EXPERIMENTAL INFECTION ON RABBITS WITH *HAEMOPHILUS SOMNUS* (NEW SPECIES)

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INTRODUCTION

An encephalitic disease of cattle, first reported as a feedlot entity in Colorado (Griner et al., 1956), was subsequently recorded as occurring in feedlots in California (Kennedy et al., 1960), Kansas (Weide et al., 1964 and Bailie et al., 1966), and Illinois (Case et al., 1965 and Gossling, 1966). In Oklahoma and Texas the disease was reported in cattle grazing pastures as well as in feedlots (Panciera et al., 1968).

The encephalitic clinical syndrome was of short duration and sudden death. The principal macroscopic lesions were single or multiple foci of hemorrhage and necrosis of various size, located in the brain without any specific anatomical pattern. Microscopic studies revealed meningoencephalitis characterized by suppurative inflammation and presence of emboli and/or thrombi (Bailie et al., 1966).

Microbiologic studies of brain tissues revealed a small gram-negative bacillus in a significant number of cases. The organism was described as Haemophilus-like (Kennedy et al., 1960 and Case et al., 1965), Actinobacillus-like (Gossling, 1966), and Actinobacillus actinoides-like (Bailie et al., 1966). Extensive studies indicated characteristics which warranted classification of the organism within the genus Haemophilus and Bailie (1969) proposed a new species designation of Haemophilus somnus.

This research was designed to test the reliability of the rabbit as a laboratory animal for pathogenic studies of this organism. The research objectives were: (1) to determine and characterize the gross and microscopic lesions of infection in rabbits; (2) to determine the method of inoculation and the infective dose necessary to reliably reproduce signs and lesions of encephalitis; (3) to determine alterations of specific hemogram and clinical parameters; (4) to test for endotoxic manifestations of the microorganism; and (5) to investigate the effects of serial passage of the microorganism in rabbits.

REVIEW OF THE LITERATURE

Griner et al., (1956) described an encephalitic syndrome in cattle as "infectious embolic meningo-encephalitis" which occurred in feedlot cattle, 12 outbreaks between 1949 and 1956, and involved 36 animals. This syndrome was characterized principally by acute progressive neurologic signs and death. Necropsy revealed that the only consistent macroscopic lesions were located in the brain and meninges. The lesions were described as "multiple reddish-brown foci of necrosis and inflammation". There was no apparent consistent pattern to location of the lesions and the overlying meninges were inconsistently involved. Histopathologic studies of the brain tissue revealed multiple lesions of inflammation, hemorrhage, and necrosis, the inflammatory reaction being primarily suppurative. Vasculitis was prominent with the formation of thrombi composed of leukocytes and fibrin.

Bacteriologic examination of brain tissues revealed Corynebacterium sp. and Streptococcus sp. as possible pathogens from several specimens, but because of lack of consistency of isolation, Griner et al., (1956) concluded that the syndrome was not related to either microorganism.

In California, Kennedy et al., (1960) reported a disease in feedlot cattle with encephalitic lesions of vasculitis, thrombosis, and necrosis similar to those reported by the Colorado workers (Griner et al., 1956). Their report offered evidence of a specific disease entity suggested by morbidity

rate, similar pathologic manifestations, and isolation of a pleomorphic, gram-negative bacterium from a significant number of cases. The authors considered the bacterial isolate to be a Hemophilus-like organism.

Weide et al., (1964) summarized the occurrence and diagnosis of bovine encephalitides in Kansas. Their report included polioencephalomalacia, listeriosis, streptococcal meningitis, and infectious embolic meningoencephalitis. The latter disease was the most frequently encountered encephalitic disease of feedlot cattle during a two-year period immediately preceeding the report. The gross and microscopic lesions were consistent with those previously reported. Microbiologic studies did not reveal the Hemophilus-like organism of Kennedy et al., (1960) nor other organisms of considered pathologic significance.

The report by Case et al., (1965) indicated the occurrence of a disease in Illinois with characteristics similar to those previously described, and a single isolation of the Hemophilus-like organism of Kennedy et al., (1960).

Gossling (1966) described characteristics of bacteria isolated from cattle in Illinois with lesions of "embolic meningoencephalitis" and suggested that ". . . this organism may probably be best described as an Actinobacillus species . . .".

