EVALUATION OF NEOMYCIN SULFATE IN THE TREATMENT OF BOVINE MASTITIS

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A. R. Drury

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THESIS

This is to certify that the

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EVALUATION OF NEOMYCIN SULFATE IN THE TREATMENT OF BOVINE MASTITIS

By

A. R. Drury

A THESIS

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TABLE OF CONTENTS

TABLE OF CONTENTS - Continued Page

INTRODUCTION

INTRODUCTION

Mastitis has been a problem to the dairyman ever since cows have been kept for milk production. Antibiotics have brought many improvements to the health and welfare of our society. Veterinarians have always been eager to explore new treatments for mastitis. The results of these investigations have created an immense volume of literature concerning the relative merits of the various treatments.

One of the more recent antibiotics is neomycin. This drug has been shown to be effective against a wide variety of organisms; therefore, it is logical that neomycin be investigated for its effectiveness in the treatment of bovine mastitis since there are several genera of causative organisms involved in this disease.

In this study I have taken neomycin which had never been used in dairy cattle and attempted to evaluate its safety, efficacy, and limitations in the treatment of bovine mastitis.

The following points have been considered: (1) The *in vitro* effects of neomycin on the causative organisms of mastitis. (2) Its effects on the udder tissues and milk. (3) Retention of the drug when infused into the udder. (4) Blood levels following a single intramuscular injection. (5) Results of its use in 405 cases of mastitis based on bacteriological findings.

REVIEW OF LITERATURE

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I. NEOMYCIN

Neomycin as an antibiotic of therapeutic significance was first brought to the attention of investigators in 1949 by Waksman and Lechevalier (1949a). They isolated the active principle from a soil organism now listed in Bergy's Manual of Determinative Bacteriology as Streptomyces fradiae. Neomycin sulfate is a basic compound. Originally it was thought to be more active in an alkaline medium. This was later shown to be inconsequential by the same workers (1950). It is soluble in water, insoluble in organic solvents, thermostable, and active against many gram positive and gram negative bacteria. It was first mentioned as an active agent against streptomycin-resistant bacteria, particularly tubercule bacilli. Waksman listed among the desirable properties of preparations of neomycin: 1. Similar activity against both streptomycin-sensitive and streptomycin-resistant microorganisms. 2. Considerable activity against various forms of Mycobacterium tuberculosis and other mycobacteria. 3. Limited or no toxicity to animals. 4. Marked activity against other bacteria in vivo, including gram negative and gram positive. 5. The development of resistance against neomycin among organisms sensitive to it has not been reported.

Waksman et al (1949b) subsequently reported that neomycin is highly effective against the ordinary pathogenic gram negative and gram positive bacteria in a dose about 1/20 - 1/50 the toxic dose. Waksman, Frankel

and Graessle (1949c) reported that a crude neomycin, varying in potency from 30 - 100 units/mg, was effective in experimental infections of Staphylococcus aureus in mice, Salmonella schottmulleri of mice and chick embryos, Salmonella pullorum of chick embryos, and Eberthella typhosa of mice. Neomycin was as effective against streptomycin-resistant strains of Staph. aureus and S. schottmulleri as against the streptomycin sensitive strains. They also recorded that neomycin exerted no serious toxic effects when instilled into the eye of a rabbit which is a standard procedure to determine irritability of a substance.

Hobby, Lenert and Dougherty (1949) produced tables showing the antibacterial spectrum of neomycin which reveals that it is highly active against many gram negative microorganisms. It was less active against gram positive organisms and showed no activity against twenty strains of pathogenic fungi. They also reported that neomycin could be readily detected in the serum of injected animals.

Harme, Bernstein and Donovich (1950) reported that a dose of neomycin of 10 mg per egg prolonged slightly the survival time of embryonated eggs infected with vaccina virus, but did not prevent their death. Warth, Chandler and Bliss (1950) reported that neomycin was the only antibiotic studied in their laboratory that was potent against both Proteus and Pseudomonas. Compared with aureomycin in vitro, neomycin was just as effective against gram negative bacilli of the coli-aerogenes group, Hemophilus influenzae staphylococci, and gram positive bacilli. It was more effective against strains of proteus and pseudomonas and was less active in inhibiting the growth of streptococci. Neomycin was valueless in infections with hemolytic streptococci and the pneumococcus.

Felsenfeld, Volini and Ishihara (1950) stated that the action of neomycin is strong, both *in vitro* and *in vivo* against gram negative rods, tubercle bacilli, *Bacillus anthracis*, *Listeria monocytogenes* and intestinal protozoa. Its action on gram positive cocci was variable. Adequate doses of neomycin *in vivo* were able to prevent experimental salmonellosis, cholera, tuberculosis, amebiosis and rickettsialpox.

A report of the committee on Medical Research and Therapy of the American Medical Association (1950) stated that neomycin, which is active against the tubercle bacillus *in vitro* and *in vivo*, is too toxic for treatment of human tuberculosis, even on an experimental basis. The unfavorable reactions attributed to neomycin treatment are deafness and renal impairment.

Welch,Reedy and Wolfson (1950) reported on nine different common antibiotics when used against experimental typhoid in mice. They placed neomycin in the middle and chloromycetin is the most successful of the group in treating cases of typhoid fever in man.

Waishren and Spink (1950) showed that neomycin was bactericidal in concentrations equal to or only slightly higher than those in which it was bacteriostatic.

Waksman, Katz, Lechevalier (1950), in an extensive study, showed a very complete antibiotic spectrum for neomycin. Neomycin is active against a variety of gram positive and gram negative bacteria, acid fast bacteria and actinomycetes, but not against fungi and viruses. It has a strong bacteriostatic and bactericidal effect upon various bacteria. The amount of the antibiotic required for complete destruction of cells is

definitely correlated with the number of bacterial cells in a given inoculum. No absolute resistance to neomycin has been observed. Neomycin is highly stable; no activity was lost when a solution of neomycin was adjusted to pH values ranging from 1.5 to 12 and kept at room temperature for twenty-four hours. Nor was there any loss in activity when a solution adjusted to pH 2 was heated for ten minutes at 60° and 100° C. A solution with a pH of 1 or 10 lost 40 per cent of its activity after ten minutes of heating at 120° C.

Felsenfeld, Volini, Young and Ishihara (1950) in a later report stated that neomycin is very effective against tuberculosis, typhoid, and other salmonella infections, Shigella, Vibrio cholerae, Pasteurella pestis, Brucella, and Entamoeba histolytica. It was not effective against the small sized viruses such as that of poliomyelitis. The same authors in another paper (1950b) reported that in 127 patients, who received neomycin, unfavorable sequelae developed in two cases. There was impairment of hearing in one case and kidney irritation in the other. They also stated that oral routes are not satisfactory for systemic therapy.

Clancy (1951) investigated the sensitivity of 171 strains of organisms commonly encountered in a hospital diagnostic laboratory. He found neomycin to be highly active against Aerobacter aerogenes, Klebsiella pneumoniae and Micrococcus aureus variety pyogenes. All the gram positive rods studied yielded except clostridia. Streptococci were rather resistant when tested in a tube test. Many proteus and pseudomonas strains were moderately sensitive to neomycin upon overnight incubation; bacteriostasis

was evident up to seven days and showed an unusual potentiality for the development of neomycin resistance.

In vitro work done by Prier (1951) with the seven commonest antibiotics showed terramycin to rank first and neomycin second in activity toward four strains of Vibrio fetus.

Waishren (1951) divulged that neomycin was used in two cases. In one case before its toxicity was established, and in the second case, when in vitro tests showed it to be by far the most active agent against an unclassified bacillus isolated from the blood of a critically ill child. He felt the neomycin probably saved the life of the patient in both instances but attributed the deafness that developed in one child to a harmful effect of the treatment.

Poth, Fromm and Wise (1951) felt that oral medication of neomycin was an excellent intestinal antiseptic as it reduced the bacterial flora of the stool to nil. They reported yeasts to be present. They also felt certain that there was no influence on the blood clotting mechanism or clotting time. They observed that neomycin promotes tissue healing in intestinal surgery.

When neomycin is administered parenterally it is found in the blood and eliminated in the urine. The only assay procedures for neomycin are those that Hanson and Collingsworth (1949) adapted from procedures used for streptomycin in blood and urine.

II. ORGANISMS CAUSING MASTITIS

According to the E. Munch-Petersen survey (1938) of the literature on bovine mastitis, credit goes to Nocard and Mollereau (1884; 1885; 1887) for the first description of a microorganism, and, more particularly, a streptococcus as a cause of mastitis. No specific generic name was assigned to the organism but it was listed as having the same biochemical behavior as the organism that is now known as Streptococcus agalactiae.

Bang (1889) described an organism identical with that described by Nocard and Mollereau as a cause of mastitis, (E. Munch-Petersen 1938).

In the book, Bovine Mastitis, by Little, Brown and Plastridge, (1946), Chapter V, it is stated that "Streptococci are regarded as responsible for at least 85 per cent of the chronic mastitis observed in dairy cattle. In about 80 per cent of these conditions the organism responsible is Streptococcus agalactiae. Other streptococci in order of importance are Str. uberis, Str. dyagalactiae and strongly hemolytic Group C and Group G streptococci (Lancefield)."

