Listeria monocytogenes in Nature

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Samples from 12 farms were examined during two successive spring and early autumn seasons. L. monocytogenes was isolated from vegetation or soil taken from 11 of the 12 farms and from 6 of the 7 nonagricultural sites. A total of 27 strains were isolated from the 19 sites. The organism was not isolated from any of the autumn collections.

The wide geographical distribution of listeric infections involving diverse species of animals (7) suggests common sources of Listeria monocytogenes shared by man and animals. The epidemiology of listeriosis is perplexing, and the habitat of L. monocytogenes is obscure. Seeliger (15) has commented on the resemblances of the biochemical and cultural characteristic of L. monocytogenes to some plant-soil inhabitants and has speculated "that there may well be a primary saprophytic life of Listeria" in which event the epidemiology and epizootology of many listeric infections would be more comprehensible.

The purpose of this investigation was to implicate the soil-plant environment as a reservoir of L. monocytogenes. In previous preliminary studies (18), L. monocytogenes was isolated from vegetation in an agricultural area where listeriosis was rare. In the present study, vegetation at the same sites was sampled during two spring and two autumn seasons of successive years. Soil was cultured from the same sites during the second year. To obtain information on the presence of L. monocytogenes in a different setting, uncultivated vegetation was examined from sites within a small area of another county. A nonagricultural, residential, suburban community with no known listeric infection was selected to minimize the possible role of livestock in Listeria dissemination. The aims were: to note possible seasonal variation in the frequency of occurrence of the organism, to observe difference or similarity of strains recovered on repeated sampling, to compare plant isolants with the strains isolated from surrounding soil, and to observe similarity or difference in isolants from nature and L. monocytogenes isolated from infections.

MATERIALS AND METHODS

Plant samples and soil were obtained in Hanover County, Va., from 12 different farms scattered throughout the county and separated by as much as 50 km. One sample per farm was collected in April 1967 from dead and decayed corn or soybean plants as described elsewhere (18). The same farm was visited during September 1967, April 1968, and September 1968 when plant samples again were obtained as near as possible to the original site. These specimens were collected from portions of the plant 50 cm above the ground. Some of the plants collected in autumn were still green, some were partially green, and some were completely brown. Also, at the time of the last three collections, a total of about 20 g of surface soil was collected to a depth of 2 to 3 cm obtained near the base of the plants being sampled.

Plant material also was collected from a nonagricultural, partially developed, suburban residential location (2 to 3 km² in area) in Henrico County. With one exception (day-lily leaves), the specimens collected here were wild grasses growing either in or at the edge of a wooded area, along the banks of a brook, or in open lots. Seven sites were selected, and each site was visited in April and again in September 1968. The April samples were dead plants which had remained standing during the winter; the September samples were still green.

The vegetation was placed in large jars with Brain Heart Infusion (BHI; Difco) as previously described (18); and held at 4°C over a period of 3.5 months (1967) to 5 months (1968) with samples of the liquor taken every 20 to 25 days (1967) to 30 days (1968). Previous experiences (Welshimer, unpublished data) in culturing vegetation without prior and prolonged cold enrichment were unrewarding; therefore, all specimens were held at least 20 days before subculturing for Listeria. The culture of the liquor obtained April 1967 has been described in detail (18) and modified as follows for the 1968 specimens: BHI (5 ml) was inoculated with 0.05 to 0.1 ml of the liquor and incubated overnight at 37°C. This culture was handled in two ways. (i) It was streaked on several plates of blood-agar base containing 1% glucose (TBG; Difco) and at the same time streaked on Mc-Bréde Listeria agar (14). Each plate was streaked by a different spreading technique to ensure good distribution of colonies. (ii) The BHI culture was placed in
the dark at 20 to 25 C. If \( L. \) monocytogenes was not recovered from the initial plating of \((i)\), the BHI cultures were held for 2 weeks before subculturing on plates of TBG agar and McBride agar. The TBG plates were incubated 37 C, and colonies were examined microscopically at 24 and 48 hr by the oblique lighting method as described by Gray (6). The McBride plates were inoculated with 4 loopsful of the BHI cultures, incubated at 37 C for 2 days, placed at 20 to 25 C for 1 to 2 days, and examined by oblique lighting for small intensely blue colonies. Soil samples (15 g) were placed into 100-mL Erlenmeyer flasks containing 50 mL of BHI adjusted to pH 7.4. These samples were held at 4 C and subcultured over a period of 3.5 months as described above for vegetation. Colonies suspected of being \( L. \) monocytogenes were picked, streaked on TBG agar plates, and incubated for 18 to 24 hr.

