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EVALUATION OF THE HEMAGGLUTINATION TEST FOR EPIDEMIOLOGIC STUDIES OF LEPTOSPIRAL ANTIBODIES IN WILD MAMMALS [1]

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Abstract: Sera from 153 wild animals of 18 species were tested for antibodies against 12 serovars of *Leptospira* by the microscopic agglutination (MA) test. Seventy-five percent of the animals tested were seropositive against one or more of the 12 serovars used. The most commonly found serovars were *pomona, autumnalis, pyrogenes, icterohaemorrhagiae, australis,* and *canicola.* Of 62 carnivores representing 7 species, 55 (89%) were seropositive, as were 46 (60%) of 77 rodents from 9 species. *Leptospira* of the serovar *copenhageni* serogroup *icterohaemorrhagiae* were recovered from kidney tissues of a Norway rat *(Rattus norvegicus).*

Of 443 wildlife sera tested by the indirect hemagglutination (IHA) test using cells sensitized with *L. illini* antigen, 47 (11%), mainly carnivores and deer, gave a heterophile reaction. Of the remaining 396 sera, 164 (41%) were seropositive for leptospirosis by the IHA test. To compare the IHA test with the MA test, 143 serum samples were tested by both methods. There was 84% concordance between the two tests.

INTRODUCTION

According to recent surveillance reports, 119 cases of human leptospirosis were reported in the United States during 1975.[1] The most common probable sources of infection were surface water and dogs. However, other probable sources of infection included cattle, swine, raccoons, deer, rodents and other wild mammals.

It seems evident that the epidemiology of leptospirosis involves wildlife as an important factor in the maintenance and spread of this disease to livestock and humans. A survey of leptospirosis was therefore done to determine the prevalence of antibodies among wildlife species and identify areas with high rates of infection for future epidemiologic studies.

The commonly used method of testing serum for leptospiral antibodies in serum specimens is the microscopic agglutination (MA) test. However, the MA test requires the use of several leptospiral serovars in the active growth phase. The maintenance of the live antigens required for the test is not practical for many laboratories. Recently an indirect hemagglutination (IHA) test using erythrocytes sensitized with an *andamana* strain has been described for serological diagnosis in humans.[8] The IHA test using sheep red blood cells sensitized with the *illini* strain antigen is being used to study its possibility as a

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presumptive test for wild animal sera. The use of this antigen for wildlife studies was evaluated by comparing results of the MA test with the IHA test.

MATERIALS AND METHODS

Wild mammals were collected throughout California. Rodents were captured by live trapping and blood was collected by cardiac puncture. Specimens from carnivores and deer were obtained through the cooperation of the U.S. Fish and Wildlife Service or commercial trappers and hunters. Serum samples were stored at -22 C until tested.

The MA test was performed with 12 live *Leptospira* antigens of the following serovars: *australis, autumnalis, ballum, bataviae, canicola, georgia, grippotyphosa, icterohaemorrhagiae, pomona, pyrogenes, tarassovi* and *wolffi.*

Serum dilutions of 1:20 to 1:2560 were made on a microtiter plate and 0.025 ml of the respective antigen was added to each well. The samples were incubated at 30 C for two h. and examined microscopically by darkfield illumination. A sample was considered positive when 50% of the *Leptospira* cells were clumped.[8] A titer of 40 was considered significant.

The IHA test was done using gluteraldehyde-fixed sheep red blood cells (SRBC) sensitized with *illini* antigen according to the method of Sulzer and Jones.[7,8] Serum samples were inactivated at 56 C for 1 h. and absorbed with non-sensitized, gluteraldehyde-fixed SRBC for 20 min. in a 37 C water bath.

Sera were screened at dilutions of 1:25 and 1:40 using a diluent of Kent buffer and at 1:25 using phosphate buffer containing 1% normal rabbit serum. The test was read after 6 to 18 h. incubation at room temperature and an agglutination reaction of 2+ or greater was considered positive. Test controls included the necessary positive and negative controls, a saline control with sensitized and non-sensitized cells, and a heterophile control using non-sensitized cells. Samples with a positive heterophile were reabsorbed and retested.