The opinions of the various workers differ as to the role of staphylococci as the etiological agent in mastitis. In the book, Bovine Mastitis it was stated: "While some reports in the early literature suggested that staphylococci were normal inhabitants of the udder, others incriminated staphylococcus as a cause of mastitis. In 1889, Lucet reported the finding of gelatin-liquefying micrococci in the secretion of 7 of 21 animals affected with mastitis. Other early investigators, including Guillebeau, Steiger, Savage, Jones, Carpenter, Hardenburg and Schlotthauer

found that staphylococci were present in a significant proportion of abnormal udder secretions in which streptococci were absent."

Miscellaneous organisms causing mastitis, described in the textbook Bovine Mastitis (1946), are: Escherichia coli, Aerobacter aerogenes, Corynebacterium pyogenes, Pseudomonas aeruginosa, Pasteurella septica, Actinomyces necrophorus, Clostridium perfringens and yeasts.

III. LOCAL IRRITATION AS EVIDENCED BY LEUCOCYTE RESPONSE

Leucocytes are normally found in freshly drawn milk. However in abnormalities involving the mammary gland, the leucocyte count is altered, both in numbers and kind. Merely striking the udder with the fist will cause an outpouring of leucocytes. They are a good indication of irritation produced by certain substances in the udder, (Bryan 1948). Even reinjection of withdrawn milk will result in a temporary increase in the white cell count. Authors do not agree as to what is a normal leucocyte count in milk, but it is generally assumed that there are less than a million leucocytes per ml in milk from a healthy, normal udder of a cow (Bryan 1948). However, in an infected udder, there is not necessarily a high cell count. Klein and Learmouth (1935) showed that irritants increase cell counts which are a measure of the irritating qualities of a product. This, then, to some degree, would be a toxicity determining factor. Many drugs create a high cell count which persists for a period of several days. Others give only slight increases of only a few days' duration. A cow will establish a pattern of numbers and will generally return to a similar level after stimulation by an irritant.

IV. DIAGNOSIS

From the many articles that have been published dealing with diagnosis, it would seem that the direct microscopic procedure is the most reliable. Particularly significant are the early reports of Plastridge (1935) dealing with microscopic diagnosis. Murphy (1932), Hucker (1942, Biddle (1944), Cone (1940), Ferguson (1940), Kelser (1950), Klien (1935, 1938), Palmer (1940), Schalm (1944), Slanetz (1939), and Udall (1947) all contributed to the development of mastitis diagnostic methods. Hotis (1936) developed the test that bears his name. Kleckner (1941, 1942a, 1942b) published a great deal on efficiency and practicability of various procedures to diagnose mastitis.

Murphy (1939, 1941, 1943a, 1943b) also published on the efficiency and practicability of some methods. Bryan (1947) modified the Hotis procedure to add convenience and accuracy. Several outstanding articles by Bryan et al (1932, 1933, 1940, 1943, 1947, 1948) both of a review nature and original contributions have appeared. The book of Little and Plastridge (1946) on bovine mastitis gives an excellent review of methods of diagnosis. The recent bulletin by Merilan, Herman, Edmondson, Tallman and Crisler (1950) from the Missouri Agricultural Experimental Station gives much information on the evaluation of diagnostic procedures.

The publication of Brown and Bryan (1950) is very interesting. They collected samples from a non-infected and an infected cow's udder every day for six months. On the basis of microscopic examination of the specimens collected they demonstrated the reliability of the direct microscopic procedure. As a matter of fact, they reported 100 per cent efficiency for this method, the only one that is so reliable. The work of Brown and

Bryan showed blood agar and Edward's Esculin Agar to be 96 per cent, the Hotis test 85 per cent and chloride content percentages as only 35 per cent accurate. The leucocyte count method was only 83 per cent accurate, giving wrong results in both positive and negative cows. Thybromol tests gave 11 per cent inaccuracies over all.

In the final analysis the results of Brown and Bryan were comparable to the results published by Merilan et al (1950), Malcolm (1942), Fay (1938), Roach (1944), Rowland (1939), Johns and Hastings (1938).

V. ASSAY

To learn something of the sojourn of a drug, in the body, assay procedures for body fluids have been developed. These must be studied when evaluating a new antibiotic. Detailed procedures for assay of penicillin are discussed by Schmidt and Moyer (1944). They described a cylinder plate method and a serial dilution procedure using Staph. aureus.

Rosenblatt, Altun, Werber, Kashdan and Loeuve (1944) gave a procedure for the determination of penicillin levels in body fluids involving Staph. pyogenes with serial dilution techniques.

Cooke (1945) developed a simple clinical method for assay of penicillin in body fluids. He first used a known concentration of the antibiotic against the organisms to establish their sensitivity. He then used the body fluids containing the unknown concentrations of penicillin against these organisms to determine the amount of antibiotic present.

Kirby and Rantz (1944) discussed the Oxford cup detection method and attempted to establish the superiority of a turbidimetric method. This

presupposes that a clear solution is to be examined. Therefore, another problem is encountered when milk is the fluid involved.

DeBeer and Sherwood (1945) evolved a paper disc agar plate method for the assay of antibiotic substances. They used absorbent paper discs and established curves with known amounts and plotted unknowns into a curve on a graph using Bacillus subtilis as a test organism. They demonstrated statistically the accuracy of this type of assay.

Price, Nielsen and Welch (1946) adapted a procedure for estimation of streptomycin in body fluids. They selected Bacillus circulans as the test organism and adapted a suitable serial dilution technique.

Loo, Shell and Thornberry (1945) published a procedure for assay of streptomycin by the paper disc method. They used a susceptible strain of B. subtilis. Standard curves were prepared from zones of inhibition on Bacto-streptomycin assay agar using a Schleicher, Schnell disc containing a volume of 0.08 ml of antibiotic delivered with a calibrated pipette of this capacity.

Hirsch (1950), writing on the assay of the antibiotic nisen, stated that because nisen does not diffuse quickly through the agar it must be assayed by dilution methods. Also, because it is bactericidal and not merely bacteriostatic it may be assayed by estimating numbers of surviving bacteria. With Str. agalactiae as the test organism, it may also be assayed in terms of acid production.

Bond and Nook (1948) could detect as little as 0.02 units per ml of bacitracin in blood serum, urine or saliva. They used a hemolytic streptococcus in penicillin assay agar plates. Their method involved a standard curve.

Watts and McLeod (1946) suggested a way to estimate penicillin levels in blood serum and milk of bovines after intramuscular injections by the use of litmus milk and susceptible acid producing strains of bacteria in various dilutions. Other outstanding articles have appeared by Foster and Woodruff (1945), Jackson and Finland (1945), Randall et al (1945) and Welch et al (1945).

The Federal Security Commission (1950) through the Food and Drug Administration maintains and publishes up-to-date information on methods, criteria and regulations pertaining to the assay of antibiotics.

MATERIALS AND METHODS

MATERIALS AND METHODS

The following procedures were used in performing the investigation.

I. SENSITIVITY TEST

A. Cultures

Cultures of Staph. aureus 26, Str. agalactiae 15, Str. dyagalactiae 22, Str. uberis 29, E. coli 30 and Ps. aeruginosa 33 were used. These were from a stock accumulated from cases of infectious mastitis by various members of the staff of Bacteriology and Surgery and Medicine over a period of many years. They have been maintained by monthly transfer on tryptose agar slants and stored in a refrigerator at 38° - 40° F.

The growth phase of these cultures was stimulated by daily transfers for several days. This tends to stimulate a constant, rapid, uniform and more vigorous growth when used in a sensitivity test.

B. Standard Solution of Neomycin

One quarter of a gram of neomycin sulfate was weighed and dissolved in 25 ml of sterile distilled water; therefore each ml contained .01 gm. Ten ml of the solution was combined with 90 ml of sterile distilled water. Thus, each ml contained 0.001 gm.

C. Method

1. A row of 25 sterile 6 inch tubes was placed in a rack.

2. One ml of Difco's nutrient tryptose broth was placed in all but the first tube of the above series.

3. Using a one ml sterile pipette, one ml of neomycin containing 0.001 gm (Step B) was placed in tubes 1 and 2. The contents of tube 2 was mixed and one ml was transferred into tube 3 and then mixed. One ml was removed from tube 3 and mixed with the contents of tube 4 and so forth, resulting in tube 1 containing .001 gm per ml; tube 2 .0005 gm per ml and tube 3 .00025 gm per ml and so forth.

4. Each series of tubes was then inoculated with a standard loopful of the 24 hour tryptose broth culture of each bacterium.

5. The tubes were incubated at 37° C. for forty-eight hours.

6. The end point was considered to have been reached in the tube which first showed growth. If, in the series, the tube to first show growth was tube 3, .0005 gm per ml was the lowest concentration of neomycin to inhibit that organism.

II. IRRITATION STUDIES

A. Animals

Normal, healthy cows were used. The animals were housed in separate box stalls, fed a good quality hay and grain ration. They were hand milked at 7:30 in the morning and 4:30 in the afternoon by the same caretaker.

Each quarter of the udder of a cow is a separate functional unit as shown by the work of Turner and his group (1948). Bryan (1948) also showed that responses to irritation with resulting changes are confined to the local area or quarter involved.

