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Source: Journal of Wildlife Diseases, 10(4) : 429-435

Published By: Wildlife Disease Association

URL: <https://doi.org/10.7589/0090-3558-10.4.429>

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CULTURAL AND SEROLOGIC EVIDENCE OF *Leptospira interrogans* SEROTYPE *Tarassovi* INFECTION IN TURTLES¹

JAMES W. GLOSSER, CATHERINE R. SULZER, MARK EBERHARDT and WILLIAM G. WINKLER

Abstract: Forty-two of 46 sera (91%) from turtles (*Pseudemys scripta-elegans*) in Georgia had microscopic agglutination titers of 200 or greater to *Leptospira* serotype *tarassovi*. Leptospirae were isolated from eight of ten hamsters (80%) inoculated with surface water collected from the settling ponds of the untreated sewage disposal system in which the turtles lived. Leptospirae were also isolated from 12 of 20 hamsters (60%) inoculated with turtle kidney suspensions and six of 20 hamsters (30%) inoculated with turtle cloacal suspension. Hamster brain appeared to be the best tissue for recovering leptospirae since 24 of the 41 isolates (59%) from the 26 culture-positive hamsters were from the brain and 17 (41%) were from the kidney. Six of the 41 isolates from hamsters that had been injected with surface water and turtle kidney and cloacal tissue were identified as being identical to serotype *tarassovi*.

INTRODUCTION

Many domestic and wild mammals have been found to be either natural reservoirs or accidental hosts for leptospirae of various serotypes. However, the potential role of non-mammalian hosts in the epidemiology of leptospirosis has received little attention. Since the epidemiology of leptospirosis is related directly to the presence and distribution of moisture as a transport medium of pathogenic leptospirae between the carrier host and susceptible animal species, amphibians and reptiles may play a role as reservoir hosts.

In recent years, agglutinating substances in turtle sera for leptospirae belonging to the Ballum and *Tarassovi* serogroups have been reported on a number of occasions.^{1,3-5,7-11,13,16,19,21,25} Whether the agglutinating substances represent specific antibody or nonspecific agglutinating factors has not been resolved, and pathogenic leptospirae were not isolated from the turtles involved in the cited studies.

However, leptospirae belonging to the Icterohaemorrhagiae serogroup and a new serotype provisionally named *rana-rum* have been isolated from frogs in Jamaica and the United States.² This report describes the isolation of serotype *tarassovi* from sewage settling ponds in Georgia as well as turtles living in the ponds and the results of serologic examination of these turtles.

MATERIALS AND METHODS

Serum specimens were obtained by cardiac puncture from 56 turtles — 46 slider turtles (*Pseudemys scripta-elegans*), eight snapping turtles (*Chelydria serpentina*), and two stink-pot turtles (*Sternotherus odoratus*). The turtles were trapped from two settling ponds at the Lawrenceville Facility, Center for Disease Control, which received untreated sewage effluent. The turtles were bled, identified, and returned to the ponds.

¹ From the Department of Health, Education, and Welfare, Public Health Service, Center for Disease Control, Bureau of Epidemiology, Atlanta, Georgia 30333 USA.

² Sulzer, C. R. 1973. Unpublished data. Center for Disease Control, Atlanta, Georgia 30333, USA.

The microscopic agglutination (MA) test using an added antigen battery of live cultures of 14 "pathogenic" and five "saprophytic" serotypes was used to detect the presence of leptospiral antibodies.⁶ Pathogenic serotypes included in the battery were *ballum*, *canicola*, *copenhageni*, M20 (Icterohaemorrhagiae serogroup), *bataviae*, *grippotyphosa*, *pyrogenes*, *autumnalis*, *pomona*, *wolffi*, *australis*, *tarassovi*, *georgia*, *patoc*, and *andamana*. Saprophytic serotypes were WA2-P438, *gent*, *sao paulo*, LT430, and *biflexa*. The leptospores were from 4- to 8-day old cultures grown in bovine albumin polysorbate (BAP) medium.¹² The serum was diluted to a 1:25 concentration in phosphate buffered saline. Equal amounts of antigen were added to the serum dilutions for a final concentration of 1:50. Agglutination of 50% or more of the leptospores constituted a positive reaction. Titer endpoints were determined for all sera reactive at the 1:50 dilution.

