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## SURVIVAL OF *Listeria monocytogenes* IN SOIL AND WATER<sup>1 2</sup>

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**Abstract:** *Listeria monocytogenes* survived over 8 weeks in pond water, with no evidence of multiplication. *Listeria* multiplied in sterilized soil during the late winter and early spring. The growth rate in soil was correlated ( $p < 0.005$ ) to the ambient air temperature. The evidence was interpreted to suggest that soil is the reservoir of *Listeria* on the George Reserve.

### INTRODUCTION

A survey of aquatic animals on the Edwin S. George Reserve in southeastern Michigan showed that *Listeria* occurred in several species of animals other than deer, but was more prevalent in the fauna of those sites used more frequently by deer.<sup>3</sup> The diversity of hosts suggested that a reservoir common to all of them (e.g., soil or water) was the source of the listeriae. The purpose of this study was to rule out the soil and water of the George Reserve as reservoirs for *Listeria*. A brief discussion of the study area has already been presented.<sup>2,3</sup>

Two experiments were done. In the first (Exp. 1), populations of a *Listeria* strain from deer were monitored in sterilized soil and water to determine if the strain could survive in the absence of normal and microbial competitors.

In the second experiment (Exp. 2), we tried to rule out unsterilized ("natural") soil and water as media capable of supporting listeriae on a long-term basis by

inoculating unsterilized soil and water of three very different sites with two very different strains of *Listeria*—based on the host source, biochemical reactions, serology, and pathogenicity. If neither strain of *Listeria* survived in the soil and water of any site, this would have indicated that an alternative to soil and water, such as carrier animals, must be responsible for the long-term maintenance of listeriae on the George Reserve. A preliminary experiment indicated that neither unsterilized soil or water were capable of supporting listeriae for more than a week.<sup>2</sup>

### MATERIALS AND METHODS

#### Bacterial Strains

Two *Listeria monocytogenes* strains were used for these experiments. Strain 2108, a type 1c supplied by Dr. Warren C. Eveland, School of Public Health, The University of Michigan, was isolated from the feces of an apparently healthy 1½ year old male deer (*Odocoileus virgini-*

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*anus*) harvested from the George Reserve in December, 1965.<sup>7</sup> It is pathogenic to mice; it ferments rhamnose in 24 hours, but does not ferment xylose in 14 days. Strain F28-71 was isolated from a leopard frog on Crane Pond collected within a few yards of the site used for the soil and water studies; it reacts strongly with type 4b antiserum. This strain is not pathogenic for mice; it ferments xylose in 24 hours, but does not ferment rhamnose in 14 days. Strain 2108 was used in Exp. 1; both strains were used in Exp. 2.

#### Sites

Animals carrying *Listeria* had been collected from all three of the sites used.<sup>8</sup> The Crane Pond site was on the north side of section b; three leopard frogs carrying listeriae had been collected there. The second site was in a small inlet of Fish-hook Marsh from which a *Listeria*-infected snail (*Helisoma* sp.) was collected. The third site was on Southwest Swamp where a *Listeria*-infected bullfrog was taken. The Crane Pond site was used in Exp. 1; all three sites were used for Exp. 2.

#### Collection and Inoculation of Soil and Water

On the morning of November 24, 1969, soil and water were collected from section b of Crane Pond for Exp. 1. The water sample was taken from the surface in a sterile jug. For the soil sample, the sod was removed from an area approximately 1,000 cm<sup>2</sup>. Soil in the upper 3 cm of this exposed area was collected in sterile flasks. The soil and water were immediately brought to the laboratory and samples of each were cultured on Bacto-Tryptose Agar<sup>®</sup> after incubation in Bacto-Tryptose broth<sup>®</sup> enriched with 3.75% potassium thiocyanate.<sup>2</sup> No listeriae were found using a Henry light apparatus.<sup>4</sup>

Ten-ml samples of the water were pipetted into 50 screw-capped tubes

(125mm x 16mm x 10mm I.D.); they were autoclaved for 15 min at 121 C and stored at 4 C. Another 50 tubes were filled with 3 g samples of the soil which were also autoclaved for 15 min at 121 C and stored at 4 C.