A period was spent establishing normals for each quarter of an udder by regular collection of milk samples and determination of their constituents. Varying amounts, as indicated in the results, were infused aseptically into the quarters.

In preparation for infecting a quarter with S. agalactiae, the same strain used in the sensitivity procedure was transferred in broth for several days. Two ml of a 24 hour broth culture was introduced with a teat canula. The udder was roughly handled to insure establishment of the infection. Daily samples were collected to insure continued infection of the quarter.

B. Sampling

Samples were collected at 8:30 A.M. and 4:00 P.M. An observation period of one week was used during which regular samples were collected. A strip cup was used in order to observe the gross appearance of the milk. The ventral aspect of the udder and particularly the teat orifices were then treated with a solution containing 400 P.P.M. available chlorine. Streams of milk were drawn directly into a 40 ml test tube and the samples were immediately taken to the laboratory. Sampling continued at regular intervals until the milk was normal in all respects. Observation and palpation of the udder were made whenever samples were collected.

C. Effect of the Drug on the Udder and Milk

1. Leucocyte Count: The procedure used was that of Bryan, Mallman and Turney (1947) devised for counting bacteria in raw milk. In brief, a flamed loop with an outside diameter of 4 mm, which was made of a 24

gauge German silver wire, bent around a 6 penny box nail, was immersed and withdrawn from the sample of milk. The milk was spread over an area 4 mm x 8 mm on a grease-free glass slide. The film was air dried and stained by the three jar procedure of Xylol, 95 per cent alcohol and methylene blue, one minute in each. The slide was washed on the reverse side of the film to remove the excess stain. After the slide was dry, the smears were examined under an oil immersion objective with a calibrated microscope. The microscope used has a factor of 300,000 so if each field has an average of one leucocyte, the milk has a count of 300,000 per ml.

2. The Methylene Blue Test: The procedure used was described in the American Public Health Association Standard Methods for the Examination of Dairy Products 9th edition. This test, when used on freshly drawn milk, is a measure of leucocyte concentration and not bacterial reduction in milk (McClement 1939 and McBride 1951).

3. The Resazurin Test: This test is a determination similar to that of the Methylene Blue Test. The procedure was described in the A.P.H.A. Standard Methods for the Examination of Dairy Products, 9th edition.

4. Hydrogen ion concentration, pH: A model H-2 Beckman pH meter was used to determine the pH of the milk. The meter was standardized prior to use with a buffered solution of pH 7.

5. Chloride content: The procedure was that of Cone (1940), using .2 ml of milk placed in a test tube. Five tenths cc of K_2CrO_3 and .5 ml of $AgNO_3$ was added. With a 1 ml pipette with .1 graduations, milk was added by .1 ml quantities until a constant lemon yellow color developed.

From the amount of milk needed for the titration the percentage of chloride in the milk was determined by reference to a chart.

6. Standard Plate Counts: The procedure used was that described in the 9th edition of the A.P.H.A. handbook for milk analysis.

III. ASSAY OF NEOMYCIN

A. Animals Used and Administration of Drugs

Healthy cows maintained under optimal conditions of herd management were used. The neomycin was dissolved in sterile distilled water. The injection site was cleansed with liquid germicidal detergent. A steam sterilized needle and syringe were used to inject the material in the gluteal muscle.

B. Samples

The jugular furrow was rubbed with an antiseptic. California bleeding needles that were steam sterilized were used to pierce the jugular vein and 10 ml of blood was allowed to flow into a sterile Kahn vial containing 3 crystals of sodium citrate (C.P.). The vials were gently shaken to prevent clotting.

C. Assay

This procedure was developed by Hanson and Collingsworth (1949) and is reproduced with their permission. The neomycin assay method follows:

1. Preparation of Plates: The plate medium contains 25 gm of Difco streptomycin assay agar per liter. The medium can also be prepared from

the ingredients listed in the reference cited above. The pH of the medium should be 8.0 ± 0.1.

After sterilization the medium is cooled to 60° C. and about 7 ml. of the B. subtilis spore suspension is added per liter. The volume of spore suspension to be used is indicated on each bottle of spores. The medium is shaken thoroughly and allowed to set until no air bubbles are present.

Five ml of inoculated agar is transferred to each flat bottom Petri dish and allowed to solidify. It is important to prepare the plates on a level surface to obtain a uniform thickness of the agar layer. The agar plates should be stored at refrigeration temperatures until used.

2. Addition of NaOH to Blood: The pH of blood samples and normal blood for diluting is adjusted by adding 0.1 ml of 0.1 molar NaOH to 5 ml of blood. About 40 ml of normal adjusted blood is required for the preparation of standard solutions and dilution of samples.

3. Preparation of Standards: An aqueous solution containing 1 mg of neomycin per ml is prepared, and 0.1 ml of this solution is added to 6.15 ml of adjusted normal blood. This blood contains 16 micrograms of neomycin per ml and this is the highest level used for the standard curve. Serial dilutions with adjusted blood give standards containing 8, 4, 2, 1 and 0.5 micrograms of neomycin per ml.

4. Preparation of Samples: Blood samples obtained after treatment are adjusted with NaOH as described above. If levels higher than 16 micrograms per ml are anticipated, serial dilutions are prepared using adjusted normal blood. Several dilutions of each sample are set up on

the assay plates so that inhibition zones of at least one dilution will be within the range of the standard curve.

5. Plating the Standards and Samples: Paper discs, having a diameter of 1/4 inch, are saturated by dipping them into either standards or samples. Excess solution is removed by touching the discs to the edge of the sample bottle.

For setting up the standard curve, discs containing the six standards are distributed on a plate and eight replicate plates are made. A disc for each of five blood samples and of the 2 micrograms per ml standard is placed on a plate and four replicate plates are prepared. The plates are placed in an incubator at 30° C. for 16 to 20 hours.

6. Estimation of Potency: After incubation, the diameters of the zones of inhibition are measured in mm and diameters of replicate zones are averaged. Zone diameters for the six standards are plotted against micrograms of neomycin per ml to obtain the standard curve. Typical readings are given below:

Neomycin micrograms/ml.	Zone Diameter mm.
16	14.1
8	12.6
4	11.6
2	10.2
1	8.6
0.5	7.4

Zone diameters for the blood samples are converted to micrograms per ml by using the standard curve. An example of a typical result is given below:

Sample	Dilution	Zone Size mm.	Potency of Dilution micrograms/ml.	Potency of Sample micrograms/ml.
1 hr.	1:2	11.8	5.0	10.0
	1:4	10.8	2.8	11.2

D. Milk

1. The preparation of the plates was identical with the procedure used for blood.

2. Preparation of Standards: An aqueous solution containing 1 mg of neomycin was added to 6.15 ml of skim milk. (This skim milk had been boiled for one half hour before use.) Milk with a normal pH of 6.7 was chosen. This milk sample then contained 16 micrograms of neomycin per ml and this was the highest level used for the standard curve. Standards with skim milk were prepared by serial dilution containing 8, 4, 2, 1 and 0.5 micrograms of neomycin per ml.

3. When levels greater than 16 micrograms per ml were anticipated, dilutions using sterile distilled water were made.

4. The balance of the procedure was the same as steps 5 and 6 as used for assay of blood.

IV. DIAGNOSIS

A. Direct Microscopic Method for Diagnosis of Mastitis

Samples were never collected within two hours after a complete milking. The ideal time as reported by Bryan (1947) was prior to a milking. A strip cup was used to discard the first streams of milk and remove the seal from the teat orifice. This also permitted a gross examination of

the milk. The cow's udder was then washed with a chlorine solution. A sample was collected in a sterile container and brought to the laboratory. Where plating and culturing was indicated, plates were made at this point. Where only direct microscopic results were indicated, samples were incubated fifteen to eighteen hours at 37° C.; films were prepared as mentioned for leucocyte counts. These films were then examined by microscope using the oil immersion objective. If any chains of five or more elements were noted, the sample would be considered infected with streptococci. If there was a high cell count and clusters of micrococci, a diagnosis of staphylococcal infection was made. Also needed was an accompanying history of some gross abnormality (Bryan 1947). When short rods and cells plus a clinical history of acute onset of infection was noted and a subsequent transfer of milk to brilliant green broth resulted in gas formation upon incubation, a diagnosis of coliform infection was made. If no gas formation occurs, a diagnosis of pseudomonas infection was tentatively made and confirmed taxonomically. In the cases showing gross abnormality of the udder with no bacteria demonstrable, a diagnosis of non-infectious mastitis was made. When no bacteria or high cell count or history of abnormality was present, the cow was considered negative, (Bryan 1947). A cow that shows non-pathogenic bacteria at 21 days was considered cured, (Federal Security Agency 1950).

B. Identification of Organisms

Primary culture was made on 5 per cent blood tryptose agar and Edward's Esculin agar. Suspicious colonies were picked and grown in

tryptose broth and then classified according to Breed's (1948) book Bergey's Manual of Determinative Bacteriology.

V. TREATMENT OF ANIMALS

A. Acute Cases

The majority of these animals were in the college herds. Excellent cooperation existed between the departments of Dairy Husbandry and Surgery and Medicine. Whenever an animal showed any abnormality involving the udder or milk a call for service was immediately made. Such cases were attended before four hours had elapsed from the previous milking. When an acutely affected animal was first seen by the veterinarian the temperature was taken, the udder and milk examined, and samples were collected. The involved quarter was milked dry.

