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LISTERIA IN AQUATIC ANIMALS[1] [2]

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Abstract: Listeria monocytogenes was isolated from seven leopard frogs *(Rana pipiens pipiens)*, three bullfrogs *(Rana catesbeiana)*, a painted turtle *(Chrysemys picta marginata)*, a leech *(Placobdella hollensis)* commonly found on turtles, and three species of snails *(Physa sayii, Helisoma* sp., *Oxyloma retusa)*. Most Listeria isolations were made from leopard frogs collected from pond sites used frequently by deer, shortly after a period of heavy rain.

INTRODUCTION

Listeria monocytogenes is a gram-positive bacterium that has infected at least 42 species of mammals and 22 species of birds, including house pets, wild, domesticated, zoo and laboratory animals.[8,9,10] Comprehensive reviews of *Listeria* are presented by Seeliger,[14] Gray and Killinger,[10] and Eveland.[5,6]

Following the repeated isolations of *Listeria* from the feces of apparently healthy white-tail deer *(Odocoileus virginiaus)* inhabiting the Edwin S. George Reserve,[12] this study was part of a program to determine how listeriae are perpetuated on the reserve. Their occurrence in the alimentary canal indicated the listeriae were ingested with food or water. This project was concerned with studying the role of the aquatic ecosystem in the long-term maintenance of *L .monocytogenes.*

STUDY AREA

The Edwin S. George Reserve is a 1200-acre tract of land in the southwest corner of Livingston County, Michigan, about 25 miles north-west of Ann Arbor. About 35% of the reserve is woodlot (predominantly *Quercus - Carya)*, about 2% is brushland, and about 40% is grassy upland; 23% is composed of ponds, swamps, bogs, and marshes.[2]

There are five relatively accessible bodies of water with frogs, turtles, and snails on the Reserve: Burt Pond, George Pond, Crane Pond, Fish-hook Marsh, and Southwest Swamp. Burt and George Ponds were studied extensively for *L. monocytogenes* in the summer of 1966.[1] Crane Pond, Fish-hook Marsh, and Southwest Swamp (Fig. 1) were studied throughout the present study. Crane Pond and Fish-hook Marsh are connected by a culvert; during periods of high water levels, Fish-hook Marsh and Southwest Swamp are also connected. These three ponds are situated at ecotones between grasslands and oak-hickory woodlots.

Crane Pond is an artificial pond of about 2 hectares situated primarily in grasslands. The substrate is composed of clay, sand, and organic muck. It was dug by dragline in 1946. The elevation is 274

[1] Taken from a dissertation submitted by R. G. Botzler in partial fulfillment of the requirements for a Ph.D. degree at the University of Michigan, Ann Arbor.

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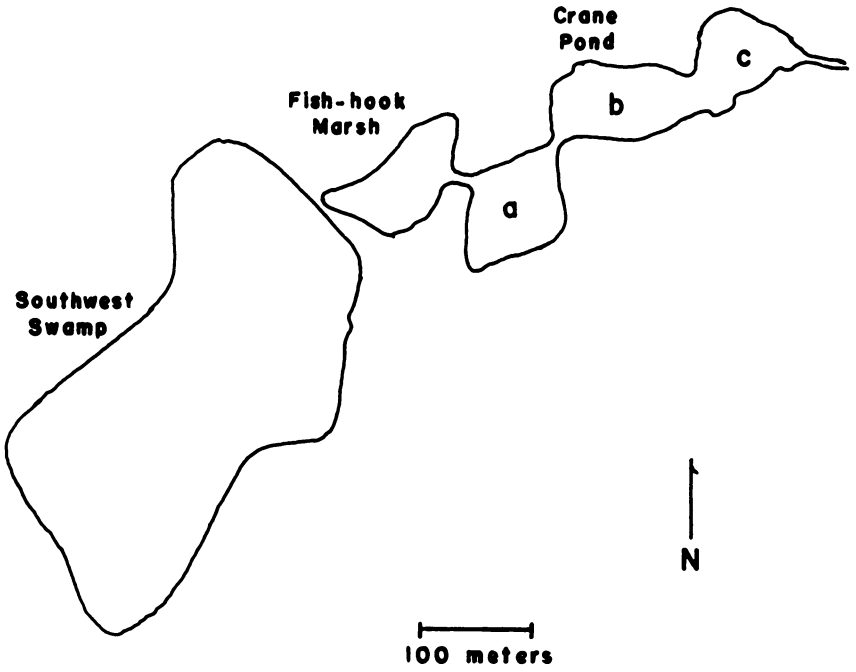


