Selective Isolation of Leptospiras from Contaminated Material by Incorporation of Neomycin to Culture Media

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Incorporation of neomycin to the culture medium was found to be effective in inhibiting Escherichia coli contaminants without interfering with the growth of serotype L. autumnalis. The growth of 12 other Leptospira serotypes was unaffected by the addition of 300 μg of neomycin per ml to Ellinghausen medium or 5 μg/ml to Fletcher medium. Neomycin-containing medium was found to be of value in the isolation of leptospiras from cultures of blood from infected laboratory animals. A higher percentage of isolates was obtained in swine kidneys from an abattoir in medium containing neomycin than resulted from the same medium without antibiotic or with 5-fluorouracil. Contaminated leptospiiral cultures growing in media with 5-fluorouracil were purified by subculturing into neomycin-containing media.

A major problem in the isolation of leptospiras from tissue and body fluids of man and animals or contaminated cultures is the concomitant presence of other microorganisms in the inoculum (2, 3, 8). The methods routinely used in attempting to obtain pure leptospiiral cultures from these materials include inoculations of laboratory animals, filtration, and inoculations of culture tubes with serial dilutions of the sample (1, 8). Relatively few reports, however, deal with the search for antimicrobial agents having no adverse effect on leptospiras in vitro and which could be added to culture media for their selective isolation.

Stuart (12) reported inhibition of contaminating organisms in leptospiiral cultures by using low concentrations of sulfadiazine and sulfaguanidine. Dominguez (5) added dihydrostreptomycin at concentrations to 100 to 200 mg/ml or chloramphenicol at 200 to 500 mg/ml to culture media to suppress contaminants. The leptospiras were then isolated by subculturing, before the lethal action of the antibiotics on the leptospiras was expressed. The pyrimidine analogue, 5-fluorouracil, was also found to control the growth of contaminants while not being toxic to leptospiiral organisms (10) and has been used in many laboratories where available. This antimicrobial agent, however, is not effective for all bacterial species and may have some growth-inhibiting action (1, 14).

Cousineau and McKiel (4) evaluated the in vitro sensitivity of L. icterohaemorrhagiae to 14 antimicrobial agents. On the basis of their observations, they suggested the addition of 50 mg of sulfathiazole, 5 mg of neomycin sulfate, and 0.5 mg of actidione per liter to standard media for the selective isolation of leptospiras.

Nevertheless, the use of neomycin or other antibiotics has not been widely used for this purpose and it was therefore deemed worthwhile to re-examine the usefulness of neomycin. In the present study, an evaluation was made of the in vitro effectiveness of neomycin in the isolation of leptospiras from contaminated cultures or from clinical material. Neomycin was selected for this study because of its wide antibiotic spectrum to both gram-positive and gram-negative organisms and its combined bacteriostatic and bactericidal action (13).

MATERIALS AND METHODS

Cultures. The 13 Leptospira serotypes selected to study were L. autumnalis strain Akiyami A, L. tarassovi strain Perepelinc, L. pomona strain Pomona, L. australis strain Ballico, L. ballum strain Castellon 3, L. grippotyphosa strain Moskva V, L. hebdomadis strain Hebdomadis, L. batavae strain Van Tienen, L. canicola strain Hond Utrecht IV, L. pyrogenes strain Salinem, L. icteroides strain RGA, L. sejroe strain M 84, and L. patoc strain Patoc 1. All of these serotypes were maintained in Fletcher (Difco) semisolide medium (7), Stuart (Difco) liquid medium
(12) containing 10% pooled rabbit serum and in
semisolid and liquid modified Ellinghausen medium
(6, 10) containing bovine fraction V (Pentex). Subcul-
tures were made at 3-month intervals if the organisms
were growing in semisolid media and weekly if in
liquid media.