If there was a fever, sulfamethazine was given orally at the rate of 1 grain per pound of body weight. If the cow remained febrile the next day, the dose was repeated. When a cow remained dull and there was atony of the rumen, ruminatorics were administered. If an animal returned to a normal temperature and did not eat on the third day, a rumen liquor transfer was administered from a rumen fistula cow on the farm.

Local treatment of the udder consisted of removal of the milk, washing the teat orifice and infusion of 0.5 gm neomycin sulfate dissolved in 10 ml of water. If the condition of the animal remained unsatisfactory, medication of the udder was repeated. Whenever material was placed in the udder, it was followed by massage to facilitate more extensive permeation

of the drug. Samples of the milk were collected at intervals and examined for the presence of bacteria following treatment. A re-check was always made at an interval of 21 days after treatment.

B. Chronic Mastitis

Only cows known to be infected were treated. All four quarters were always treated and quarter samples were collected at the time of treatment to determine the quarters actually infected, and, when cows were retested, quarter samples were collected from treated cows and subsequent treatments were administered to infected quarters. Cows were treated at a time when there would be at least a 10-hour time lapse before a milking. The amount of the aqueous solution used varied from 5 ml to 30 ml in which 0.25 to 1 gm of neomycin was dissolved. The ointments contained 0.5 gm of neomycin per 3.5 gms of vehicle.

The udder and teat orifice were always washed with a bactericidal solution before anything was placed in the udder.

RESULTS AND DISCUSSION

RESULTS AND DISCUSSION

I. IN VITRO STUDIES

When the organisms from infectious mastitis were exposed to the dilutions of neomycin as outlined in the procedure, Staph. aureus showed no growth until tube 20; therefore, 0.000000039 gm or 0.039 micrograms of neomycin sulfate per ml inhibited Staph. aureus strain 26 for 48 hours. Str. agalactiae grew first in tube 21 so 0.000000020 gm (0.02 micrograms) per ml inhibited its growth. Str. dyagalactiae grew first in tube 21 but failed to grow in tube 20, so in this case 0.02 micrograms inhibited this organism. Str. uberis was less vigorous as it failed to grow in tube 21 but grew in the remaining tubes. Thus 0.010 micrograms inhibited this strain of the organism. E. coli #30 was less susceptible; showing growth from tube 17 on, therefore 0.31 micrograms of neomycin was needed to inhibit the growth of this organism. Ps. aeruginosa grew from tube 15 on, therefore 1.25 micrograms per ml was needed in this determination.

This series was duplicated using the same procedure and only the end point of E. coli varied, which went out another tube. A third determination was made using 5 less tubes and diluting stock solutions of neomycin 1 ml to 99 ml or 1-100 instead of the 1-10. The results are set forth in the Table I. All discrepancies can be attributed to normal variations in the dilution technique. These results show the small concentration of neomycin that was sufficient to control the growth in vitro of the

principal organisms which are at present known to be the etiological agents of infectious bovine mastitis. Since some of the organisms are gram positive and some gram negative it shows the effectiveness of the drug for both groups.

II. IRRITATING EFFECT ON THE UDDER

In eventual acceptance of a drug for mastitis treatment, little or no irritating effect on the udder is of prime importance. Changes in the milk with respect to gross appearance, pH, chloride content and leucocyte counts are indications of the irritating effect of a product when placed in the udder, (Jackson 1950).

Table II shows leucocyte counts, chloride content, pH, resazurin class and standard plate counts respectively for an animal. The milk from this cow was normal in appearance on strip cup examination at all times. Normal leucocyte counts were from 60,000 per ml to 300,000 per ml before any material was introduced into the udder. The animal was free from bacterial infection. The cow was a grade Holstein, giving about 20 pounds of milk a day. Her butterfat test was 3.3.

After injection of: one gram neomycin in the right front quarter, 0.5gm in the left front, 0.25 gm in left rear and 10 ml of water in the right rear quarter which was the vehicle used for all infusions. The leucocyte counts seven hours after treatment were 15 million, 15 million, 10 million and 100 thousand respectively. Twenty-four hours later counts were still high, showing 10, 5, 3 millions and 100 thousand respectively.

TABLE I

Number of Micrograms of Neomycin Sulfate Required to Inhibit Mastitis-causing Organisms for 48 Hours *in vitro*

Test Organism	1st Trial	2nd Trial	3rd Trial
Str. agalactiae #15	0.02	0.02	0.039
Str. dyagalactiae #22	0.02	0.02	0.02
Str. uberis #29	0.01	0.01	0.01
Staph. aureus #26	0.04	0.04	0.04
E. coli #30	0.31	0.155	0.31
Ps. aeruginosa #33	1.25	1.25	1.25

By 96 hours the cell counts were nearly normal. At no time was there any gross abnormality of the milk.

During the period preceding injections the chloride content was less than .14 per cent. The samples collected seven hours after infusion showed a chloride content of over .20 per cent. There was a gradual return of the chloride content until a normal was reached after 96 hours.

The pH of the milk showed a pattern very similar to the chloride content in terms of hours before returning to normal.

The resazurin class of the milk returned to one after 72 hours.

The standard plate counts of the milk during the observation period varied from a low of zero to a high of 140 bacteria per ml. The logarithmic average would be 50 per ml. The counts on milk from treated quarters showed no bacteria at 24 and 48 hours and only 10 per ml at 72 hours.

There did not appear to be any regularity in the response to the various dosage levels in the different quarters.

On a second cow, Table III, 5 grams, 3 grams, 2 grams and 1 gram of neomycin in 10 ml of sterile distilled water were placed in the quarters of the udder. The 1 gram dose in the RR quarter was repeated 24 hours later. The milk from all quarters remained normal in appearance as viewed in a strip cup. When judged by the leucocyte count, pH, resazurin class, chloride content and standard plate count, the quarter receiving 5 grams was normal 104 hours later. The quarter receiving 3 gms was normal at 96 hours as was the one receiving 2 gms. The quarter receiving 1 gm and another gm 24 hours later was normal on the morning of the fourth day from the last treatment.

The preceding experiments indicate that no lasting harmful sequelae followed the infusion of amounts of neomycin ranging from 0.25 gm to 5 gms. In order to determine if an infected udder would respond, a cow was experimentally infected with the test organism, Str. agalactiae 15. In Table IV there is a complete summary of the status of this animal before infection, during infection and following treatments.

Six days after artificial infection, treatment was instituted by infusing 10 ml sterile water solutions containing 0.25 gm, 0.5 gm and 1 gm of neomycin into the RF, LF, and LR respectively and the 0.5 gm dose repeated 24 hours later in RR. Response was favorable in all the quarters. The favorable response in treatment was attributed to the fact that they were administered soon after infection took place and there was limited induration of the udder. Some retention data were also assembled from this series of treatments and indicated that neomycin remained in the milk in detectable amounts for 72 hours.

All quarters were negative at the next milking and remained so for the next 21 days. The irritation from the treatment was not as severe as the infection when judged on the basis of the leucocyte count, chloride content, pH and resazurin class.

III. RETENTION OF NEOMYCIN

Milk

Specific indications of concentration and persistence of neomycin in the milk of a normal healthy cow were obtained. The subject was in the

TABLE II

Irritation Determinations
(Cow H60)

Date	Leucocyte Counts RF	LF	LR	RR	Chloride Content RF	LF	LR	RR
3-6-50	100T	300T	300T	200T	.14	.14	.14	.14
3-7-50	200T	200T	300T	100T	.14	.14	.14	.14
3-8-50 9 A.M.	100T	100T	200T	100T	.14	.14	.14	.14
3-8-50 4 P.M.	60T	40T	100T	60T	.14	.14	.14	.14
3-9-50 9 A.M.	100T	200T	300T	200T	.14	.14	.14	.14
3-9-50 9:15 A.M.*	-	-	-	-	-	-	-	-
3-9-50 4 P.M.	15M**	15M**	10M**	100T**	.20	.20	.20	.15
3-10-50 9 A.M.	10M	5M	3M	100T	.20	.18	.18	.14
3-10-50 4 P.M.	2M	3M	1M	50T	.18	.19	.18	.14
3-11-50 9 A.M.	1M	1M	1M	100T	.16	.16	.16	.14
3-11-50 4 P.M.	1M	1M	1M	100T	.17	.16	.16	.14
3-12-50 10 A.M.	1M	1M	1M	100T	.16	.16	.16	.14
3-13-50 9 A.M.	1M	600T	1M	100T	.16	.15	.16	.14
3-13-50 4 P.M.	300T	300T	720T	200T	.14	.14	.14	.14
3-14-50 9 A.M.	400T	400T	300T	100T	.15	.15	.14	.14
3-14-50 4 P.M.	100T	100T	100T	60T	.14	.14	.14	.14
3-15-50 9 A.M.	200T	200T	300T	100T	.14	.14	.14	.14
3-15-50 4 P.M.	100T	100T	100T	100T	.15	.15	.14	.15
3-16-50 9 A.M.	200T	100T	200T	100T	.14	.14	.14	.15

* One gram RF, .5 gm LF, .25 gm LR in 10 ml of water for each and 10 ml water in RR.

