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 leptospiral 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 leptospiral 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 leptospiral 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 contaminantos while not being toxic to leptospiral 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 Akiyama A, *L. tarassovi* strain Perepelin, *L. pomona* strain Pomona, *L. australis* strain Ballico, *L. ballum* strain Castellon 3, *L. grippotyphosa* strain Moskva V, *L. hebdomadis* strain Hebdomadis, *L. bataviae* 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) semisolid 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). Subcultures 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 neomycin 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 distilled 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 leptospirese 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 preparing replicate decimal dilutions in tubes containing 4.5 ml of Ellinghausen semisolid medium. Estimates on growth were based upon the highest dilution showing leptospires microscopically after 14 days of incubation.

**Effect of neomycin on 13 leptospiral serotypes.** The effect of neomycin on the growth of the 13 leptospiral serotypes was studied in both Ellinghausen and Fletcher semisolid media with and without 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 cultures.** Ten *Escherichia coli* strains recently isolated from bovine fecal material, maintained on *Brucella* (Albiml) 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 *Enterobacteriaceae* (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 Ellinghausen liquid medium, and viable numbers were determined by plate counts. Approximately 3 × 10⁴ *E. coli* organisms were used as inocula to contaminate the tubes which contained neomycin in final concentrations 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 leptospiras to multiply and the bactericidal 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* (Albiml) broth with and without bovine fraction V and Tween 80 (at the concentrations used in Ellinghausen medium). Twofold serial dilutions of neomycin were made in each of the media ranging from 500 to 1.9 μg/ml, inoculated with 3 × 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 leptospiras 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 × 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 leptospiras.

**Leptospiral isolation from abattoir material.** The potential value of neomycin for the isolation of leptospiras 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 leptospiras.

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, medulla, 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 Soerensen 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 on 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 media 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*  | -                                         | +                                     |     |     |     |     |
| *Castellon 3*          | +                                         | -                                     |     |     |     |     |
| *L. grippotyphosa*     | -                                         | +                                     |     |     |     |     |
| *Moskva V*             | +                                         | -                                     |     |     |     |     |
| *L. tarassovi*         | +                                         | +                                     |     |     |     |     |
| *Perepelicin*          | +                                         | -                                     |     |     |     |     |
| *L. hebdomadis*        | -                                         | +                                     |     |     |     |     |
| *Hebdomadis*           | +                                         | -                                     |     |     |     |     |
| *L. batavica*          | +                                         | -                                     |     |     |     |     |
| *Van Tienen*           | +                                         | +                                     |     |     |     |     |
| *L. canicola*          | -                                         | +                                     |     |     |     |     |
| *Hond Utrecht IV*      | +                                         | -                                     |     |     |     |     |
| *L. pyrogenes*         | +                                         | -                                     |     |     |     |     |
| *Salinem*              | +                                         | -                                     |     |     |     |     |
| *L. icteroides RGA*    | +                                         | +                                     |     |     |     |     |
| *L. sejroe*            | -                                         | +                                     |     |     |     |     |
| *M 84*                 | +                                         | -                                     |     |     |     |     |
| *L. autumnalis*        | +                                         | +                                     |     |     |     |     |
| *Akiyami A*            | +                                         | -                                     |     |     |     |     |
| *L. pato*              | -                                         | +                                     |     |     |     |     |
| *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 leptospiras. 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 leptosomal 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 leptosporal 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 leptospiras 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 leptosporal media aided in the control of contaminants. The use of other antibiotics for the selective culture of leptospiras (5) required rapid subculture into antibiotic-free media to prevent the expression of toxic effects on the leptosporal 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 leptosporal serotypes in high concentrations, is both bacteriostatic and bactericidal (13). This wide spectrum antibiotic is inexpensive and readily available. A contaminated leptosporal 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-

**Table 3. Comparative value of incorporating neomycin to Fletcher medium in the isolation of leptospiras and the control of contaminants in swine kidney cultures**

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

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

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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 \( \mu g/ml \), 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 \( \mu g \) or neomycin per ml may be equally adequate.

Subsequent studies (unpublished) with bacterial sensitivity disks (Difco) containing 10 and 30 \( \mu g \) 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 \( \mu g \) 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 \( L. tarassovi \) growing in Ellinghausen medium and the possibility of a similar action on other serotypes is being investigated.

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