Isolation, enumeration and survival methods for the study of Leptospira in the environment
by Thomas Dean Roberts

A thesis submitted in partial fulfillment of the requirements for the degree of MASTER OF SCIENCE in Microbiology
Montana State University


Abstract:
Cultural media and methods for leptospiral growth were reviewed to find a system to promote growth from a small inoculum size, to obtain high cell densities in a short time period, and to detect leptospiral growth early in culture. Ellinghausen and McCullough's albumin-tween medium was found to be the best media for culturing and growth.

A 70% isolation success from natural water was achieved using natural leptospiral motility across a 0.22 µm pore size membrane into semisolid culture medium. The addition of 100 µg/ml 5-fluorouracil to the semisolid recovery medium helped control background contaminants.

A purification system which relied on leptospiral motility and drugs, in particular colistin, was used. This was found to be satisfactory for removing the major problem contaminants, which were small spiral organisms. Further purification was accomplished by membrane passage and other conventional methods.

Survival studies were made with membrane side-wall, diffusion chambers. Leptospiral hardjo and L. pomona were found to survive for periods of a week or more when they were suspended in water environments. Leptospires were also found to survive and reproduce in algal supernatant.

Leptospiral recovery was possible from moist soils and most natural waters. Areas of high pollution showed higher leptospiral numbers than those of lower pollution, with "pristine" sites showing no leptospiral recovery. The ability of leptospires to survive in water and algal supernatant as well as recovery from water and moist soil reflects a wide distribution. Harboring of leptospires in these various environments may assure a continuous infectivity of surface waters with leptospires.

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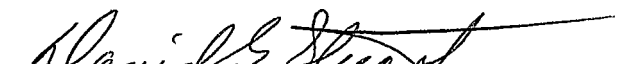
Approved:



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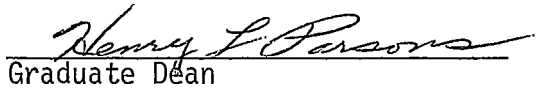

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# ABSTRACT

Cultural media and methods for leptospiral growth were reviewed to find a system to promote growth from a small inoculum size, to obtain high cell densities in a short time period, and to detect leptospiral growth early in culture. Ellinghausen and McCullough's albumin-tween medium was found to be the best media for culturing and growth.

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# CHAPTER 1

## INTRODUCTION

The increased use and reuse of the water resource has intensified interest in water quality and in methods for evaluating water quality. The search for a biological indicator which can be easily assayed and which gives a clear correlation with specific types of pollution has long been sought. The fecal coliforms are widely used organisms for detecting animal and human fecal pollution, which could contain water-borne pathogens. The interrelation of the fecal coliforms and other fecal pollution indicators with water-borne pathogens remains obscure, although probable pathogenic contamination can be suspected when fecal coliform counts are high.

Difficulties in obtaining definitive information concerning the potential health hazards of pathogenic bacteria through the use of indicator systems has increased interest in the survival and virulence of pathogenic bacteria in the environment. Of the hundreds of diseases involving animals there are more than 150 which are zoonotic (16). Some of the better known ones are salmonellosis, brucellosis and leptospirosis.

The complex epidemiology, the increasing recreational exposure (80) and the improved diagnosis of previously unrecognized mild forms of leptospirosis (47) have made leptospirosis a disease of public significance. Leptospires have been shown to survive in water (66,8,4),

soil (59,37) and animal wastes (16,17), however little is known of their actual abilities to endure hostile environments and to maintain their virulence apart from their host animals.

The information gleaned about leptospiral behavior in the environment has largely been gathered through laboratory experiments, through utilization of host animals, and by following leptospirosis cases. Methodology enabling *in vitro* and *in situ* observation and experimentation needs to be expanded to fully explore the interactions and impact of pathogenic leptospires in the environment.

## Statement of Purpose

The aim of this research is to develop methods and to utilize these methods for *in situ* and *in vitro* examination of leptospires. In particular, the goals are to look at the survival of leptospires in water as compared to indicator organisms and to recover leptospires from the natural water systems.

# CHAPTER 2

## LITERATURE REVIEW

The first observance of leptospira was in 1905 by Stimson who, in examining tissue from a "yellow fever" case, noted long thin spiral organisms. It was realized later that these thin spiral organisms were icteric-producing leptospires and were themselves the cause of the "yellow fever." Stimson called these organisms *Spirochaeta interrogans* (74). In 1913, S. B. Wolbach and C. A. L. Binger described the same type of organisms from fresh water sources. They were unable to cultivate these organisms (84). Later researchers, working with a pathogenic spiral organism, were able to cultivate them in sera-containing media. They named their isolates *Leptospira icterohaemorrhagiae*, and identified it as the etiological agent of Weil's disease described in 1886 (40,57).

Increasing isolation of these thin spiral organisms gave evidence that the epidemiology of leptospiral infections is complex. There are now over 190 serovars described and of these 150 are named and recognized (68,82). These serovars are placed in 18 serogroups based on cross-agglutination and in two complexes based on their parasitic or saprophytic character. The "Interrogans Complex" includes the serovars which have been isolated from sick animals or carriers. They are pathogenic to some and may be commensal to other animals. The "Biflexa Complex" includes about 70 nonpathogenic, free-living serovars

which have been isolated from surface waters and moist soils.