** Samples collected before milking.

TABLE II A

Irritation Determinations
(Cow H60)

Date	pH				Resazurin Class			
	RF	LF	LR	RR	RF	LF	LR	RR
3-6-50	6.7	6.7	6.7	6.7	1	1	1	1
3-7-50	6.7	6.75	6.7	6.65	1	1	1	1
3-8-50 9 A.M.	6.7	6.7	6.7	6.7	1	1	1	1
3-8-50 4 P.M.	6.6	6.7	6.7	6.7				
3-9-50 9 A.M.	6.7	6.7	6.7	6.7	1	1	1	1
3-9-50 9:15 A.M.*								
3-9-50 4 P.M.	6.4	6.45	6.4	6.65	4	4	4	2
3-10-50 9 A.M.	6.4	6.5	6.5	6.7	4	4	4	1
3-10-50 4 P.M.	6.5	6.55	6.5	6.7				
3-11-50 9 A.M.	6.6	6.6	6.6	6.65	3	2	2	1
3-11-50 4 P.M.	6.6	6.65	6.65	6.7				
3-12-50 10 A.M.	6.6	6.7	6.7	6.7				
3-13-50 9 A.M.	6.7	6.65	6.7	6.65	2	1	1	1
3-13-50 4 P.M.	6.6	6.7	6.7	6.7				
3-14-50 9 A.M.	6.7	6.75	6.7	6.7	1	1	1	1
3-14-50 4 P.M.	6.7	6.7	6.7	6.7				
3-15-50 9 A.M.	6.75	6.75	6.75	6.75	1	1	1	1
3-15-50 4 P.M.	6.7	6.7	6.7	6.7				
3-16-50 9 A.M.	6.7	6.7	6.7	6.65				

* One gram RF, .5 gm LF, .25 gm LR in 10 ml of water for each and 10 ml water in RR.

** Samples collected before milking.

TABLE II B

Irritation Determinations
(Cow H60)

Date	Standard Plate Counts RF	LF	LR	RR
3-6-50	120	140	60	0
3-7-50	20	100	80	40
3-8-50 9 A.M.**	80	10	0	20
3-8-50 4 P.M.***				
3-9-50 9 A.M.	60	40	30	80
3-9-50 9:15 A.M.*				
3-9-50 4 P.M.	0	10	0	40
3-10-50 9 A.M.	0	0	0	60
3-10-50 4 P.M.				
3-11-50 9 A.M.	20	0	10	110
3-11-50 4 P.M.				
3-12-50 10 A.M.				
3-13-50 9 A.M.	10	30	20	40
3-13-50 4 P.M.				
3-14-50 9 A.M.	20	0	40	0
3-14-50 4 P.M.				
3-15-50 9 A.M.	60	70	20	80
3-15-50 4 P.M.				
3-16-50 9 A.M.				

* One gm RF, .5 gm LF, .25 gm LR in 10 ml of water for each and 10 ml water in RR.

** Samples collected after A.M. milking.

*** Samples collected before P.M. milking.

TABLE III

Irritation of the Udder with Neomycin
(Cow H70)

Date	Leucocyte Counts RF	LF	LR	RR	pH RF	LF	LR	RR
4-2-51 A.M.	200T	100T	60T	100T	6.7	6.7	6.65	6.7
4-2-51 P.M.	300T	200T	100T	100T	6.75	6.7	6.7	6.7
4-3-51 A.M.	200T	100T	100T	200T	6.7	6.7	6.7	6.7
4-3-51 P.M.	200T	50T	50T	100T	6.7	6.7	6.65	6.7
4-4-51 A.M.	100T	100T	75T	200T	6.7	6.7	6.7	6.7
4-6-51 A.M.	60T	100T	100T	100T	6.7	6.7	6.7	6.7
4-9-51 A.M.	100T	200T	100T	200T	6.7	6.65	6.7	6.7
Animal infused with neomycin after A.M. milking with 5 grams RF, 3 grams LF, 2 grams LR, 1 gram RR. Each was contained in a 10 ml solution. The 1 gram was repeated the next morning for RR.								
4-9-51 P.M.	20M	20M	20M	15M	6.6	6.6	6.65	6.6
4-10-51 A.M.	20M	20M	20M	20M	6.5	6.5	6.5	6.5
4-10-51 P.M.	15M	15M	15M	15M	6.4	6.4	6.4	6.5
4-11-51 A.M.	10M	10M	10M	10M	6.5	6.5	6.6	6.5
4-11-51 P.M.	10M	8M	8M	7M	6.6	6.6	6.6	6.6
4-12-51 A.M.	5M	5M	2M	1M	6.5	6.65	6.7	6.5
4-12-51 P.M.	1M	1M	500T	500T	6.7	6.7	6.7	6.7
4-13-51 A.M.	500T	200T	300T	400T	6.7	6.7	6.7	6.7
4-13-51 P.M.	200T	100T	60T	60T	6.75	6.7	6.7	6.7
4-14-51 A.M.	100T	200T	100T	100T	6.7	6.8	6.7	6.7
4-15-51 A.M.	200T	100T	60T	40T	6.7	6.7	6.7	6.7
4-16-51 A.M.	100T	200T	100T	100T	6.7	6.7	6.7	6.7

TABLE III A

Irritation of the Udder with Neomycin
(Cow H70)

Date	Resazurin Test				Chloride Content				Standard Plate Count			
	RF	LF	LR	RR	RF	LF	LR	RR	RF	LF	LR	RR
4-2-51 A.M.	1	1	1	1	.14	.14	.14	.14	80	20	40	100
4-2-51 P.M.	1	1	1	1	.14	.14	.14	.14				
4-3-51 A.M.	1	1.	1	1	.14	.14	.14	.14	60	90	70	80
4-3-51 P.M.	1	1	1	1	.14	.14	.15	.14				
4-4-51 A.M.	1	1	1	1	.14	.14	.14	.14				
4-6-51 A.M.	1	1	1	1	.14	.14	.14	.14	100	20	80	20
4-9-51 A.M.	1	1	1	1	.14	.14	.14	.14	40	60	30	80
Animal infused with neomycin after A.M. milking with 5 grams RF, 3 grams LF, 2 grams LR, 1 gram RR. Each was contained in a 10 ml solution. The 1 gram was repeated the next morning for RR.												
4-9-51 P.M.	4	4	4	4	.20	.20	.20	.20	0	0	0	0
4-10-51 A.M.	4	4	4	4	.20	.20	.20	.20	0	0	0	0
4-10-51 P.M.	4	4	4	4	.20	.20	.20	.20				
4-11-51 A.M.	4	4	4	4	.20	.20	.20	.20	0	10	0	10
4-11-51 P.M.	4	4	3	3	.20	.20	.19	.19				
4-12-51 A.M.	4	3	2	2	.20	.20	.18	.18	40	30	20	20
4-12-51 P.M.	2	2	1	2	.16	.15	.17	.16				
4-13-51 A.M.	2	1	1	1	.15	.14	.14	.14	20	60	80	40
4-13-51 P.M.	1	1	1	1	.15	.14	.14	.14				
4-14-51 A.M.	1	1	1	1	.14	.14	.14	.14	60	10	20	30
4-15-51 A.M.	1	1	1	1	.14	.14	.14	.14				
4-16-51 A.M.	1	1	1	1	.14	.14	.14	.14				

TABLE IV

Biological and Biochemical Response of Cow Experimentally Infected with Str. agalactiae #15 and Treated with Neomycin Sulfate
(Cow J-22)

Date	Direct Microscope				Leucocyte Counts				Chloride Content				pH				Resazurin Class			
	RF	LF	LR	RR	RF	LF	LR	RR	RF	LF	LR	RR	RF	LF	LR	RR	RF	LF	LR	RR
4-7-51	-	-	-	-	100T	300T	200T	100T	.14	.14	.14	.14	6.7	6.8	6.7	6.7	1	1	1	1
4-10-51	-	-	-	-	200T	400T	100T	100T	.14	.14	.14	.14	6.7	6.75	6.7	6.7	1	1	1	1
4-10-51	All quarters injected 2 ml of 24 hour broth culture of Str. agalactiae #15																			
4-11-51	+	+	+	+	20M	15M	12M	15M	.20	.20	.18	.20	6.65	6.7	6.7	6.7	4	4	4	4
4-13-51	+	+	+	+	15M	14M	15M	14M	.20	.20	.20	.20	6.7	6.75	6.8	6.8	4	4	4	4
4-16-51	+	+	+	+	10M	8M	10M	5M	.20	.19	.20	.20	6.8	6.7	6.8	6.85	4	4	4	4
4-16-51	.25 gm neomycin RF; .5 gm LF; 1 gm LR; .5 gm RR repeated in RR 4-17-51. All injections in 10 ml sterile distilled water.																			
4-17-51	*	*	*	*	15M	10M	10M	10M	.20	.20	.20	.19	6.8	6.8	6.8	6.8	4	4	4	4
4-18-51	*	*	*	*	12M	10M	12M	10M	.20	.20	.20	.20	6.8	6.85	6.8	6.8	4	4	4	4
4-19-51	*	*	*	*	10M	8M	8M	10M	.20	.20	.20	.20	6.8	6.8	6.7	6.8	4	4	4	4
4-20-51	o	o	o	*	7M	8M	10M	8M	.20	.20	.20	.20	6.8	6.8	6.8	6.8	4	4	4	4
4-21-51	o	o	o	o	1M	1M	1M	600T	.16	.15	.15	.14	6.8	6.7	6.7	6.6	2	1	1	1
4-23-51	-	-	-	-	400T	200T	100T	100T	.14	.15	.15	.14	6.75	6.7	6.7	6.6	1	1	1	1
4-24-51	-	-	-	-	200T	300T	200T	100T	.14	.14	.15	.14	6.7	6.7	6.7	6.6	1	1	1	1
5-7-51	-	-	-	-	60T	100T	100T	60T	.14	.14	.14	.14	6.7	6.65	6.7	6.6	1	1	1	1

