rikihisa, 1991). The organism is transmitted through the bite of infected ticks. *Ixodes scapularis* (black-legged tick) is a principal vector species in the northeastern and upper midwestern USA (Pancholi et al., 1995).

Human granulocytic ehrlichiosis is a febrile illness with symptoms ranging from moderate to severe. The most common clinical features are headache, myalgia, chills, and malaise. Clinical laboratory findings frequently include leukopenia and thrombocytopenia in later stages (Bakken et al., 1996a).

In the USA, the majority of HGE cases

has been reported from Connecticut, Massachusetts, Minnesota, New York, Rhode Island, and Wisconsin (McQuiston et al., 1999). However, evidence for human infection occurs in California and in mid-Atlantic states south to Florida (Walker and Dumler, 1996; Comer et al., 1999). Most HGE cases are reported during the months of April through September when nymphal ticks are most active; however, cases do occur into December (Comer et al., 1999). The principal vector, *I. scapularis*, also transmits *Borrelia burgdorferi* (Burgdorfer et al., 1982) and *Babesia microti* (Spielman et al., 1985), and some aspects of the epidemiology and the geographic distribution of these diseases are similar.

The range of reservoir hosts of the HGE agent is still under investigation. The white-footed mouse (*Peromyscus leucopus*) is a competent reservoir in the eastern USA (Telford et al., 1996), and naturally infected white-footed mice have been found in Minnesota (Walls et al., 1997) and Connecticut (Magnarelli et al., 1997).

Other mice of the genus *Peromyscus* (e.g., *P. gossypinus* and *P. truei*) also have been found infected with granulocytic ehrlichiae, by either serologic or molecular (polymerase chain reaction; PCR) techniques (Nicholson et al., 1998, 1999), but the role of mice other than *P. leucopus* as reservoirs remains undefined. In California, the dusky-footed woodrat (*Neotoma fuscipes*) is a reservoir for granulocytic ehrlichiae and *I. pacificus* has been implicated as a vector (Nicholson et al., 1999).

The potential role of the black bear (*Ursus americanus*) in the natural history of granulocytic ehrlichiae has not been previously investigated. In this study a serological test was used to detect antibodies reactive with the HGE agent in bear sera, and PCR was used to amplify and identify granulocytic ehrlichial DNA.

MATERIALS AND METHODS

Collection sites

The study sites were areas from 25 counties of Pennsylvania (USA). The Pennsylvania Game Commission (PGC) requires that all hunter-killed black bears, within 24 hr of being shot, be examined and tagged by PGC personnel at check stations located throughout the state (Alt, 1994). Bear blood and serum for this study were collected at 11 PGC check stations on 24 November 1997. These sites were selected based on the high volume of bears brought into the stations in past hunting seasons (Alt, 1994) and on the areas where Lyme borreliosis has been previously shown to be endemic (J. G. Humphreys, unpub. data).

In this study bear blood samples were obtained from the following counties: Armstrong (40°81'N, 79°46'W), Bradford (41°79'N, 76°51'W), Cambria (40°40'N, 78°71'W), Cameron (41°44'N, 78°20'W), Centre (40°91'N, 77°81'W), Clarion (41°19'N, 79°42'W), Clearfield (41°00'N, 78°47'W), Clinton (41°24'N, 77°63'W), Elk (41°41'N, 78°36'W), Fayette (39°92'N, 79°64'W), Forest (41°52'N, 79°23'W), Indiana (40°65'N, 79°08'W), Jefferson (41°12'N, 78°99'W), Lackawanna (41°44'N, 75°61'W), Lycoming (41°34'N, 77°06'W), McKean (41°80'N, 78°56'W), Monroe (41°05'N, 75°33'W), Pike (41°32'N, 75°03'W), Potter (41°74'N, 77°89'W), Somerset (39°97'N, 79°03'W), Susquehanna (41°82'N, 75°80'W), Tioga (41°77'N, 77°25'W), Venango (41°40'N,

79°76'W), Wayne (41°65'N, 75°30'W), and Westmoreland (40°30'N, 79°46'W) (Fig. 1).

Blood and serum collection

At the check stations, 10–15 ml of blood and serum that had collected in the body cavity of eviscerated bears were placed in nonsterile, 15 ml screwcapped vials. Blood was kept on ice in the field. In the laboratory, the samples were centrifuged for 15 min at 400 × G; then serum was removed and frozen at –20 C until analysis. Information including the PGC field number, sex of the animal, age (juvenile or adult as determined by length of canine teeth) (Willey, 1974), and the county and township where the bears were killed was recorded.