Epidemiology

Leptospirosis is cosmopolitan in nature. Fifteen of the serogroups have been confirmed or suspected as infective agents by serological means (47). Before 1948, *L. icterohaemorrhagiae* was recognized as the major infective serovar, with *L. canicola* recognized to a lesser extent. After 1948, with improved isolation, culturing and maintenance, the complexity of leptospiral epidemiology became apparent. The rat leptospire *L. icterohaemorrhagiae* was displaced by *L. canicola* as the most frequent etiological agent. In 1971 and 1972 the five major common source outbreaks occurred in Minnesota, Texas, Missouri, New York and Oregon, and were attributed to the serogroups Autumnalis, Canicola, Interohaemorrhagiae, Pomona and Autumnalis, respectively. The outbreak in Minnesota was attributed to riding go-carts in a farm yard after seasonal rains. In Texas the outbreak was among children playing in pools of water with infected dogs. The outbreak in Missouri was associated with infected dogs that were shedding leptospires in their urine. The outbreak in New York was attributed to contact with urine of infected cattle, and that in Oregon with an infected dog and a moist environment (31). The mild anicteric forms of leptospirosis are recognized as more prevalent than the severe icteric forms.

Pathogenic leptospires are found as parasites of both wild and

domestic animals. One or more host species will act as the reservoir for a specific leptospiral infection with other animals, including man, being infected in localized outbreaks. The Norway rat is a carrier and shedder of *L. icterohaemorrhagiae*. Counts of $10^9$ organisms per milliliter have been shown in the urine of the asymptomatic Norway rats (3). *Leptospira pomona* is usually associated with swine, and to a lesser extent, with cattle and sheep. Dogs are the major carriers of *L. canicola*. The rodent population has been found to be the major source reservoir in feral populations. Leptospires have been isolated from nonmammalian hosts such as frogs (22) and turtles (36). Insects do not appear to play a role as carriers or vectors of infective leptospires (62).

Transmission

Transmission occurs through contact with infected blood, urine, tissues and organs. Leptospires are indirectly transmitted through contaminated natural fresh water systems, soils, muds, vegetation and food stuffs. Mucosal membranes of the nose, muzzle or the mouth, skin abrasions, and conjunctiva are the sites of entry. A study on guinea pigs, whose abdominal hair was shaved and the abdomen then exposed to slowly flowing water containing *L. pomona*, indicates that, given time, the leptospires can penetrate intact skin (33). Direct contact occurs when handling infected hosts and their fresh carcasses and organs.

Other important routes of transmission are coitus and transplacental infection of the fetus (62,80).

Leptospiral infection in man is often dependent on occupational hazards. Veterinarians, farmers, hunters, slaughter house workers and miners are examples of the types of workers developing leptospiral infections. While occupational disease incidence has been decreasing, except that of the farmer, recreational activities are accounting for increased numbers of leptospiral cases. Swimming, wading and contact with infected pets (in particular, dogs) have become the major sources of leptospirosis in man.

Reported cases of leptospirosis have been increasing in frequency in the last five decades. Sixteen cases in 1925-1934, 230 in 1935-1944, 267 in 1945-1954, 705 in 1955-1964 and 791 in 1965-1974 have been reported (47). The increase in numbers of cases is probably due to improved recognition of mild cases of leptospirosis and to increased recreational exposure especially swimming and wading in contaminated areas.

The eradication of leptospirosis appears to be unachievable due to the complexities of host reservoirs and the potential survival of leptospires in water. Some control has been accomplished through vaccination of domestic herds (such as cattle and swine) using multivalent antigens of the types most prevalent in that area. However, the efficacy of vaccines and cross reactions among the pathogenic leptospires

are not fully understood. Vaccination of human populations does not seem practical because of the low incidence of leptospirosis in man. Some high risk vocations may warrant vaccination (75).

Pathogenicity

Leptospirosis is protean in nature, dependent upon the host and the infecting serovar as well as other poorly defined characteristics. The classical Weil's leptospirosis is usually considered to follow three separate stages. After penetration through mucosal membranes or abraided skin, the leptospires quickly enter the blood stream. They then spread throughout the visceral tissue with the kidneys and liver bearing the brunt of the infection. After this primary leptospiremia, the populations increase in size and a fever will occur usually 6 to 8 days after infection. The septicemic stage is characterized by generalized muscular aches and pains, and exquisite tenderness of muscles, particularly the gastrocnemisis muscles. Headache, nausea and vomiting reflect meningeal irritation. Occasional intense conjunctival congestion and hemorrhage may occur as well as petechial and ecchymotic hemorrhages appearing on the skin.

In the third to seventh day, the toxic or icteric stage begins. Jaundice appears in about 2/3 of the cases, with an enlarged and tender liver. The jaundic condition lasts for a few days and gradually decreases over the remaining course of the disease. At the end of the

first week, oliguria may appear and progress to anuria leading to uremic death.

The convalescent stage shows the fever slowly falling and urine output returning to normal. The jaundice gradually fades and the patient generally returns to normal (35,64).