Bailie et al., (1966) reported the occurrence of "infectious thromboembolic meningoencephalitis" in feedlot

cattle as ". . . a disease of primary importance . . ." in Kansas. The clinical features, gross and histopathologic lesions were similar to those previously described (Griner et al., 1956; Kennedy et al, 1960; Weide et al., 1964; and Case et al., 1965). In addition, they isolated a gram-negative bacillus from a significant number of cases. This organism was similar to both the Hemophilus-like organism of Kennedy et al., (1960) and Case et al., (1965) and to Actinobacillus actinoides. Similarities to the latter organism were considered significant and a tentative identification as Actinobacillus actinoides-like organism was made.

In 1968, Panciera et al. described a septicemia occurring in cattle in Oklahoma and in the Texas Panhandle caused by a Hamophilus-like organism, which was manifested by acute, subacute, and chronic syndromes involving the central nervous system, the respiratory system, and joints. The central nervous system manifestations parallelled those reported previously as embolic or thromboembolic meningoencephalitis. Their report provided evidence of the disease entity occurring in animals pastured on winter wheat as well as in feedlots. In addition, their report suggested significant lesions in tissues other than those in the central nervous system and the extraneural lesions appeared related to vascular injury. The Oklahoma workers considered that these lesions were not attributable to embolism, but to a vasculitis which may lead

to thrombosis. Their report indicated that polyarthritis, polyserositis, and psuedomembranous or ulcerative laryngitis were commonly associated with central nervous system lesions. The authors successfully reproduced the disease experimentally in 2 cattle utilizing a Hamophilus-like organism isolated from the brain of a naturally-occurring field case.

Bailie (1969) summarized the occurrence of thromboembolic meningoencephalomyelitis (TEMEM) among the suspected bovine encephalitis cases which he investigated. He reports TEMEM occurring in 60.5% (193) of 259 brains with encephalitic lesions. The cases were from Kansas (157), Nebraska (20), Illinois (6), Texas (4), Oklahoma (4), and Missouri (2). Extensive morphologic, physiologic, and biochemic examinations of a microorganism isolated from brain tissue of affected animals were conducted by the author and he concluded that the microorganism ". . . possesses much in common with the genus Haemophilus and therefore the name Haemophilus somnus (new species) is proposed."

MATERIALS AND METHODS

Rabbits

A total of 116 ten to twelve week-old New Zealand white rabbits of similar breeding were used in the experiments. Random distribution of rabbits according to the 4 primary research objectives was:

Experiment I: Optimal Infective Dosage and Hemogram Alteration Study (32).

Experiment II: Optimal Inoculation Method Study (51).

Experiment III: Endotoxin Study (21).

Experiment IV: Serial Passage Effect on Virulence Study (12).

The rabbits were housed individually in cages, under as nearly identical conditions as possible, in isolation facilities. All animals received a commercial rabbit food and water ad libitum. An equal number of males and females were selected by random sampling for each test group and each group included an appropriate number of control animals.

The normal health status of each rabbit was determined for a minimum of 2 weeks immediately preceeding each test. It was evaluated by daily examination of general appearance, appetite, and rectal temperature. A minimum of 2 fecal samples were collected from each rabbit during this period and examined by the zinc sulfate flotation method (Ewing, 1967) for evidence of parasitic infection. For those tests to evaluate specific hemogram alterations, blood samples were collected as described under hematologic examination. Only

rabbits which appeared healthy and whose status was considered normal were utilized in this investigation.

Hematologic Examination

One phase of the experimental design was to evaluate specific hemogram alterations following inoculation of H. somnus (n. sp.) described under inoculum preparation. During the 2 week period preceeding testing a minimum of 4 blood samples were collected by intracardiac puncture. The skin puncture site was prepared by shaving the hair and cleansing with 70% alcohol. A one inch 20 gauge needle with a 2 cc. glass syringe was used. Dipotassium ethylenediamine tetra-acetate* was utilized as the anticoagulant. The examination of each blood sample included: hemoglobin, hematocrit, total erythrocyte count, total leukocyte count, differential leukocyte count, and bacteriologic culture. Following inoculation of the test rabbits, and according to the experimental design, blood samples were collected and examined as described.

Hemoglobin was determined by the photometric cyanmethemoglobin method (Wintrobe, 1961) and the packed cell volume by the microhematocrit method (Schalm, 1965). The total erythrocyte and total leukocyte counts were made with the

*Mallinckrodt Chemical Works, St. Louis, Missouri.

