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Isolation of *Listeria monocytogenes* from Vegetation

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A rural area in Virginia, in which clinical listeriosis of man and animals has been rare, was selected for this study. Vegetation, which had died and remained in the fields over the winter, was collected and examined for *Listeria monocytogenes* by Gray's cold-holding procedure. Twelve fields were sampled and cultured, and eight strains of *L. monocytogenes* were isolated. Only two of the strains were pathogenic for the mouse. Compared with strains of *L. monocytogenes* isolated from listeric humans and animals in Virginia and the United States as a whole, these represented serotypes of low frequency.

The widespread geographical distribution of overt infection with *Listeria monocytogenes*, occurring in man and more than 40 species of animals (5), wild and domestic, suggests to some workers (10) that there is a possible natural reservoir of the organism common to all hosts. The role of vegetation as a source of the organism deserves consideration, since, over a period of years, many workers (8) have remarked on the appearance of *L. monocytogenes* in ruminants concurrent with silage feeding. In Iceland, a disease of sheep, long known as "Votheysveiki" or silage disease, was ascribed to *L. monocytogenes* (11), following the isolation of the organism from guinea pigs injected with extracts of poor quality grass silage. Gray (3) isolated *L. monocytogenes* from mice which had been injected with extracts of oat silage that was implicated in an outbreak of listeriosis of sheep in Montana. Krüger (8) provided an extensive review of listeric infections and silage feeding, in addition to his own studies implicating poor quality silage in listeriosis of sheep.

Since *L. monocytogenes* has been isolated from poor-grade silage, the organism would be expected to be present in natural vegetation. If such is the case, this should be of epidemiological significance. The purpose of this investigation was to determine whether, and to what extent, *L. monocytogenes* is associated with natural vegetation in a rural area where listeriosis has been rare.

MATERIALS AND METHODS

All plant samples were obtained from Hanover County, Va.: one sample per farm, from 12 different farms. Eleven of the samples, obtained in April 1967, consisted of pieces of stalks, leaves, and tassels of corn from crops which had been planted the previous year. Part of each sample was obtained by stripping the stalks that remained standing during the winter and part was obtained from portions of the plant which dropped to the ground. One sample (S-8) was obtained from a soy bean crop which had been standing since the previous summer. Each sample was collected with sterile rubber gloves, shredded as much as possible, placed in a plastic bag, and sealed with plastic tape for transport to the laboratory.

Sterile, screw-cap, wide-mouth glass jars (15 × 24 cm) were filled from 1/3 to 1/2 volume with each sample, and 750 ml of Brain Heart Infusion (BHI; Difco), *p*H 7.4, was added to each jar. The lids were tightened and the jars were shaken vigorously to thoroughly moisten the plant material with the BHI. The broth remained at a depth of about 5 cm in the bottom of the jar, leaving some of the vegetation above the level of this substance. The jars were placed at 4 C, with the lids sufficiently loosened to permit escape of gases that may have built up pressure but tight enough to minimize evaporation. At intervals of from 20 to 25 days, over a period of 3.5 months, each jar was shaken and 1 ml of the liquor was removed with a sterile pipette for cultivation. The liquor specimen from each jar was handled in three ways. (i) Each specimen was directly streaked on a plate of Tryptose Blood Agar Base containing 1% glucose (TBG; Difco). Several plates were streaked with an inoculating loop, each by a different spreading technique, to ensure good distribution of colonies. (ii) BHI, 5 ml, was inoculated with 0.05 to 0.1 ml of the liquor and incubated at 37 C overnight before streaking on TBG agar as in (i). (iii) The remainder of the 1-ml sample was incubated at 37 C overnight and then planted on TBG agar as in (i). All plates were incubated at 37 C and observed at 24 and 48 hr. Plates were examined microscopically by Henry's oblique lighting method for fluorescent colonies, as described by Gray (2). All colonies suspected of being *L. monocytogenes* were picked and streaked on TBG agar plates and incubated for 18 to 24 hr. Gram-stained smears were made, and all typical colonies, showing gram-positive small rods, were inoculated into two tubes of BHI broth (one tube

was held at room temperature and the other at 37 C for 18 hr). Those isolants demonstrating good growth at both temperatures and prominent motility (observed microscopically in hanging drop) at room temperature, but rare or absent motility at 37 C, were screened further for positive catalase response. The isolants were also tested for their reactions on the following sugars: glucose, lactose, sucrose, salacin, maltose, rhamnose, melizitose, mannitol, and esculin. Sugars were prepared in concentrations of 1% in Nutrient Broth (Difco) with bromocresol purple as the indicator. The sugar tubes were held at 37 C and were observed daily for 8 days for typical reactions, as described by Seeliger (12).