Indirect immunofluorescence assay

Sera were examined for antibodies reactive with the HGE agent by using an indirect immunofluorescence assay (IFA), as developed at the Viral and Rickettsial Zoonoses Branch, (Centers for Disease Control and Prevention [CDC], Atlanta, Georgia, USA; Nicholson et al., 1998). Spot slides were coated with human promyelocytic cells (HL-60) infected with the USG3 isolate of the HGE agent (kindly provided by Aquila Biopharmaceuticals, Cambridge, Massachusetts, USA).

All serum samples were centrifuged at 10,000 × G for 5 min to reduce the amount of bacterial contamination. An initial 1:16 screening dilution was made in dilution buffer containing 0.01 M phosphate-buffered saline (PBS) at pH 7.4, 1% bovine serum albumin, and 0.1% sodium azide. Slides frozen at –70 C were placed in a vacuum desiccator and warmed to room temperature. The slides were removed, soaked for 5 min in PBS to rehydrate the antigen wells, and allowed to dry.

Positive, negative, and diluent controls were included in each series of tests. The positive control was empirically obtained by screening serum samples for antibody reactivity from bears collected in Lyme-endemic areas of Pennsylvania (J. G. Humphreys, unpub. data). The negative control serum was obtained from archival bear serum samples taken from a long-term resident at the National Zoological Park (Washington D.C., USA; kindly supplied by R. Montali). Slides containing serum samples were placed in a humidified chamber, incubated at 37 C for 30 min, rinsed in PBS, washed with stirring in PBS three times (5 min each), and allowed to dry. First detector antibody (unlabeled, goat-anti-bear whole serum (ICN Pharmaceuticals, Inc., Irvine, California, USA) was added to each well at a predetermined op-