For cultural studies, 40 of the slider turtles were retrapped and killed. Cloacal and kidney tissues were collected and pooled into a 10 ml (two turtle cloacae/tube, two turtle kidneys/tube) of liquid BAP medium containing approximately 200 mg of 5-fluorouracil for transport to the laboratory.¹⁴ At the laboratory, the tissues were macerated by being forced through the barrels of 5 ml disposable syringes into another tube of liquid BAP medium. From each of these suspensions, 0.5 ml aliquots were placed in semisolid BAP medium as well as inoculated intraperitoneally in male golden Syrian hamsters³ (40 cultures and 40 hamsters). Five uninoculated male hamsters from the same lot served as controls.

The pond water was examined by inoculating each of ten male golden Syrian hamsters weighing approximately 40 g intraperitoneally with 1 ml water specimens collected from both ponds (5 specimens/pond). In addition, each of ten tubes of semisolid BAP medium with 5-fluorouracil were inoculated with approximately 0.03 ml of water from the ponds (five tubes/pond).

The water and turtle cultures were incubated at 29 C for 6 weeks and examined weekly by darkfield microscopy during the incubation period. The hamsters were observed daily during the same time interval. At 6 weeks, the hamsters were killed, and serum, brain, and kidney specimens were collected for serologic and cultural studies. The brain and one kidney from each hamster were ground and inoculated into semisolid BAP medium, using the same technique described for the turtle tissues.

Six leptospiral isolates from hamsters inoculated with surface water, turtle kidney, and turtle cloacal tissue were definitively serotyped by the cross-agglutinin-absorption technique.¹⁵ One brain and one kidney isolate from hamsters receiving each type of inoculum were serotyped by this procedure.

RESULTS

Fifty-four of the 56 turtle sera tested (96%) agglutinated leptospores at the 1:50 dilution or greater (Table 1). However, agglutination reactions to parasitic leptospores were observed only in the stink-pot (*Sternothaerus odoratus*) and slider turtle (*Pseudemys scriptaelegans*) sera. The highest rate of agglutinins to a parasitic serotype was noted in the sliders, with 42 of the 46 sera tested (96%) having an MA titer to *tarassovi* of 1:200 or greater. The distribution of *tarassovi* titers expressed as the reciprocal of the serum dilution within this group was: 200 (1), 400 (0), 800 (3), 1600 (6), 3200 (4), 6400 (8), 12,800 (8), 25,600 (10), and 51,200 (4). Titers of 1:50 or greater to serotypes *ballum*, *copenhageni*, *wolffi*, *patoc*, and *andamana* were noted in the snapping turtles (*Chelydria serpentina*) and the slider turtles (*Pseudemys scriptaelegans*). The snapping and slider turtles had the highest antibody rate for saprophytic leptospores, with reactivity being found to serotypes WA2-P438, *gent*, and *sao paulo*.

³ Outbred Strain Lak: LVG, (SYR), Lakeview Hamster Colony, Newfield, New Jersey 08344, USA.

TABLE 1. Leptospiral Agglutinin Rates by Serotype in Turtle Sera Collected at the CDC Lawrenceville Facility, 1972.