Coulter Counter.* The differential leukocyte counts were made from smears utilizing standard techniques (Coles, 1967) and Wrights stain.**

Bacteriologic Examination

Bacteriologic examination of the blood samples was done by inoculating 0.5 ml. into 10.0 ml. of brain-heart infusion (BHI) broth.*** An aliquot from this was then inoculated into thyoglycolate broth for anaerobic incubation. The BHI broth sample was divided into 2 eaual parts, one was incubated aerobically and the other was incubated in an atmosphere of 10% CO_2.**** The broths were incubated for 48 hours at 37 C. One drop aliquots from each tube was then inoculated into blood agar***** plates and incubated under one of the 3 atmospheres described above. Plates were checked for growth at intervals of 48, 72, and 168 hours. Negative plates were discarded after 168 hours.

Inoculum Preparation

The inoculum was prepared from bovine brain

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*Coulter Electronics, Hialeah, Florida.

**Matheson Coleman and Bell, East Rutherford, New Jersey.

***Difco Laboratories, Detroit, Michigan.

****NAPCO, National Appliance Co., Portland, Oregon.

*****Sterile defibrinated sheep blood.

tissues* from which H. somnus (n. sp.) had been previously isolated. The brain tissue was cultured on typtose agar containing 20% sterile defibrinated sheep blood.

The cultures utilized had the following reactions: gram-negative; growth under microaerophilic conditions (approximately 5-10% CO_2), but without growth under aerophilic conditions; catalase-negative; production of acid fermentation in OF media with serum; oxidase-positive; and Mac Conkeys agar growth negative.

A bacterial suspension was prepared by washing the growth from the surface of the agar plates with sterile saline. The inoculum was standardized in a spectrophotometer** to conform with MacFarland nephlometer tube no. 3 equivalent to 3×10^6 organisms per ml. and confirmed by poured plate counts. Each inoculum was utilized within one hour after preparation.

Inoculation Procedure

The study was designed in 4 experiments and the inoculation procedure was determined for each experiment. A total of 116 rabbits were utilized with various numbers of rabbits in each experiment according to the investigation objectives. Without regard to the objectives of a specific experiment, each rabbit was examined at the termination of the experiment

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*Veterinary Diagnostic Laboratory, Kansas State University, Manhattan, Kansas.

**Coleman, Jr. Coleman Instruments Corp. Maywood, Illinois.

by the methods described for necropsy, histopathologic, and bacteriologic examinations.

Experiment I

Optimal Infective Dosage and Hemogram Alteration Study

The aim was to establish the optimal infective dosage and any alteration of the specific hemogram parameters.

Thirty two rabbits were divided into 4 equal groups and each consisted of 4 males and 4 females, 3 test and 1 control of each sex. Each group received a different dosage calculated as milliliters of standardized inoculum per kilogram of body weight. Each pair of a group were further identified as "High Dose" (HD), "Mid Dose" (MD), "Low Dose" (LD), and "Control" according to dosage. The rabbits were inoculated as follows:

Rabbit Number	Identification	Dosage (ml./kg. of body wt.)
Group I		
001 005	Control	0.2
002 006	LD	0.1
003 007	MD	0.2
004 008	HD	0.3

Group II		
009 013	Control	0.45
010 014	LD	0.40
011 015	MD	0.45
012 016	HD	0.50
Group III		
017 021	Control	0.60
018 022	LD	0.40
019 023	MD	0.60
020 024	HD	0.80
Group IV		
025 029	Control	0.60
026 030	LD	0.40
027 031	MD	0.60
028 032	HD	0.80

Each rabbit was injected via the lateral marginal ear vein with a one-half inch 26 gauge needle and 1.0 ml. tuberculin syringe. Prior to inoculation, the hair was clipped from the injection site and the area cleansed with

70% alcohol. The control rabbits were injected in the same manner with an equal volume of the inoculum suspending medium.

Experiment II

Optimal Inoculation Method Study

Prior to the initiation of this experiment, a preliminary trial was conducted to evaluate the viability and pathogenicity of the available bacterium. The perliminary study utilized 3 rabbits which were inoculated intracerebrally. Two rabbits received a standarized inoculum and one received the suspending medium of the inoculum and served as a control.

The preliminary study group of rabbits were inoculated as follows:

Rabbit	Identification	Dosage (ml. of stand. inoculum)
A	Control	0.15
B	LD	0.1
C	HD	0.2

Forty-eight rabbits were divided into 4 groups of 12. Each group consisted of 3 dose levels, with 3 rabbits per dose level and 3 animals served as controls. The dosage was calculated as milliliters of standardized inoculum per kilogram of body weight. The control rabbits in each group were inoculated with the inoculum suspending medium. The amount of inoculum and method of inoculation corresponded to

that for each group.