Maintenance

Media for cultivating and maintaining leptospiral cultures are of three types. The original successful growth media contained sera (40). Different sera were used with varying success, with rabbit becoming the serum of choice. A pool of rabbit serum made up of sera from at least 20 leptospiral negative rabbits has become the most successful medium additive. Sera-containing media consist of a basal salts buffered portion with serum added to form 8 to 10% of the volume. Some of the serovars may require as much as 20% serum for growth to occur. Examples of this type of media are Fletcher's 1928 (29), Korthof's 1932 (49), Stuart's 1946 (76) and modifications of these (81).

Leptospiral media in wide use at present include those containing albumin fractions and polysorbates. Earlier attempts to utilize albumin fractions of sera proved unsuccessful, because in these media formulations, the importance of only one vitamin was stressed and the requirements for lipids was not known. With the addition of B12 and and lipids in the form of polysorbates, the albumin media became suc-

cessful in cultivating leptospires (21). The most widely used albumin-tween media are those of Ellinghausen and McCullough and a modification by Johnson and Harris (25,45).

The third type of medium is the chemically defined serum-free synthetic medium. Several researchers have described media of this type. Shenberg (1967) has had the most success and was able to sub-culture 52 strains belonging to 12 serogroups (68). Work is still incomplete in formulating chemically-defined media capable of sustaining all the serovars. Thus, most media used in routine culturing consists of the first two types.

Three forms of media are used for various culturing needs. Liquid medium is essential for growth of antigens for serum testing as flakes of agar interfere with test results (81). Semisolid agar media are the media of choice for maintenance of stock cultures. Growth of leptospires occurs faster, and with greater success in semisolid media. Some of the serovars, especially those of the Hebdomadis serogroup, are difficult to culture without the use of semisolid media. The nutrient value or other beneficial properties of agar are not understood in culturing leptospires (21). Solid agar media are used to study colony morphology and as aids in isolation and purification. The saprophytic leptospires will usually become visible within 3 to 4 days on solid 1% agar media. The parasitic leptospires may require 14 to 20 days with some of the serovars being very difficult to observe on solid agar (73).

Incubation of leptospires is generally done in the dark with a temperature of 27-30° C. Some researchers claim primary incubation at 37° C for a 2-3 day period enhances leptospiral growth, but other workers disagree (81). Lower temperatures tend to decrease the growth of pathogenic leptospires, while 56° C for a few minutes will kill leptospires (81). Johnson and Harris have suggested the use of growth at lower temperature as a means to differentiate saprophytic and parasitic leptospires (45). Culturing is also carried on at room temperature.

Survival of leptospiral cultures usually depends upon transfer to fresh media at 3-4 week intervals. Cultures have remained viable for over a year in Ellinghausen and McCullough semisolid medium. Freeze drying may be the method of choice for maintaining cultures over long time periods since subculturing often results in loss of virulence and may result in antigenic alterations giving false information in serological examination (81).

Morphology

Leptospires are Gram-negative, long, thin, tightly-spiraled organisms. They are 4-20 μm long and approximately 0.1 μm in diameter. Under certain conditions, they may obtain a length of 30-40 μm. The coils have 0.2-0.3 μm overall diameter and a pitch of 0.3-0.5 μm. There are two axial filaments, one inserted at each end in terminal disks with the free filament ends meeting at the center of the organism.

A sheath or envelope encloses both the axial filaments and the cytoplasmic body (42,55). Three forms of movement can be seen. A to-and-fro shunting with a short rest at the change in direction and rapid spinning of the hooked ends about the axis can be seen in liquid medium. In semisolid agar, leptospires move in a serpentine fashion, boring through the medium (58).

Observation

Dark-field microscopy is the method of preference for viewing leptospires. The high, dry objective of 43X is generally acceptable for most observations. Oil immersion can be used for better resolution. Phase contrast microscopy can be used, although, due to the leptospire's long thin shape, they are not very phase dark and do not show up without a great deal of focal plane adjustment. Electron microscopy has been used for gross morphology and viewing transverse and longitudinal sections (70). Light-field microscopy is of limited value for observing leptospires, however, leptospires may be rendered visible through the use of silver impregnation and aniline dyes (5,69).

Macroscopic observation depends on the type of media used for culturing. Growth in semisolid media generally appears as thin disks 3-5 mm below the surface. Several disks often appear with the number of discrete disks dependent upon the age of the culture. In some types of media or in certain batches of media, these thin disks do not appear, although growth of motile leptospires can still be observed upon micro-

scopic examination. Dinger first wrote about these thin disks of growth in 1932; thus, this growth phenomenon has come to be called the Dinger ring (15).

Leptospiral growth in liquid media is recognized by increasing turbidity and a shot silk appearance upon agitation. Growth on solid agar was described by Cox and Larson in 1956 (12). Appearance on solid agar depends upon several parameters outlined by Cox in 1966 (11). Since the leptospires have such a long generation time and incubation must be carried out over an extensive period of time, it is necessary to pour plates of sufficient depth to prevent drying. Care must be taken in streaking to allow formation of colonies. Plates should be incubated for at least 3-4 weeks before they are considered negative. Some strains, due to their fastidiousness, may not grow on the solid agar used. The concentration of agar is very important; 1% agar appears to be the best concentration, although some leptospires form thin veils of growth at this agar concentration and may need 1.3-1.4% agar.

