1957

Listeria monocytogenes

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LISTERIA MONOCYTOGENESS

by

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A thesis submitted
in partial fulfillment of the requirements for the
degree Master of Science at South Dakota
State College of Agriculture
and Mechanic Arts

October 1957

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This thesis is approved as a creditable independent investigation by a candidate for the degree, Master of Science, and acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Professor in charge of the thesis

Head of the major department

ACKNOWLEDGMENT

The author realizes that this paper could not have been as accurate and complete as it is without the cooperation of several people. He is, therefore, grateful to Dr. H. E. Calkins, Dr. E. C. Berry and Dr. J. B. Taylor for invaluable criticisms and suggestions and to John Morrell and Company for their cooperation in collecting the serum. Last, and by no means least, the writer takes this occasion to express his sincere appreciation to his wife, Marlene, for helping to set up the agglutination tests and for typing the manuscript.

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INTRODUCTION

Organisms of the genus Listeria have stimulated a great deal of interest as a result of their pathogenicity and affinity for the central nervous system. The clinical condition produced by this organism is known as listeriosis, or in ruminants as "circling disease".[34] The organism was described by Murray, Webb and Swann in 1926 as the causative agent of an epizootic infection in laboratory rabbits and guinea pigs. Their observations showed that the disease produced emaciation, abundant exudate in serous cavities, necrotic foci in and on various organs, and a marked increase in monocytes circulating in the blood stream. The name Bacterium monocytogenes was used to describe the organism.[22]

In 1927 Pirie isolated an organism from gerbilles which he called Listerella hepatolytica referring to the numerous necrotic foci found on the liver.[30] The comparative studies of Murray and Pirie showed that the organisms which they had worked with were identical, and the name of the genus was changed to Listeria, its present form.[22,31] The word "Listeria" was actually derived from the name of the famous English surgeon and bacteriologist, Joseph Lister.[15]

While it may be rather precarious to attempt prognostication at this time, it is nevertheless a possibility that listeriosis may exhibit a history similar to Malta fever. When Sir David Bruce discovered Micrococcus melitensis as the causative organism of Brucellosis, there was little evidence to support the occurrence of a new disease in humans. Yet, it is a well-recognized fact that one of the public health problems today is undulant fever. There is reason for contemplating that the

genus Listeria now occurring in twenty species of animals in nearly all the Northern States and fifteen foreign countries is adapting itself to man.[8,11,12] It does not seem too remote that in the future we may witness many human cases of listeriosis rather than isolated cases as now occur.

Recently a culture of Listeria monocytogenes was sent to this laboratory for verification from St. John's Hospital in Rapid City, South Dakota. The culture was taken from the spinal fluid of a fifty-two year old man, a station agent for the Milwaukee Railroad at Faith, South Dakota. Biochemical and serological tests proved that the bacterium was Listeria monocytogenes. It is believed that this is the first diagnosed case of human listeriosis in South Dakota. From the period between 1951 and 1953 thirteen cases occurred in humans in the State of California.[8] Prior to this time one case had been reported. Linsert reports that during the interval between 1953 and 1956 eighteen human cases were bacteriologically confirmed by the Bezirk Hygienic Laboratory of Germany.[20]

Whether or not the sudden appearance of many listeriosis cases in man is a matter of better technique in diagnostic procedure is certainly a factor to consider, but seems rather doubtful since it could hardly account for the large percentage of the total number. It is quite possible that there exists an epidemiological connection between man and animal, although this is strictly a matter of conjecture on the part of the investigator. It is hoped that these studies, directly or indirectly, may contribute to solving this problem.

From the standpoint of farm economics the two most important animals involved are sheep and cattle. It is not uncommon that listeriosis in sheep reaches epizootic proportions. An outbreak in flocks of 3,000

may cause losses totaling as high as 200 to 500 animals. Fewer than two percent of the animals showing definite clinical symptoms recover. Listeriosis in cattle has been sporadic and usually involves only a small number of animals, although twenty percent of a large beef or dairy herd may be infected.[3]

The exact mode of transmission under field conditions is not understood. Bolin and Osebold report that Listeria cells pass into the urine and feces of rodents, therefore raising the question of food and water contamination of domestic animals.[4,27] On the other hand, clinical listeriosis is often detected when animals are kept in close quarters. Examples of this are in feed-lot cattle and in sheep at lambing time. Osebold further suggests that the opportunity for consumers' meat to be infected with Listeria could likely exist since many animals that are involved show no apparent infection, therefore not decreasing their commercial value.[27] Since it appears that many of the infected animals show no symptoms of listeriosis, it would seem advantageous to conduct a systematic study to determine the degree of infestation, hence giving information on the pathogenicity of the organism, its prevalence, and the number of potential carriers resulting from previous infections.

REVIEW OF LITERATURE

Listeria monocytogenes is an infectious bacterium producing a disease known as listeriosis which is often referred to in ruminants as "circling disease" because infected animals retract their heads and walk in circles.[34]

Synonyms for Listeria monocytogenes are Bacterium monocytogenes, Listerella hepatolytica, Listerella monocytogenes, Corynebacterium parvulum and Bacillus monocytogenes. Listeria monocytogenes is classified according to the following taxonomic scheme.[5]

Class:	Schizomycetes
Order:	Eubacteriales
Sub order:	Eubacterieneae
Family:	Corynebacteriaceae
Genus:	Listeria
Type species:	Listeria monocytogenes

Listeria monocytogenes is a small gram positive nonspore-forming bacillus capable of producing smooth and rough colonies. In the smooth phase small rods predominate measuring 0.5 microns in width by 1.0 to 2.5 microns in length and occur singly or in short chains. Palisade formations are often noticed, but this is by no means a determinative characteristic of the organism. The rough phase of the organism produces filaments measuring up to 60 microns in length.[10] Its motility is characterized by a "tumbling" or spiral movement brought about by peritrichous flagella when the organism is grown at ordinary room temperatures, and it has a tendency to be nonflagellated or monotrichous when grown at 37 degrees centigrade. Its oxygen requirements are those of a facultative aerobe. Listeria is generally described as being nonencapsualted.[5]

A thin transparent layer typifies the growth of Listeria on

nutrient agar but with the addition of ascitic fluid or defibrinated blood the growth is greatly improved. On rabbit, horse, or human blood agar plates a small zone of beta hemolysis appears around each colony.[6] Cultivation on artificial media apparently lowers the organism's pathogenicity, and serial passages through laboratory animals will restore its virulence.[2]

In the smooth phase the colonies appear almost transparent and are very small, measuring 0.8 millimeters in diameter, and possess a smooth, slightly flattened surface. Colonies in the rough phase exhibit a granular center and are slightly larger than those of the smooth phase. The growth in broth cultures appears as a flocculent sediment with profuse turbidity.[10]

The greatest variation in described characteristics of Listeria is found in biochemical reactions of carbohydrate fermentations.[6,7,5,10,15,23] There is complete agreement on the production of acid without gas from glucose, rhamnose and salicin, and also no fermentation of ducitol, inulin and inositol. The following carbohydrates have been reported as producing acid and no gas by at least one author: maltose, lactose, arabinose, galactose, xylose, mannitol, melizitose, soluble starch, dextrin, glycerol, trehalose and sorbitol. Other biochemical tests show that acid production and reduction of the dye occur in litmus milk without coagulation or digestion. Indole, hydrogen sulfide and acetyl methyl carbinol are not produced. Nitrites are not formed from nitrates and gelatin is not liquified. The methyl red test indicates acid production.

According to Patterson the antigenic structure of Listeria monocytogenes includes four serological types, each containing one type

specific and at least one species specific factor. The species specific determinant is found in the flagellar antigen and is responsible for cross agglutinations between strains.[20,29] Julianelle divides the species into two serological groups which he believes may be correlated with host species.[18] It is interesting to note that Harvey and Faber discovered a correlation between Patterson's serological groupings and melizitose fermentations.[15,29]

Nearly all mammals and many birds are affected.[11] In sheep, cattle, and goats the disease manifests itself as an acute specific infectious encephalitis with spontaneous abortions sometimes occurring. The infection in rodents for the most part is generalized with mononucleosis and glandular and hepatic involvement. In carnivorous animals the disease appears to affect the respiratory system, resembling distemper in dogs. Myocarditis with necrosis is the important manifestation of the infection in fowl. In man, Listeria invades the central nervous system, producing mening-encephalitis with a fatality of seventy percent.[16]

Most cases of listeriosis are reported between the months of March and July with few exceptions in February indicating that the seasonal incidence is in early spring and middle summer.[20]

The route by which the organism reaches the brain has not been definitely determined. Osebold suggests several pathways to the central nervous system; namely, intranasal, due to the proximity of the olfactory tracts to the nasal mucosa; neuronal via the trigeminal nerve; and by means of the vascular system.[27]

Graham and Dunlap conducted research to determine whether or not they could produce listeriosis by inoculating different animals at dif-

ferent sites. Their results show that death was produced in sheep, heifer, horse, and chickens by intracerebral inoculations. Death was also produced in sheep, cat, pig, and chickens as the result of intravenous inoculations, but only illness and then recovery in a yearling steer, horse and a pig. A dog similarly exposed suffered a slight attack of the disease. Cerebral involvement similar to that witnessed in field cases was produced by intravenous inoculation of a lamb.