Typical colonies of catalase-producing, gram-positive, evenly staining, small rods, motile at 20 to 25 C, were tested on the following carbohydrates: glucose, salacin, lactose, trehalose, esculin, rhamnose, maltose, melezitose, mannnitol, and sucrose. These carbohydrates were held at 37 C and observed for 8 days for reactions characteristic of \( L. \) monocytogenes (8, 15). Organisms with the morphological, cultural, and biochemical reactions of \( L. \) monocytogenes were tested serologically in Richmond by macroscopic tube agglutination tests with rabbit antisera developed against the major serotypes and then forwarded to Utrecht where, in addition to repeating the biochemical and cultural reactions, an extensive analysis was made of each strain by using monospecific antisera developed against the individual \( H \) and \( O \) antigen factors of the various serotypes of \( L. \) monocytogenes.

Pathogenicity of the isolants was based on the response of mice (Rockland Farm SW) weighing 16 to 18 g, intraperitoneally inoculated with suspensions of 2 X 10\(^8\) \( L. \) monocytogenes. Isolants failing to kill sets of mice in 3 weeks were considered avirulent. At this dose, the mice either died within 5 days or survived the 3-week observation period. The dead mice were autopsied and the liver and spleen were cultured.

Isolants were streaked on sheep and rabbit blood-agar with 5% blood in Tryptose-blood-agar base, incubated at 37 C, and observed at 24 and 48 hr.

After mixing and holding the soil or vegetation in BHI at 4 C for the first 18 to 24 hr, some of the broth was streaked on eosin-methylene blue plates.

RESULTS

\( L. \) monocytogenes was not recovered from vegetation or soil collected from the agricultural area (Hanover) during September 1967 or September 1968. \( L. \) monocytogenes was not recovered from vegetation collected from the smaller suburban area (Henrico) during the single September collection, but it was isolated from the spring collections in both areas. Eight strains of \( L. \) monocytogenes were isolated from vegetation at 7 of the 12 sites in the spring of 1967, and 9 strains were isolated from vegetation of 9 of these sites 1 year later. \( L. \) monocytogenes was isolated from vegetation at three sites where none was found the previous year. Only at one site were the organisms isolated the first year and not the second year. Specimens simultaneously collected from soil and the adjacent vegetation provided contrasting results: \( L. \) monocytogenes was isolated from soil at four sites, three of which also yielded \( Listeria \) from the vegetation; however, the soil strain and the vegetation strains were antigenically distinct. A virulent soil strain was isolated from a site where previously no \( Listeria \) were isolated. Of the 12 farms examined, \( L. \) monocytogenes was recovered from vegetation, soil, or both at each of 11 sites either in April 1967 or 1968. \( L. \) monocytogenes was isolated in the spring from six of the seven nonagricultural sites.

The usual \( H \) and \( O \) antigens of \( L. \) monocytogenes with an additional \( O \) factor, XV, were found in 17 strains. This factor previously was encountered (Donker-Voet, unpublished data) and designated as "XV" in \( L. \) monocytogenes W-Li 93/65 received by one of us (D-V.) from H. Seeliger, Würzburg, originally isolated by H. E. Larsen, Copenhagen, from chicken feces. Strains with factor XV closely resembled subtypes of type 4 \( L. \) monocytogenes; e.g., in addition to \( H \) factors A, B, C and \( O \) factors III and XV, seven strains contained \( O \) factors V, VII, and IX as does type 4a; five of the strains contained factors VI and IX; and five strains contained factors V, VI, and IX, thus resembling 4ab and 4e but lacking factor VII or VIII associated with these serotypes. One strain, with the unusual combination of factors I and IX, was indeed anomalous. The nine remaining \( Listeria \) strains were type 1a or 1b; two strains were avirulent. Factor XV strains were all avirulent.