While immunofluorescence has been used to detect leptospires, this technique at present has limitations due to cross-reaction problems. When demonstration of leptospires is the only goal, fluorescent antibody techniques may be the methods of choice (37).

Enumeration

In Chang's early work on the survival of *Leptospira icterohaemorrhagiae* counts were made by placing a drop of a leptospiral suspension upon a slide from a calibrated capillary. A standard cover slip was then placed over the drop and it was examined with the high dry objective of a dark-field microscope. Twenty fields were examined for each preparation. Chang had to resort to this type of count because no counting chamber was available which was thin enough to use on a dark-field application (8). With the advent of the Petroff-Hausser bacterial counting chamber, researchers began making direct counts of leptospires. Plate counts have also been obtained using spread plate techniques (51). Later Schiemann had success generating survival curves using most probable number procedures (67). Measurement of turbidity has also been used to monitor the growth of leptospires. Greene used a Klett-Summerson photoelectric colorimeter (34) and Ellinghausen used nephelometry as a measure of growth (27). Since nephelometry measures only scattered light the color of the medium does not interfere with measurements, thus turbidity measured via nephelometry correlates well with direct microscopic counts. Nephelometry has become the turbidity measurement of choice in enumeration of leptospires.

Isolation

Leptospires can be isolated from the tissue of an infected host.

Infected tissue is removed aseptically, ground, diluted in buffer and then placed in replicate tubes of semisolid media (77). If care is taken, pure cultures can be obtained in this manner (60). Surface waters can also be sampled for leptospires by inoculating an appropriate host, such as guinea pigs and hamsters in the weanling stage, intraperitioneally with the sample. After a period of incubation, tissue is then harvested and placed in the appropriate medium (19,32). Isolation through the use of host animals has been very successful, but cost, expertise and time are all high when using *in vivo* isolation techniques.

*In vitro* isolation has met with some success. Researchers such as Cox were able to cultivate leptospires from surface waters by placing a drop of filtered water on solid agar medium and then watching for leptospiral growth on the advancing edge of bacterial growth (11). Filtration has also been used as a method of isolation (6,63,79). These methods usually consist of filtration through decreasing pore size membrane filters until the final filtrate has passed a 0.45 or 0.22 μm pore size membrane. The filtrate is then inoculated into culture media. A method described by Smibert to decontaminate a leptospiral culture has also been used to isolate leptospires (17,70). This method consists of placing a sterile membrane of 0.22 or 0.45 μm pore size on a solid agar surface and sealing a sterile ring to the top of the membrane. The sample is then placed inside the ring. After several days of incubation, the membrane and ring are removed and the agar checked for

leptospiral growth. *Leptospira canicola* was isolated from the urine of an infected dog using this method (P. Thomson, personal communication).

## Purification

A problem in isolation and cultivation of leptospires is contamination by other bacteria. Since leptospires are capable of passing a 0.22 μm pore size filter, the methods of Smibert, Rittenberg and Braun can be utilized to eliminate some contaminating bacteria (6, 63,71). The addition of drugs such as neomycin (57), 5-fluorouracil (46), furazolidone (56), sulfathiazole (10), acidione (10), singly and in combination has also been used for decontamination (10,56).

Streak plates and dilution have also been used for purification, however, due to slow growth and the failure of some leptospires to grow on agar media, these methods may be limited in value. Purification may also be accomplished through animal passage, which maintains virulence or may revive lost virulence (69).

## Survival

Survival of leptospires in natural systems plays an important part in transmission to host animals. Some of the early work by Chang indicated that survival times may be quite long (8). At 25-27° C and pH 7.0, *L. icteroharmorrhagiae* survived for 30-32 days in sterile tap water and 98-102 days in sterile tap water plus 1% serum. Diesch showed

that motile leptospires could be found after six days in selas candles suspended in a ditch of manure (16). Other studies show survival times of a few hours to several weeks and in some cases several months depending on the temperature, pH and type of suspending fluid (59,65). Survival in fresh water systems is dependent on a pH range of 6.8-8.0 and a temperature range of 7-30° C (13).

Cattle have been shown to shed as many as $10^8$ leptospires per ml in their urine (32). Periods of heavy rain and flooding have also been shown to increase the concentration of leptospires in water (4). Thus demonstration of survival and transmittance in water systems is dependent upon the number of leptospires shed into the system as well as the ability to detect leptospires through *in vivo* and *in vitro* techniques. Another factor confusing leptospiral survival studies is the saprophytic leptospiral population interfering with the recovery of specific leptospiral serovars (42).

# CHAPTER 3

# MATERIALS AND METHODS

## Organisms Utilized

Three serovars representing three serogroups of leptospires were utilized in these studies. *Leptospira pomona* and *Leptospira hardjo*, Communicable Disease Center (CDC) serovars, representing the Pomona serogroup and the Hebdomadis serogroup, respectively, were kindly supplied by Mrs. Kay Newman from the Montana State Department of Livestock Diagnostic Laboratory in Bozeman, Montana. *Leptospira pomona* was used for most of the studies. The saprophytic serovar *Leptospira patoc* (American Type Culture Collection #23582B), representing the Semaranga serogroup, was used in comparative studies. Leptospiral isolates from the springs and streams surrounding Bozeman were also used in some of the studies.

