Two Strains of Listeria Monocytogenes (Pirie) Isolated from Feral Sources in Washington

MARION BACON AND NORMAN G. MILLER

Department of Microbiology, College of Medicine
University of Nebraska, Omaha, Nebraska

IN THE course of the investigation of wild animals in eastern and central Washington for the presence of bacterial pathogens two strains of *Listeria*-like bacteria were isolated. One of these was cultured directly from the spleen of a blacktailed jack rabbit, *Lepus californicus wallawalla* Bachman, collected in February, 1951, Schrag, Adams Co. (Miller and Drake, 1954). The other was obtained by the inoculation of white mice from the pooled livers and spleens of five voles, *Microtus montanus* Peale, collected in October, 1953, Pullman, Whitman Co. (Bacon, *et al.,* 1958).

Listeria monocytogenes (Pirie) has been identified on the basis of several criteria. It is regarded as motile and in this way may be separated from *Erysipelothrix* and other diphtheroids apt to be isolated from animals. It ferments a number of different carbohydrates with the production of acid but no gas. Murray, *et al.* (1926) reported a characteristic increase in large, mononuclear leucocytes in rabbits infected with this organism. Julianelle and Pons (1939) recorded a characteristic type of kerato-conjunctivitis in rabbits and guinea pigs infected with *L. monocytogenes* by ocular instillation. These two tests of pathology have often been used as supplements to the biochemical and other tests. The organism has been regarded as usually pathogenic to rabbits, guinea pigs, and white mice. Paterson (1939) reported that *Listeria* was strongly antigenic with both flagellar and somatic antigens; he studied several strains which gave strong cross-agglutination reactions, but which also possessed individual antigens that permitted their classification into three or four distinct groups.

The tests for determining the present isolates from *Lepus* and *Microtus* included a consideration of their animal pathogenicity and a direct comparison with known strains of *Listeria* as to biochemical and antigenic properties, morphology, and motility.

Methods and Procedures

Four strains of *Listeria monocytogenes* were obtained from the American Type Culture Collection. These had the ATCC designations of 984, 4428, 7644, and 7646. Strain 984 had been originally obtained from a gerbille (Mitchell *et al.,* 1927); 4428 was from a rabbit (Murray, *et al.,* 1926); 7644

was from a fatal human case (Gibson, 1935); and 7646 was of ovine origin (Graham, *et al.*, 1939).

The Lepus and Microtus strains together with the ATCC strains were tested for comparative microscopic morphology, gram staining, motility, colony formation, and hemolysis on blood agar. They were also inoculated into nitrate broth, litmus milk, and gelatin as well as diagnostic carbohydrates. The carbohydrates were added to Phenol Red broth base (*Difco*) to form a 1 per cent solution. Each test was run at least three times and the results read by two different individuals. In the case of the carbohydrates with which results were not clear cut or seemed to disagree with the reports of other workers, the tests were repeated using the Seitz filter instead of the autoclave for sterilization. Cultures for the nitrate test, litmus milk test, sugar-fermentation test, and hemolysis test were incubated at 37°C. Some of the cultures for the motility test and the gelatin liquefaction test were incubated at room temperature.

In the agglutination tests laboratory rabbits were first tested serologically to make sure that they were negative for antibodies against the strain of *Listeria* with which they would be inoculated. Six rabbits were then inoculated by a series of intravenous injections of formalin-killed bacteria, one rabbit for each of the strains to be compared. At the end of three weeks the rabbits were bled from the heart and their serums tested by the tube-agglutination method against the formalinized antigens of each of the six strains. The serum of a rabbit which had been inoculated with a living culture of the Lepus strain was also tested against the six strains of antigens.

The Lepus and Microtus strains had previously been tested, or were tested, at this time for pathogenicity by intravenous inoculation into experimental rabbits, by the intraperitoneal or subcutaneous inoculation into laboratory white mice, and by the ocular instillation of rabbits or guinea pigs.

Results

Results on the comparative biochemical tests are listed in Table 1. Maltose, sucrose, lactose, glycerol, dextrin, dulcitol, arabinose, galactose, and inulin were sterilized by filtration, the other carbohydrates by autoclaving at 10 pounds for 10 minutes. All six strains gave essentially the same sugar-fermentation pattern with the exception of the Lepus and 7646 strains; these were alike in that they did not ferment sucrose, whereas the other four strains showed a delayed acidic reaction. All six strains failed to reduce nitrate or to liquify gelatin. All produced a prompt reduction of litmus milk followed by a slight acidity without coagulation.