* Antibiotics present
o Antibodies are absent

middle of her lactation period, giving approximately 15 pounds in the morning and 8 pounds in the evening. The quarters were infused with 1 gm of neomycin in 10 ml sterile distilled water in the RF; 0.5 gm in LF; 0.5 gm ointment in LR and 0.25 gm ointment in RR. Milk was collected and assayed at 7, 24, 32, 48, 55, 72 and 80 hours. The peak concentration was found at 7 hours and progressively decreased to 72 hours; when only a small zone of inhibition was evident from foremilk. At 80 hours there was a discernible ring of inhibition but it was not measurable. The 7 and 24 hour assays were made on complete milkings, the remainder only on foremilk samples. The results are summarized in Chart 5A.

A second cow, Table V B, was infused into the udder with 0.25 gm neomycin sulfate in 20 ml sterile distilled water in RF; 0.5 gm neomycin sulfate in 20 ml sterile distilled water in LF and 0.5 gm neomycin in 20 ml aluminum monostearate in LR. Samples of foremilk were collected at 1, 2, 4, 8, 8:10, 12, 24, 28, 32, 48 and 72 hours. A high concentration was evident 1 hour afterward. This was followed by a rapid decrease in neomycin concentration in both quarters that received the aqueous solutions. The quarter receiving the aluminum monostearate never did reach levels as high as the quarters that received the aqueous infusion. The length of time during which the drug was detectable was somewhat less in this quarter (48 hours), whereas, detectable amounts were seen in the other two quarters at 72 hours. It is rather curious that a detectable zone of inhibition appeared in the RR quarter at the 2 and 4 hour collections, since this quarter received no neomycin. The last few ml of milk at the 8 hour collection time had less antibiotic per ml than the

foremilk collected at the same time or the foremilk taken four hours later.

The udder of a third cow, represented in Table V C, was infused with the same amounts of neomycin sulfate dissolved in only half the volume of vehicle used in the previous experiments. The data in the table show that the first sample collected after treatment contained substantially greater quantities of neomycin. This can be explained by the fact that since dilutions were so high, more of the residual antibiotic in the teat canal was removed in taking the first samples. Subsequent samples showed nearly the same potency as those in the previous experiments. Levels were nearly as high and lasting when either volume was infused.

Table V D shows results from a S. agalactiae infected cow infused with 0.5 gm neomycin in 10 ml water. Retention was evident at 60 hours but not at 72. This cow was in a herd milked by machines, while all other animals were hand milked. The animal remained negative for S. agalactiae following this treatment.

From the amount of neomycin per ml required to inhibit the growth of S. agalactiae in the test tube and the results showing the concentration of neomycin in the udder at various time intervals, it seems that should a follow-up treatment be indicated the time to give it is after a 48 hour period.

Blood

A total of four cows received the antibiotic by intramuscular injection and their blood was assayed for neomycin at 1, 2, 3, 4, 5, 6, 7,

8, 9, 11 and 24 hours. Results are shown in Table VI. One animal (J25) was a Jersey cow weighing 800 pounds. This subject received two gms of neomycin sulfate in 10 ml of sterile distilled water injected into the gluteal muscle. A detectable level persisted for 8 hours. Another animal was a Holstein cow weighing 950 pounds, which was given 2 gms in 10 ml of aluminum monostearate by the same method of injection. The levels did not reach as high and did not remain any longer than in the cow injected with neomycin in an aqueous vehicle. Two additional animals (H75 and H80) received only 1 gm of neomycin, the former in water and the latter in aluminum monostearate. Lower levels of neomycin were attained in both instances and did not persist in the blood, for as many hours. One animal was killed one week later and no abnormal macroscopic findings were evident in the carcass at the site of the injection.

From these limited studies, it would appear that if systemic blood levels are desired for a prolonged period, the drug apparently must be injected at eight hour intervals when used in aqueous solvents. Milk was collected several times during these trials and did not show amounts of neomycin sufficient for detection.

TABLE V A

Assay of Neomycin in the Milk Expressed as Micrograms per ml
(Cow H22)

Amount of Drug and Vehicle	Intervals by Number of Hours							
	0	7*	24	32	48	55	72	80
1 gm neomycin 10 ml water RF	0	60	30	16	12	2	.8	+
.5 gm neomycin 10 ml water LF	0	50	25	10	3	.8	.4	+
.5 gm teatube neomycin ointment LR	0	60	30	10	3	.8	.4	+
.25 gm teatube neomycin ointment RR	0	40	30	6	3	.4	.4	+

TABLE V B
(Cow B-20)

Amount of Drug and Vehicle	Intervals by Number of Hours											
	0	1	2	4	8	8**	12	24	28	32	48	72
.25 gm neomycin 20 ml water RF	0	700	156	156	120	60	80	39	10	10	.3	.3
.5 gm neomycin 20 ml water LF	0	1000	312	156	156	60	120	156	20	20	5	.6
.5 gm aluminum monostearate 20 ml water LR	0	1620	60	20	39	10	20	2.5	2.5	.3	0	0
0 in RR	0	0	>.3	>.3	0	0	0	0	0	0	0	0

* Complete milking
** Last few ml of milk of the 8 hour milking

TABLE V C

Assay of Neomycin in the Milk Expressed as Micrograms Per Ml
(Cow C)

Amount of Drug and Vehicle	Intervals by Number of Hours									
	0	1	2	4	8	12	24	32	48	72
.25 gm neomycin 10 ml water RF	0	630	156	156	104	90	40	10	.3	.3
.5 gm neomycin 10 ml water LF	0	900	312	156	156	156	80	30	.10	.6
.5 gm neomycin 10 ml aluminum monostearate LR	0	1260	60	40	40	25	20	2.5	.3	>.3
.25 gm neomycin 10 ml aluminum monostearate RR	0	920	40	20	20	2.5	2.5	>.3	.0	.0

TABLE V D
(Cow A-61)

Amount of Drug and Vehicle	Intervals by Number of Hours					
	12	24	36	48	60	72
(A-61) cow + Strep in RR quarter						
RR quarter .5 gm neomycin 10 ml water	< 1024 units	64	32	4	.12	0
LR .25 gm neomycin 10 ml	512	16	.25	.12	0	0

D M was negative to strep for period following treatment.

TABLE VI

Blood Levels of Neomycin Expressed as Micrograms Per Ml Following Intramuscular Injections

Amount and Vehicle Cow Number and Body Weight	Time in Hours 0	1	2	3	4	5	6	7	8	9	11	24
2 gm neomycin 5 ml water J-25, 750 lbs.	0	12	15	7.7	3.4	2.2	1.4	1.2	1	>1	0	0
2 gm neomycin 5 ml aluminum monostearate H-95, 950 lbs.	0	7.9	8.1	4.2	4.2	2.6	1.2	1	1	>1	0	0
1 gm neomycin 5 ml aluminum monostearate H-75, 750 lbs.	0	6	7	6	3	1.8	1.5	>.5	>.5	>.5	0	0
1 gm neomycin 5 ml water H-80, 975 lbs.	0	6.5	7	10	8	6	2.8	.5	>.5	>.5	0	0

Micrograms of neomycin in the blood following intramuscular injections.

CLINICAL RESULTS AND DISCUSSION

CLINICAL RESULTS AND DISCUSSION

These studies on neomycin show that the drug was: (1) effective in in vitro studies against organisms causing mastitis, (2) innocuous to the udder as judged by irritation trials in healthy cattle, (3) successful in the treatment of experimentally infected animals, and (4) retained in the udder for 72 hours. With this information at hand, it seemed logical to extend these studies to cows in the field.

Throughout this work all preliminary diagnoses were based on direct microscopic examination of aseptically collected, and incubated milk samples. Retests were made at 7 to 10 days and 21 to 30 days. If an animal or quarter was positive seven days after the last treatment, the infected quarter of the udder was retreated at this time. If the quarter of the udder was negative, it was retested 14 to 20 days later and if still negative, considered cured. The word cured is used with qualifications. In chronic streptococcus infections, "cured" means that the cow was no longer shedding streptococci of five elements or more. In most instances the streptococcus infections treated were in the chronic stage. Some of the cows were not economically practical subjects for treatment as judged by a physical examination of the udder. However, in this study these were also included.

Some cases were not treated with a sufficient dose in the early phase of the treatment. Many chronic cases were cured by one or more treatments. Also a few cases did not respond to three treatments with neomycin but did

respond to other antibiotics. Conversely, some cases that were refractory to other antibiotics, responded to neomycin therapy.