Antimicrobial agents. The evaluation of neomy-
cin sulfate was performed by using a dry powder
(Squibb, control no. 1G167568) with a potency of 586
µg/mg. The concentrations used were calculated from
the potency of the sulfate. The dry powder was diluted
with phosphate-buffered distilled water (pH 7.2),
Seitz filter-sterilized, and either incorporated directly
into test media or stored at -25 C until ready for use.
The pyrimidine analogue, 5-fluorouracil, used for
comparison was kindly supplied as a dry powder by
Hoffman-La Roche (Nutley, N.J.) and stored at 4 C.
The dry powder was diluted with the buffered dis-
tilled water, filter-sterilized, and used as described by
Johnson and Rogers (10). The stock solution was
stored at -25 C.

Effect of neomycin on serotype autumnalis
growth. In determining the number of viable leptos-
pires present in cultures of L. autumnalis containing
various concentrations of neomycin, the tube dilution
method (11) was used. An actively growing 4-day-old
culture of L. autumnalis organisms in Ellinghausen
liquid medium was diluted 1:20 with the same
medium. A 9.5-ml amount of the diluted leptospiral
suspension was dispensed into sterile culture tubes,
and 0.5 ml of stock solutions of neomycin was added to
produce final concentrations of 500, 200, and 100
µg/ml. One tube containing 9.5 ml of the culture
dilution plus 0.5 ml of antibiotic diluent was used as a
control. The cultures were incubated at 30 C and
titered at 1, 5, 7, and 9 days later. Titrations were
made by removing 0.5 ml of the cultures and prepar-
ing replicate decimal dilutions in tubes containing 4.5
ml of Ellinghausen semisolid media. Estimates on
growth were based upon the highest dilution showing
leptospires microscopically after 14 days of incuba-
tion.

Effect of neomycin on 13 leptospiral serotypes.
The effect of neomycin on the growth of the 13
leptospiral serotypes was studied in both Elling-
hausen and Fletcher semisolid media with and with-
out neomycin or 5-fluorouracil. In these tests, two
tubes, containing 5.0 ml of either media having a final
concentration of the antibiotic ranging from 5 to 500
µg/ml, were inoculated with 0.1 ml of a 3-day-old
culture of each serotype. Tubes of antibiotic-free
culture media were used as controls. Evidence of
unrestricted growth was determined by comparing
their ability to produce the characteristic Dinger
zones within 1 week after incubation at 30 C.

Inhibition of contaminants from leptospiral cul-
tures. Ten Escherichia coli strains recently isolated
from bovine fecal material, maintained on Brucella
(Albimi) agar, were used to determine the optimal
neomycin concentration inhibitory to these organisms
without adversely affecting leptospiral growth. E. coli
was selected for study rather than an array of common
contaminating bacteria because if was more resistant
to the action of neomycin than other Enterobacteri-
aceae (13), and was frequently associated with
contaminated clinical material.

Culture tubes containing 5.0 ml of Ellinghausen
and Fletcher semisolid media were inoculated with
0.1 ml of a 3-day-old L. autumnalis strain. Ten
18-h-old E. coli cultures were suspended in Elling-
hausen liquid medium, and viable numbers were
determined by plate counts. Approximately 3 x 10⁴ E.
coli organisms were used as inocula to contaminate
the tubes which contained neomycin in final concen-
trations ranging from 3.12 to 500 µg/ml. All cultures
were incubated at 30 C and examined daily for E. coli
for 1 week. After incubation for 7 days, a transfer
was made into fresh tubes of Fletcher and Ellinghausen
semisolid media without antibiotic to establish the
ability of the leptospires to multiply and the bacteri-
cidal action of neomycin on the E. coli strains.