Serotype	<i>Chelydria serpentina</i>	MA Titer Range	<i>Sternothocerus odoratus</i>	MA Titer Range	<i>Pseudemys scripta-elegans</i>	MA Titer Range
<i>ballum</i>	0/2*		1/8 (13)	100**	7/46 (15)	50-100
<i>copenhagani</i>	0/2		2/8 (25)	100	10/46 (22)	50-200
<i>wolffi</i>	0/2		2/8 (25)	50-100	1/46 (2)	400
<i>tarassovi</i>	0/2		1/8 (13)	50	42/46 (91)	200-51,200
<i>patoc</i>	0/2		1/8 (13)	100	3/46 (7)	50
<i>andamana</i>	0/2		2/8 (25)	50-100	7/46 (15)	50-100
WAS-P438 †	0/2		3/8 (38)	200-400	15/46 (33)	50-800
<i>gent</i> †	0/2		0/8 (—)	—	41/46 (90)	100-25,600
<i>sao paulo</i> †	1/2 (50)	200	6/8 (75)	100-800	42/46 (91)	50-1,600
LT430 †	0/2		8/8 (100)	50-400	26/46 (57)	50-200
<i>biflexa</i> †	0/2		6/8 (75)	50-100	18/46 (40)	50-100

* No. positive with a microscopic agglutination (MA) titer of 1:50 or greater/total No. tested (percent); all sera non-reactive to *canicola*, *bataviae*, *grippolyphosa*, *pyrogenes*, *autumnalis*, *pomona*, *australis*, and *georgia*.

** Expressed as the reciprocal of the highest serum dilution where 50 percent or more of the leptospirae are agglutinated.

† Saprophytic serotypes.

TABLE 2. Leptospiral Cultural and Serologic Results of Hamsters Inoculated with Surface Water and Kidney and Cloacal Tissues from Slider Turtles (*Pseudemys scripta-elegans*), CDC Lawrenceville Facility, 1972.

Inoculum	Hamster Culture Results			Serologic Results		
	Total*	Brain	Kidney	Frequency of Seropositivity	Titer Range	
Main Pond Water	4/5 (80)**	4/5 (80)	2/5 (40)	4/5 (80)†	50-3200	
Accessory Pond Water	4/4 (80)	3/5 (60)	3/5 (60)	4/5 (80)	3200-6400	
Turtle Kidney	12/20 (60)	12/20 (60)	11/20 (55)	10/20 (50)	50-1600	
Turtle Cloaca	6/20 (30)	5/20 (25)	1/20 (5)	2/20 (10)	50	
Total	26/50 (52)	24/50 (120)	17/50 (34)	20/50 (40)	50-6400	
Uninoculated Controls	0/5 (—)	0/5 (—)	0/5 (—)	0/5 (—)	—	

* Excluding duplicates when both kidney and brain cultures were culture positive in the same animal.

** No. Culture positive/No. cultured (%).

† No. hamsters having a microscopic agglutination titer $\geq 1:50$ against *tarassovi*/No. tested (%). None of the hamsters had titers against the other 18 serotypes in the test battery.

No deaths occurred in any of the hamsters inoculated with either the water or tissue specimens in the 6-week observation period. However, at necropsy, leptospire were isolated from eight of ten hamsters (80%) inoculated with surface water and 18 of 40 hamsters (45%) inoculated with turtle tissues (Table 2). The distribution of infection in the 40 turtles based on the site of recovery was kidney only in nine (23%), kidney and cloaca in four (10%), and cloaca only in two (5%). Forty-one of the 100 cultures (41%) were positive for leptospire; and, of these, 24 (59%) were hamster brain isolations. Isolations were made from both the brain and the kidney of 14 hamsters (28%); from brain only of nine (18%), and kidney only of three (6%).

The leptospiral isolates made from hamster brains and kidneys were more-phenologically different. In every instance, 95% or more of the leptospire in the brain isolates were straight in contrast to those from the kidney which were virtually all hooked.

Twenty of the 50 hamsters inoculated with pond water or turtle tissues had MA titers ≥ 50 against *tarassovi*, including eight of ten inoculated with pond water and 12 of 40 inoculated with turtle tissue (Table 2). None of the 50 hamsters had titers against the remaining 18 serotypes in the test battery. All the seropositive hamsters were also culture-positive, but six of the culture-positive hamsters were seronegative.