When tested on agar plates with 5 per cent human blood the Microtus, 984, 7644, and 7646 strains produced beta hemolysis. The Lepus strain showed an alpha hemolysis and strain 4428 a weak alpha hemolysis. The Lepus and Microtus strains resembled the four known strains in gram staining, morphology, type of growth, and colony formation. These were in accordance with

TABLE 1. RESULTS OF CARBOHYDRATE-FERMENTATION TESTS FOR THE SIX STRAINS OF BACTERIA

Carbohydrate	Lepus	Microtus	984	4428	7644	7646
Dextrose	prompt* A	prompt A	prompt A	prompt A	prompt A	prompt A
Maltose	prompt A	prompt A	prompt A	prompt A	prompt A	prompt A
Sucrose	neg.	slow** A	slow A	slow A	slow A	neg.
Lactose	slow A	slow A	slow A	slow A	slow A	slow A
Glycerol	slow A	slow A	slow A	slow A	slow A	slow A
Dextrin	prompt A	prompt A	prompt A	prompt A	prompt A	prompt A
Mannitol	neg.	neg.	neg.	neg.	neg.	neg.
Inositol	neg.	neg.	neg.	neg.	neg.	neg.
Rhamnose	prompt A	prompt A	prompt A	prompt A	prompt A	prompt A
Salicin	prompt A	prompt A	prompt A	prompt A	prompt A	prompt A
Dulcitol	neg.	neg.	neg.	neg.	neg.	neg.
Arabinose	neg.	neg.	neg.	neg.	neg.	neg.
Galactose	prompt A	prompt A	prompt A	prompt A	prompt A	prompt A
Xylose	neg.	neg.	neg.	neg.	neg.	neg.
Inulin	neg.	neg.	neg.	neg.	neg.	neg.

*acid formed within 24 hours.

**acidity first perceptible 7 to 13 days after inoculation.

the description of *Listeria monocytogenes* given by Breed, *et al.* (1948) and for *"Erysipelothrix monocytogenes"* by Wilson and Miles (1955).

The Lepus and Microtus strains showed motility at room temperature and at 37°C when tested soon after they had been isolated. After a period of subculture the Lepus strain seemed to have lost its motility; however, when it was cultured in motility medium (*Difco*) containing 1 per cent Dextrose and subsequent subculture was made from the periphery of the zone of growth on this medium, motility seemed to have been fully restored. The Microtus and the four ATCC strains were observed to be motile.

The serum of each rabbit inoculated with one of the six strains of antigen was tested by tube agglutination against a suspension of formalinized antigen for each of the six strains. These antigens were made up to a turbidity of the McFarland #4 nephalometer. The results of these tests are listed in Table 2. Mutually high cross-agglutination titers occurred among all of the strains except in the case of 7646. Its antiserum gave a high reading with its homologous antigen, but a very low or negative reading with heterologous antigens. Also, the antigen from strain 7646 gave comparatively low readings when tested against the antiserums for the other strains.

Experiment rabbits inoculated intravenously with the Lepus strain seemed either to be unaffected or to show chronic infections of from 4 to 18 months' duration; the infective organism was isolated from the tissues after the death of these chronically infected animals (Miller and Drake, 1954). Miller (1953) reported an increase in large, mononuclear leucocytes in a rabbit inoculated with the Lepus strain. In the present investigation a slight increase in cells

TABLE 2. RESULTS OF AGGLUTINATION TEST. TITERS STATED AS RECIPROCALS OF DILUTIONS OF SERUM

Antigens:	Lepus	Microtus	984	4428	7644	7646
Serums						
Lepus	1280±	1280	640	1280	1280	80
Lepus (living)	640	640	160	320	640	80
Microtus	1280±	2560	640	1280	1280	40±
984	640±	1280±	640	640±	640±	160
4428	1280±	2560±	640	1280	1280	80±
7644	1280	1280	1280±	1280	1280	80
7646	40	neg.	20	neg.	20	1280

of this type was observed in another inoculated experiment rabbit. Attempts were made to produce conjunctivitis in rabbits and guinea pigs by using the method of Julianelle and Pons (1939), but results were negative. No pathogenicity for guinea pigs or white mice could be shown by the injection of the organism by the intraperitoneal route, nor did mice inoculated in this way show any persistence of the organisms in their tissues when they were sacrificed and autopsied.