In addition, Staphylococcus aureus was used as a
sensitive indicator strain to determine the relative
potency of neomycin in diverse media. A culture of S.
aureus was isolated from a human and maintained in
the same medium as the E. coli cultures. In these tests
the sensitivity of this organism to the antibiotic was
determined in the various leptospiral media, Brucella
(Albimi) broth with and without bovine fraction V
and Tween 80 (at the concentrations used in Elling-
hausen medium). Twofold serial dilutions of neomy-
cin were made in each of the media ranging from 500
to 1.9 µg/ml, inoculated with 3 x 10⁴ viable S. aureus
organisms, and incubated at 37 C for 24 h. Culture
tubes were then examined for turbidity as evidence of
growth.

Laboratory animal isolations. The suitability of
neomycin for the isolation of leptospires from the
blood of experimentally infected hamsters and its
usefulness in the isolation of reduced numbers of these
organisms was evaluated. Eight 21-day-old hamsters
were inoculated intraperitoneally with 0.5 ml of a
3-day-old culture of L. tarassovi contaminated with 3
x 10⁴ E. coli organisms. Blood was obtained 24 h later
by cardiac puncture as described by Galton et al. (8).
One drop of heparinized blood was cultured in three
tubes of Ellinghausen semisolid medium without
antibiotic; with 5-fluorouracil at 200 µg/ml; and with
neomycin having a final concentration of 300 µg/ml.
Culture tubes were incubated at 30 C and examined
weekly by dark-field examination to detect lepto-
spires.

Leptospiral isolation from abattoir material.
The potential value of neomycin for the isolation of
leptospires from cultures of clinical material was
evaluated by using swine kidneys collected from a
local abattoir. A high percentage of swine kidneys
from this abattoir had previously been found to
contain leptospires.

Isolations were performed as described by Galton et
al. (8) with several variations. The entire kidney was
first immersed for 10 min in a 1:10,000 solution of
Hyamine (Rohm & Haas Co.) to disinfect the exterior
surface and was dried with a sterile towel. The kidney
was then cut longitudinally exposing the cortex, me-
dulla, and renal hilus with a sterile knife. A suspension
was obtained from the inner exposed surface of the
kidney by scraping with a sterile metal Coca-
Cola soda bottle cap which had previously been
punched with holes to act as a scraper. The suspen-
sion was prepared with sterile Sorensen phosphate-buffered saline (pH 7.2) in 10⁻¹ to 10⁻⁴ dilutions, and 0.5 ml of each dilution was used to inoculate triplicate tubes of Fletcher semisolid media. This medium was chosen since previous experience with swine kidneys using both Fletcher and Ellinghausen semisolid media resulted in a higher number of isolations in the former medium.

Stock solutions of neomycin and 5-fluorouracil were added to one culture tube of each dilution to provide final concentrations of 25 and 200 μg/ml of the antibiotics, respectively. In addition, one culture tube of each of the dilutions was used without antimicrobial agents. Cultures were incubated at 30 C and examined weekly for 6 weeks by dark-field microscopy to detect the presence of leptospirosis. When growth was detected, successive transfers were made in Stuart liquid medium until a growth satisfactory for use as antigen was obtained. Each leptospiral isolate was identified by testing it against a battery of hyperimmune rabbit antisera to the above-mentioned leptospiral serotypes, using the technique described by Galton et al. (8).

RESULTS

Results on titrations of viable L. autumnalis organisms growing in liquid Ellinghausen media containing 100, 200, and 500 μg of neomycin per ml are shown in Fig. 1. The log titer (base 10) was in proportion to the concentration of neomycin when compared to that of the antibiotic-free culture. The viable titer in the 5th day of the culture containing 500 μg of neomycin per ml increased 4 log higher than the control. By the 9th day both the culture without antibiotic and that with 500 μg of neomycin per ml had reached a plateau with a 3-log difference. To rule out nonspecific effects, such as pH or variations in media constituents, the same lot of medium was used for all titrations in this experiment. An illustration of the variations in leptospiral growth at different antibiotic concentrations in Ellinghausen semisolid medium is shown in Fig. 2.