A sheep and horse developed a temporary illness when the organism was introduced into the stomach. Subcutaneous injection of chickens elicited no reaction. Two lambs that were kept in the same pen with inoculated sheep which later died showed no effect from the exposure.[13]

Since the experimental section of this paper deals mainly with the occurrence of Listeria infection in sheep, a description of their symptoms will be given as reported by Olafson. The first observable symptom of listeriosis in sheep is listlessness, as is true with most infectious diseases of animals. The animal separates itself from the flock and seems quite unaware of its surroundings. The sheep then may lean against a building or fence for long periods of time. On the second or third day the animal starts to move in a circle which may be in either direction, but an individual case will always circle the same way. Facial paralysis, characterized by a drooping ear or partially closed eyelids may develop along with a profuse nasal discharge. The head begins to retract and deviate to one side. Whenever the retracted head is forcibly returned to its normal position, it immediately flies back to the bent and twisted posture; hence indicating tetanus of the muscle. (See Plate 1, page 8) On about the third or fourth day the animal will have trouble standing

Plate 1. A LISTERIA-INFECTED SHEEP

A sheep with typical listeriosis, showing facial paralysis and characteristic position of the head.

and eventually falls down, not being able to get up. Shortly after the sheep has fallen it may show a tendency to move its legs as if it were trying to run. The course of the disease is usually completed within three to seven days.[23] The similar clinical symptoms produced by Listeria and the tapeworm Multicep multiceps (gid) makes differential diagnosis difficult, hence causing error when distinguishing the two diseases.

In order to diagnose the disease bacteriologically it is necessary to isolate the organism from the blood stream or cerebral-spinal fluid. Care must be taken to avoid confusing the Listeria organism with beta hemolytic streptococci and diptheroids.[10] Serological diagnosis is not of too much value due to the short course of the disease which allows very little time for the production of antibodies and since high agglutinating titers sometimes exist in clinically normal animals.

A more definite identification procedure is described by Julianelle and Moor where Listeria is instilled into the conjunctival sac of a guinea pig or rabbit. This produces a conjunctivitis and corneal involvement. Monkeys (Macaca rhesus) respond in the same manner as do rabbits and guinea pigs except that their response is irregular due to a higher degree of resistance; therefore they cannot be depended upon to give a consistently true test.[18,19]

The clinical course of the opthalmic reaction is initiated by an intense conjunctivitis with an increased secretion, purulent exudate, swelling, chemosis, and edema of both eyelids. The cornea appears quite normal with a steamy appearance at its margins. After several days the cornea will take a more active part exhibiting turbidity, vascularization and eventually a heavy pannus formation which originates at the entire

circumference and gradually progresses to the center. The exudate contains for the most part monocytes and polymorphonuclear leukocytes. The ballooning of the conjunctiva is great and eventually is responsible for the inability of the animal to part the eyelids.

The reaction reaches a maximum intensity in four to seven days. Iritis and corneal ulceration sometimes occur with resultant hypopyon, but is by no means to be considered an established characteristic of the opthalmic reaction. The course of the eye infection gradually subsides taking at least three weeks to heal. Upon recovery the eye of the animal shows a local acquired resistance to subsequent Listeria infections, unaccompanied by generalized immunity as determined by intravenous injections.[19]

It may be pointed out that the opthalmic reaction cannot be initiated by inoculating any sites other than ocular. Also no gross lesions are produced on any organs other than the eye as the result of an ocular inoculation.

Bacteriological isolations and identifications after the animal has died are usually attempted by plating out brain tissue on suitable media such as tryptose agar or blood agar and incubating at 37.5 degrees centigrade for twenty-four to forty-eight hours. It is often difficult to isolate Listeria from brain tissue due to focal distribution. Beister partially overcame this problem by grinding up larger amounts of the brain and then inoculating a small portion of it into suitable media. Olson indicates that positive cultures are more often obtained from the medulla or cerebellum rather than from the cerebrum.[1,25]

It would seem that at least three factors are instrumental in

determining whether or not successful isolations are obtained. One factor has already been mentioned, that of focal distribution. The other two possible factors are choice of the correct medium and the occurrence of contaminants.

Olson, Cook and Bagdonas, and Levine and Graham report that little, if any, protection is afforded to animals as the result of living vaccines and bacterins. Olson injected half of a flock of 540 sheep with living Listeria monocytogenes cultures during an outbreak of listeriosis. After one week one case of the vaccinated group developed the disease. A second dose of the vaccine afforded no protection since more sheep in the vaccinated group developed listeriosis than those of the nonvaccinated control group.[24,14]

Eveleth reports that the results of numerous trials using three different types of bacterins yielded a low degree of immunity which he indicates is sufficient to protect sheep exposed under field conditions. He recommends vaccination in flocks where outbreaks occur, but considers wide scale vaccination programs impractical.[12] The use of antisera of high agglutinating titer offers little, if any, protection in mice.[18] From the above information it is quite obvious that conflicting reports exist; therefore it is desirable that more research be done to develop an effective vaccine.

Treatment of the disease has progressed from hopeless to slightly successful. Most of the drugs and antibiotics have not proven to be entirely effective. Webb reports that sulfonamide treatment promises success.[34] Recovery was brought about in two cases of listeriosis in sheep by use of prontosil and sulfanilamide. Olafson suggests that

antibiotic treatment must be started early and the dosage must be large.[23] It appeared in one instance that penicillin possibly prolonged the life of sheep in two cases of field listeriosis.[1] The efficacy of nine antibiotics is given below in the following table:[33]

Penicillin	Usually ineffective
Erythromycin	Ineffective in vitro
Streptomycin	Ineffective in vitro
Carbomycin	Ineffective in vitro
Tetracycline	Ineffective in vitro
Chlortetracycline	Effective clinically
Oxytetracycline	Effective clinically
Chloramphenicol	Effective clinically
Neomycin	Effective in vitro

It is interesting to note that the male human case mentioned in the introduction was treated with the antibiotics penicillin, aureomycin and dihydrostreptomycin, and according to his physician, Dr. Dzentars of Faith, South Dakota, the patient made a complete recovery.

Since the mode of transmission of the infection is not definitely known, one is likely to suspect that animals previously infected act as natural reservoirs for the disease. Linsert conducted research in East Mecklenburg, Germany dealing with the agglutinin titers of normal and Listeria-infected animals in order to provide a picture of the number of potential carriers. A high percentage of positive agglutinations was observed, although Linsert did not believe that such a high saturation existed. Therefore he concluded that normal agglutinins against Listeria existed in titers of 1:400 which he claims are probables, and that titers of 1:800 and up should be considered as positives and that any concentration lower than 1:400 is of no importance. By declaring the above boundaries for probable and positive tests Linsert found five to eight percent positives in infected flocks of sheep, and 3.7 percent in

noninfected flocks. However, in cattle only 1.7 percent were found to be positive.[20] According to the literature, and to the best knowledge of the investigator, no survey of this type has been conducted in the United States; therefore a systematic study was carried out and is reported in the experimental portion of this paper.

CULTURES AND METHODS

The strains of Listeria monocytogenes utilized in this study conformed to the description of Murray, Webb and Swann.[22] The chief characteristics chosen were the colonial size, 1 to 2 millimeters, and the size of the organism, 0.5 x 1 micron, retention of the gram stain, production of beta hemolysin, virulence for mice, motility, and biochemical activity. These characteristics will be amplified later in this report.

There were sixteen strains in all which were collected from five different laboratories. The location of these laboratories and the animal from which the individual strains were isolated is given below:

Strain number	Animal isolated from	Place acquired from
4-115A	Sheep	N.D.S.C., Fargo, N. D.
52A-51	Sheep	N.D.S.C., Fargo, N. D.
8A-51	Sheep	N.D.S.C., Fargo, N. D.
5-66A	Sheep	N.D.S.C., Fargo, N. D.
3-118A	Sheep	N.D.S.C., Fargo, N. D.
5-531A	Swine	N.D.S.C., Fargo, N. D.
3-443A	Swine	N.D.S.C., Fargo, N. D.
736W	Skunk	N.D.S.C., Fargo, N. D.
821A-51	Bovine	N.D.S.C., Fargo, N. D.
Nebraska	Sheep	U. of Nebr., Lincoln, Nebr.
Moor's A	Sheep	Washington U., St. Louis, Mo.
D82N	Human	Washington U., St. Louis, Mo.
SDSC #1	Sheep	S.D.S.C., Brookings, S. D.
SDSC #2	Sheep	S.D.S.C., Brookings, S. D.
SDSC #3	Sheep	S.D.S.C., Brookings, S. D.
SJH	Human	St. John's Hospital, Rapid City, S. D.