All strains showing typical morphological, cultural, and biochemical characteristics of *L. monocytogenes* were tested serologically by the macroscopic tube agglutination method, with rabbit antisera developed against the major serotypes. The cultures were subsequently submitted to the Bacterial Serology Center of the Communicable Disease Center, Atlanta, Ga., for antigenic study with factor sera.

Overnight cultures grown on TBG agar slants at 37 C were suspended in dilute BHI broth (diluted 1:8 with distilled water). The suspension was diluted, and two sets of mice, six mice per set (Rockland Farm SW, weighing 16–18 g), were inoculated intraperitoneally with 0.2 ml of suspension. One set received approximately 2×10^9 organisms and the other set, 2×10^7 organisms. The mice were held for 3 weeks and those mice dying within that period were autopsied. Lungs, liver, and spleen were examined by Gram stain and cultured on TBG agar. Blood-agar plates (sheep blood-agar and rabbit blood-agar) were streaked with the isolants to observe hemolytic reactions. These plates were prepared with 5% blood in Tryptose Blood Agar Base, incubated at 37 C, and observed at 24 and 48 hr.

After 120 days of refrigeration, some of the liquor from each specimen was removed and passed through a 0.45-μ membrane filter. The *p*H was determined with a combination glass electrode (A. H. Thomas, Co., Philadelphia, Pa.).

RESULTS

As indicated in Table 1, *L. monocytogenes* was isolated from vegetation samples from 7 of the 12 farms. Two strains of *L. monocytogenes*, S-2 and S-2a, were isolated from one farm. Of the eight strains isolated, only two were lethal for mice at dosages which previously proved lethal with all but one of the many strains of *L. monocytogenes* isolated and tested in this laboratory. The two lethal strains killed all of six mice, inoculated at each dosage level, within 48 to 72 hr after inoculation. *L. monocytogenes* was observed on direct smears and was also recovered from the spleen, liver, and lungs. The surviving strains failed to kill mice within the 3-week observation period. One of the lethal strains, S-2a, was isolated from a specimen which also harbored a nonlethal strain, S-2. All the nonlethal isolants failed to hemolyze sheep blood-agar at 48 hr; however, the two lethal strains both produced good hemolysis on this medium. When plated on rabbit blood-agar, all but one of the strains demonstrated hemolysis, and this strain failed to hemolyze both rabbit and sheep blood.

TABLE 1. *Occurrence and characteristics of strains of Listeria monocytogenes isolated from old vegetation sampled from 12 farms*

Farm sample	*L. monocytogenes* isolated	Serotype	Hemolysis		Lethal for mouse	*p*H (120 days, 4 C)
			Rabbit	Sheep		
S-1	Yes	4b	+	−	0/12	8.2
S-2	Yes	4d	+	−	0/12	8.2
S-2a	Yes	1a	+	+	12/12	
S-3	No					8.1
S-4	No					8.4
S-5	No					8.4
S-6	No					8.3
S-7	Yes	1b	+	+	12/12	8.2
S-8	Yes	4b	+	−	0/12	7.8
S-9	Yes	4a	+	−	0/12	8.4
S-10	No					8.4
S-11	Yes	4a	+	−	0/12	8.3
S-12	Yes	1b	−	−	0/12	8.0

The earliest time of recovery of *L. monocytogenes* was after 20 days of holding the plant material in BHI at 4 C. Three samples were positive when cultured after refrigeration for 48 days and three samples were positive after 77 days of refrigeration. No new recoveries were made from samples when plated between the 77th day of refrigeration and the termination of sampling after 3.5 months of refrigeration.

L. monocytogenes colonies were best detected on plates streaked from BHI broth which had been inoculated with a small amount of the liquor and incubated at 37 C overnight; six of the strains were isolated in this way. Plates streaked from the incubated liquor accounted for two isolants, whereas no recoveries were made from plates streaked directly from the refrigerated specimens.