The growth of 13 leptospiral serotypes in neomycin-containing semisolid Ellinghausen and Fletcher media and in Fletcher medium with 5-fluorouracil is shown in Table 1. Addition of neomycin at levels of 5 and 300 μg/ml to Fletcher and Ellinghausen media, respectively, and 200 μg of 5-fluorouracil per ml to Fletcher medium resulted in the unrestricted growth of all leptospiral serotypes. Increasing the neomycin concentration of Fletcher and Ellinghausen media to 10 and 500 μg/ml, respectively, inhibited the growth of some stock leptospiral serotypes. At 25 μg/ml, no leptospiral growth was detected in cultures from the collection growing in Fletcher medium. The antigenic identity of the serotypes remained unaltered by this treatment.

The marked differences observed in the sensitivity of leptospirosis to neomycin when cultured in Fletcher and Ellinghausen media suggested a neutralizing effect by the constituents of the latter medium. The neomycin sensitivity of Staphylococcus aureus grown in different leptospiral media and in Brucella (Albimi) broth was used as a model to investigate this possibility. Between 62.5 and 125 μg of the antibiotic per ml were required for inhibition of S. aureus when the cultures were grown in Ellinghausen semisolid or liquid media (Table 2). When the organisms were grown in Fletcher, Stuart, or Brucella (Albimi) media, however, neomycin concentrations between 7.8 and 15.6 μg/ml were equally effective in suppressing their growth.

The requirement for a higher neomycin concentration in Ellinghausen media for S. aureus inhibition suggested that a neutralizing action on the antibiotic by some of the components of the medium was involved. Table 2 shows that the same neomycin concentration was required to inhibit S. aureus in Brucella (Albimi) broth containing bovine fraction V (at a concentration equivalent to that in Ellinghausen medium) as was necessary when the cultures were grown in Ellinghausen medium. Addition of Tween 80 to Brucella (Albimi) broth had no inhibitory effect on the action of the antibiotic.

The 10 E. coli cultures which were used to determine the neomycin concentration inhibitory to these organisms without affecting the growth of L. autumnalis in Ellinghausen or Fletcher semisolid media showed that a final antibiotic concentration of 100 μg/ml to Ellinghausen semisolid medium was effective in inhibiting the growth of 4 of 10 strains of E. coli. At antibiotic concentrations of 200 to 500 μg/ml, no growth of E. coli was detected. By contrast, 7 of the 10 strains were inhibited in Fletcher
semisolid medium containing a neomycin concentration of 12.5 µg/ml, and no growth of E. coli resulted in the cultures containing from 25 to 100 µg of the antibiotic per ml.

In all E. coli-negative cultures grown in Ellinghausen medium and in the 7 E. coli-negative cultures grown in Fletcher medium containing 12.5 µg of neomycin per ml, the leptospiral ring growth was evident at 96 h. No growth of E. coli was detected 7 days after subculture into antibiotic-free media. Visual observation as evident by the turbidity of the leptospiral cultures growing in the Ellinghausen medium with neomycin revealed an increased growth of leptospires with increasing concentrations of the antibiotic.

Table 1. Growth of 13 leptospiral serotypes in neomycin-containing Fletcher and Ellinghausen semisolid media and in Fletcher semisolid media with 5-fluorouracil