## Media

Water Utilized. The water used for media and buffers was either double distilled water stored in glass or water obtained from a Milli-Q activated carbon, ion-exchange system (Millipore Corp., Bedford, MA) following single distillation.

Buffers. Sorensen's buffer and peptone-phosphate buffer were used for washing, resuspending and diluting organisms. Sorensen's buffer was made by adding 8.33 g $Na_2PO_4$ (anhydrous) and 1.09 g $KH_2PO_4$ (monobasic) to one liter of distilled water. The pH was then adjusted

to the range of 7.4-7.6 with 2N NaOH. Peptone-phosphate buffer was made by adding 1.25 ml of phosphate stock solution (2) to one liter of distilled water and then adding peptone (Difco) to 0.1%. The pH was then adjusted to 7.4 with 2N NaOH.

Solid, Semisolid and Liquid Media. Three types of media were used for these studies. Semisolid medium made with a 0.25% agar concentration (BBL, Agar Purified) was used for maintenance, isolation and enumeration. Solid medium at a 1% agar concentration (BBL, Agar Purified) was used for isolation, purification and enumeration. Liquid medium was used to grow leptospires for survival studies. The semisolid and liquid media were stored at room temperature in 100 ml amounts and dispensed as needed. The solid media were plated and stored at 4° C. All media were checked for sterility before use by streaking on Tryptic Soy Agar (Difco) and by visual inspection. The various media utilized are shown in Table 1.

Bovine Serum Albumin Media. Ellinghausen and McCullough medium (EM) and the Johnson and Harrison modification (EMJH) thereof were prepared as described (25,45). A modification in preparation of Ellinghausen and McCullough medium was made as follows: to 879 ml of water, 40 ml of 25X buffer, 50 ml of 20X salts, 1 ml $CuSO_4 5H_2O$, 10 ml $ZnSO_4 \cdot 7H_2O$ and 20 ml $FeSO_4 \cdot 7H_2O$ were added. This was stirred for 5 min and then 200 mg L-cystiene was added and stirring continued for another 5 min. This basal mixture was then filtered through a 0.8 μm pore size

Table 1. Leptospiral Growth Media Used in These Studies and the Major Components Other Than Basal Salts

| Media | BSA[a] | Serum | Tween-80 | MEM[b] | Oleic Acid | Tryp[c] $PO_4$ |
|---|---|---|---|---|---|---|
| Ellinghausen McCullough (EM) | X | | X | | | |
| Johnson Harrison Modification of EM (EMJH) | X | | X | | | |
| Minimum essential medium oleic (MEM-BOH) | X | | | X | X | |
| Minimum essential medium tween-80 (MEM-BT) | X | | X | X | | |
| Fletcher | | X | | | | |
| Stuart | | X | | | | |
| Cox (SM 1) | | | X | | | X |
| Cox (SM 2) | | | X | | | |

[a]Bovine Serum Albumin Fraction V.

[b]Minimum essential medium, Dulbecco's modification.

[c]Tryptose phosphate.

membrane filter and the pH adjusted to 7.4 with 2N NaOH. This solution was either autoclaved for 15 min at 15 lb or filter sterilized through a 0.22 μm pore size membrane. Agar was also added to the basal mixture if semisolid or solid medium was desired.

The albumin solution was made by adding 12.5 g bovine serum albumin fraction V (Pentex) to 250 ml of distilled water. While stirring 1.2 ml of tween 80 (Baker), 0.1 ml thiamine stock and 0.2 mg B12 were added. The pH was adjusted to 7.4 with 2N NaOH. The medium was then filter sterilized by sequentially passing through 0.8 μm, 0.45 μm and 0.2 μm pore size membranes. The albumin solution was then added to the basal solution.

Minimum Essential Media. Two types of media utilizing minimum essential tissue culturing media were used. Both contained Dulbecco's modified Eagle medium (Cat #D714, International Scientific Ind.) The minimum essential medium containing bovine serum albumin oleate was prepared as described by Finn and Jones (MEM-BOH) (28). The other minimum essential medium with bovine serum albumin and tween 80 (MEM-BT) was prepared by adding 13.43 g Dulbecco's modified Eagle medium, 5 g bovine serum albumin, 1.2 ml of tween 80, 1.1 ml glycerol and 0.2 mg B12 to 500 ml of water. The mixture was adjusted to a pH of 7.4 with 2N NaOH. This mixture was then filter sterilized with a 0.22 μm pore size membrane filter (Millipore). To another 500 ml of distilled water 0.5 g $Na_2HPO_4$, 0.2 g $KH_2PO_4$ and 0.25 g $NH_4CL$ were added. This portion

was either autoclaved or filter sterilized. Agar was added for solid or semisolid media. The pH was adjusted to 7.4 with 2N NaOH. The two portions were then added to give a total of one liter.