Group I

The rabbits were inoculated intravenously via the lateral marginal ear vein as follows:

Rabbit Number	Identification	Dosage (ml./kg. of body wt.)
101	LD	0.3
102	LD	0.3
103	MD	0.5
104	MD	0.5
105	HD	0.7
106	HD	0.7
107	LD	0.3
108	MD	0.5
109	HD	0.7
110	Control	0.3
111	Control	0.5
112	Control	0.7

The volume of inoculum was injected at a rate which required 30 seconds to complete.

Group II

The rabbits were inoculated via the conjunctiva by swabbing each eye 3 times with a dry sterile cotton swab 2 minutes prior to inoculation. One-half of the calculated dose of inoculum was instilled into the conjunctival sac of each eye, with the head and eyelids held in a manner which provided retention of the maximal contact of the inoculum with the conjunctiva for 2 minutes.

The 12 rabbits in this group were inoculated as follows:

Rabbit Number	Identification	Dosage (ml./kg. of body wt.)
201	LD	0.08
202	LD	0.08
203	MD	0.12
204	MD	0.12
205	HD	0.16
206	HD	0.16
207	LD	0.08
208	MD	0.12
209	HD	0.16
210	Control	0.08
211	Control	0.12
212	Control	0.16

Group III

Nasal inoculation was performed by inserting a sterile catheter 2 cm. into each nasal passage and injecting one-half of the calculated dose into each nostril.

The 12 rabbits were inoculated as follows:

Rabbit Number	Identification	Dosage (ml./kg. of body wt.)
301	LD	0.07
302	LD	0.07
303	MD	0.14
304	MD	0.14
305	HD	0.21
306	HD	0.21
307	LD	0.07
308	MD	0.14
309	HD	0.21
310	Control	0.07
311	Control	0.14
312	Control	0.21

Group IV

This group was inoculated orally by intubation. A sterile catheter was inserted per os into the stomach and the calculated dose was injected, followed by 2 ml. of sterile physiological saline.

The rabbits were inoculated as follows:

Rabbit Number	Identification	Dosage (ml./kg. of body wt.)
401	LD	1.0
402	LD	1.0
403	MD	2.0
404	MD	2.0
405	HD	3.0
406	HD	3.0
407	LD	1.0
408	MD	2.0
409	HD	3.0
410	Control	1.0
411	Control	2.0
412	Control	3.0

Experiment III

Endotoxin Study

Twenty-one rabbits were utilized to test for the presence of endotoxin which was suggested by preliminary studies. Endotoxin was evaluated by the local and generalized Shwartzman reactions (Shwartzman, 1937 and 1953).

Local Shwartzman Reaction:

Twelve rabbits were divided into 4 groups of 3. Each

rabbit was initially injected intradermally and then 24 hours later intravenously. The inoculum used was different for each group and is described below. The intradermal site was located approximately 2 cm. lateral to the dorsal midline of the third lumbar vertebrae on the left side. This site was selected as it offered less opportunity for self-mutilation which could mask skin changes. The injection site was prepared by clipping and shaving a 5 cm. circular area, and cleansing with 70% alcohol. The injection was accomplished with a one-half inch 26 gauge needle and a 1.0 ml. tuberculin syringe. The intravenous injection was given via the lateral marginal ear vein after clipping and cleansing with 70% alcohol.

Group 1 was injected intradermally with 0.25 ml. of a filtrate obtained by Seitz-filtration* of a standardized inoculum followed 24 hours later by 0.1 ml. intravenously. The intradermal site was examined carefully for any visible change for a 48 hour period following the intravenous or provoking injection.

Groups 2, 3, and 4 were injected similarly except that group 2 received standardized inoculum, group 3 was given bacterial suspension which had been heated for 5 minutes in a water bath at 100 C, and group 4 was injected with a filtrate obtained by the filtering process described for group 1 utilizing the heated suspension used for group 3.

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*Republic Seitz Filter Corp., Milldale, Conn.

Generalized Shwartzman Reaction:

Nine rabbits were divided into 3 groups of 3. Each rabbit was injected intravenously and 24 hours later received a second intravenous injection.

Group 1 was injected with standardized inoculum. Group 2 was injected with the heated suspension previously described. Group 3 was injected with a filtrate of heated suspension which was filtered as previously described.

The rabbits were inoculated as follows:

Rabbit Number	Inoculum No. 1	Inoculum No. 2
Group 1		
701	S.I.,* 1.0 ml.	Saline
702	S.I., 1.0 ml.	S.I., 1.0 ml.
703	S.I., 1.0 ml.	S.I., 2.0 ml.
Group 2		
704	S.I., heated at 100 C for 5 min.	Saline
705	S.I., heated at 100 C for 5 min.	As Inoc. No. 1, 1.0 ml.
706	S.I., heated at 100 C for 5 min.	As Inoc. No. 1, 2.0 ml.
Group 2		
707	S.I., filtered	Saline
708	S.I., filtered	As Inoc. No. 1, 1.0 ml.
709	S.I., filtered	As Inoc. No. 1, 2.0 ml.