By using the above strains it is evident that a reasonably broad distribution was obtained. For purposes of storage and to prevent loss of virulence all strains were sealed in small vials and frozen at minus 56 degrees centigrade until needed.

Cultivation of Strains:

As a rule the growth of Listeria was scanty, although there was some variation between strains. This variation was related to the length of time the particular strain had been grown on artificial media. In other words, those strains which were more recently isolated seemed to produce a more luxuriant growth. The nutritional demands of Listeria were satisfied by using tryptose broth or tryptose agar.[25] Defibrinated rabbit blood was sometimes added to a concentration of five percent to the above medium as an enrichment as well as an indicator of beta hemolysis.[6]

PRELIMINARY STUDIES

Biological Experiments:

While a number of investigators (Burn, Murray and Swann, and Harvey and Faber) have determined the effect of *Listeria* on carbohydrates, there appears to be some disagreement in their results.[6,15] It was necessary to utilize the sixteen strains, listed on page 14, to restudy this question. Thirteen different carbohydrates were inoculated in duplicate with each strain and incubated for two weeks at 37 degrees centigrade. For a control an uninoculated tube was incubated with the active culture. Any test which showed no reaction after two weeks was considered to be negative.[21]

A basal medium of nutrient broth plus phenol red indicator adjusted to a pH of 7.4 was used with the addition of 0.5 percent carbohydrate.[9] According to the MANUAL OF METHODS FOR PURE CULTURE STUDY OF BACTERIA, carbohydrate fermentation media may be prepared by separately sterilizing the basal medium and a highly concentrated dissolved form of the carbohydrate and then mixing them together.[21] The investigator believes that hydrolysis can be further reduced by sterilizing the carbohydrate in its dry form and then mixing it with the basal medium in the proper concentration. This was done, and the solution was then drained from its container by an apparatus shown in Figure 1, page 17. Each tube was then stoppered and incubated at 37 degrees centigrade for three days to determine its sterility. They were then placed in a refrigerator for storage until used.

Nearly all strains acted uniformly except for a difference in

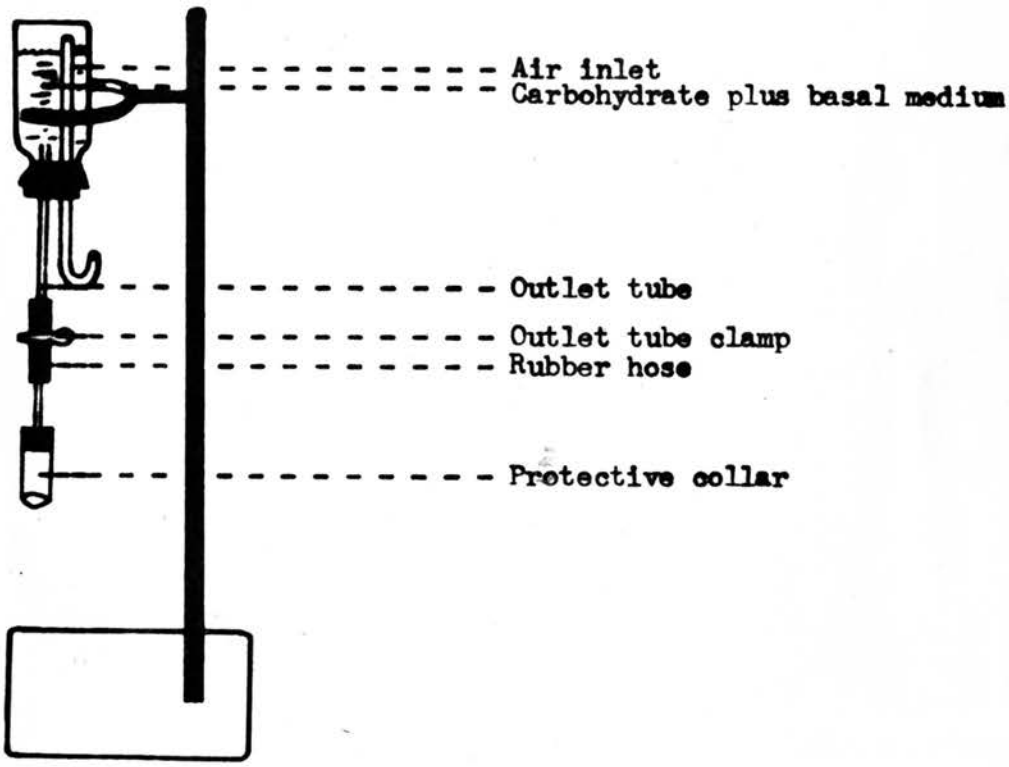


Figure 1. APPARATUS USED FOR TRANSFERRING THE STERILE CARBOHYDRATE SOLUTION INTO STERILE TEST TUBES. (Supplied by Dr. R. J. Baker)

xylose and melizitose fermentation. The only other divergence was the amount of acid production from different carbohydrates. The results of the carbohydrate fermentations are given in Chart 1, page 19. None of the sixteen strains produced indole from trypophan, acetyl methyl carbinol from Methyl Red Voges Proskauer medium, nor hydrogen sulfide from lead acetate agar. In the case of hydrogen sulfide a narrow black ribbon was formed on the line of the inoculation, but did not extend into the surrounding agar. In litmus milk, Listeria caused reduction of the dye and acid production with no coagulation. Nitrites were not formed from nitrates and the methyl red test gave a red color reaction.

Pathogenicity:

The possibility that Listeria might be pathogenic was first suggested by Murray, Webb, and Swann when an outbreak of the infection occurred in laboratory rabbits.[22]

In this investigation the test animals were nine white mice and strains UN and SDSC #1 and #2 were chosen. The tail vein and the intraperitoneal cavity were used as the inoculating sites. The dosage given for the intravenous injection was 0.01 milliliter and for the intraperitoneal injection 0.5 milliliter. The intervals before death of all nine mice ranged from one and one-half days to sixteen days. There seemed to be no correlation between the site of inoculation and the number of days before the animals died. The first symptoms noticed were that they refused feed, appeared inactive, humped over, and trembled quite violently. Their breathing was labored and the eyes were grayish, watery and partly closed. These symptoms continued, along with emaciation, until death.

Chart 1. RESULTS OF CARBOHYDRATE FERMENTATION

No. of Strain	Glucose	Rhamnose	Salicin	Galactose	Soluble Starch	Maltose	Lactose	Arabinose	Xylose	Melisitose	Mannitol	Inulin	Dulcitol	Inositol
4-115A	+++24	+++24	++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	+++48	-14da.	-14da.	-14da.	-14da.
52A-51	+++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	+++48	-14da.	-14da.	-14da.	-14da.
8A-51	+++24	+++24	++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	+++48	-14da.	-14da.	-14da.	-14da.
5-66A	+++24	+++24	++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+ 48-72	-14da.	-14da.	-14da.	-14da.	-14da.
3-118A	+++24	+++24	++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	+++48	-14da.	-14da.	-14da.	-14da.
5-531A	+++24	+++24	++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	-14da.	-14da.	-14da.	-14da.	-14da.
3-443A	+++24	+++24	++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	-14da.	-14da.	-14da.	-14da.	-14da.
736W	++24	+++24	++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+14da.	+++48	-14da.	-14da.	-14da.	-14da.
821A-51	+++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	-14da.	-14da.	-14da.	-14da.	-14da.
Moor's A	+++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	-14da.	+++48	-14da.	-14da.	-14da.	-14da.
D82N	+++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+14da.	+++48	-14da.	-14da.	-14da.	-14da.
SDSC #1	+++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+ 48-72	-14da.	-14da.	-14da.	-14da.	-14da.
SDSC #2	+++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+ 48-72	-14da.	-14da.	-14da.	-14da.	-14da.
SDSC #3	+++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+ 48-72	+++48	-14da.	-14da.	-14da.	-14da.
UN	++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+ 48-72	+++48	-14da.	-14da.	-14da.	-14da.
SJH	++24	+++24	+++24	++48	-14da.	+++ 24-30	++ 30-48	+ 48-60	+ 48-72	+++48	-14da.	-14da.	-14da.	-14da.

Unless otherwise indicated all figures represent hours.

+++ Signifies sufficient acidity to turn the phenol red indicator bright yellow.