| Leptospiral serotypes          | Growth on Ellinghausen medium with neomycin | Growth on Fletcher medium with neomycin | Growth on Fletcher medium with 5-fluorouracil |
|-------------------------------|---------------------------------------------|----------------------------------------|----------------------------------------------|
|                               | 500*                                        | 300                                    | 25                                           |
|                               |                                             |                                        | 10                                           |
|                               |                                             |                                        | 5                                            |
|                               |                                             |                                        | 200                                          |
| *Leptospira pomona* Pomona    | −                                           | +*                                     | −                                            |
| *L. australis* Ballico        | +                                           | +                                      | −                                            |
| *L. ballum* Castellon 3       | +                                           | +                                      | −                                            |
| *L. grippotyphosa* Moskva V   | −                                           | +                                      | −                                            |
| *L. tarassovi* Perepelicin    | +                                           | +                                      | −                                            |
| *L. hebdomadis* Hebdomadis    | −                                           | +                                      | −                                            |
| *L. bataujae* Van Tienen      | −                                           | +                                      | −                                            |
| *L. canicola* Hond Utrecht IV | −                                           | +                                      | −                                            |
| *L. pyrogenes* Salinem        | −                                           | +                                      | −                                            |
| *L. icteroides* RGA           | +                                           | +                                      | −                                            |
| *L. sejroe* M 84              | −                                           | +                                      | −                                            |
| *L. autumnalis* Akiyami A     | +                                           | +                                      | −                                            |
| *L. paso* Pactoc 1            | −                                           | +                                      | −                                            |

* Final antibiotic concentration (micrograms per milliliter).
* Indicates the formation of Dinger zones in the cultures within 1 week of incubation at 30 C.

Table 2. Effect of the culture medium on the antimicrobial action of neomycin on a strain of Staphylococcus aureus

| Medium                           | Growth on medium with final neomycin concn (µg/ml) |
|----------------------------------|--------------------------------------------------|
|                                  | 500  | 250  | 125  | 62.5 | 31.2 | 15.6 | 7.8  | 3.9  | 1.9  |
| Fletcher semisolid               | 0    | 0    | 0    | 0    | 0    | +a   | +    | +    | +    |
| Stuart liquid medium             | 0    | 0    | 0    | 0    | 0    | +    | +    | +    | +    |
| Ellinghausen semisolid           | 0    | 0    | 0    | +    | +    | +    | +    | +    | +    |
| Ellinghausen liquid medium       | 0    | 0    | 0    | +    | +    | +    | +    | +    | +    |
| *Brucella* (Albimi) broth        | 0    | 0    | 0    | 0    | 0    | +    | +    | +    | +    |
| Albimi broth and 1% bovine fraction V | 0    | 0    | 0    | +    | +    | +    | +    | +    | +    |
| Albimi broth and 1.25% Tween 80  | 0    | 0    | 0    | 0    | 0    | +    | +    | +    | +    |
| Albimi broth and 1% bovine fraction V and 1.25% Tween 80 | 0    | 0    | 0    | +    | +    | +    | +    | +    | +    |

*a Growth at 24 h of culture.
The eight hamsters experimentally infected with *L. tarassovi* were used to evaluate the use of neomycin in blood culture isolations of leptospiras. Both 300 µg of neomycin per ml and 200 µg of 5-fluorouracil per ml were equally effective in the isolation of leptospires. In these cultures and in Ellinghausen semisolid medium without either antibiotic, leptospires were demonstrated 10 days after culture from the same animals in cultures of one drop of blood taken 24 h after infection. All cultures were free of *E. coli*, and the lepospiral ring growth was detected only in media containing neomycin.

The comparative value of neomycin-containing media in the isolation of leptospiral organisms from swine material is shown in Table 3. The smallest number of contaminated cultures and the largest number of leptospiral isolations and positive culture tubes were obtained with Fletcher semisolid medium containing 25 µg of neomycin per ml. The largest number of isolates was detected at the first of the weekly microscopic examinations of cultures in neomycin-containing media. The 12 leptospiral isolates were identified as 10 of the cultures being *L. tarassovi* serotypes and 2 cultures (pig no. 8 and 9) as serotype *L. pomona*. Although leptospires were not isolated in neomycin-containing media from three kidneys, the use of Fletcher semisolid medium at a final concentration of 25 µg/ml increased the chances of recovering leptospiros from the contaminated specimens.

Subculture from a contaminated *L. tarassovi* isolate (pig no. 20) growing in Fletcher medium with 5-fluorouracil into Fletcher semisolid medium with 25 µg of neomycin per ml yielded a pure culture. When the subculture was performed in the former medium, the growth of contaminants was not inhibited. Contaminants comprised both gram-positive bacilli and streptococci.