The following morning, an 18 hour culture of strain 2108 was washed and re-suspended in 0.85% saline; 0.1 ml of this stock suspension was pipetted into each tube of soil and water. Plate counts were done on serial dilutions of two randomly chosen tubes from each type of substrate to determine the bacterial input. The remaining tubes for each type of substrate were placed in a test tube rack which in turn was placed inside a wire cage for protection and returned to the Reserve in the afternoon of November 25, 1969. The cages with the soil samples were placed directly on the bare plot of soil from which the samples were originally derived. The cages with the water samples were placed at a depth where the water level outside approximated that inside the tubes. The maximum and minimum temperature were recorded for each week.

In the morning of January 12, 1970, soil and water were collected from all three sites for Exp 2. The methods described in Exp. 1 were used, except that none of the samples were autoclaved. The samples were returned to their respective sites on the afternoon of January 13, 1970.

#### Monitoring the Population Changes

The number of surviving bacteria was determined at weekly intervals in two randomly chosen samples from each soil and water sample. The medium used was Bacto-Tryptose Agar<sup>®</sup> enriched with 3.38% potassium thiocyanate (Tr-3).

The samples of sterilized and unsterilized water were thoroughly mixed and successive 10-fold dilutions of each original sample were inoculated in duplicate onto Tr-3 plates. The plates were incuba-

<sup>®</sup> Difco Laboratories, Detroit, Michigan.

ted at 37 C for 40-48 hours; listeriae counts were made on the plates with a Henry light apparatus.<sup>4</sup> All figures were recorded as listeriae/ml of the water samples.

Each 3 g sample of sterilized soil was suspended in 9 ml of sterile 0.85% saline and thoroughly mixed; successive 10-fold dilutions of the soil suspensions were cultured in duplicate. Listeriae counts were made after 40-48 hours incubation. The results were recorded as listeriae/ml of the suspended soil sample. A sample was cultured from each population of bacteria presumptively identified as *Listeria* with the Henry light apparatus. These cultures were then biochemically confirmed as *Listeria*.

## RESULTS

### Exp. 1

Based on the replicate samples cultured shortly after inoculation, all sam-

ples had a concentration of about  $10^5$  listeriae/ml of suspension. The average concentration of listeriae was  $8.8 \times 10^4$ /ml in the sterilized water; the sterile soil samples had an average of  $1.1 \times 10^5$  listeriae/ml of soil suspension.

The listeriae survived poorly in sterilized water. After an interval of 1 week, one of the samples had 78 listeriae/ml; the other sample had no detectable listeriae. After finding no detectable listeriae in the water samples for 3 consecutive weeks, they were discarded.

The survival pattern of *Listeria* in the sterilized soil is shown in Fig. 1. Following an initial decline from  $10^5$ , a level of about  $10^{2.5}$  bacteria/ml was maintained from the 2nd week (Dec. 9, 1969) to the 9th week. Two periods of rapid growth between the 9th and 16th week, brought the population to about  $10^{6.5}$  listeriae/ml. After the 16th week, the listeriae stabilized at a level of about  $10^7$  bacteria/ml of soil suspension.