FIGURE 1. Study area on the Edwin S. George Reserve.

m above mean sea level. The hills along the southeast shoreline reach 23 m above the Crane Pond water level. Dredging in 1965 resulted in a steep incline from the shore to the lake bottom along most of the edge.[15] The contours of the land on which the pond is situated naturally divide it into three smaller areas (Fig. 1): sections a, b, and c. Dense clumps of *Salix* sp. grow along the shore of section a. An oak-hickory woodlot grows at the juncture of sections b and c. A small creek at the east end of section c drains Crane Pond; it joins with Honey Creek and eventually empties into the Huron River.

Fish-hook Marsh has an overall area of about 0.6 hectare that is suitable for frogs, turtles, and aquatic snails. A bog is on the north and west sides; there is a heavy growth of shrubs, trees (*Carya* sp. and *Quercus* sp.) and vines (*Vitis* sp.) on the shores of the south and east sides. In general, there is a very shallow incline from the shore to the center of the pond.

Southwest Swamp covers an area of about 6 hectares; a swampy island in the center reduces the area of open water to about 3-4 hectares.[15] Remnants of an oak-hickory forest are found along most of the shore of this swamp; an oak-hickory woodlot occurs at the north end. The incline of the shore into the pond is very shallow; periods of dry weather produce extensive mudflats along the edge. In 1946, a semicircular moat was dug which always contains water — even in dry periods.

MATERIALS AND METHODS

Field Collection

In 1968, we collected aquatic animals between May 1 and August 28 from Crane Pond, Fish-hook Marsh, and Southwest Swamp. These included leopard

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frogs *(Rana pipiens pipiens)*, bullfrogs *(Rana catesbeiana)*, green frogs *(Rana clamitans melanota)*, midland painted turtles *(Chrysemys picta marginata)* and common snapping turtles *(Chelydra serpentina serpentina)*.[3] The snails were identified as *Physa sayii*, *Helisoma* sp., *Lymnaea (Stagnicola) palustris elodes*, and *Oxyloma retusa* (E. Berry, 1968, pers. comm.). The leech was identified as *Placobdella hollensis* (D. Klemm, 1969, pers. comm.). The collections are summarized in Table 1. Limited collections were also made of *Oxyloma retusa* and water samples between May 2 and September 20, 1969.

TABLE 1. Animals collected and their distribution on the Edwin S. George Reserve.

Animals Collected	Source: Crane Pond	Source: Fish-hook Marsh	Source: Southwest Swamp	Total
		1968		
Frogs				
Leopard	16	4	4	24
Bullfrog	14	4	2	20
Green	0	0	3	3
Turtles				
Painted	15	4	4	23
Snapping	0	1	1	2
Snails (pooled)				
Physa	9	2	4	15
Helisoma	3	13	6	22
Lymnaea	2	7	4	13
Oxyloma	0	1	2	3
Leech (pooled)				
Placobdella	1	0	0	1
		1969		
Snails (pooled)				
Oxyloma	3	3	13	19
Water samples	9	1	0	10

Processing Collected Material

Each frog was pithed and opened ventrally to expose the internal organs. After checking for parasites, lesions and any other abnormality, about 1 ml of fecal material was transferred aseptically into a screw-capped tube containing 10 ml Trypticase Soy Broth[5] with 0.5% yeast extract and 50 μg/ml of potassium tellurite (TY-50).

Each turtle was decapitated and the plastron was removed with a bone saw. After checking the viscera for parasites and abnormalities, about 1 ml of fecal material was transferred aseptically to a tube of TY-50.