The MA and IHA were compared by testing the same sample by both techniques.

Isolation of *Leptospira* was attempted from kidney tissues of 96 animals by one of the three following methods: (1) A biopsy sample was removed aseptically using a pasteur pipette, (2) crushing and expelling a sample through a syringe and needle, or (3) by grinding the tissue by mortar and pestle. A small portion of the sample was inoculated into Fletchers semi-solid medium.[2,5,7,9] The cultures were incubated at 29 C and examined weekly by dark field microscopy. Antisera were used to tentatively identify the strain of *Leptospira* isolated. Specimens that were negative for leptospiral isolation were discarded after 90 days incubation.

RESULTS

MA test. Of 153 samples from the 18 species of wildlife tested by the MA test, 115 (75%) were positive for leptospiral antibodies (Table 1).

Carnivores proved to have the highest rate of infection. Of 62 carnivores from 7 species that were tested, 55 (89%) had antibodies against one or more of the *Leptospira* types used.

The prevalence of seropositives among carnivores was 100% in 12 coyotes and 10 raccoons, 86% of 7 skunks, 85% of 20 bobcats, and 80% of 10 foxes. The MA titer for the serovars with the highest value in each animal was quite evenly distributed in that 29% of the carnivores had titers in the low range (40 to 80), 38% had titers in the midrange (160 to 1640) and 33% had titers in the high range from 1280 to 2560 (Table 1). The highest titers ($\geq$2560) were found in coyotes, raccoons and skunks.

TABLE 1. Prevalence of microagglutinating antibodies against *Leptospira* among wildlife in California, 1975-76.

Species	Tested	Seropositive		Titers*						
	No.	No.	%	40	80	160	320	640	1280	≥2560
Carnivora	62	55	89							
Bobcat *(Lynx rufus)*	20	17	85	6	2	2	3	2	1	1
Coyote *(Canis latrans)*	12	12	100	1	1	6		1		3
Grey Fox *(Urocyon cinereoargenteus)*	10	8	80		2		4		1	1
Mountain Lion *(Felix concolor)*	2	1	50							1
Raccoon *(Procyon lotor)*	10	10	100	2	1	1		1	1	4
Skunk *(Mephitis mephitis)*	7	6	86		1					5
Domestic cat *(Felis domestica)*	1	1	100			1				
Rodentia	77	46	60							
Deer mouse *(Peromyscus maniculatus)*	9	4	44	1	2					1
House mouse *(Mus musculus)*	8	2	25	1						1
Meadow mouse *(Microtus californicus)*	5	5	100	2	2					1
Pocket mouse *(Perognathus parvus)*	4	2	50	2						
Black rat *(Rattus rattus)*	10	0	0							
Norway rat *(Rattus norvegicus)*	15	10	67	7	2					1
Kangaroo rat *(Dipodomys heermanni)*	1	1	100							1
Muskrat *(Ondatra zebethica)*	18	16	89	5	3	1	2	2	1	2
Ground Squirrel *(Otospermophilus beecheyi)*	7	6	86	2	1	1				2
Lagomorpha										
Black-tailed hare *(Lepus californicus)*	2	2	100					1		1
Artiodactyla										
Black-tailed Deer *(Odocoileus hemionus columbianus)*	12	12	100	1	3	2	1		1	4
TOTALS	153	115	75	30	20	14	10	7	5	29

*Reciprocal titer of serovar with highest value when seropositive for more than 1 type.

Of the 77 rodents (9 species) tested, 46 (60%) were seropositive for leptospiral antibodies by the MA test (Table 1). The highest prevalence was among the 18 muskrats (89%), 7 ground squirrels (86%) and 5 meadow mice (100%). The lowest prevalence was for 8 house mice (25%) and black rats; none of the 10 black rats tested reacted to any of the 12 serotypes used. Unlike the relatively even distribution of titers ranging from low to high among carnivores there was a tendency for most of the titers for rodents to be in the low range. Sixty-five percent of the titers were in the low range, 13% were in the medium range and 22% were in the high range. The muskrats were unique among the rodents in that their titers were distributed throughout the entire range tested.