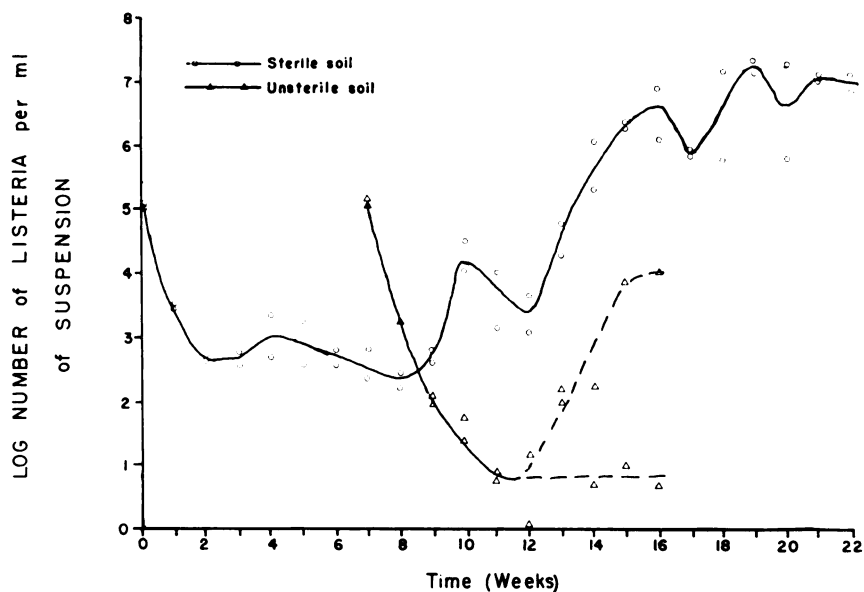


FIGURE 1. Growth of *Listeria monocytogenes* in Crane Pond soil.

### Exp. 2

The water samples inoculated with strain F28-71 had a concentration of  $1.2 \times 10^5$  listeriae/ml; the strain 2108 inoculum was  $1.3 \times 10^5$  listeriae/ml. The soil samples inoculated with strain F28-71 had an average of  $1.2 \times 10^5$  listeriae/ml of soil suspension, and those samples inoculated with strain 2108 had an average of  $1.3 \times 10^5$  listeriae/ml of suspension.

The population changes in the water samples are shown in Fig. 2. Neither strain had detectable levels in Crane Pond water at 1 week (Jan. 20, 1970) or thereafter. In Fish-hook Marsh water, both strains were detected up to 5 or 6 weeks. Both strains had detectable levels in water from Southwest Swamp until the 9th week. In general, the deer strain (2108) appeared to survive better in the water than did the frog strain (F28-71). The population changes in the infected soil samples are shown in Fig. 3. In the Fish-hook Marsh soil, both strains survived until the 9th week. In Southwest Swamp, both strains appeared to be maintaining a stable population after 8 or 9 weeks—perhaps with a very slow decline in numbers. In Crane Pond soil, the populations of both strains not only fluctuated erratically from week to week, but for any given week, the sample variance for either strain was often very large. The frog strain generally survived at higher levels than the deer strain in the soil.

### DISCUSSION

Culturing suspensions of soil, water and feces directly onto Tr-3 and using a Henry light to check for listeriae has been shown to be a more sensitive means of detecting the presence of *Listeria* than first enriching the sample in tryptose broth with 3.75% potassium thiocyanate for 48 hours before culturing it on tryptose agar.<sup>2</sup> It has the added advantage of allowing the number of listeriae in the sample to be quantified, and of taking less laboratory time.

Data from Exp. 2 showed that *Listeria* can be monitored over a period of several

weeks in soil and water containing a normal complement of microorganisms, even when the listeriae are present in low levels. Preliminary studies on both soil and water showed that only 1% of the soil and water bacteria growing on tryptose agar were able to grow on Tr-3 agar.<sup>2</sup> This reduction in organisms made it possible to observe and count individual colonies on plates with little or no dilution of the soil and water samples.

It is also evident from Exp. 2 that soil generally supported both strains of *Listeria* at higher levels and for a longer time than water, the soil generally supporting *Listeria* at levels of 100 to several thousand per gram over the 9 weeks of the experiment. Although the frog strain usually survived at higher levels than the deer strain, there appeared to be more variation between the sites than between the strains.

From Exp. 1, it was evident that in the absence of the normal soil competitors, the deer strain of *Listeria* not only survived, but multiplied under winter conditions. At  $10^7$  bacteria/ml of soil suspension, there were about 100 times as many listeriae as originally inoculated. One sample on the 19th week (April 7) had  $2.14 \times 10^7$  listeriae/ml; this was nearly 200 times the original population. The increase from the population low of  $10^{2.3}$  at the 8th week (Jan. 20) to the high of  $10^{7.2}$  at the 19th week represents nearly a 100,000-fold increase in the *Listeria* population.

The rate of *Listeria* growth in Exp. 1 was regressed on temperature. Because all temperatures fell well below the optimum (30-37 C),<sup>5</sup> it was believed that the maximum temperatures would have the greatest influence on population growth. The rapid decline of the first 2 weeks (Fig. 1) was interpreted as an initial adjustment of a *Listeria* strain adapted to laboratory conditions being returned to a non-laboratory environment; these data were omitted. The data after the 16th week were discarded because of the strong possibility that the population had exhausted some vital nutrient (reached its "K") and was incapable of increasing further.