The Microtus strain showed a high virulence for experiment rabbits and white mice (Bacon, *et al,* 1958); since one rabbit died 24 hours and another 72 hours after intravenous inoculation, no further attempts were made toward producing a monocytosis. Rabbits were inoculated by swabbing the everted eyelid. No conjunctivitis resulted on two attempts, but on the third attempt a rabbit developed a severe ocular inflammation on the second day after inoculation. On the seventh day recovery from this infection seemed to be almost complete, but typical microorganisms were observed in a smear of pus taken at this time. Inoculation of captive *Microtus montanus* with this strain seemed to result either in a rapidly fatal infection or an asymptomatic persistence of the bacteria in the liver and spleen (Bacon, *et al.,* 1958).

Discussion

The results of the biochemical tests conducted here were essentially the same for the Lepus and Microtus strains as for the four known *Listeria* strains. According to several works including Bergey's manual (Breed, *et al.,* 1948), *Listeria* does not ferment galactose. However, some other authors have reported this sugar to have been fermented by *Listeria.* In the present study results were variable when autoclaved galactose was used, but with galactose newly obtained from distributor (*Difco*) and sterilized by filtration, acid formation was consistently prompt for all six strains. The reports of various workers in the field on sugar-fermentation patterns for *Listeria* have shown enough disparity to suggest either a variability in or among the strains involved or else a variability in methods used in conducting the fermentation tests. But it is probable that a useful diagnostic criterion could be worked out for this organism on the basis of the sugars for which it shows a prompt acid formation and those sugars for which it shows negative results. Smith, *et al.* (1955) proposed a method by which *Listeria* could be differentiated from *Erysipelothrix* on the basis of sugar fermentation and nitrate reduction. *Listeria* produces acid from maltose, sucrose, and salicin and does not reduce nitrate; *Erysipelothrix* produces no acid from these three sugars and reduces nitrate.

Motility is still one of the useful criteria for differentiating *Listeria* from *Erysipelothrix* and the diphtheroids. The occurrence of the Lepus strain in a nonmotile phase as observed in this study suggests that the use of a medium for inducing motility should always be considered in carrying out tests for such a differentiation.

Both the Lepus and Microtus strains gave very high cross-agglutination reactions with each other and with the 984, 4428, and 7644 strains. The failure of 7646 to show high cross-agglutination titers with the other strains is in accordance with the findings of Paterson (1939) in regard to antigenic variability among various *Listeria* strains. Such a variability would suggest that it might be well to keep a number of known strains of *Listeria* available where newly isolated organisms are being identified.

Because of their similarities both antigenically and biochemically to known strains, the Lepus and Microtus strains are unquestionably *Listeria monocytogenes*. The authors believe that these are the first isolations of the organism to have been made from either *Lepus californicus* or *Microtus montanus,* or in either of these two genera as far as New World locations are concerned. *Listeria* has been reported in a European hare (Wramby, 1944), and Levy (1948) described the isolation of several strains from *Microtus agrestis,* a European vole. *Listeria* is probably world wide in its distribution, and it has been isolated from a great number of different host species including man and both wild and domestic animals. Olson (1954) in a review of the distribution records of *Listeria* said it had been found in 23 different species of animals and in 20 different countries of the world.

There is no indication as to whether the Lepus or Microtus strains had been indigenous in eastern Washington or recently introduced. These two strains represent the only isolations of *Listeria* made from a total of 1041 rabbits, 1846 rodents, and 23 shrews collected over a period of six years. White mouse inoculation was the principal means used in detecting bacteria in the tissues of these animals so of course some *Listeria* strains of low pathogenicity could have been missed. Although its incidence may have been low at the time of the collections, the presence of *Listeria* in such animals as jack rabbits and field mice may have significance in regard to its veterinary and medical importance, since these animals so often are the most numerous elements in wild mammalian populations in the West. It is well known that listeriosis infection may be acquired by ingestion of the pathogen.

The Lepus strain seemed to have a very low general pathogenicity or to have an extremely long persistence in a living animal; it was indicated that the Microtus strain could have asymptomatic persistence in the tissues of

Microtus (Bacon, *et al.*, 1958). These characteristics may be important in maintaining listeriosis in an enzootic condition. Pirie (Mitchell, *et al.*, 1927) believed that a chronic form of the disease existed among populations of wild gerbilles. Osebold and Inouye (1954) said that some experiment rabbits can become latently infected shedders of *Listeria* bacilli. According to Morris and Norman (1950) *Listeria* organisms could persist in the tissues of apparently healthy ferrets.