In instituting therapy the ideal time to administer treatment is shortly after a complete milking. Cows were never treated unless a minimal time period of three hours could elapse before the next milking. Generally a 10 ml amount of sterile distilled water was used as a vehicle to facilitate placement of .5 gm of neomycin in each quarter. In treating chronic mastitis, all four quarters of the udder were infused. When a quarter was retreated, two half-gram doses, 36 or 48 hours apart, were given. Cows that did not respond to a series of three treatments were treated with other antibiotics or removed from the milking line, either for slaughter or to serve as nurse cows. Some quarters were dried up. A summary of results of treatments for various infections is provided in Table VIII.

A total of 261 cases of streptococcus mastitis were treated with aqueous neomycin sulfate. Two hundred and eight eventually were cured with neomycin. Of these, the first five were treated with only 0.25 gm of neomycin and none responded. All were later subsequently treated with 0.5 gm and they responded. Twenty-nine were treated on successive days with 0.25 gm, 24 of these treatments proved successful. Of the remaining 227 treated, all were given an initial treatment of 0.5 gm. One hundred and seventy-seven of these were negative at the 7 - 10 day test period and still negative at the 21 - 30 day test period. Of the remaining fifty, eight were treated with 0.5 gm at 7 to 10 days. Retest results showed cure in three cases. The five remaining cattle were retreated with 0.5 gm

on two successive days. Two of these responded favorably and three did not. These three were treated on successive days with 100,000 units of penicillin. One responded favorably and two did not. These two were treated with aureomycin and subsequently with tyrothrycin to no avail. They were finally given up as incurable. One animal was lactating in only two quarters; the others were lost, presumably, as the result of earlier infections. There was a great deal of induration evident in all quarters. The reason for this cow still being available was that she was a foundation cow of a herd and had produced some outstanding progeny. The second cow was just one of those cows which some farmers seem to keep as her milk seldom showed any abnormality.

The twenty-six cows treated for staphylococci infections are difficult to discuss except from the standpoint of individual case history. The generalizations that can be drawn are: All cows had shown an acute syndrome along with the presence of the organisms and a high cell count in the excretions from the udder. A half gram of neomycin was instilled into an affected quarter as early as possible. Confirmation of the type of infection was gained later. When the cows had an elevation of temperature, supportive treatment involving sulfamethazine orally or intravenously was utilized. When the cow was off feed ruminatorics were administered. When the cow remained dull and showed evidence of atony of the rumen for a couple of days, rumen liquor transfers were effected. In some instances penicillin was given intramuscularly. Of this group only three cows died. It is not known how many lost the function of the afflicted quarter but some did. There was also some impairment of udder

function in some cows that were considered cured. This cannot easily be measured.

The encouraging results in these cases of Staphylococci infections may have been due in part to early medication. Also, since staphylococci vary so greatly in pathogenicity, the possibility of a chance encounter with a high percentage of strains of low pathogenicity should not be overlooked.

In the course of these investigations, eighteen cases of coliform mastitis were encountered. Treatment consisted of infusion of 0.5 gm quantities of neomycin into each quarter on succeeding days until external evidence of acute inflammation had subsided. Animals were always febrile so supportive treatment with sulfamethazine or penicillin was given parenterally. When indicated, rumen stimulants were freely used. Cases were always treated prior to specific diagnosis and confirmations was made later from the samples collected at the time of treatment. The majority of the cases were treated early in the course of the infection. This may partially explain the excellent record of only one death in the entire series of coliform infected animals. Several cows were sold for salvage after recovery because they would be uneconomical producers. In many cases the return to normal pre-infection milk production and udder consistency was slow.

Fourteen cases with Pseudomonas infection were encountered. With one exception, these were in herds treated by the farmers with agents obtained from sources other than veterinarians. Animals infected with pseudomonas were given 0.5 gm of neomycin on two successive days. Milk

was usually thick and stringy at the time of treatment. This condition persisted for a considerable period of time following treatment. Eventually eight animals became useful producers again, and six were removed from the herds as incurable cases.

Neomycin was the most effective in treating this infection. The drugs used included penicillin, aureomycin, streptomycin, bacitracin, tyrothrycin and sulfa preparations.

During the course of this study only four cases of yeast infections were encountered. These were all in one herd of thirty Holsteins. The herdsman had noted the irregular appearance of flakes in his strip cup. Analysis of milk samples showed nothing significant except a possible contamination with yeast cells. The owner had previously treated all animals showing flakes in the milk. He had used penicillin but to no avail. Subsequently, carefully collected milk samples disclosed two cows to be shedding yeast cells. Treatment by infusion of 0.5 gm of neomycin corrected the condition permanently in these two cows. Later a diagnosis of yeast infection was made on two more cows in the same herd and thought to be successfully cured with the same treatment. The owner had changed from chlorine to quaternary ammonium compound for dairy sanitation prior to his difficulties. After going back to chlorine, following the last appearance of yeast cells in the milk, he has experienced no such difficulties.

On initial call, there have been 22 instances in which clinical cases of mastitis had been treated. Subsequent laboratory findings failed to show any known pathogenic organism. All such cases eventually recovered.

Sixty-five cases of mastitis were treated with a preparation of Teatube-Neomycin which is neomycin in a water-miscible base, furnished in a tube with attached canula.

The results of 40 cases of streptococcic mastitis showed that twenty responded to one treatment of 0.5 gm neomycin in 7.5 gms of vehicle. Fourteen others, receiving a series of treatments of 0.5 gm 48 hours apart, also recovered. Of the six that did not respond, three yielded to penicillin therapy and three did not.

Eight cases of staphylococcus infection were also treated with Teatube Neomycin. All animals survived and ceased to shed the organisms in the milk. However, three of these failed to milk from the involved quarter during that lactation period. It was not possible to observe whether this condition persisted in subsequent lactation periods.

Six cases of coliform infection were encountered. All were treated and survived. Udder secretions became negative for the organism. Four returned to nearly normal milk production during the lactation period. Two cows dried up in the affected quarter.

The eleven cows that turned out to have non-infectious mastitis eventually recovered.

Because of the problems inherent in clinical results, one must follow the case history of a few cows listed in the appendix in order to gain full appreciation of the meaning of the results.

TABLE VII

Summary of the 405 Quarters Treated with Neomycin Sulfate

Type of Infection	Number of Cows Treated	Number in Which Infection Overcome	Died	Remarks
Streptococci	261	208	0	
Staphylococci	26	23	3	
Coliform	18	17	1	
Pseudomonas	14	8	0	6 sold for beef
Noninfectious	22	22	0	
Yeast	4	4	0	
Total	345	282	4	

Summary of Cows Treated with Teatube Neomycin

Streptococci	40	34		
Staphylococci	8	8		3 lost function of quarter
Coliform	6	6		1 lost function of quarter
Noninfectious	11	11		
Total	65	59		

SUMMARY

SUMMARY

Neomycin *in vitro* at very low levels inhibited the growth of the six common causative microorganisms associated with infectious bovine mastitis.

The results of this study with neomycin indicate a minimum of local irritation in the udder as judged by leucocyte counts, chloride content, resazurin class, pH and strip cup observation.

The agent was found to remain in the udder for 80 hours or longer when an aqueous solution was infused.

Neomycin when injected intramuscularly was found to give demonstrable blood levels for at least 8 hours.

The results in treating 405 cases of mastitis indicate this drug to be very effective against infections caused by streptococci, staphylococci, coliforms and a yeast. Neomycin was also of value in eight of fourteen pseudomonas infections.

There appeared to be no difference in the effectiveness of neomycin when employed in either an aqueous or water-miscible base.

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APPENDIX

Selected Individual Mastitis Cases Treated With Neomycin Sulfate

Date	Cow	Diagnosis	History	Treatment	Remarks & Results
3-2-50	C-302	Strept.	Monthly check on college herd	.25 gm neomycin sulfate 10 ml sterile distilled water each quarter	*plus on 3-7-50
3-2-50	C-331	Strept.	Monthly check	.25 gm neomycin sulfate each quarter	plus on 3-7-50
3-2-50	C-73	Strept.	Monthly check	.25 gm neomycin sulfate each quarter	plus on 3-7-50
3-7-50	C-302	Strept.	Still plus from previous treatment	.25 gm 2-8-50 .25 gm 2-9-50	+ Neg. 3-17-50 and at 4-4-50
3-7-50	C-331	Strept.	Still plus from previous treatment	.25 gm neomycin 3-8-50 .25 gm neomycin 3-9-50	Neg. 3-17-50 and 4-4-50
3-7-50	C-73	Strept.	Still plus from previous treatment	.25 gm neomycin 3-8-50 .25 gm neomycin 3-9-50	Neg. 3-17-50 and 4-4-50
3-11-50	C-350	Coliform RF	RF quarter, watering, swollen, Temp. 103.6 came up from 5:30 A. M.-8:00 A.M.	gave .5 gm neomycin sulfate RF at 8:30 A.M.	Neg. sample 3-12-50 milk normal, quarter returning normal Temp. 101.2, eating
3-14-50	C-350	Coliform LF	3-14-50 - LF quarter swollen, garget Temp. 104.0, not eating	Gave .5 gm neomycin LF .25 gm LR, RR, RF - discovered at 8:30 A.M. treated promptly. Cow was eating at 4:30 gave .25 gm at 4:30 each quarter	3-15-50 - milk grossly abnormal all quarters, but neg. on test, blood & mic. Temp. 101.6 was neg. at next monthly test 4-4-50