The study of plates under the microscope, by oblique lighting, was excellent for screening plates and detecting *L. monocytogenes* colonies in the presence of large numbers of other organisms. Although some colonies closely resembled *L. monocytogenes*, they usually were eliminated on the basis of Gram stain. No problems were encountered with molds despite heavy mycelial development in some of the jars.

The *p*H of the liquor ranged from 7.8 to 8.4, after cold-holding for 120 days.

DISCUSSION

Gray's refrigeration and holding procedure (7) served as an excellent enrichment method for isolation of *L. monocytogenes* from plant material shaken in broth.

L. monocytogenes is considered pathogenic for laboratory animals and it may seem unusual to have recovered predominantly nonpathogenic strains. However, this is less surprising when one considers that the majority of *L. monocytogenes* strains studied were those which were isolated from humans or animals with acute and often lethal infections. In this laboratory, of 53 other strains of *L. monocytogenes* isolated in Virginia (comprising 32 strains from acutely infected humans, 16 strains obtained postmortem from infected animals, and 5 strains isolated from silage), only one strain was not lethal for mice and that was a silage strain. Comparison of the frequency of the serotypes of the above human and animal strains with the vegetation strains showed some differences. The serotypes (as confirmed by Gray, Seeliger, or Donker-Voet) of the human isolants consisted of 26 strains of type 4b and six strains of type 1 (subtype not determined), whereas one animal strain was untypable and the remaining 15 strains were all type 4b. It is remarkable that all but 6 of the 52 typable infectious strains are type 4b, and only 2 of the plant isolants are type 4b. Both mouse pathogens are type 1. It is interesting to note that of the five silage isolants (from other areas in Virginia), three were serotype 4a and these were mouse pathogenic. Gray (4) found only four *L. monocytogenes* type 4a strains in 417 cultures isolated from patients. The occurrence of type 4a strains in a much smaller number of samples of vegetation and silage tempts one to extrapolate and suspect that 4a serotypes are peculiar to the "free-living" or saprophytic state rather than a parasitic existence. Krüger's study (8) of *L. monocytogenes*, isolated from silage in East Germany, showed 15 type 1 and three type 4 strains (the subtypes were not determined). Krüger found that all of his silage strains were comparable in mouse pathogenicity to animal strains isolated in the area. Perhaps the differences in pathogenicity, found by this investigator and by Krüger, are a reflection of the differences in the incidence of infection in the two areas studies. Krüger examined silage from a locale where listeriosis in sheep is very high, whereas Hanover County was selected because listeriosis is rare (one human case and one bovine case are known to the author).

The isolation of *L. monocytogenes* is favored by a neutral-to-alkaline reaction, since, as Krüger noted (8), the organism was isolated from poorly fermented silage low in acidity. It is difficult to conceive that any silage prepared in this area could be devoid of *L. monocytogenes* at the time of preparation, although adequate fermentation and mixing, to insure high acidity throughout the silo, should render the silage safe.

Man and animals in the area must be regularly exposed to *L. monocytogenes* and must harbor the organism, at least transiently. The existence of undetected infection in humans has been demonstrated by Bojsen-Møller (1), who isolated *L. monocytogenes* from the stools of asymptomatic meat packers in Denmark. Listeric infections in humans usually fall into one of three catagories (6, 12): (i) the pregnant, with infection frequently inapparent or unrecognized until the fetus is aborted, (ii) the newborn, (iii) the over-forty debilitated adult. This observation reveals the opportunist character of *L. monocytogenes* which successfully infects susceptible individuals but is resisted by others.

In this report, it is difficult to assess the role of soil since, in each sampling, part of the plant material was retrieved from the ground. Under experimental conditions, Lehnert (9) and Welshimer (13) have demonstrated the ability of *L. monocytogenes* to survive in soil for long periods of time. However, there was no evidence of multiplication. It is conceivable that plants, in varying stages of decomposition after remaining in the fields through the winter, may in their partially decayed state support multiplication of the organisms. The portions of plants on the ground, which are protected from desiccation and sunlight, may particularly support multiplication.

The same sites studied in this report are now being examined with sampling of soil and vegetation performed at different seasons.

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