There was evidence of a high rate of leptospiral infection among deer. All 12 of the deer had agglutinins against *Leptospira* (Table 1), and 5 (42%) of them had titers in the higher range.

Both of the two jackrabbits (black-tailed hares) tested were seropositive for *australis* which was considered unusual in view of few reports of this serovar in any single species.[4] Twenty-five percent of the bobcats were also positive for this serotype indicating a possible predator-prey relationship.

Samples were collected in 20 of the 58 counties of California. It appeared that a higher prevalence of leptospiral antibodies was present among wildlife in the coastal and central valley areas than in the mountain areas. Whether this was a function of climatic conditions related to rainfall, irrigation or temperature or merely reflected the species of animals collected is unknown.

Serovars. The results of the MA test show that antibodies noted most commonly among the 306 positive agglutinations were in response to the following serovars, in descending order: *pomona* (14%), *autumnalis* (14%), *pyrogenes* (12%), *icterohaemorrhagiae* (11%), *australis* (11%), *canicola* (9%), and *wolffi* (8%) (Table 2). Among the remaining 5 serovars *tarassovi, bataviae, grippotyphosa*, and *ballum* accounted for only 3% to 6% of the positive seroreactions. The least prevalent seroreactions were against *georgia* for which only 2 rodent serums were positive.

The most common serologic activity for the bobcats were to the serovars *pyrogenes* and *australis* whereas the predominating serovars noted for coyotes were *pomona* and *autumnalis* (Table 2). Over 60% of the coyotes, foxes, raccoons, or skunks reacted to *autumnalis*. From 60% to 75% of the raccoons, coyotes and skunks were also seropositive for *pomona*. From 54% to 81% of the deer were seropositive for *canicola, pomona* and *pyrogenes*.

The most common serovars serologically seen among the rodents were *autumnalis, tarassovi, icterohaemorrhagiae, australis*, and *canicola* (Table 2). Nearly half of the Norway rats tested were positive for *icterohaemorrhagiae* whereas only one muskrat was seropositive to this serovar. However, nearly all of the muskrats were seropositive for *bataviae*.

The muskrats were seropositive for the greatest number (11) of the 12 serovars tested. The coyotes, foxes, Norway rats, bobcats, ground squirrels and deer were also seropositive for at least 9 of the 12 strains. This high serologic reactivity possibly denoting either current or recent infection by one of the serovars noted.

Most of the animals tested had MA antibodies against more than one serovar. Twenty-five percent of the individual animals were seropositive for 2 serovars while 18% were positive for only one serovar and 14% were positive for 3 serovars (Table 3). Two skunks, two muskrats, a bobcat, and a deer were each seropositive for 6 serovars. One muskrat was test-positive for 7 serovars which was the highest number of agglutinins detected in a single animal.

TABLE 2. Distribution of antibodies to leptospiral serovars noted in California wild mammals using the microagglutination test, 1975-76.

Species	Bobcat	Coyote	Fox	Raccoon	Skunk	Mountain Lion	Feral Cat	Deer Mouse	House Mouse	Meadow Mouse	Pocket Mouse	Norway Rat	Black Rat	Kangaroo Rat	Muskrat	Ground Squirrel	Rabbit	Deer	Total
Number tested	20	12	10	10	7	2	1	9	8	5	4	15	10	1	18	7	2	12	153
Negative	3	0	2	0	1	1	0	5	6	0	2	5	10	0	2	1	0	0	35
Serovar																			
australis	7	4	1	3	2	1			2	2		1			6	1	2	1	33
autumnalis	2	7	6	6	5			1		1	1	2		1	4	4		3	43
ballum	4				1							3			2				10
batavia		1	1	2	2					1					10	1			18
canicola	3	2	1	1			1	1		2	1	1			4	1	1	10	29
georgia												1				1			2
grippotyphosa	1		2					2		2		1		1	3	1		1	14
icterohaemorrhagiae	6	5	2	2	3			1		3	1	7			1			2	33
pomona	6	9	2	6	5				1		1	2			5	1		7	45
pyrogenes	9	3	1	3	4		1		1	2		2			4	1		7	38
tarassovi		2					1	2				2		1	7			1	16
wolffi	2	4	3		3		1	1	1		1				5	1		3	25
Total Serovar-positives	40	37	19	23	25	1	4	8	5	13	5	22	0	3	51	12	3	35	306
Number of serovars	9	9	9	7	8	1	4	6	4	7	5	10	0	3	11	9	2	9	—