*S.I. = Standardized Inoculum.

Beginning 24 hours after the second inoculation, a rabbit from each group was killed and necropsied. A rabbit from each group was similarly examined at 48 hours and 72 hours following the second or provoking injection.

Experiment IV

Effect of Serial Passage on Virulence Study

Twelve rabbits were utilized to investigate the virulence of H. somnus (n. sp.) for rabbits following serial passage. A culture of H. somnus (n. sp.) was obtained by isolation from the brain tissue of a rabbit (103) used in Experiment II. The rabbit exhibited manifestations of central nervous system disturbances and the brain tissue had microscopic lesions of nonsuppurative meningoencephalitis. A standardized inoculum was prepared from this culture by the method previously described.

Twelve test rabbits were divided into 2 groups of 6, 4 test and 2 control. The first group were inoculated with standardized inoculum intravenously and the 2 control rabbits received suspending medium.

The animals were inoculated intravenously as follows:

Rabbit Number	Identification	Dosage (ml./kg. of body wt.)
801 804	Control	0.4
802 805	LD	0.3
803	HD	0.5

The second group were injected with a culture obtained from the brain tissue of one rabbit (802) from group 1. Six

rabbits were inoculated intravenously as follows:

Rabbit Number	Identification	Dosage (ml./kg. of body wt.)
807 810	Control	0.4
808 811	LD	0.3
809 812	HD	0.5

Post-Inoculation Procedures

General physical examinations were made of all rabbits as frequently as dictated or at intervals not exceeding 12 hours. The basic examination included general appearance and rectal body temperature. Changes in feed and/or water consumption rates were noted. In the first experiment blood samples were collected at specific times according to the method described.

Disposition of Rabbits

Test rabbits were necropsied immediately after death or if *in extremis*. Test rabbits which survived were killed by carbon dioxide narcosis and exsanguination, and then necropsied. The control rabbits were killed and necropsied at the time as the surviving test rabbits.

Necropsy examination was conducted on each rabbit and the following tissue sections were collected and fixed in 10% buffered neutral formalin: all macroscopic pathologic lesions, brain, spinal cord, eyes, nasal mucous membranes and turbinates, liver, kidney, stomach, duodenum, lung, heart, and lymph nodes (parotid, retropharnygeals, mandibular, gastric, and mesenteric). Representative sections of all macroscopic pathologic lesions, central nervous system tissues, liver, kidney, and lung were frozen and held at -70 C* for subsequent bacteriologic culture.

Histopathologic Examination

Emphasis of the histopathologic examination was placed on tissues from the central nervous system. Sections from the cerebral cortex, caudate nucleus, hippocampus, thalmus, hypothalmus, corpora quadrigemina, pons, medulla oblongata, cerebellum, and spinal cord were processed by the paraffin block method, sectioned at 5-6 microns, and routinely stained with hematoxylin and eosin. Sections with lesions were stained with the Brown and Brenn modification of the Gram stain for bacteria (Thompson and Hunt, 1966).

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*Revco Inc., Industrial Products Division, Deerfield, Michigan.

RESULTS

Experiment I

Optimal Infective Dosage and Hemogram Alteration Study

Group I

The average pre-inoculation values for the specific hemogram parameters were determined (Appendix, Table I). The values, for each of 7 samples of blood collected during this period, were considered within normal limits for all rabbits. Rectal body temperatures were recorded for each rabbit twice daily at 12 hour intervals (Appendix, Table II). Each rabbit was observed daily to determine the normal clinical attitude of movement, alertness, color of mucous membranes, and respiratory rates. No abnormalities were noted. Examination of fecal samples by the zinc sulfate flotation method revealed oocysts of hepatic coccidia (Eimeria sp.) in 4 rabbits (004, 006, 007, and 008) and intestinal coccidia (Eimeria sp.) oocysts in 2 (004 and 006). Microbiologic examination of blood samples collected from this group revealed Staphylococcus aureus in the sample taken 4 days prior to inoculation of animal 005. This was considered a contaminant because the rabbit had no clinical signs of disease.

The post-inoculation (P.I.) values of the specific hemogram parameters were determined (Appendix, Table I). At 8 hours P.I., an increase in the number of large lymphocytes was observed in all rabbits receiving H. somnus (n. sp.).