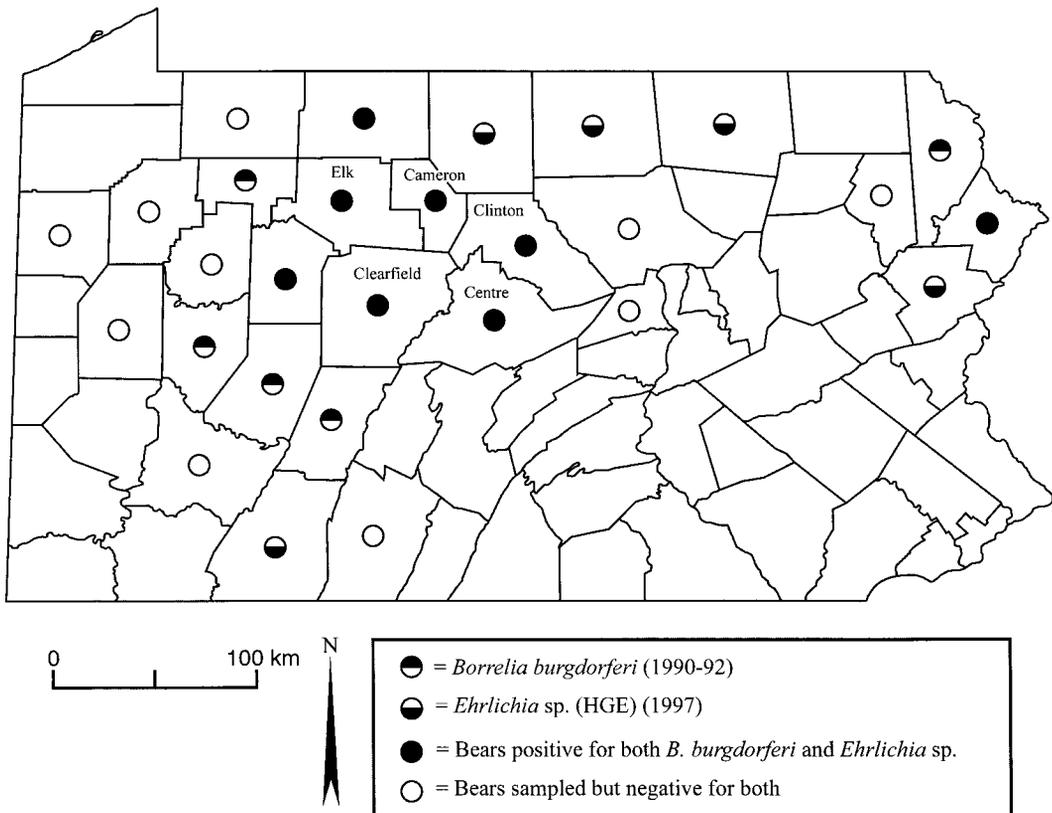


FIGURE 1. Geographical distribution of the human granulocytic ehrlichiosis agent (1997) and *Borrelia burgdorferi* (1990–1992) among black bears from Pennsylvania as determined by serological testing.

timal dilution of 1:500. Slides were then incubated, rinsed, and washed, as done before.

When dry, a predetermined optimal dilution of 1:200 fluorescein isothiocyanate (FITC)-labeled, rabbit-anti-goat heavy and light chain, partially purified serum (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Maryland, USA) was added to each appropriate well. The conjugate solution was centrifuged at $10,000 \times G$ for 5 min to remove complexes of FITC-labeled antibodies that may have formed during storage. The slides were incubated, rinsed, and washed. Six drops of 1.65% Eriochrome Black T counterstain (Sigma Chemical Co., St. Louis, Missouri, USA) in distilled water were added during the second wash to reduce cellular autofluorescence (Schenck and Churukian, 1974). A small drop of antifading mounting medium (DABCO: 4-diazabicyclo [2.2.2] octane) (Aldrich Chemical Co., Milwaukee, Wisconsin, USA) in glycerol-PBS [pH 9.0], was added to each well before a coverslip was affixed (Beutner et al., 1987). The slides were then examined with a Zeiss Axiophot ultraviolet epifluorescence microscope (Zeiss, Wetzlar,

Germany) at $\times 250$ and $\times 400$ magnification for fluorescence of the distinct individual ehrlichial organisms or morulae.

All serum samples were then screened at a 1:16 dilution and then serial twofold dilutions were used to ascertain endpoint titers. Endpoint titers were recorded as the reciprocal of the last dilution showing distinct fluorescence to the HGE antigen.

Polymerase chain reaction assay

Polymerase chain reaction amplification was performed by using commercial amplification kits (Applied Biosystems, The Perkin-Elmer Corp., Foster City, California). The DNA was extracted from 81 seropositive and 100 seronegative bear blood clots and/or pellets (ca. $3 \times 3 \times 3$ mm) resulting from centrifugation of the blood samples by adding 180 μ l of 10 mM Tris saline buffer (pH 8.0) and 20 μ l of proteinase K stock (pH 7.5) (Boehringer Mannheim Biochemicals, Indianapolis, Indiana, USA). The tubes were incubated at 55 C for 1 hr to digest the tissues. The DNA solution was

extracted (QiaAmp tissue kits, Qiagen, Chatsworth, California) three times and washed three times with 10 mM Tris saline buffer. The extracted DNA solution was refrigerated until it was tested by PCR assay.

Five μ l of DNA template were used in a 50- μ l PCR mixture containing HGE agent primers HS1a and HS6a (Biotechnology Core Laboratory, CDC) (Sumner et al., 1997) in the first round of amplification. The reaction mixture was overlaid in 50 μ l of mineral oil and cycled (Thermal Cycler, Perkin-Elmer Cetus, Norwalk, Connecticut, USA) for three preliminary cycles (94 C for 1 min, 55 C for 2 min, and 72 C for 1.5 min), for 37 cycles (88 C for 1 min, 55 C for 2 min, and 70 C for 1.5 min), followed by an extension period of 5 min at 70 C. A nested reaction was conducted using HGE agent primers, HS43, described in Sumner et al. (1997), and HSVR, described in Lotric-Furlan et al. (1998) (Biotechnology Core Laboratory, CDC). Two microliters of DNA template was used in a 50- μ l PCR mixture during the second round of amplification, totaling 40 cycles. During the three preliminary cycles, the annealing temperature was lowered to 48 C and the extension period temperature to 68 C. Each PCR assay included a known positive control HGE agent DNA (USG3 strain) and a control with no template DNA (irradiated water). All PCR assays were performed under strict conditions to minimize the risk of contamination.