With the exception of the *Oxyloma* snails, each pool of snails was ground with a mortar and pestle after a rinsing in absolute alcohol. The leeches were cut

[5] Baltimore Biological Laboratories, Baltimore, Maryland.

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in half and ground with a mortar and pestle. The remains of each pooled sample were added to TY-50.

The *Oxyloma* snails were scrubbed in a solution of 200 ppm Iosan[6] in tap water. After rinsing in sterile distilled water, each pool of snails was ground with a mortar and pestle. Half of the remains was stored in a screw-capped tube containing 10 ml of trypticase soy broth, 0.5% yeast extract, and 25 μg/ml of potassium tellurite (TY-25); the other half was stored in screw-capped tubes containing 10 ml of trypticase soy broth and 0.5% yeast extract (TY-0).

The water samples were shaken thoroughly and filtered through a cellulose triacetate membrane with a pore size of 0.45 microns.[7] The membrane was divided into two parts; one part was stored in a tube of TY-25 and the other was stored in TY-0.

All tubes were mixed thoroughly and stored at 4 C for approximately one year.

Listeria Isolations

One ml of each sample was incubated 48 hours at 26 C in Bacto-Tryptose Broth[8] with 3.75% potassium thiocyanate. The selective solid media used for the initial isolations of Listeria were Bacto-Tryptose Agar[8] and 5% sheep blood agar (BAP). BAP was composed of 5% packed sheep red cells added to a base of Trypticase Soy Agar[9] with 0.5% yeast extract. Potential *Listeria* colonies were detected on the tryptose plates with a Henry light apparatus.[11,7]

Biochemical Characterization

All motile, catalase positive isolates were characterized by methods outlined in Cowan and Steel.[4] One percent concentrations of most carbohydrates were used; two carbohydrates, soluble starch and salicin, were prepared at 0.5% final concentration. Soluble starch and glycogen were autoclaved for 10 minutes at 116 C; all others were filter-sterilized and added aseptically to Bacto-Purple Broth Base.[4]

Serological Characterization

The isolates were characterized by direct fluorescien-labelled antibody methods.[13] Dr. Warren C. Eveland provided labelled antisera for serotypes 1 and 4b.

Pathogenicity To Mice

The pathogenicity of the *Listeria* isolates was determined at two levels; 3 x 10^8 and 3 x 10^9 bacteria, using 18 hour cultures of each isolate grown at 26 C. Three 17-20 g Balb male mice were injected intraperitoneally with each dose of bacteria in 0.1 ml. Deaths were recorded over a 10-day period. Attempts were made to recover *Listeria* from the heart blood of the mice that died.

Deer Pellet Surveys of Ponds

Deer fecal pellet surveys were made alongside each pond as an index of the extent each pond was frequented by deer. Three by six meter plots were randomly placed along each pond; one of the 3 m sides was placed along the shore of the pond and the plots extended back 6 m from the shore. The deer fecal pellet groups on each plot were counted. A similar comparison was made between the three sections (a, b, c) of Crane Pond.

RESULTS

Sixty-five isolates of *L. monocytogenes* were obtained from two snails, a leech, a turtle, and ten frogs (Table 2) from the 1968 collections. Five isolates were obtained from a pool of five snails *(O. retusa)* collected in 1969. The colonies appeared gray or blue-gray on tryptose

[6] West Chemical Company, Long Island City, New York.
[7] Gelman Instrument Company, Ann Arbor, Michigan.
[8] Difco Laboratories, Detroit, Michigan.
[9] Baltimore Biological Laboratories, Baltimore, Maryland.

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TABLE 2. Variation among **Listeria** isolates from the George Reserve.