TABLE 3. Distribution of numbers of leptospiral serovars against which individual animals had agglutinating antibodies.

No. of Test-Positive Serovars	Bobcat	Coyote	Fox	Raccoon	Skunk	Mountain Lion	Feral Cat	Deer Mouse	House Mouse	Meadow Mouse	Pocket Mouse	Norway Rat	Black Rat	Kangaroo Rat	Muskrat	Ground Squirrel	Rabbit	Deer	Total	%
none	3		2		1	1		5	6		2	5	10		2	1			38	25
1	5	2	3	3	1	1				1		2			3	4	1	1	27	18
2	7	1	1	5	1			4	1	2	1	5			3		1	5	37	24
3	1	4	2		1				1	1	1	2		1	5	1		3	23	15
4	3	4	2	1			1					1			2			1	15	10
5		1								1						1		1	4	3
6	1			1	2										2			1	7	5
7					1										1				2	1
total animals	20	12	10	10	7	2	1	9	8	5	4	15	10	1	18	7	2	12	153	

Isolations. Of the 73 kidney samples that were cultured from freshly killed animals, one isolation was made. The positive culture, identified as serovar *copenhageni* serogroup *icterohaemorrhagiae* was recovered from a Norway rat.

Attempts to recover the organism from 23 kidney samples (from bobcats, coyotes, or other animals) that had been kept in a refrigerator or frozen for several days were unsuccessful.

Indirect Hemagglutination. Four hundred forty-three wildlife serum samples were tested by the IHA test. Of these, 47 (11%) gave a positive heterophile reaction against the unsensitized SRBC (Table 4). Reabsorbtion with stabilized cells did not clear the heterophile reaction when the samples were retested. However, many of the sera that gave a heterophile response in this IHA test were positive by the MA test. The majority of positive heterophile reactions were with serum from bobcats, foxes, coyotes and deer.

Due to heterophile reactions we eliminated 47 sera leaving 396 sera to be compared to the MA test results. Of these 164 (41%) were positive for leptospiral antibodies by the IHA test (Table 4).

One hundred forty-three serum samples were tested by both the MA test and the IHA test. There was 84% concordance between the tests with 85.5% sensitivity and 77.8% specificity of the IHA test relative to the MA test (Table 5). The 23 discordant results were essentially randomly distributed; as tested by chi-square ($p>0.10$).

DISCUSSION

The high prevalence of leptospiral antibodies found among carnivores in this study is in agreement with other reports,[3,4,6] as is the high prevalence among muskrats.[4,6] However, the deer tested showed a higher antibody prevalence than has been reported for white-tailed deer.[6] This is believed to be due in part to the location of the populations sampled and their access to contaminated water. For instance, the seronegative black rats tested in this survey were all collected on an enclosed pheasant farm where only fresh tap water was available whereas the seropositive Norway rats were collected from areas where only stagnant water was available. The association between water and leptospirosis is well known, and is amply demonstrated by the high (89%) prevalence of seropositives and large number (10) of serovars serologically noted among the muskrats.

The MA test is considered the standard for leptospirosis testing.[7,9] However, the test is difficult and time consuming. Furthermore, maintenance of live antigens is tedious and constitutes a risk of infection. On the other hand, the IHA test promises to be a valuable preliminary test which is superior to the MA test with respect to speed, convenience, and safety. Many sera can be tested daily by IHA by screening first with 2 dilutions.