++ Signifies the appearance of a flesh-like color in the phenol red, indicating moderate acidity.

+ Refers to a barely perceptible change in the indicator.

Several animals were autopsied to determine the pathology. As the longitudinal incision was made there existed a considerable quantity of fluid in the peritoneal cavity, although there seemed to be no distention or swelling of the stomach or gut. The abdominal blood vessels showed signs of congestion and the spleen manifested well-developed petechial hemorrhages. The adrenals were greatly enlarged while the lymph nodes appeared to be intact, although it was difficult to state definitely due to their relatively small size. Miliary abscesses were found on the liver. These were lanced and the contents were inoculated on blood agar plates. After twenty-four hours of incubation at 37 degrees centigrade, typical Listeria colonies appeared. Smears of these colonies were observed microscopically and typical small gram positive bacilli were present. Also impression slides which were made of the spleen and liver were stained with methylene blue, gram's stain and Wright's stain. Microscopic examination revealed the presence of small gram positive rods plus contaminants which might be expected since death occurred several hours earlier. A number of mice that died comparatively early (two days) showed no pathology. This might be expected since a relatively short time was allowed for the development of active symptoms.

Two white rats were injected, one intravenously and one intraperitoneally, with strains SDSC #1 and UN. The animals showed no symptoms after one month. They were then sacrificed, and upon autopsy showed no pathological condition. This probably is another example of the high resistance displayed by the rat and explains his high rate of survival.

Histopathological studies of a Listeria-infected sheep's brain revealed perivascular cuffing, focal infiltrations, and accumulations

of monocytes and polymuclear leucocytes with necrosis as shown in Plate 2, page 22.

Opthalmic Infection:

Closely related to virulence and pathogenicity is the diagnostic test, previously described in the review of literature section, in which Listeria is injected into a guinea pig's or rabbit's eye. Two strains (52A-51 and Moor's A) representing the two serological groups as described by Julianelle were inoculated into the conjunctival sacs of two rabbits.[18] The infecting dosage was one to two drops of an eighteen-hour tryptose broth culture. After the instillation gentle massaging of the closed eyelid insured complete contact and prevented the animal from eliminating the culture by winking or increased lachymal flow. In approximately twenty-four to thirty hours the eyes of both rabbits formed a watery secretion and in forty-eight hours a purulent exudate with slight swelling of the conjunctivas. The exudate was inoculated on blood agar plates and after the incubation period produced hemolytic colonies, typical of Listeria. In three to four days the lids were three-fourths closed, and if they were pried apart, one could readily see the involvement of the cornea which was distinctly cloudy with the presence of tiny capillaries. Thus, the reaction continued in intensity until approximately the sixth day when photographs were taken. See Plates 3 and 4, page 23.

Blood was drawn from the animal's heart to obtain serum samples for antibody titer determinations. Serum samples showed a titer of 1:640 for Moor's A strain and 1:160 for the 52A-51 strain. The severity of both reactions appeared to be of the same magnitude. The rabbits

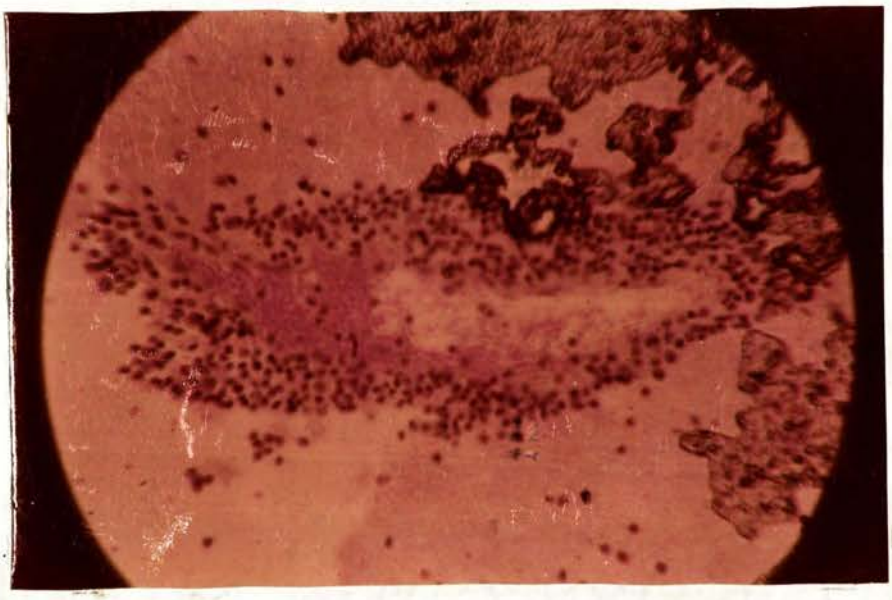


Plate 2. PERIVASCULAR CUFFING

The above photograph shows brain tissue from a sheep infected with Listeria showing perivascular cuffing. (haematoxylin-eosin stain)

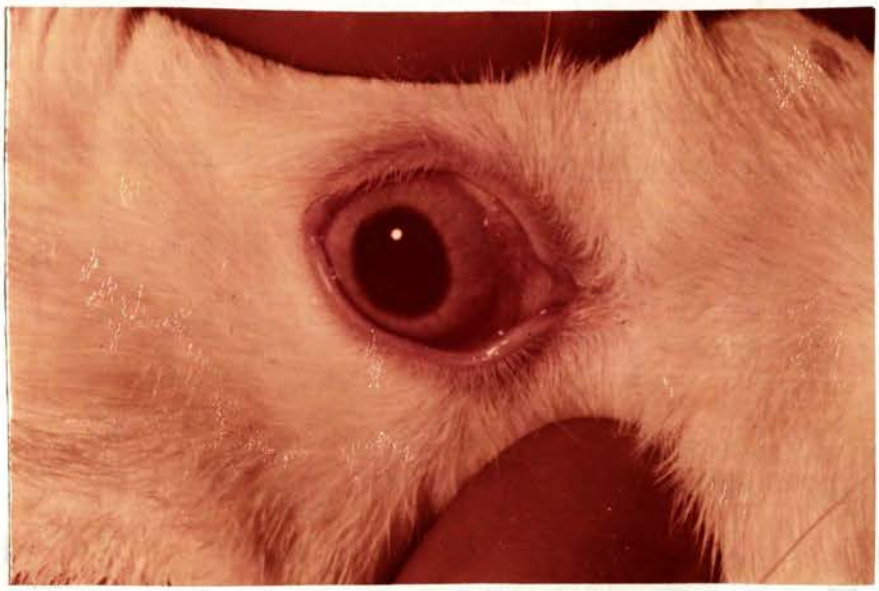


Plate 3. NORMAL EYE OF A RABBIT

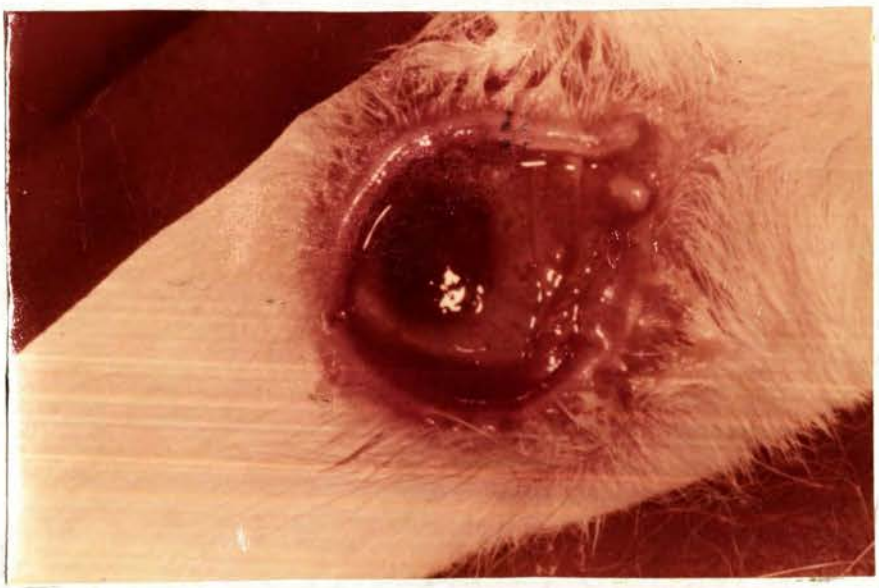


Plate 4. LISTERIA-INFECTED EYE OF A RABBIT

Please note panoptholmitis with pannus formation on the right margin of the eye, swelling, vascularization, and pus formation.

were sacraficed and upon autopsy showed no macroscopic pathology.