Serum-Containing Media. Fletcher's and Stuart's media formulations were used for serum-containing media. They were prepared as described in Leptospirosis Methods in Laboratory Diagnosis (77). Sterile serum was added to form 8-10% of the volume. Fetal calf serum was supplied by Dr. Malcolm H. Smith at the Montana State Veterinary Science Research Laboratory. Rabbit serum was obtained from research rabbits used for the production of anti$\mu$. Some rabbit serum was also obtained from Dr. Smith. The Fletcher's and Stuart's media were adjusted to a pH range of 7.4-7.6 and agar was added for semisolid or solid media.

Serum-Free Defined Media. Cox described simple serum-free media for isolation and cultivation of "water-leptospires." Cox's SM1 and SM2 media were made as described (11). Stock solutions were made up for rapid preparation of SM1 and SM2. A 10X solution of the salts of SM1 was made in a liter volume. Two stock solutions were made for SM2 medium. The first 10X solution included $NH_4Cl$, KCl, $CaCl_2$ and $MgSO_4$. The second stock solution included the remaining salts in a 100X solution. SM1 and SM2 were made up in one liter volumes and the pH adjusted to 7.4. Agar was added if desired. Cox's media were sterilized by autoclaving at 15 lb for 15 min.

Cultivation and Observation

Leptospire cultures were incubated at 27° C. On some occasions the cultures were incubated for a period of 3-4 days until macroscopic growth could be seen. They were then removed and maintained at room temperature in the dark. Macroscopic observations were made by noting the Dinger ring phenomenon in semisolid media. Liquid cultures showed macroscopic growth in the form of turbidity and the so-called shot silk appearance upon agitation of the culture tube. Macroscopic colonial growth in solid agar appeared as spreading translucent growth descending throughout the depth of the agar. Discrete colonies were round and usually subsurface with a puff ball appearance. The type of macroscopic growth in solid media depended upon the type and concentration of agar used.

All culture manipulations were done in a bacteriological hood to prevent culture contamination and spread of leptospires. All laboratory surfaces were disinfected with amphyl (National Laboratories Lehn and Fink Industrial Products Division).

Equipment

All glassware and filtering equipment were taken from general laboratory stocks. They were machine washed, rinsed with distilled water and air dried. All acid washing was done by soaking in 10% HCl for a minimum of 30 min, rinsing six times with tap water and then rinsing with double distilled water or reagent grade Milli-Q water.

Any equipment treated as for tissue culturing was prepared by boiling in 7X (LINBRO Chemical Co., Inc.), three rinses of tap water were followed by boiling in double distilled water and rinsing in double distilled water.

Dark-Field Microscope. Microscopic observations were made with a Bausch and Lomb microscope with a dark field condenser. Wet mounts were prepared for observation by placing a drop of the culture on a slide and covering with a #1 cover slip. Care was taken to prevent any bubbles from forming because of the light scattering affecting observations. Most of the observations were made using the 43X objective, while oil emersion was used when needed.

Phase Microscope. Occasional observations were made with a Leitz phase microscope using a 100X objective to obtain greater resolution. This was done on isolated cultures to help determine whether or not they were leptospires based on morphology. Blendon and Goldberg silver impregnation stain (5) was used with some success for observation of leptospires on a light field microscope. Dark field microscopy was the preferred method for observing leptospiral growth.

Bacterial Counting Chamber. A Petroff-Hausser bacterial counting chamber was used to obtain direct cell counts. This chamber had been used by other leptospiral researchers and was chosen because it could be used with dark field applications due to the thin glass and chamber depth of 0.2 mm. At least 128 squares were counted, and on

occasion, duplicate enumerations were made.

Spectrophotometry. A Varian Techtron model 635 spectrophotometer set at 420 nm with a slit width of 0.2 nm was used for all spectrophotometry. Coleman cells with a 10 nm light path and a volume of 1 ml were used for small sample sizes.

Nephelometry. Nephelometry was done with a Turner Designs Nephelometer with a 4 dram vial sample chamber. The Turner nephelometer was adapted to a sample size of 5 ml by placing a five ml vial containing the sample in the Turner vial and filling the Turner vial with water until the surface of the 5 ml vial was covered. An adaptor was made from a 50 ml sorvall centrifuge tube to hold 25 x 200 mm test tubes for measuring turbidity in continuous culture.

Swinnex Modification 1. A millipore swinnex 13 mm membrane filter holder (Figure 1) was cut on the needle side leaving a hole in which an 8 x 29 mm fermentation tube would fit snugly. The swinnex was then unscrewed and a 0.22 μm pore size membrane (Millipore) placed over the top of the fermentation tube. The swinnex was then reassembled and autoclaved at 15 lb for 15 min. After the assembly cooled, it was reopened and semisolid leptospiral medium was placed in the fermentation tube. The swinnex was then reassembled and the syringe side was inoculated with 0.1 ml of a sample. The swinnex assembly was then incubated at 27° C and was checked daily for growth of leptospires.