As shown in Table 1, the *L. tarassovi* and *L. pomona* serotypes from the culture collection were inhibited in Fletcher semisolid media containing 25 µg of neomycin per ml, whereas, with the relatively smaller number of leptospires in the 10⁻² dilution of kidney tissue, there was unrestricted growth of these same strains in the same medium. These results suggest that a differential growth requirement for newly isolated strains and for those maintained in stock cultures may be involved.

**DISCUSSION**

The data reported here demonstrate that the addition of neomycin sulfate to leptospiroplas media aided in the control of contaminants. The use of other antibiotics for the selective culture of leptospiros (5) required rapid subculture into antibiotic-free media to prevent the expression of toxic effects on the lepospiral organisms. Adding the pyrimidine analogue, 5-fluorouracil, has several limitations. It is ineffective in large amounts with grossly contaminated material (10) and in some countries it is not easily obtained. Furthermore, in our experience, cultures purified with this agent must be subcultured several times in 5-fluorouracil-containing media before the cultures are cleared of contaminants.

Neomycin, although like 5-fluorouracil in that it is not effective against all species of bacterial contaminants and may adversely affect the growth of some leptospiroplas serotypes in high concentrations, is both bacteriostatic and bactericidal (13). This wide spectrum antibiotic is inexpensive and readily available. A contaminated lepospiral culture grown in 5-fluorouracil-containing media was successfully cleared of contaminants when subcultured into media with neomycin. Cultures of *E. coli* inhibited by neomycin did not grow when subcul-

| Pig no. | No antibiotic* | 5-Fluorouracil, final concn 200 µg/ml | Neomycin sulfate, final concn 25 µg/ml* |
|--------|---------------|---------------------------------|--------------------------------------|
| 1      | C             | C                              | C                                   |
| 2      | C             | C                              | C                                   |
| 3      | C             | C                              | C                                   |
| 4      | C             | 3*                             | C                                   |
| 5      | C             | C                              | C                                   |
| 6      | 3             | 1                              | 2                                   |
| 7      | C             | C                              | C                                   |
| 8      | C             | C                              | C                                   |
| 9      | C             | 1                              | 1                                   |
| 10     | C             | C                              | C                                   |
| 11     | C             | C                              | C                                   |
| 12     | C             | C                              | C                                   |
| 13     | C             | C                              | C                                   |
| 14     | C             | C                              | C                                   |
| 15     | C             | C                              | C                                   |
| 16     | C             | 1                              | 1                                   |
| 17     | C             | 2                              | 3                                   |
| 18     | C             | C                              | C                                   |
| 19     | C             | C                              | C                                   |
| 20     | C             | C                              | C                                   |

* Six isolations.
* Eight isolations.
* Nine isolations.
* Contaminated culture tube.
* Number represents week at which leptospiros were detected by dark-field microscopy.

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No antibiotic* 5-Fluorouracil, final concn 200 µg/mlb Neomycin sulfate, final concn 25 µg/mlc

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tured into media without this antibiotic, demonstrating its bactericidal action. Furthermore, the addition of various concentrations of neomycin to media appeared to stimulate leptospiral growth. Neomycin-containing media was also found to be of value in the isolation of leptospiras from cultures of blood from infected laboratory animals and from swine kidney material.

The data presented here show that by using Fletcher semisolid medium with final neomycin concentrations of 25 and 5 μg/ml, an optimal control of contaminating organisms (including *E. coli*) may be achieved without affecting leptospiral survival. The use of Ellinghausen semisolid medium with a final concentration of 300 μg or neomycin per ml may be equally adequate.