* Plus animal shedding organism
Neg. animal not shedding organism

Continued next page

Date	Cow	Diagnosis	History	Treatment	Remarks & Results
3-11-50	285	Strept. LR	Flakes on strip cup	Gave .5 gm on 3-12-50 neomycin	plus on 3-16-50
3-17-50	285	Strept. LR	Flakes gone	Gave .25 gm 3-17-50 3-18-50	plus on 3-27-50
3-17-50	EA-37	Strept.	Monthly check	.5 gm neomycin each quarter	Neg. on 3-24-50 also 4-11-50
4-17-50	C-75	Staph.	Temp. 105.4, off feed, RR quarter swollen, hard	Gave .25 gm neomycin LR, LF, RF, gave .5 gm RR, gave 1000 gr sulmet 4-17-50 orally 5-17-50, gave RR .5 gm neomycin 5-18-50	Was neg. 4-18-50, 4-19-50, 4-27-50, 5-3-50 - cow returned to good production
5-3-50	C-169	Strept.	Monthly check	Gave .25 gm 5-4-50 Gave .25 gm 5-5-50	Plus on 5-13-50
5-14-50	C-169	Strept.	Still positive, previous treatment	Gave .25 gm 5-14-50 Gave .25 gm 5-15-50	Neg. on 5-15-50, 5-19-50, 5-26-50, 6-6-50
5-1-50	BK-20	Strept.	Monthly check	Gave .25 gm 5-2-50 Gave .25 gm 5-3-50	Plus on 5-8-50
5-9-50	BK-20	Strept.	Still plus after previous treatment	Gave .5 gm 5-9-50	Neg. on 5-15-50, 5-26-50 and 6-6-50
5-9-50	C-358	Staph.	Garget, Temp. 104.5	Gave .25 gm neomycin all quarters on 5-9-50, 5-10-50 infusion udder. Gave 1250 gr sulmet 5-9-50 orally	Neg. on 5-19-50, 6-6-50 cow returned to fair production

Continued next page

Date	Cow	Diagnosis	History	Treatment	Remarks & Results
6-9-50	5757	Strept.	Herd survey - RF neg. LF plus, LR plus, RR plus	.5 gm neomycin 6-12-50 all quarters	6-19-50 RF neg, LF plus, LR plus, RR neg.
6-9-50	5855	Strept.	Herd survey RF plus, LF neg, LR neg, RR neg.	.5 gm neomycin 6-12-50 all quarters	6-19-50 RF LF LR RR neg. neg. neg. neg.
6-9-50	5901	Strept.	Herd survey - RF plus, LF neg, LR neg, RR neg.	.5 gm neomycin 6-12-50 all quarters	neg. neg. plus neg.
6-9-50	5633	Strept.	Herd survey - RF plus, LF plus, LR plus, RR plus	.5 gm neomycin 6-12-50 all quarters	plus plus plus neg.
6-9-50	5682	Strept.	Herd survey - RF plus, LF neg, LR plus, RR plus	.5 gm neomycin 6-12-50 all quarters	plus plus plus neg.
6-9-50	5681	Strept.	Herd survey - RF plus, LF 0, LR plus, RR plus	.5 gm neomycin 6-12-50 all quarters Chronic marked induration	plus 0 plus neg.
6-9-50	5801	Strept.	Herd survey - RF plus, LF plus, LR plus, RR plus	.5 gm neomycin 6-12-50 all quarters Chronic marked induration	neg. plus plus plus
6-20-50	5757	See prev. under Remarks	Continued treatment	.5 gm neomycin, all quarters	6-30-50: neg. neg. plus neg. 7-13-50: neg. neg. neg. neg.

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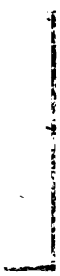

Date	Cow	Diagnosis	History	Treatment	Remarks & Results
6-20-50	5901	See prev. under Remarks	Continued treatment	.5 gm neomycin, all quarters	6-30-50 RF LF LR RR neg. neg. neg. neg. 7-13-50 neg. neg. neg. neg.
6-20-50	5633	See prev. under Remarks	Continued treatment	.5 gm neomycin, all quarters	6-30-50 plus neg. neg. neg. 7-13-50 neg. neg. neg. neg.
6-20-50	5682	See prev. under Remarks	Continued treatment	.5 gm neomycin, all quarters	6-30-50 plus neg. plus neg. 7-13-50 plus neg. plus neg.
6-20-50	5681	See Prev. under Remarks	Continued treatment	.5 gm neomycin, all quarters	6-30-50 plus 0 neg. neg. 7-13-50 plus 0 neg. neg.
6-20-50	5801	See Prev. under Remarks	Continued treatment	.5 gm neomycin, all quarters	6-30-50 neg. plus plus plus 7-13-50 neg. plus plus plus
			7-3-50: Treated all plus quarters 1. gm neomycin		
6-21-50	C-522	Pseudo-monas	Garget, RF quarter monthly check	.5 gm neomycin	Neg. 7-28-50 and 9-12-50
6-21-50	C-459	Pseudo-monas and Strept.	RR flakes	.5 gm neomycin	Neg. 7-28-50 and 9-12-50

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Date	Cow	Diagnosis	History	Treatment	Remarks & Results
6-21-50	C-46	non-infest mastitis	Flakes on strip cup	.5 gm neomycin	Flakes disappeared
6-21-50	C-230	non-infest mastitis	Flakes on strip cup	.5 gm neomycin	Flakes disappeared
7-24-50	Goldy Lox	plus strept	Monthly check	.5 gm neomycin all quarters	Neg. 8-1-50 and 9-14-50
7-24-50	Red Rose	plus strept	Monthly check	.5 gm neomycin all quarters	Neg. 8-1-50 and 9-14-50
7-25-50	C-459	plus strept	Monthly check	.5 gm neomycin all quarters	Neg. 8-1-50 and 9-13-50
7-25-50	C-73	plus rod	Monthly check - Flakes on strip cup	.5 gm neomycin all quarters	Neg. 8-1-50 and 9-13-50
7-25-50	C-331	plus Strept	Monthly check	.5 gm neomycin all quarters	Neg. 8-1-50 and 9-13-50
9-13-50	C-451	Strept (1)*	Monthly check	.5 gm neomycin 9-13-50 9-15-50	Neg. 9-25-50 10-12-50
9-13-50	C-295	Strept (1)*	Monthly check	.5 gm neomycin 9-13-50 9-15-50	Neg. 9-25-50 10-12-50
9-13-50	C-424	Strept (1)*	Monthly check	.5 gm neomycin 9-15-50	Neg. 9-25-50 10-12-50
9-13-50	C-302	Strept (2)*	Monthly check	.5 gm neomycin 9-15-50	Neg. 9-25-50 10-12-50

* Number of quarters positive

Continued next page

Date	Cow	Diagnosis	History	Treatment	Remarks & Results
9-13-50	C-335	Strept (1)*	Monthly check	.5 gm neomycin 9-15-50	Neg. 9-25-50
9-13-50	C-367	Strept (1)*	Monthly check	.5 gm neomycin 9-15-50	10-12-50
9-13-50	C-169	Strept (2)*	Monthly check	.5 gm neomycin 9-13-50 9-15-50	Plus 9-25-50 and Neg. 10-12-50
9-14-50	C-169	Strept (1)*	Still plus one quarter from prev. treatment	.5 gm neomycin 9-26-50 9-27-50	Neg. 10-12-50
9-14-50	Gem	Plus Strept LR, RR	Monthly check	.5 gm neomycin 9-15-50 9-16-50	Neg. 9-22-50 and 10-19-50
9-14-50	Spotty	Plus Strept RR	Monthly check	.5 gm neomycin 9-15-50 9-16-50	9-22-50 - sold before 10-19-50
9-14-50	Bell	Plus Strept LR	Monthly check	.5 gm neomycin 9-15-50 9-16-50	Neg. 9-22-50 10-19-50
9-14-50	Susan	Plus Strept	Monthly check	.5 gm neomycin 9-15-50 9-16-50	Neg. 9-22-50 10-19-50
9-14-50	Penny	Plus Strept	Monthly check	.5 gm neomycin 9-15-50 9-16-50	Neg. 10-19-50
9-14-50	Queen	Plus Strept	Monthly check	.5 gm neomycin 9-15-50	Neg. 9-22-50 10-19-50
9-14-50	Olivet	Plus Strept	Monthly check	.5 gm neomycin 9-16-50	Neg. 9-22-50 10-19-50

* Number of quarters positive

Concluded next page

Date	Cow	Diagnosis	History	Treatment	Remarks & Results
10-30-50	C-508	Strept	Temp 106.8 RR quarter swollen, milk yellow serumy	.5 gm neomycin 10 ml sterile distilled water infusion into udder, 1250 grains sulmet orally. Repeated .5 gm neomycin 11-31-50	Temp 102.0 at 4:30 (8 hrs. later) Cow was negative to direct mic. and blood agar 11-8-50 also 11-20-50
10-31-50	EA-62	Strept	Monthly check	.5 gm neomycin 11-1-50	11-9-50 neg.