Antibiotic Sensitivity:

To secure a measure of in vitro sensitivity the 16 strains were subjected to fourteen individual antibiotics according to the following procedure:

Tryptose agar plates were inoculated by spreading twenty-four hour tryptose broth cultures over the surface with a sterile bent glass rod. Bacto Sensitivity Discs were then placed on the inoculated surface and incubated for forty-eight hours after which time they were classified according to the distance across the inhibition zone.[9] The boundaries set up for these classifications are as follows:

2.5 to 3.0 millimeters.....Very sensitive
2.0 to 2.5 millimeters.....Sensitive
1.0 to 2.0 millimeters.....Slightly sensitive
Less than 1.0 millimeters.....Resistant

The results observed in this experiment are shown in Chart 2, page 25.

Chart 2. THE EFFECT OF VARIOUS ANTIBIOTICS ON THE DIFFERENT STRAINS OF LISTERIA MONOCYTOGENES

	4-115A	52A-51	8A-51	5-66A	3-118A	5-531A	3-443A	736W	821A-51	Moor's A	D82N	SDSC#1	SDSC#2	SDSC#3	UN	SJH
Aureomycin	VS	VS	VS	VS	VS	S	VS	VS	VS	VS	VS	VS	VS	VS	S	VS
Chloromycetin	S	SS	SS	S	SS	S	VS	VS	SS	S	S	S	SS	S	SS	S
Di'streptomycin	R	R	SS	R	SS	SS	R	R	R	R	SS	SS	R	SS	R	SS
Erythromycin	VS	S	VS	S	S	S	S	S	S	S	S	SS	S	VS	S	VS
Magnamycin	SS	SS	S	S	SS	SS	SS	SS	SS	SS	SS	SS	SS	S	SS	SS
Neomycin	SS	SS	S	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	S	SS	SS
Novobiocin	S	S	S	SS	S	SS	SS	S	SS	S	SS	S	SS	S	SS	SS
Olendomycin	SS	SS	S	SS	SS	SS	SS	SS	SS	SS	SS	SS	SS	S	SS	SS
Penicillin	SS	SS	SS	SS	SS	SS	SS	S	SS	S	SS	S	SS	S	SS	S
Polymixin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R
Streptomycin	R	R	SS	R	R	SS	R	R	R	R	SS	SS	R	SS	SS	SS
Terramycin	SS	SS	S	SS	S	S	SS	S	S	S	SS	SS	SS	S	S	S
Tetracycline	VS	VS	VS	VS	VS	VS	VS	VS	VS	VS	VS	VS	VS	VS	SS	VS
Viomycin	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R

VS - Very Sensitive - 2.5 mm. to 3.0 mm.
S - Sensitive ——— 2.0 mm. to 2.4 mm.
SS - Slightly Sensitive - 1.0 mm. to 1.9 mm.
R - Resistant ———— less than 1.0 mm.

EXPERIMENTAL SEROLOGICAL INVESTIGATIONS

Antigen Preparation:

One milliliter of a twenty-four hour tryptose broth culture was inoculated on a tryptose agar slant in a 4" x 1½" x1" antigen bottle. After it had incubated for twenty-four hours at 37 degrees centigrade the cells were washed from the agar with ten milliliters of a 0.3 percent formalin solution, buffered at pH 7 with Na_2HPO_4 and KH_2PO_4. Since intact cells were used the antigen consisted of both flagellar and somatic antigenic structures. The formalinized cells were incubated for four hours and then stored in a refrigerator until needed. Prior to use the antigen suspension was diluted with an equal volume of 0.9 percent saline in order that the optimum density could be obtained.

Sterility checks were made on each new lot of antigen by streaking a loop full on a tryptose agar slant. If no growth occurred after several days the antigen was considered safe to use. The antigen was standardized by washing the bacteria from one of the slants into buffered saline and plating serial dilutions of this suspension on tryptose agar. The plates were incubated at 37 degrees centigrade for twenty-four hours, and the colonies were then counted on a Quebec Colony Counter. The counts indicated approximately 2,000,000,000 cells per milliliter of finished antigen.

The density of a portion of the antigen suspension was measured on a Bausch and Lomb Spectronic 20 photoelectric colorimeter. The original sample was diluted ten times with water in order to obtain a reading near the middle of the scale. Using a 1:10 dilution and a wave

length of 540 millimicrons the percentage of transmittance ranged from 45 to 47 and the optical density between 0.325 and 0.350.

Serological Typing:

In order that an epidemiological survey might be accomplished, it was necessary to locate representatives of the two existing serological groups corresponding to Julianelle's strains which he designated as I and II. Two antisera, corresponding to the above-mentioned types, were obtained through the courtesy of Dr. Moor, a co-worker of the late Dr. Julianelle at Washington University, St. Louis, Missouri. The two antisera were designated as D82W (type I) with a titer of 1:320, and 206 (type II) with a titer of 1:160.[18]

Antigens, prepared as described on page 26, were made from each of the sixteen strains mentioned earlier in this report. The individual antigens were then subjected to an agglutination test using each type of positive antiserum and a negative serum which acted as the control. In order to convey rapidly the results of the agglutinations a summary of all the tests is given in Chart 3, page 28.

Julianelle suggests a natural division in serological groups between strains from ruminants and rodents.[18] It would be dangerous to formulate a similar concept from the above since there seems to be no particular division and since the number of strains studied is not sufficiently large to draw a definite conclusion.

Sheep Survey:

The representative strains for the survey were chosen according to the agglutinin titer, biochemical conformity, colony formation, and

Chart 3. SEROLOGICAL TYPES

Origin of Strain	Strain	Type I				Type II			
		1/40	1/80	1/160	1/320	1/40	1/80	1/160	1/320
Sheep	4-115A	+	+	+					
Sheep	52A-51	+	+						
Sheep	8A-51	+	+						
Sheep	5-66A	+	+						
Sheep	3-118A	+	+						
Swine	5-531A	+	+	+					
Swine	3-443A	+	+						
Skunk	736W	+	+						
Bovine	821A-51		+						
Sheep	UN					+	+	+	+
Sheep	Moor's A					+	+	+	+
Human	D82N					+	+	+	+
Sheep	SDSC #1	+	+	+					
Sheep	SDSC #2	+	+						
Chinchilla	SDSC #3	+	+	+					
Human	SJH					+	+	+	+

A summary of serological types I and II of the sixteen strains of Listeria monocytogenes utilized in this study.

the recentness of isolation. The strains which most accurately satisfied the above qualifications were SJH and SDSC #1 representing types I and II respectively. An antigen was prepared from each of the two strains twice weekly; hence a fresh supply was always available for the agglutination tests to be described.

The survey was made using blood sera from 1018 sheep, 72 cattle and 62 humans. A more complete study was made of sheep since they are most often, of all the domestic animals, subjected to listeriosis. The individual sheep serum samples were obtained at John Morrell and Company in Sioux Falls, South Dakota, a slaughtering and meat-packing plant. The collections, made weekly, were started June 12, 1957 and ended July 15, 1957. Sheep were taken from twenty separate Morrell lots which possibly contained animals from thirteen Western, mid-Western and South Western states; namely, South Dakota, Wyoming, New Mexico, Utah, Washington, Iowa, North Dakota, Montana, Texas, California, Idaho, Arizona and Minnesota. It was nearly impossible to determine where a sheep originated since buyers and feeders never kept individual flocks separate. Of the 1018 sheep, 32 were considered as "mutton" or older animals, and the rest were lambs. The reason for such an unbalanced ratio lies in the fact that the collection period and the best economical time for farmers to sell their lambs coincided.

Collections were made by catching the blood in a nonsterile Kahn test tube after the animal's jugular vein had been cut. The blood samples were transported to South Dakota State College without refrigeration and allowed to clot. The clot was then removed and the remaining erythrocytes were eliminated by centrifugation. By this procedure very

little hemolysis occurred.