Subsequent studies (unpublished) with bacterial sensitivity disks (Difco) containing 10 and 30 μg of neomycin implanted into tubes containing 5.0 ml of Fletcher medium were as effective as 5-fluorouracil in the isolation of leptospiras from swine kidney material. The merit of bacterial sensitivity disks for these purposes suggests their convenient, low-cost, and efficient application in the laboratory.

Cysteine, yeast nucleic acid, and hydroxyamine as well as the type of media involved are known to inhibit the activity of neomycin (13). In the present studies, a higher concentration of the antibiotic was required for contaminant inhibition in Ellinghausen medium when compared with Stuart and Fletcher media. Further study revealed that the bovine fraction C component of the former medium was involved in the neutralization of the antibiotic action.

The use of 25 μg or neomycin per ml in Fletcher medium permitted isolation of leptospiras from clinical material, whereas under the same culture conditions the growth of stock strains was inhibited. By using this medium, a lower antibiotic level was required for the unrestricted growth of the 13 leptospiral serotypes from the culture collection. The isolates from the swine kidney suspensions were identified as *L. tarassovi* and *L. pomona* serotypes which were among the stock cultures examined. These observations suggested the possibility of a differential growth requirement for newly isolated strains and those maintained by continual subculture. Further studies, however, are required to clarify this point.

The interesting observation on the growth-promoting effects of neomycin on serotypes *L. autumnalis* and *L. tarassowi* growing in Ellinghausen medium and the possibility of a similar action on other serotypes is being investigated.

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LITERATURE CITED

1. Alexander, A. D. 1970. Leptospira, p. 244-250. In J. E. Blair, E. H. Lennette, and J. P. Truant (ed.), Manual of clinical microbiology. American Society for Microbiology, Bethesda, Md.
2. Alston, J. M. and J. C. Broom. 1958. Leptospirosis in man and animals. E & S. Livingstone Ltd., Edinburgh and London.
3. Babudieri, B. 1961. Laboratory diagnosis of leptospirosis. Bull. W.H.O. 24:45-58.
4. Cousineau, J. G., and J. A. McKiel. 1961. In vitro sensitivity of Leptospira to various antimicrobial agents. Can. J. Microbiol. 7:781-788.
5. Dominguez, O. R. 1969. Los antibióticos en el aislamiento de leptospiras. Invest. Genad. (B. Aires) 6:129-138.
6. Ellinghausen, H., C., and W. G. McCullough. 1965. Nutrition of *Leptospira pomona* and growth of 13 other serotypes: fractionation of oleic albumin complex and a medium of bovine albumin and polysorbate 80. Amer. J. Vet. Res. 26:45-51.
7. Fletcher, W. 1928. Recent work on leptospirosis, taumagamushi disease and tropical typhus in the federated Malay States. Trans. Roy. Soc. Trop. Med. 21:265-287.
8. Galton, M. M., R. W. Menges, E. B. Shotts, A. J. Nahmias, and C. W. Heath. 1962. Leptospirosis: epidemiology, clinical manifestations in man and animals, and methods in laboratory diagnosis. Communicable Disease Center, Atlanta, Ga.
9. Johnson, R. C., and V. G. Harris. 1967. Differentiation of pathogenic and saprophytic leptospires. I. Growth at low temperature. J. Bacteriol. 94:27-31.
10. Johnson, R. C., and P. Rogers. 1964. 5-Fluorouracil as a selective agent for growth of leptospires. J. Bacteriol. 87:422-426.
11. Stalheim, O. H. V. 1966. Chemotherapy of renal leptospirosis in hamsters. Amer. J. Vet. Res. 27:803-807.
12. Stuart, R. D. 1946. The preparation and use of a simple culture medium for leptospira. J. Pathol. Bacteriol. 60:343-349.
13. Waksman, S. A. 1953. Neomycin. Rutgers University Press, New Brunswick, N.J.
14. World Health Organization. 1967. Current problems in leptospirosis research. W.H.O. Tech. Rep. Ser. 380 (Geneva).