#	Source: Species	Source: Pond	Date Collected	FA: 1 or 4b	Carbohydrate Fermentation (14 days): Xylose	Rhamnose	Sucrose	Melezitose	Sorbitol
			PATHOGENIC STRAINS						
			1968 Collections						
N49	*Helisoma* sp.	F	7/1	4b	—	+	+	+	+
F16	*Rana catesbeiana*	S	7/1	4b	—	+	+	+	+
F37	*Rana catesbeiana*	Cc	7/3	4b	—	+	+	—	+
					—	+	+	+	+
F12	*Rana catesbeiana*	Cb	6/29	4b	—	+	+	+	+
F19	*Rana pipiens*	Cb	7/1	4b	—	+	+	+	+
					—	+	+	+	—
FA	*Rana pipiens*	C	7/1 or 7/2	4b	—	+	+	+	+
FC	*Rana pipiens*	C	7/1 or 7/2	4b	—	+	+	+	+
N20	*Physa sayii*	Cc	5/15	4b	—	+	+	—	—
					—	+	+	—	+
					—	+	+	+	—
					—	+	+	+	+
C1	*Placobdella hollensis*	C	5/25	4b	—	+	+	+	+
					—	+	+	+	—
			1969 Collections						
N66	*Oxyloma retusa*	S	7/5	4b	—	+	+	+	+
					—	+	+	—	+
			NONPATHOGENIC STRAINS						
			1968 Collections						
T3	*Chrysemys picta*	Cb	6/29	4b	+	—	+	+	—
F22	*Rana pipiens*	Cb	7/1	4b	+	—	+	+	—
F28	*Rana pipiens*	Cb	7/2	4b	+	—	+	+	—
F20	*Rana pipiens*	Cb	7/1	1	+	—	+	—	—
					+	—	—	—	—
F27	*Rana pipiens*	Cb	7/2	1	+	—	+	+	—
F37	*Rana catesbeiana*	Cc	7/3	1	+	—	+	+	—

Pond:
F: Fish-hook Marsh
S: Southwest Swamp
C. Crane Pond (Cb = section b, Cc = section c).
FA: Serological type (1 or 4b) based on fluorescent antibody methods.
—: negative reaction
+: positive reaction

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agar with the Henry light. The isolates on BAP had hemolysis under the colonies; some colonies had faint rings of hemolysis around their edges.

All isolates were gram-positive diptheroid rods. All were positive for the catalase, methyl red, and acetylmethylcarbinol reactions. None produced cytochromoxidase, urease, indol, H_2S, or phenylalanine deaminase. All were motile at room temperature. No spores were observed.

Based on the O-F test, all strains fermented glucose in 2-7 days. In purple broth base, all strains fermented glucose and salicin in 24 hours; lactose was fermented in 1-3 days. None of the strains utilized glycogen, soluble starch, mannitol, or inositol within 14 days. Reactions with xylose, rhamnose, sucrose, melezitose, and sorbitol varied (Table 2).

Based on pathogenicity, the isolates fell into two basic groups. The pathogenic isolates were recovered from three species of snails (N20, N49, N66), three bullfrogs (F12, F16, F37), three leopard frogs (F19, FA, FC), and a pool of leeches (C1) commonly found on turtles. All pathogenic strains reacted with type 4b antiserum. They all fermented rhamnose in 24 hours, but did not ferment xylose in 14 days. All strains killed the three mice when injected at doses of $10^{8.5}$; most strains killed some of the mice when injected at doses of $10^{6.5}$ bacteria.

The nonpathogenic isolates were recovered from a painted turtle (T3), four leopard frogs (F20, F22, F27, F28), and one bullfrog (F37). All animals came from Crane Pond; with one exception all came from section b of Crane Pond. These strains did not kill any of the mice at doses of $10^{6.5}$ or $10^{8.5}$ bacteria. Serologically, the nonpathogenic *Listeria* belonged to two groups; some reacted strongly with type 4b antiserum and the others reacted with type 1 antiserum. Regardless of serologic reaction, all nonpathogenic strains had a xylose-rhamnose fermentation pattern opposite to the pathogenic strains; all nonpathogens fermented xylose in 24 hours, but did not ferment rhamnose in 14 days. One bullfrog (F37) carried a nonpathogenic strain reactive with type 1 antiserum and a pathogenic *Listeria* reactive with type 4b antiserum.