Summary

Two strains of bacteria isolated in Washington, one from *Lepus californicus* and one from *Microtus montanus*, were identified as *Listeria monocytogenes* by comparing them with four known strains. All six strains gave essentially similar results when tested with nitrate broth, litmus milk, gelatin, and 15 different carbohydrates. It was proposed that the carbohydrate-fermentation pattern for *Listeria* strains is consistent enough to justify its use in routine identification of the organism. It was further suggested that the use of a special bacterial motility medium be considered in carrying out tests for separating *Listeria* from *Erysipelothrix* and other diphtheroids on the basis of motility.

The Lepus and Microtus strains showed high cross-agglutination titers with each other and with three of the four known strains.

Both the Lepus and Microtus strains showed some tendency to persist in the tissues of living animals. A very low pathogenicity was indicated for the Lepus strain and a relatively high one for the Microtus strain. It was suggested that *L. monocytogenes* may be more common in wild animal populations of the West than the records reveal.

Acknowledgement

Acknowledgement is made to Dr. Francis D. Smith, Department of Pathology, University of Nebraska, College of Medicine, for invaluable assistance and advice in the hematological procedures carried out in this work.

Literature Cited

Bacon, M., R. F. Bacon, and C. H. Drake. 1958. Bacterial infections in wild rodents of eastern and central Washington. J. Infect. Dis., 102: 5-13.

Breed, R. S., E. G. D. Murray, and O. P. Hitchens. 1948. Bergey's manual of determinative bacteriology, Sixth Edition. Baltimore. The Williams and Wilkins Co. pp. iii-1529.

Gibson, H. J. 1955. A pathogenic diphtheroid bacillus from a fatal case of meningitis. J. Path. and Bact., 41: 239-252.

Graham, R., G. L. Dunlap, and C. A. Brandly. 1938. Ovine and bovine listerellosis in Illinois. Science, 88: 171-172.

Julianelle, L. A. and C. A. Pons. 1939. Identification of *Listeria monocytogenes*. Proc. Soc. Exp. Biol. Med., 40: 362-363.

Levy, M. L. 1948. *Listeria monocytogenes* in voles. Vet. J., 104: 310-312.

Miller, N.G. 1953. Infectious diseases in native wild animals in the Columbia Basin, Washington. Doctoral thesis. State College of Washington, Pullman, Washington. vii-83.

Miller, N. G. and C. H. Drake. 1954. Infectious diseases in native wild animals in the Columbia Basin, Washington. Northwest Sci., 28: 135-156.

Mitchell, H. A., J. H. H. Pirie, and A. Ingram. 1927. The plague problem in South Africa. Publ. South African Inst. Med. Res., 3: 86-256.

Morris, J. A. and M. C. Norman. 1950. The isolation of *Listeria monocytogenes* from ferrets. J. Bact., 59: 313-314.

Murray, E. G. D., R. A. Webb, and M. B. R. Swann. 1926. A disease of rabbits characterized by a large, mononuclear leucocytosis, caused by a hitherto undescribed bacillus, *Bacterium monocytogenes* (N.sp.) J. Path. and Bact., 29: 407-439.

Olson, C. 1954. Public health aspects of listeriosis. Proc. Amer. Vet. Med. Assoc. 1954: 450-455.

Osebold, J. W. and T. Inouye. 1954. Pathogenicity of *Listeria monocytogenes* infections in natural hosts. I. Rabbit studies. J. Infect. Dis., 95: 52-66.

Smith, R. E., I. M. Reynolds, and R. A. Bennett. 1955. *Listeria monocytogenes* and abortion in a cow. J. Amer. Vet. Med. Assoc., 126: 106-110.

Wilson, G. S. and A. A. Miles. 1955. Topley and Wilson's principles of bacteriology and immunity. Fourth Edition. Vol. 1. Baltimore. The Williams and Wilkens Co. xi-1106.

Wramby, G. O. 1944. *Listerella monocytogenes*. Bacteriology and occurrence of *Listerella* infections in animals. Skand. Vet-tidskr., 34: 277-290. Cited by Olson (1954).