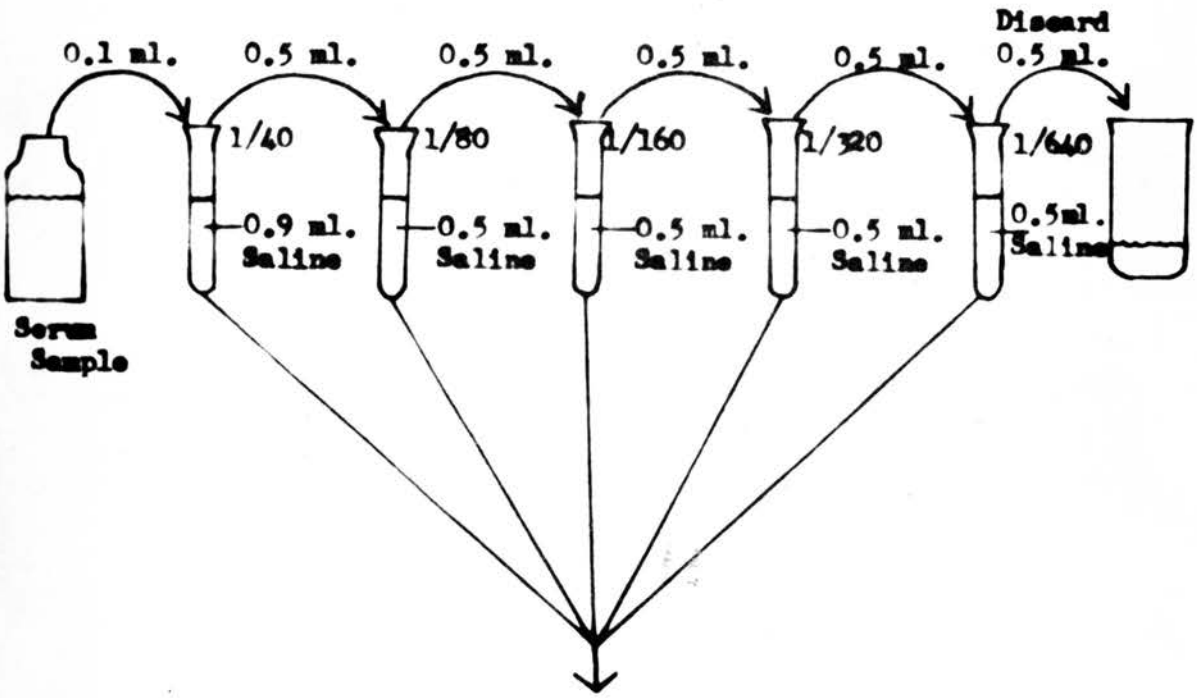
Screening Test:

On the day following centrifugation an agglutination screening test was made on each serum sample. The antigen used for the test was divalent, consisting of equal portions of type I and type II strains. Four dilutions, 1:40, 1:80, 1:160 and 1:320 were set up for each serum sample as shown in Figure 2, page 31.

The tests were incubated eighteen hours in a 37 degree centigrade incubator. Agglutination reactions were read against a background of natural daylight with the unaided eye. A definite sediment, which after a gentle shaking produced small floccules that were resistant to resuspension, was considered as positive. Those tubes, with or without a sediment, which after gently shaking yielded a fine suspension were considered as negative. Whenever a positive agglutination was encountered, the corresponding serum sample was placed in a separate rack and stored until the next day when it was typed with the individual antigens. The condensed results of the screening test showing the number of positive serums and titers are shown in Graph 1, page 32.

Typing Procedure:

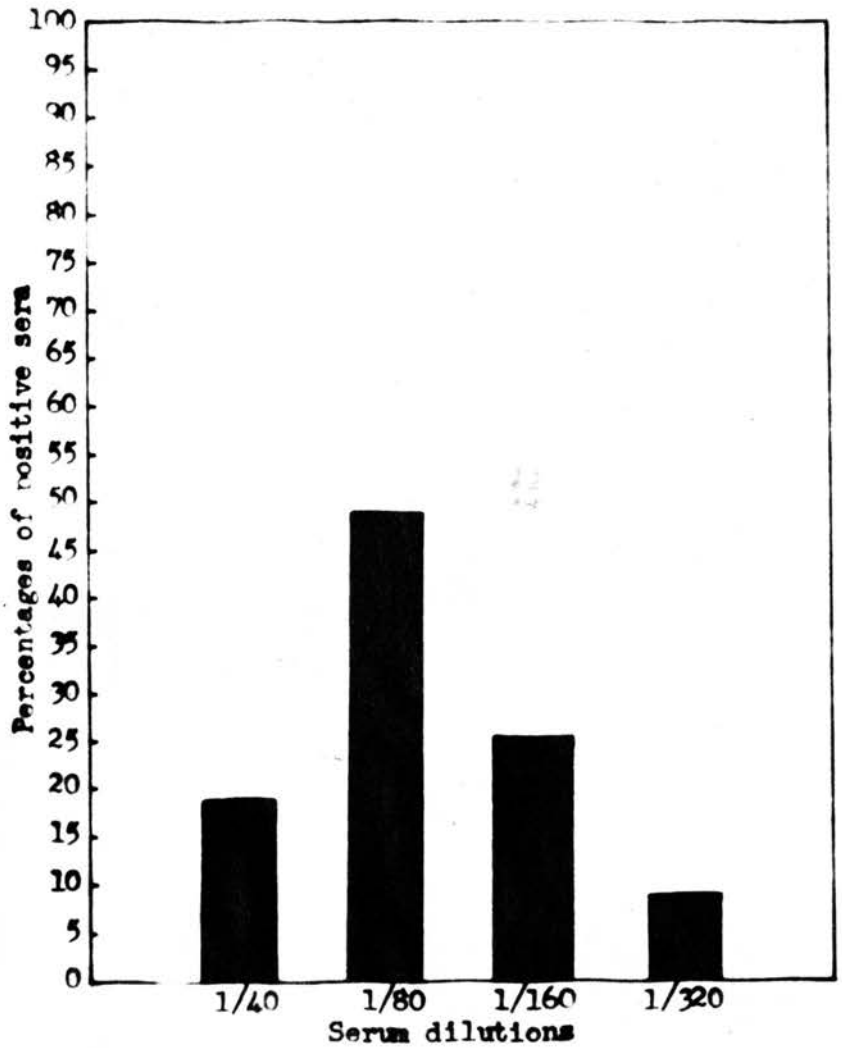
Those serum samples which showed a positive agglutination in the screening test were subjected to a typing procedure using monovalent antigens. Two additional dilutions were used in the typing test beyond those used in the screening test. The reason for adding the two extra dilutions was that occasionally, for reasons not understood, a serum would show a higher titer when exposed to the monovalent antigen than



Add 1.0 ml. saline and 0.5 ml. antigen bringing the first serum dilution to that marked to the right of each tube.

Figure 2. SCHEME USED FOR MAKING SERUM DILUTIONS.

Graph 1. RESULTS OF THE SCREENING TEST WITH SHEEP SERA



The above graph shows the percentage of the positive sera showing agglutination at the indicated dilutions.

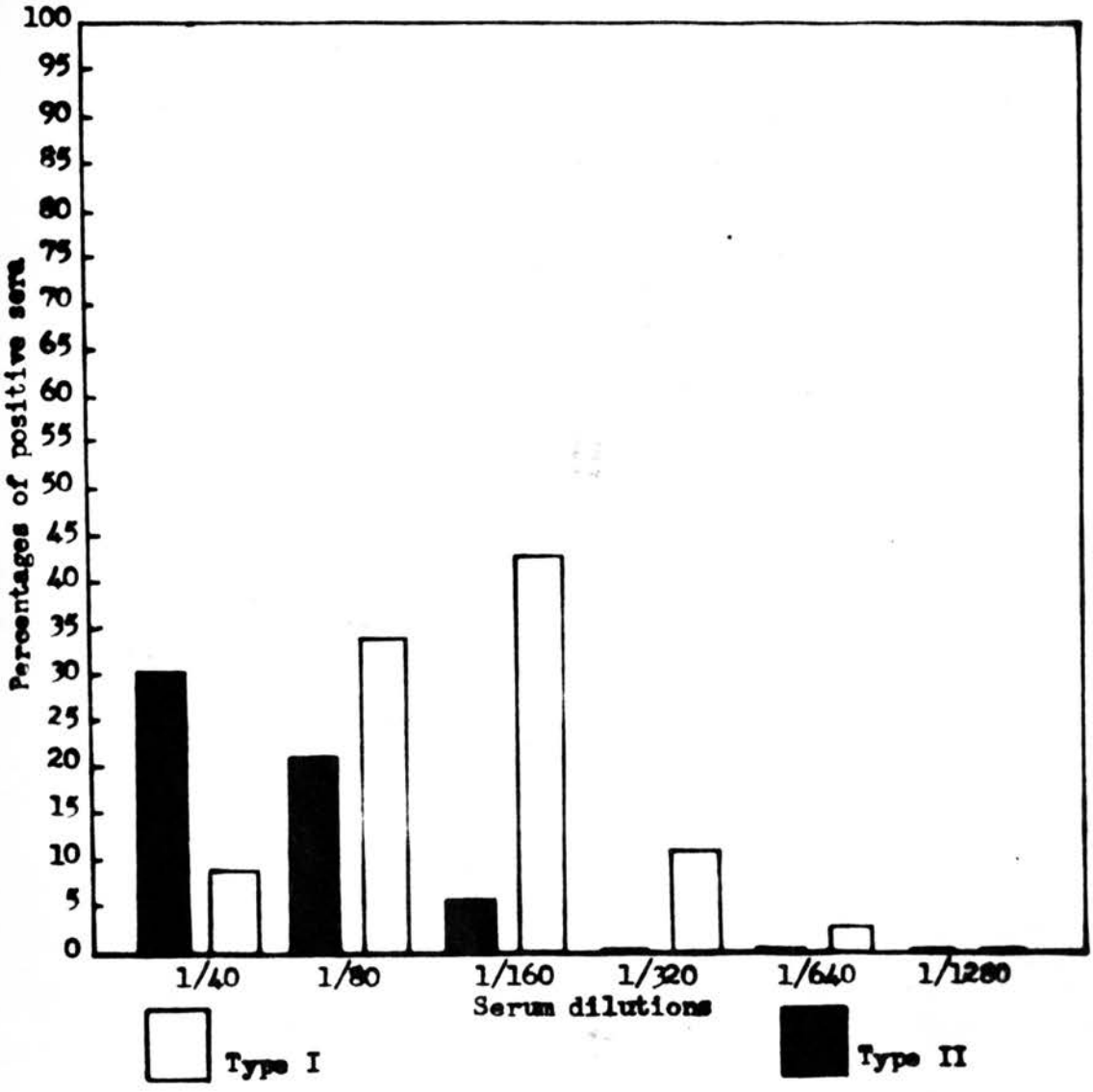
when exposed to the divalent antigen. The reverse of this was sometimes true where the titer shown in the typing test would be lower than the titer displayed in the screening test and occasionally would show no titer at all; hence this was termed as a false positive.