The IHA technique using pyruvic- and gluteraldehyde-treated red blood cells sensitized with the *andamana* serovar has been used successfully for diagnosis of leptospirosis in humans. From the results reported here, testing with the *illini* serovar appears to be a successful method to use for epidemiologic studies in wildlife. Utilizing the IHA technique, approximately 200 samples can conveniently be screened for antibodies in one day. The positive reactors can be titered for endpoints the following day. The disadvantages are that the serovars are not identified, heterophile and cross-reaction occurs with some of the sera, and the sensitized cell preparation loses sensitivity in storage after a few weeks.

The 84% agreement between the MA and IHA test found here could possibly be improved if fresh sheep red blood cells were used to absorb heterophile reacting sera. Using more than the 12 antigens in the MA test also would improve agree-

TABLE 4. Wildlife sera tested by the indirect hemagglutination test and retested by the microagglutination test.

Species*	IHA						MA		
	Tested	Heterophile		Non-Heterophile			Tested	Positive	
	No.	reactors	%	No.	Pos.	%	No.	No.	%
Bobcat	57	11	19	46	25	54	20	17	85
Fox	17	11	65	6	4	67	10	8	80
Coyote	79	7	9	72	37	51	12	12	100
Mt. Lion	2	1	50	1	1	100	2	1	50
Domestic Cat	4	1	25	3	3	100	1	1	100
Bear	3	1	33	2	1	50	ND	—	—
Beaver	4	0	0	4	0	0	ND	—	—
Muskrat	37	0	0	37	15	41	18	16	89
Raccoon	23	2	9	21	19	90	10	10	100
Opossum	3	0	0	3	0	0	ND	—	—
Skunk	17	1	6	16	8	50	7	6	86
Deer mouse	29	1	3	28	4	14	9	4	44
House mouse	13	0	0	13	2	15	8	2	25
Norway rat	16	0	0	16	1	6	15	10	67
Black rat	14	1	7	13	0	0	10	0	0
Ground Squirrel	19	0	0	19	5	26	7	6	86
Meadow mouse	15	0	0	15	3	20	5	5	100
Deer	60	8	13	52	30	58	12	12	100
Badger	1	0	0	1	1	100	ND	—	—
Kangaroo rat	12	1	8	11	0	0	1	1	100
Pocket mouse	4	0	0	4	0	0	4	2	50
Black-tailed Hare	14	1	7	13	5	36	2	2	100
Total	443	47	11	396	164	41	153	115	75

*See Table 1 for scientific names.

TABLE 5. Comparison of the microagglutination (MA) test and the indirect hemagglutination (IHA) test for *Leptospira* antibodies in wildlife sera.

		MA test +	—	total
IHA test	+	92	8	100
	—	15	28	43
	total	107	36	143

$\frac{120}{143}$ = 84% concordance

$\frac{92}{107}$ = 85.5% sensitivity*

$\frac{28}{36}$ = 77.8% specificity*

*IHA test relative to MA test.

ment between the tests by detecting antibodies in serum that was positive in the IHA test but negative in the MA test.

Most of the samples where results did not agree between tests were of 2 categories. The first area of disagreement was samples positive on the IHA test that were negative by the MA test. This could be due to the fact that the IHA test detects antibodies much earlier than does the MA test. The other area of disagreement was with rodent sera; the IHA test appeared to be less sensitive than the MA test, for titers that were high with the MA test were negative by the IHA test. Thirteen of the 15 samples positive by the MA test and negative by the IHA test were from rodents. The negative IHA results among rodents may have been due in part to old infections for which the IHA test may not detect antibodies since the IgM antibody fraction usually decreases at a faster rate than IgG antibodies.

The type of buffering agent used in the IHA test does not appear to be a major factor as long as it contains serum. Good agreement was noted between sera tested at 1:25 with either Kent buffer or phosphate buffer.

Further work is needed using the IHA antigen with animal sera to determine whether IgM or IgG antibody fractions are involved in the seroreaction with various species. Continued studies also are needed to determine geographic and climatic conditions that influence infection of wildlife and transmission of the disease to livestock and humans.

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