None of the five uninoculated hamsters had cultural or serologic evidence of a leptospiral infection (Table 2).

None of the water specimens inoculated directly into the semisolid BAP medium were culture-positive. However, the heavy bacterial contamination initially occurring in all these cultures may have prevented the recognition or growth of the organisms. Transfer of the cultures at 2-4 day intervals into new medium containing 5-fluorouracil did rid most cultures of other bacteria. Also, the small inoculum (0.03 ml) of pond water per culture severely limits the sensitivity of this procedure.

Six leptospiral isolates, representing one brain and one kidney isolate from hamsters inoculated with pond water, turtle kidney, and turtle cloaca, were selected for identification based on the two-way agglutinin-absorption test. All isolates were serologically identical to serotype *tarassovi*.

DISCUSSION

The results of this study demonstrate the limitation and danger of utilizing only serologic methods in epidemiologic investigations of leptospiral infections. Serologic methods used for the detection of leptospiral antibody offer only presumptive evidence of infection. In this instance, the serologic data from the turtles would have been regarded as nonspecific by some investigators. Recent studies have shown that some species of freshwater turtles have nonspecific leptospiral agglutinating factors.^{18,24} In fractionated sera, this fraction behaved like gamma globulin by immunoelectrophoretic tests, had a low sedimentation coefficient, and was sensitive to treatment with 2-Mercaptoethanol. Moreover, this fraction was identified in turtles which were never exposed to leptospire. In this study, it was deemed necessary to perform cultural studies to determine if the leptospiral agglutinating factor was responsible for the serologic response noted, or if this was indeed an immune response because of infection.

Based on the number of turtle kidney pools which were culture-positive, the infection rate in the turtles was in the range of 33-66%. Whether one or both of the turtles represented in a given kidney pool were positive cannot be determined. Because the cloacal isolates may have represented either cloacal infection or contamination by pond water, turtles which were not cultured at sites other than the cloaca were not included in the estimation of the infection rate.

The source of leptospiral contamination of the pond water is unknown. Two possibilities for the contamination are: (1) infected turtles may have introduced the leptospire to the pond and served

as an amplifying host, or, (2) contaminated urine from other infected animals including wildlife or laboratory animals at the Lawrenceville facility.

The isolation of leptospire from brain specimens from hedgehogs and other wildlife species has been reported previously.^{20, 22, 23} In one report, the brain was second in importance after the kidneys for isolation of leptospire.²³ The turtle study was the first evidence at CDC of brain infection in hamsters inoculated with field material. Also of interest was the morphological variation noted in the isolates between brain and kidneys of the same animal. In every instance, 95% or more of the leptospire from the brain were straight in contrast to those from the kidney, which were virtually all hooked. However, in the six isolates identified, they were serologically identical based on the agglutinin-absorption test. The significance of the morphological variation and, more important, the ecologic significance of leptospiral brain infections is unknown. Additional research must be performed to answer these questions.

The epidemiologic significance of *tarassovi* in turtles is unknown. However, isolation of these leptospire from surface water suggests that infections with this serogroup may be prevalent in the other animal species in the southeastern United States. This is unknown since few, if any, laboratories other than CDC include *tarassovi* in the antigen battery for screening human and other animal sera. The ability of water to serve as the vehicle for the indirect transmission of *tarassovi* was recently suggested in the case of leptospirosis in a United States citizen vacationing in Mexico.¹⁷ His activities included swimming in the ocean and in a remote jungle stream frequented by many cattle. He reported no other contact with livestock other than saddle horses. Also, he had little or no contact with animals in the United States. Previously, a limited serologic survey of swine in this area of Mexico showed evidence of *tarassovi* infection.²⁴ Surveillance of animal and human populations for leptospiral infections may demonstrate *tarassovi* to be a significant cause of such infections.

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Received for publication 21 June 1974