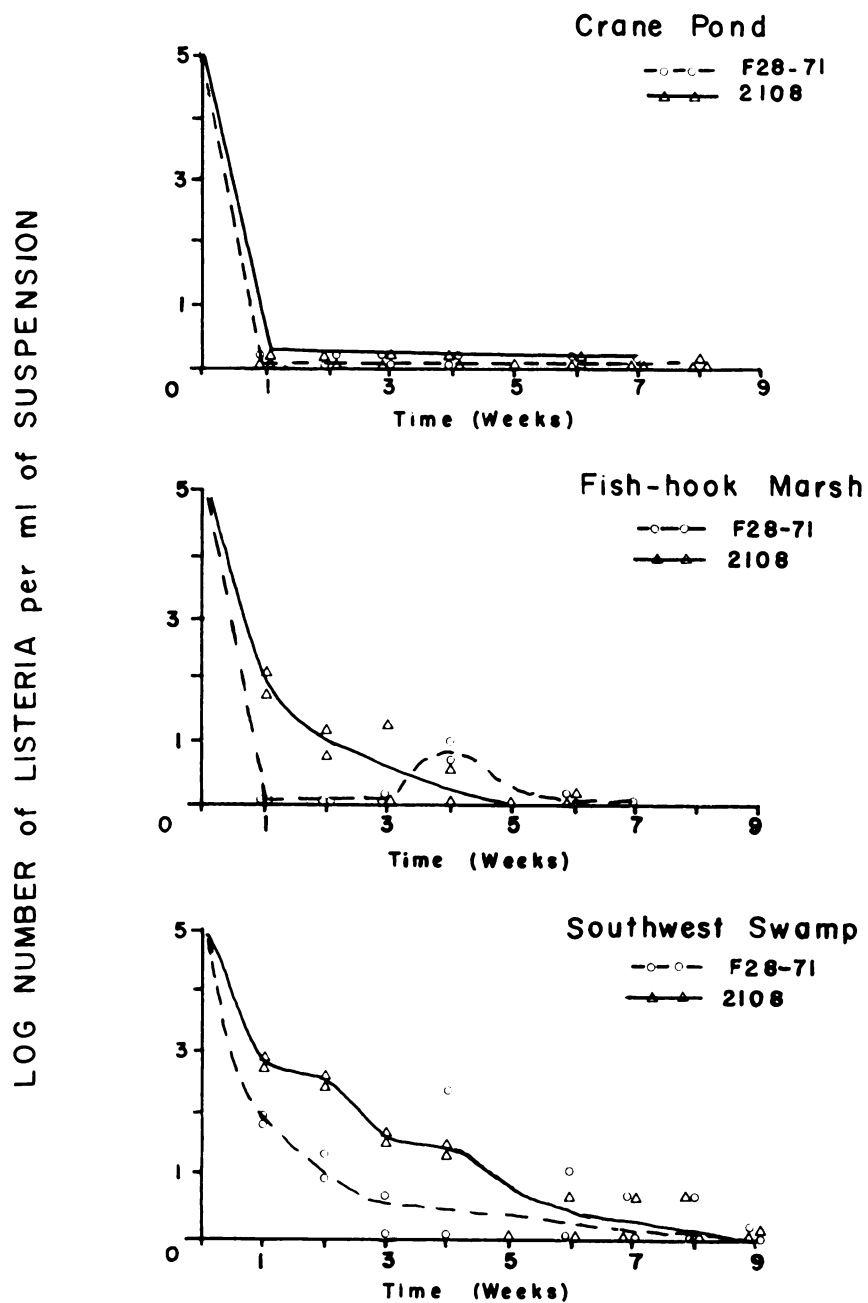


FIGURE 2. Survival of *Listeria monocytogenes* in unsterile water.