Most positive serums agglutinated both antigen types, indicating cross agglutination between strains, or that the serum contained both antibodies. This phenomenon seemed to be ruled by a fixed relationship since in most serum which showed a higher agglutinin titer, (1:160 and up) the type I antigen would agglutinate one to three dilutions higher than type II. The reverse of this was quite often true in cattle serum. While in serum below 1:160 only one antigen would usually show agglutination, although occasionally both types would agglutinate to the same titer; hence being nontypable. The antigen which was agglutinated at the highest titer determined the serum type. With 47 exceptions of which four fell into type II and 43 were nontypable, all 524 positive sheep sera fell into type I. Graph 2, page 34 indicates the number of sera showing titers of 1:40, 1:80, 1:160, 1:320, 1:640 and 1:1280.

Cattle Survey:

Seventy-two cattle sera were obtained from the Brucellosis Laboratory, Aberdeen, South Dakota. Their distribution entailed eight different South Dakota communities. They were collected during the period between July 14, 1957 and July 19, 1957. The individual sera were subjected to the same routine investigation as the sheep serum. By using this procedure 44 percent of the animals showed a positive titer, 28.1 percent falling into type I, 43.7 percent into type II, and

Graph 2. RESULTS OF THE TYPING TEST WITH SHEEP SERA



The above graph shows the percentage of positive sera reacting with types I and II antigens at the indicated serum dilutions.

28.1 percent were nontypable. Graphs 3 and 4, pages 36 and 37 show the percentage of individual titers of the screening and typing tests respectively.

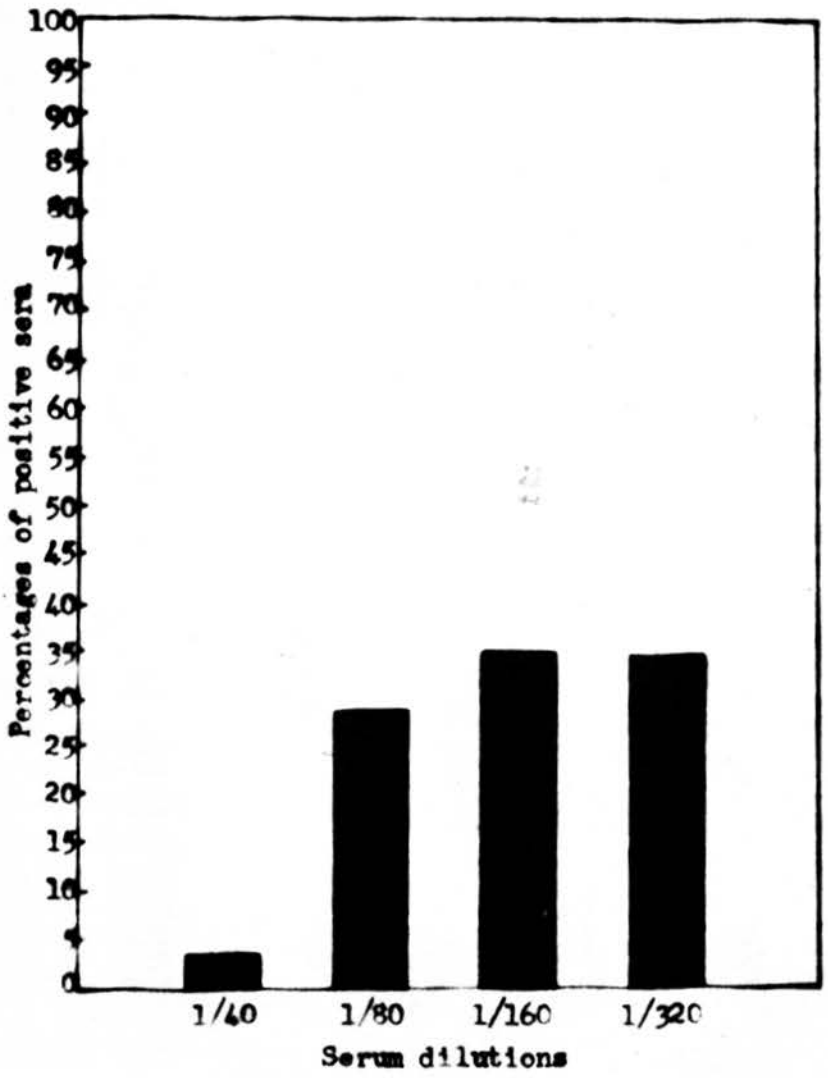
The cattle serum reacted differently from the sheep serum in that there existed a much higher percentage of type II sera and that a large percentage of the sera showed a fine flocculation of the same density in all dilutions. In other words there was no gradual change in size and number of floccules as one progressed from the lower to a higher dilution, such as would be expected in a true agglutination test. Those sera which acted in this manner were considered as negative.

The possibility that Brucella antibodies would cause a cross-agglutination with Listeria was considered and all serum samples were divided into brucellosis reactors, suspicious reactors and nonreactors. The results of the agglutination tests showed no correlation between listeriosis-positive serum and brucellosis-positive serum.

Human Serum:

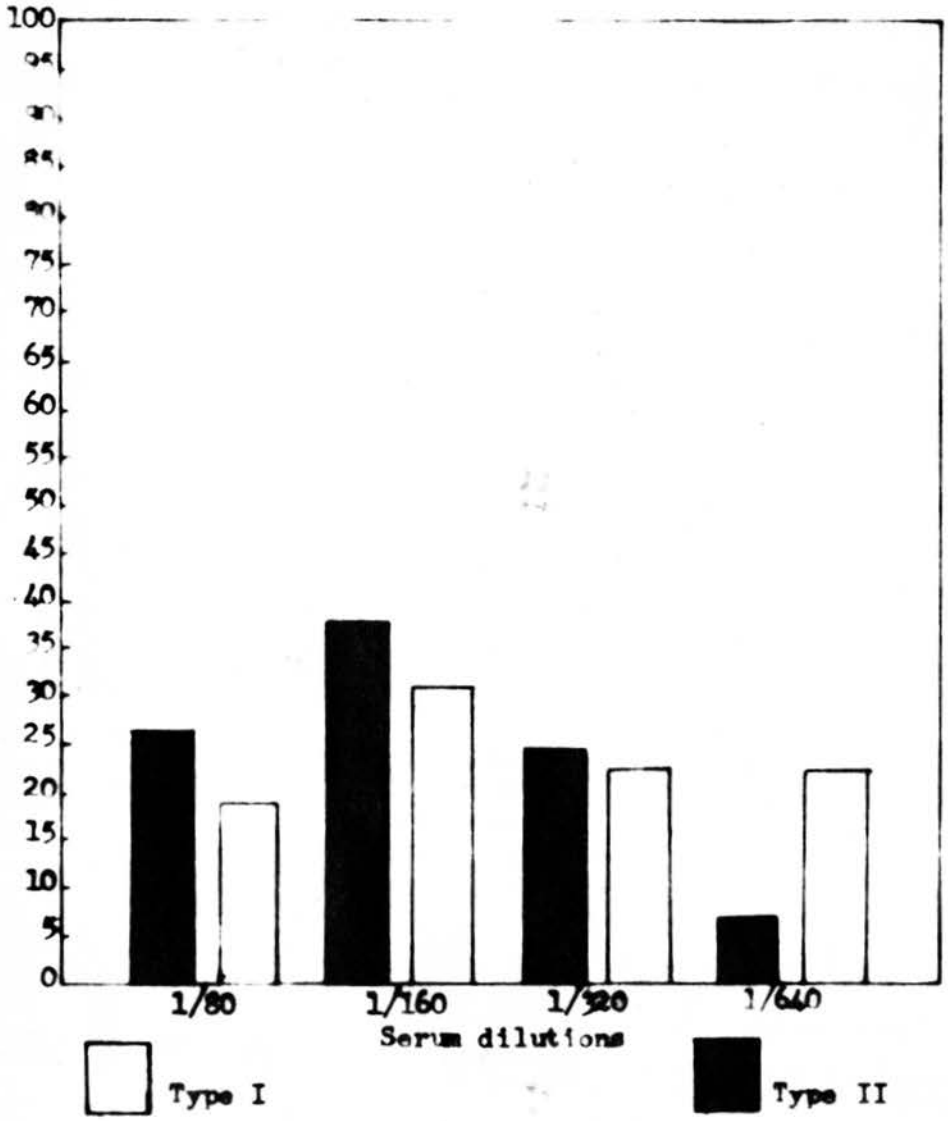
Mentioned in the introduction is the possibility of listeriosis becoming a major problem in humans. It seemed quite possible that if some animals do not show symptoms, but still display an antibody titer, that man might also, hence a study of 62 sera was undertaken to help answer this question. The human sera was acquired equally from Brookings and Mitchell, South Dakota. A screening test was performed, as described earlier in this report, on each sample. This showed that all 62 sera did not agglutinate the antigen at any titer. For reasons which are obvious these sera were not typed. Whether or not any definite conclusion can be drawn from these results due to such a small number tested is highly