Mice inoculated with the virulent strains presented the hepatic lesions and overwhelming septicemic response characteristic of mice inoculated with human animal strains of \( Listeria \). All strains were tested on both rabbit and sheep blood-agar. The virulent strains hemolyzed sheep blood-agar, but the avirulent strains did not. Rabbit blood was hemolyzed by all of the virulent strains and six of the avirulent strains. The avirulent strains which hemolyzed rabbit blood produced a darkening or alpha-like hemolysis on sheep blood.

The method of subculturing the refrigerated specimens collected in 1968 resulted in earlier detection of \( L. \) monocytogenes than previously accomplished (18). Of the 19 positive isolations of \( L. \) monocytogenes in 1968 from the different specimens, 11 were obtained from specimens cultured after refrigerating for 1 month, and 8 isolations were obtained after refrigerating the specimens
for 2 to 5 months. The four soil strains were isolated after 1 month of refrigeration. Nine of the isolations were obtained from BHI subculture of the refrigerated liquor held at 20 to 25 C for 2 weeks after the initial overnight incubation at 37 C (method ii).

All of the plant specimens obtained in the agricultural area during the spring gave lactose-positive colonies on eosin-methylene blue-agar. The number varied from 3 to 4 colonies per plate to uncountable numbers. No Escherichia coli was observed, for these were all Klebsiella-Enterobacter types of colonies. Only one sample of soil from the same 12 sites contained any lactose-positive colonies. At the nonagricultural sites, lactose-positive colonies were found in two of the seven plant samples. Autumn specimens were comparable to spring specimens with respect to the numbers and distribution of Klebsiella-Enterobacter type colonies. No E. coli colonies were observed in any of the 52 specimens examined during 2 years.

**DISCUSSION**

Decaying moist vegetation favors the support of L. monocytogenes, for none was isolated from the green or recently dead vegetation collected in early September although the same plant growth yielded Listeria in the spring after standing over winter.

The dryness of the surface soil may explain the absence of organisms from that source in the autumn collection; experimental studies (13, 17) have shown that survival of Listeria in soil is influenced by moisture content.

The morphology, colony appearance, biochemical reactions, growth characteristics, antigenic composition, and mouse response to inoculation with the virulent plant-soil strains of Listeria were indistinguishable from L. monocytogenes strains isolated from infected humans and animals. The avirulent strains likewise were similar except for the presence of factor XV in many of the strains and the absence of beta-hemolytic activity on sheep blood-agar. These properties should not exclude the strains from the species, for the organisms do possess other H and O factors associated with L. monocytogenes (5). In the absence of less definitive antigenic analysis, some of these strains might have been placed within the group 4 serotypes as was the case in an earlier report (18). Bojesen-Møller (2) found that 12% of the strains of L. monocytogenes which he isolated from human feces grew without hemolysis on a variety of blood-agar media.

*L. monocytogenes* has been isolated from feces of healthy animals and healthy human carriers (1, 3, 10, 11, 16), leading the Danish workers (1, 3, 10) to postulate that the oral route of listeric infection is important, with the animal gut acting as a reservoir of the agent.

In our studies, the absence of *E. coli* indicates that there was no marked fecal contamination of the soil at the time of sampling; however, it does not exclude the possibility of earlier deposition with death of the less hardy *E. coli*. The isolation of *Listeria* from the suburban community indicates that the organism is not peculiar to agricultural pursuits nor restricted to association with farm animals in the area, but one can not exclude the possible role of birds and feral animals such as squirrels, chipmunks, and oppossums.

The ability of Listeria to multiply at low temperature, its ability to survive for long periods in soil (13, 17), and its recovery from decaying vegetation implies a saprophytic existence wherein the plant-soil environment may serve as a reservoir. Rather than attribute the presence of *Listeria* in nature solely to past contamination with animal feces to the exclusion of an independent role as a free-living organisms, one might liken *Listeria* to the Klebsiella-Enterobacter organisms which extensively exist as free-living forms on plants and soil yet inhabit the gut of man and animals and under appropriate circumstances produce disease.

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