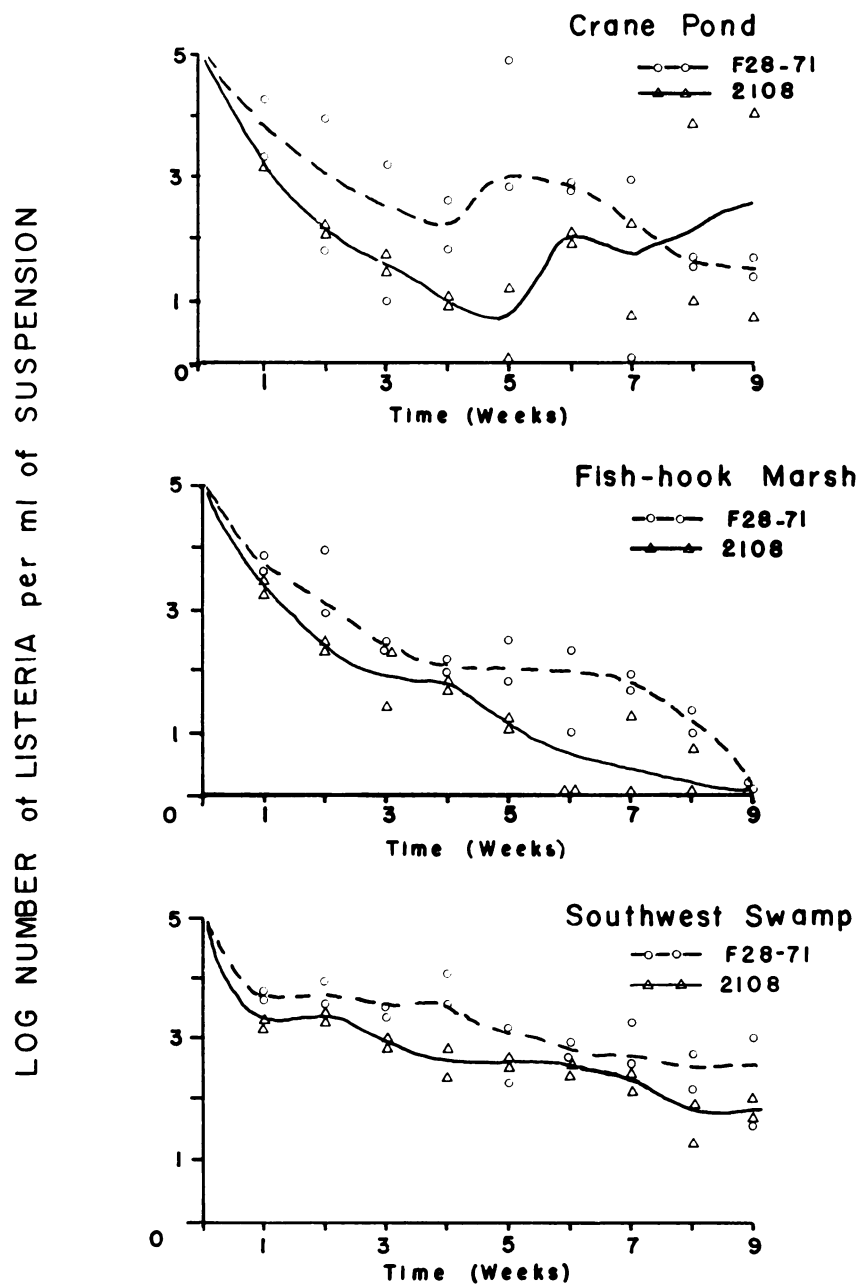


FIGURE 3. Survival of *Listeria monocytogenes* in unsterile soil.

Based on data from Table 1, the regression equation for *Listeria* growth between the 3rd and 16th week was:

$$\hat{Y}' = -0.276 + 0.135x$$

where  $\hat{Y}'$  = the signed difference between the mean log population for a given week and that of the previous week.

x = the maximum temperature (C) recorded during that week.

The calculated  $F(1, 13) = 12.61$  ( $p < 0.005$ ). Thus, from these data it is hypothesized that the maximum ambient temperature has an important influence on the rate of *Listeria* growth in soil during the winter and early spring. In this sample, variation in the maximum temperature accounted for 50% ( $R^2 = 0.513$ ) of the variation observed in the rate of *Listeria* growth.