Swinnex Modification 2. A 25 mm Millipore Swinnex membrane

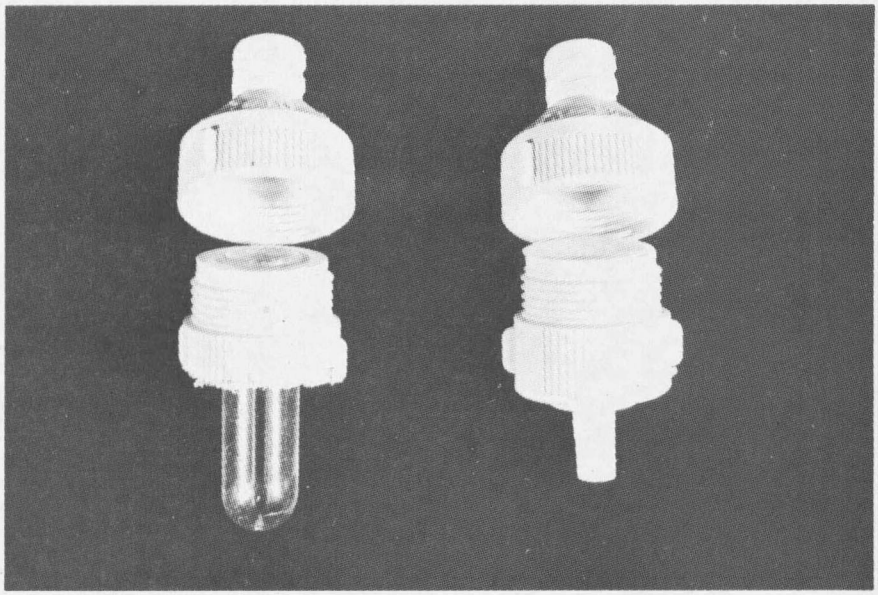


Figure 1. Swinnex Modification 1 Used for Isolation Attempts and Purification With an Unaltered Swinnex (Right) for Comparison

holder was taken apart and the membrane stage removed. A hole was then bored through the needle side to fit snugly around a 10 x 40 mm culture tube. The edge of the tube was placed even with the membrane shelf and the swinnex reassembled. This unit was then autoclaved at 15 lb for 15 min. After cooling, semisolid leptospira medium was placed in the tube filling to the top. A 0.22 μm pore size membrane filter (Millipore) was then placed on the top of the filled tube and the swinnex screwed back together. The needle side was then inoculated with a 1 ml sample. The unit was incubated at 27° C and observed daily for growth of leptospires.

Polycarbonate Cell. A 25 mm polycarbonate cell (Figure 2) (Millipore Cat #XX42 025 10) was fitted with a 25 mm swinnex membrane filter holder (Millipore). The bottom of the needle side of the swinnex was removed. A 0.22 μm pore size membrane filter was set in place at the needle side and the unit assembled. The syringe side was plugged with a plastic syringe tip which had been cut off and filled with plastic cement. This unit was then autoclaved for 15 min at 15 lb. After cooling the cell was filled with semisolid leptospiral medium through the syringe side of the swinnex. A board was drilled with 5/16 inch holes to hold the unit. The plugged syringe side was inserted in the board. A 10 ml sample was then placed in the skirt above the membrane. The unit was then incubated at 27° C and checked daily for macroscopic growth in the semisolid medium.