Several interesting relationships were evident with the 1968 isolates. Most *Listeria* strains were isolated from animals collected when the water level was at its maximum height. Forty-six animals were collected between June 26 and July 2. This period immediately followed a week of heavy rains which raised the water level 13-15 cm above the spring (May 15-25) level. Eleven of these forty-six animals carried *Listeria;* only three of the eighty animals collected when the water level was lower carried *Listeria.* A chi-square test was used; a significantly ($p < .005$) higher frequency of animals collected when the water level was at its maximum height carried *Listeria* than when the water level was lower.

None of the three ponds was free of *Listeria.* Twelve of 60 animals collected in Crane Pond carried *Listeria,* compared to one of 36 from Fishhook Marsh and one of 30 from Southwest Swamp. A chi-square test showed that the *Listeria* occurred with a significantly higher ($p < .01$) frequency among the animals of Crane Pond than among the animals of the other two ponds.

Considering Crane Pond alone, the site of capture was known for 56 animals. None of the 16 animals from section a carried *Listeria;* but 8 of 31 animals from section b, and 2 of 9 animals from section c carried *Listeria.* A chi-square test indicated some difference ($p < .10$) between the isolation rate from animals of section a and those of sections b and c.

From these results it appeared that the animals of Crane Pond carried *Listeria* at a higher frequency than the animals of the other ponds. In Crane Pond, there was also a difference in the carrier rate of the animals in the three sections. We then tried to correlate the frequency with which the aquatic animals of a pond carried *Listeria* with the frequency that the pond was used by deer. Seven of the twelve plots on Crane Pond contained fecal pellet groups, compared to one of twelve on Fish-hook Marsh and none of twelve on Southwest Swamp. Studying Crane Pond alone, one of the ten plots on section a of Crane Pond contained

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deer fecal pellets while seven of ten on section b and three of ten on section c had pellets. These results demonstrate that those areas with the highest frequency of animals carrying *Listeria* were the ones used most often by deer.

Leopard frogs carried *Listeria* at a greater frequency than any other group of animals. Seven of 24 leopard frogs carried *Listeria* compared to 3 of the 23 other frogs, 2 of 53 snails, and 1 of the 25 turtles sampled. A chi-square test indicated that the isolation rate was significantly ($p < .005$) higher from leopard frogs than from the other animals.

The pattern of collections was such that a large number of samples, predominantly leopard frogs, were taken from Crane Pond shortly after the heavy rains. Because of this sampling artifact, it is difficult to determine whether the increased rate of *Listeria* isolations at this time is independently correlated to each of these three variables (water level or rainfall, pond site, species of carrier), to two of the three, or to only one of the three.

DISCUSSION

The finding that *Listeria* was more prevalent in the aquatic fauna from pond sites used most frequently by deer suggests a relationship between *Listeria* in the deer and in the aquatic animals. It is possible that the relationship is merely coincidental. However, the hypothesis of a more direct relationship is not contradicted by the finding that most isolations of *Listeria* occurred after heavy rains. The deer are known to carry *Listeria* in their feces and some areas on the ponds have a higher frequency of deer pellet groups. The higher prevalence of *Listeria* in those areas could, in part, be the result of the *Listeria* being washed in directly from contaminated deer feces.

The presence of *Listeria* in *O. retusa*, an intermediate host of the deer meningeal worm *(Parelaphostrongylus tenuis)* on the reserve, suggests that these snails can be potential carriers of *Listeria* from infected to uninfected deer. The populations of *Oxyloma*, however, did not occur near the areas where most animals harboring *Listeria* were collected. Also, isolation of *Listeria* from only one pool of five snails from the total of 19 pools containing 193 snails suggests that these populations of *Oxyloma* do not play important roles as carriers of *Listeria* between deer.

It was noted that *Listeria* was isolated at a higher rate from leopard frogs than from any other group of animals. One behavioral difference observed among the leopard frogs was that, in contrast to all other species sampled, they were nearly always observed and caught on land rather than in the water.

All of these observations taken together suggest that the terrestrial ecosystem may be just as vital as the aquatic ecosystem for the long-term maintenance of *Listeria*.

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