Ten μ l of PCR product was added to each sample well of gel containing 1% agarose in 0.04 M Tris-acetate-0.01 M EDTA (TAE) buffer (pH 8.4) and stained with 2.5 μ l of ethidium bromide (10 mg/ml). Gel electrophoresis was conducted at 100 V in TAE buffer for 1 hr and gels were photographed under ultraviolet illumination.

Data analysis

Two-way chi-square tests of independence (Linton and Gallo, 1975) were used to determine statistical associations of antibody prevalence with the sex and age of bears.

RESULTS

Detection of antibodies reactive to the HGE agent

Antibodies reactive to the HGE agent at titers ≥ 16 were detected in 21% (81/381) of bear samples (Table 1). Endpoint antibody titers ranged between 16 and 8,192 (Table 1) with a geometric mean titer (GMT) of 582. Reactive serum samples were identified from 56% (14/25) of Penn-

TABLE 1. Seroprevalence of human granulocytic ehrlichiosis antibodies by indirect immunofluorescence assay in serum samples of black bears in Pennsylvania from 1997. Percentages of seroreactive bears are shown in parentheses.

County	Number tested	Number reactive	Range of reciprocal antibody endpoint titer ^a
Armstrong	2	0 (0)	0
Bradford	2	0 (0)	0
Cambria	6	0 (0)	0
Cameron	4	3 (75)	16–514
Centre	1	1 (100)	2048
Clarion	5	0 (0)	0
Clearfield	38	18 (47.3)	32–8,192
Clinton	23	11 (47.8)	64–4,096
Elk	15	12 (80)	64–4,096
Fayette	1	0 (0)	0
Forest	2	0 (0)	0
Indiana	18	0 (0)	0
Jefferson	9	3 (33.3)	128–512
Lackawanna	1	0 (0)	0
Lycoming	30	1 (3.3)	32
McKean	34	9 (26.5)	16–8,192
Monroe	7	4 (57.1)	1,024–2,048
Pike	29	8 (27.6)	128–8,192
Potter	56	7 (12.5)	64–1,024
Somerset	16	1 (6.3)	64
Susquehanna	1	0 (0)	0
Tioga	37	2 (5.4)	512–2,048
Venango	19	1 (5.3)	256
Wayne	17	0 (0)	0
Westmoreland	8	0 (0)	0
Total	381	81 (21.3)	16–8,192

^a A titer of ≥ 16 was considered reactive.

sylvanias counties from which samples were obtained (Fig. 1). In counties where seroreactive bears were found, the sample sizes were highly variable and antibody prevalence ranged between 3% (Lycoming, $n = 30$) to 100% (Centre, $n = 1$). The majority of reactive samples (51%, $n = 41$) were obtained from three counties in northcentral Pennsylvania (Clearfield, Clinton, and Elk), some of the same areas previously shown to be endemic for Lyme borreliosis (J. G. Humphreys, unpubl. data) (Fig. 1).

PCR analysis

None of the 181 blood clots from both seropositive ($n = 81$) and seronegative (n

= 100) bears tested by PCR assays was found to be positive upon amplification. No bands of the expected 1,297-bp size were amplified from blood clots by using the 16S rRNA primers HS43 and HSVR in the nested PCR.

Demographic characteristics of bears with reactive sera

No significant difference ($\chi^2 = 1.667$; $P = \text{ns}$) in reactive antibody to the HGE agent was found between male and female bears. Of the 185 male bears tested, 45 (24%) had antibodies reactive to the HGE agent, while 36 (18%) of 196 female bears had reactive antibody. However, significantly ($\chi^2 = 7.668$; $P = <0.01$) more adult bears (76/316, 24%) than juveniles (5/65, 8%) had sera reactive to the HGE agent.

DISCUSSION

This is the first study indicating that antibodies reactive to granulocytic *Ehrlichia* sp. are present in black bears in the USA. Reactive antibody to the HGE agent could indicate previous exposure to an ehrlichiae antigenically related to the HGE agent. In the northcentral counties of Pennsylvania, prevalence of antibody frequently exceeded 40% of the bears tested suggesting high levels of exposure.