There is some evidence that multiplication of both strains occurred in some of

TABLE 1. Log Number of *Listeria* per ml of Soil Suspension and Air temperature in Exp. 1.

Week	Mean Log of <i>Listeria</i> Population	Air temperature (C)	
		Minimum*	Maximum*
0	5.04	—	—
1	3.49	—12	9
2	2.69	—12	5
3	2.67	—17	4
4	3.03	—15	3
5	2.91	—25	1
6	2.69	—18	2
7	2.60	—22	—2
8	2.33	—26	5
9	2.71	—29	3
10	4.26	—19	9
11	3.54	—22	4
12	3.35	—28	0
13	4.49	—16	9
14	5.67	—18	7
15	6.29	—13	9
16	6.50	—17	3
17	5.90	—5	12
18	6.41	—12	9
19	7.19	—6	9
20	6.51	3	17
21	7.00	1	17
22	6.92	1	27

\* Minimum and maximum temperatures since previous week



the unsterile soil samples. Using the *t* test, the Crane Pond samples of strain 2108 on the 6th week were significantly ( $p < 0.02$ ) higher than the samples taken the 5th week after inoculation (Fig. 3). High levels of both 2108 and F28-71 were found sporadically in Crane Pond soil; a sample of 2108 had a population near 70% of its original level after 5 weeks, and samples of F28-71 were observed at levels of about  $10^4$  listeriae/ml of soil suspension after 8 and 9 weeks. It is not certain that these high levels were due to multiplication; it is clear, however, that *Listeria* can survive at high concentrations in the soil for several weeks despite severe weather conditions and competition from normal soil flora.

A comparison between the population changes of strain 2108 in the unsterile Crane Pond soil in Exp. 2 and the sterilized Crane Pond soil of Exp. 1 is shown in Fig. 1. After the 11th week (4th week of Exp. 2), the variance between the samples in the unsterile soil increased very rapidly. One explanation is that two populations of *Listeria* were present in the unsterile soil. By plotting them separately, one hypothetical population remained at a low level while the other increased at the same rate and for the same time as the listeriae in their sterilized soil. Another explanation is that some microorganism capable of inhibiting *Listeria* occurred in some, but not all, soil samples. A similar increase in variance occurred among the listeriae in the sterilized soil samples between the 16th and 20th week.

The major multiplication of *Listeria* in the sterilized soil occurred in the late winter and early spring, between the 9th week (Jan. 27) and the 16th week (March 17). This multiplication coincides with the season when most animal outbreaks have been found to occur.<sup>6,8</sup>

The studies on the George Reserve suggest that the long-term maintenance of *Listeria* is related to the terrestrial ecosystem. In all cases, *Listeria* survived longer in artificially infected soil than in water. Leopard frogs carried *Listeria* with a much higher frequency than any other aquatic animal studied;<sup>3</sup> in contrast to all other animals studied, most leopard frogs observed and collected were on land. These observations are supported by the finding that the greatest frequency of *Listeria* isolations were made after heavy rains;<sup>3</sup> it is at this time that the aquatic ecosystem has its greatest exposure to materials from the terrestrial ecosystem.

The evidence does not support the hypothesis that animals are reservoirs of *Listeria* on the reserve. Most listeriae were isolated from leopard frogs 2 to 6 days following a period of heavy rains.<sup>2,3</sup> When the leopard frogs were inoculated *per os* with *Listeria*, the majority of detectable listeriae were shed between 2 and 6 days after infection.<sup>2</sup> Rather than the frogs being reservoirs, the data indicate that any listeriae ingested by frogs are passively shed without becoming established in the intestine.

The evidence suggests that soil is important. Over a 9 week period in winter, *Listeria* survived in unsterile soils better than in unsterile water on the George Reserve. There was no satisfactory evidence for multiplication of *Listeria* in frogs or water; but over a 5 month period, from late fall to early spring, *Listeria* survived and multiplied in sterilized soil. The results indicate that *Listeria* may have multiplied in the unsterile soil too. It has been suggested that soil is the natural reservoir of *Listeria*.<sup>1</sup> The survival and multiplication of *Listeria* in soil over a period of months suggests that soil is the reservoir, and natural habitat, of *Listeria* on the George Reserve.

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