Graph 3. RESULTS OF THE SCREENING TEST WITH CATTLE SERA



The above graph shows the percentage of the positive sera showing agglutination at the indicated dilutions.

Graph 4. RESULTS OF THE TYPING TEST WITH CATTLE SERA



The above graph shows the percentage of positive sera reacting with types I and II antigens at the indicated serum dilutions.

debatable, although it most certainly indicates that if humans do possess antibodies against Listeria, the frequency is very low.

The serum of the male listeriosis patient, mentioned in the introduction, displayed a titer of 1:80 for a heterologous antigen and 1:160 for a homologous antigen. It is of interest to note that the investigator tested his own serum to determine if he might have acquired the infection while working with it, and found no Listeria antibodies.

DISCUSSION

The observations described in the present communication are for the most part straight-forward enough, and consequently require no explanatory comment. This is especially true of the data pertaining to cultural characteristics, biochemical reactions and pathogenicity. Instead the main part of this discussion is devoted to the high percentage of agglutinin titers, which is certainly of immunological interest. It must be emphasized that the agglutination test for listeriosis is of little value to the practitioner because the duration of the infection is relatively short, hence very little time is allowed for antibody production. It is hoped that this test will be of considerable value in epidemiological studies pertaining to the occurrence and distribution of the infectious agent.

At the present time the agglutination test for listeriosis is of questionable efficacy; therefore it is desired that this paper should act as a ready source of pertinent information to those who are interested in this problem. Probably one of the main reasons for doubting the ability of the agglutination test to detect Listeria antibodies lies in the fact that, by using it as a means of determination, a tremendously high rate of infection is found in clinically normal animals. Investigation of 1018 sheep and 72 cattle show the following positive agglutinin titers:

	SHEEP		CATTLE	
Titer	With type I antigen	With type II antigen	With type I antigen	With type II antigen
1:40	8.6%	30.0%	0.0%	0.0%
1:80	32.8%	21.2%	18.7%	25.0%
1:160	43.7%	5.5%	31.2%	37.5%
1:320	11.3%	0.6%	21.8%	25.0%
1:640	2.6%	0.0%	21.8%	6.2%
1:1280	0.4%	0.0%	0.0%	0.0%

Linsert's investigations in East Mecklenberg, Germany of three flocks of sheep designated as A, B and C, and a herd of 270 cattle also indicated a high percentage of reactors as follows:[20]

SHEEP

Titer	Flock A	Flock B	Flock C
1:400 or less	90.00%	91.30%	87.50%
1:800 one plus	2.00%	3.70%	8.75%
1:800 two plus	8.00%	5.00%	3.75%

CATTLE

Titer	Number of animals	Percentage
1:200	180	66.6%
1:400	71	26.3%
1:800	14	5.2%(one-plus)
1:800	5	1.9%(two-plus and higher)

It must be determined whether one is dealing with specific Listeria agglutinins or with "nonspecific antibodies" before an accurate interpretation can be placed on the above results. Julianelle ran experiments to determine whether or not a closely related organism to Listeria, Erysipelothrix, would produce cross-agglutination.[17] His results show that Erysipelothrix is a single group antigenically and differs from the two immunological types of Listeria. Linsert states that Seeliger set up agglutination experiments using Listeria and antiserum of common skin saprophtes and other common organisms, such as

E. coli and M. pyogenes, to determine whether a cross-agglutination would occur.[20] This experiment showed the same as Julianelle's in that a nonspecific reaction was not produced.

The author attempted to answer this question by conducting heating experiments to destroy natural antibodies of several positive sera thinking that possibly they might be involved in a nonspecific reaction. Temperatures of 60 and 65 degrees centigrade were used for a thirty minute period. An agglutination test was then performed which indicated no reduction in agglutinin titers. Serum was also heated at 70 degrees centigrade for thirty minutes, but could not be used since it caused coagulation of the serum protein.

It would seem more practical from the clinical viewpoint to set up strict limits on what titer a serum must agglutinate before it is considered as positive. In other words, most veterinarians treat a very small number of Listeria-infected animals; hence for all practical purposes the true agglutination test would begin at one of the higher dilutions which would correspond more closely to the incidence of the disease.

Linsert's viewpoint coincides with the above reasoning since he considers titers of 1:400 (two plus) to 1:800 (one plus) as doubtful reactors, titers of 1:800 (two plus) as positive reactors and anything below 1:400 as a nonspecific reaction. He states that the basis for setting up such boundaries is his past experience with Listeria.[20] The investigator in no way doubts the efficacy of Linsert's judgment, but believes that, by using this as a measuring stick, he leaves many questions unanswered. It is, nevertheless, an odd circumstance that type

specific antibodies, of high agglutinating potency, confer very little, if any, measurable degree of protection.

Whether or not such a high percentage of animals are or have been infected is certainly questionable. It is believed by the investigator that until there is definite proof of nonspecific reactions, there is no basis for declaring one titer as positive and another as negative; hence the only course of action is to assume that a tremendous saturation of the disease exists. If one is to make this assumption, the study becomes one in epidemiology, and the distinction between infection and disease is very significant. The existence of an infection of comparable magnitude is by no means rare since 50 to 90 percent of the adult urban population have at one time or the other been infected with the tubercle bacilli, as proven by the tuberculin test. Nearly all adults show positive titers to the poliomyelitis virus and it has not been too many years ago that whole herds of cattle were eradicated due to Brucella abortus infections. The above-cited examples by no means indicate that all infected hosts showed symptoms. It is then highly possible that listeriosis falls into the same category, since no control measures have been put into effect to control this insidious organism.

It is difficult to state any definite reasons why one animal will display symptoms and another will not. Olson claims that before Listeria can produce symptoms, it must be accompanied by an enhancing factor which he claims is nonbacterial, without which the organism is not successful.[26] Osebold suggests that the degree of contact between host and parasite in the choroid plexuses and vessels in the brain probably determines whether an animal will display symptoms and die, or whether it will undergo a

transient infection.[28] In other words a great deal depends on the ability of the organism to reach the central nervous system, which effect probably is controlled by many factors. The number of organisms in the infecting dose would play an important part in lowering the bacterial resistance of the host. It is a well-known fact that diet plays an unquestionable part. Topely and Wilson state that deficiencies of vitamins A, B, and C increase susceptibility and experimental evidence indicates that fatigue will contribute to the acquisition of an infection.[32] All the above factors plus many more probably play an important role in determining whether a Listeria infection will terminate fatally.

SUMMARY AND CONCLUSION

Representative strains of Listeria monocytogenes have been studied in the laboratory and in experimental animals with reference to their physiology and pathogenicity. Agglutination tests were conducted with samples of sera from sheep, cattle and humans for the determination of the presence of agglutinins reacting with the organism. Exposure to Listeria monocytogenes is a common occurrence among sheep and cattle available for this study. The investigator believes that the agglutinins found are acquired antibodies, and not natural protective substances. The relation of this high exposure rate to the rather rare occurrence of the clinical disease casts a shadow of doubt on the generally held belief that Listeria monocytogenes is highly pathogenic.

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