Unfortunately we were unable to derive PCR amplicons to assist in identifying the genetic characteristics of the ehrlichiae infecting black bears. Our inability to amplify ehrlichial DNA from samples obtained from bears was not surprising. Samples were obtained late in November, after the peak of *Ixodes scapularis* activity. Unless the ehrlichiae infections causing the antibody responses persisted in blood, many of the bears presumably would have cleared their bacteremic phase. Also, our inability to amplify ehrlichial DNA from the blood of antibody reactive animals using PCR suggests that most infections are not current and that ehrlichiae have been cleared from the blood. In some species, such as *Neotoma fuscipes*, both antibody and ehrlichiae, as

measured by PCR of specific DNA, co-exist (Nicholson et al., 1999). It is possible, although unlikely, that the PCR primers used on the bear blood samples might have failed to prime DNA from the infecting ehrlichiae and given false-negative findings. These primers were selected as they readily amplify *Ehrlichia* species belonging to genogroup I and genogroup II (Anderson et al., 1991). It will remain for further investigations to determine if the detected organism is actually the HGE agent or an antigenically related *Ehrlichia* sp.

Black bears may serve as reservoirs for granulocytic ehrlichiae or they may simply provide a blood meal for *I. scapularis* and become "deadend" hosts of the bacteria. Wildlife that have been identified as either infected with a granulocytic ehrlichiae or as potential reservoir hosts for the HGE agent include: *P. leucopus* (Telford et al., 1996) and other species of *Peromyscus* (Nicholson et al., 1999); eastern chipmunks (*Tamias striatus*) and southern red-backed voles (*Clethrionomys gapperi*) (Walls et al., 1997); and white-tailed deer (*Odocoileus virginianus*) (Belongia et al., 1997). Most of these species have been identified solely on the basis of antibody studies; the identity of the ehrlichiae causing the antibody response has not been determined. Additionally, the presence of antibody does not necessarily indicate an ability to act as a competent reservoir for maintaining the natural cycle of ehrlichiae transmission.

Regardless of the exact identity of the infecting organism, black bears had both a high antibody prevalence (21%, 81/381) (Table 1) and an overall high antibody GMT (1:582) to the HGE agent. Other large animals, such as white-tailed deer in Wisconsin (USA), have had an antibody prevalence that was considerably lower (8%, 14/187) (Belongia et al., 1997). Walls et al. (1997) reported that 10% (12/119) of *P. leucopus* in Minnesota (USA) had antibodies reactive to HGE, while overall, 12% (131/1095) of *Peromyscus* species

sampled from 14 states of the US had reactive antibodies (Nicholson et al., 1999). The comparatively high prevalence antibody reactive to HGE in black bears reflects either the long duration of antibody in these potentially long-lived animals or a high incidence of infection.

In Pennsylvania, work involving black bears and Lyme borreliosis has documented a prevalence of antibody reactive to *B. burgdorferi* in bears of 33% (50/150) (J. G. Humphreys, unpubl. data; Cameron 2/2 bears, Centre 2/2, Clearfield 15/22, Clinton 5/5, Elk 9/9, other counties 17/117) (Fig. 1). The counties (Cameron, Centre, Clearfield, Clinton, and Elk) where the highest prevalences of antibody reactive to HGE were found, also were those areas where antibody reactive to *B. burgdorferi* occurred and where *I. scapularis* has been recovered (Stewart et al., 1996). The concordance of these findings suggests that the granulocytic ehrlichiae causing infection in black bears were also being transmitted by *I. scapularis*.

There was no significant difference between male and female bears in the prevalence of antibodies reactive to the HGE agent. However, adult bears were significantly more likely to be infected than juveniles. This pattern may reflect the greater cumulative exposure time for adults. Juvenile bears spend the first 2 to 3 mo after birth in the den (Alt, 1984) and are presumably at low risk for tick exposure during this time.

Black bears are widely distributed throughout Pennsylvania and are used as a source of food for humans. Bear management programs maintain the population at approximately 7,500 with an average kill of about 1,500 during the 3-day hunting season in November (Alt, 1990). Bears are normally field dressed by hunters before arrival at the PGC check stations. Hunters, along with any individual in contact with potentially infected bear blood, should use protective gloves to minimize the risk of exposure to blood-borne pathogens. Although exposure to bear

blood as the source of HGE is purely speculative, three confirmed cases of HGE have been reported in individuals after exposure to deer blood (Bakken et al., 1996b).

This survey provides strong serological evidence that black bears are infected with a granulocytic ehrlichiae, but the exact nature of their role, if any, in the maintenance of the HGE agent remains undefined and merits further investigation.

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