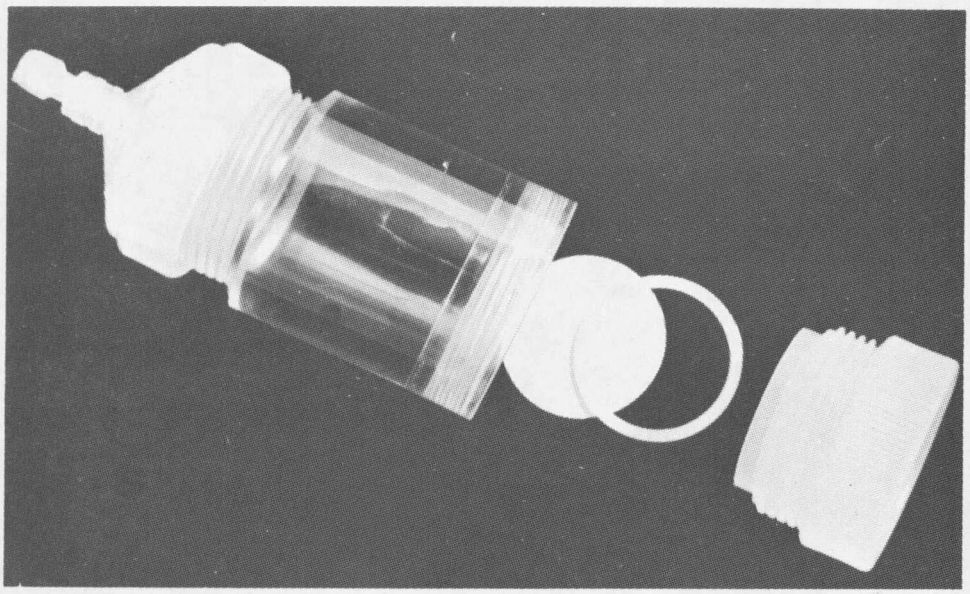


Figure 2. Polycarbonate Cell Used for Isolation Attempts and Purification

Isolation Vial. A four dram vial (Figure 3) was used for leptospiral studies. A 1/2-inch hole was drilled through the center of the cap. Polycarbonate tubing 5 cm long of 1/2-inch outside diameter was inserted in the hole and brought flush with the top side of the cap. Epoxy (Duro) was used to cement the tube in place. The end of the tube was smoothed by sanding with #100 sand paper and a 13 mm 0.22 µm pore size membrane filter glued in place, with MF cement (Millipore). The cap with the tube in place was then screwed back on the vial and autoclaved at 15 lb for 15 mins. After cooling, semisolid leptospiral medium was placed in the vial until it touched the membrane filter on the end of the tube. Two ml water samples were then placed in the polycarbonate tubing through the hole in the outside. The water was evaporated by blowing air across a lamp and then across the vials. This procedure allowed fast evaporation while maintaining the vials at 27° C. After evaporation to about 1/3 the original volume, the vials were incubated at 27° C until macroscopic growth was observed. At this time, the cap was replaced with an unaltered cap and microscopic observations made of the medium. Isolates were then maintained in these vials.

Diffusion Chambers. Chambers with membrane filter side walls as described by McFeters and Stuart (54) were used in some of the survival studies. Double membrane layers were used on each side of the chambers with tear-resistant microweb membranes of 0.45 µmpore size

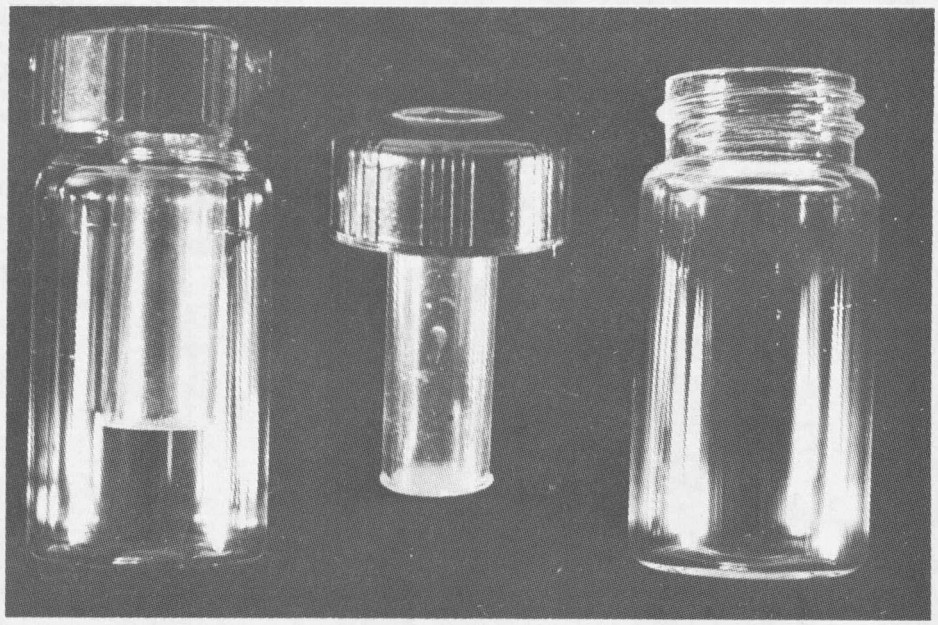


Figure 3. Isolator Vial Used for Isolation Attempts and Purification. A 0.22 μm pore size membrane is glued to the bottom edge of the inter-tube (center).

(HAWP 304FO, Millipore Corp.) on the outside and 0.1 μm pore size membranes on the inside (E442A6, Millipore). These membranes were obtained in 12-inch by 12-inch sheets and then cut into circular disks of 7.5 or 11.5 cm diameter depending on the size of chamber used. The chambers were assembled with the membranes sealed in place with stop-cock grease (Dow Corning). They were then autoclaved for 15 min at 15 lb, with the sample ports left open. The autoclave was allowed to cool without exhausting to prevent membrane rupture from expansion. After cooling to room temperature sterile plugs were placed in the sample ports and the chamber bolts were tightened. The sterile chamber was then stored until needed.

Isolation

Filtration. One liter water samples were taken from irrigation systems, natural streams and cattle watering ponds. These samples were then filtered through Whatman #1 paper filters to remove the larger particulate matter. The samples were then filtered through 0.45 μm pore size membranes (Millipore), and one ml aliquots of the filtrate placed in 3 tube replicates of semisolid leptospiral medium. The tubes were then incubated at 27° C and observed daily for leptospiral growth. Some of these samples were spun at 5,000 r.p.m. for 30 minutes and most of the water poured off. The remaining water sample was then remixed and filtered through a 0.45 μm pore size membrane and the supernatant treated as above.

Ten ml water samples were forced through 0.45 and 0.22 μm membranes (Millipore) with 25 mm swinnex membrane holders (Millipore). Two five ml washes of EM medium were passed through these same membranes. The sample filtrate was placed in three replicate tubes in 1 ml amounts. All the tubes, including the five ml washes, were incubated at 27° C and checked periodically for leptospiral growth and contamination. This procedure was similar to those described by Braun et al. (6), Rittenberg et al. (63) and Tripathy et al. (2, 79).

Isolation by Natural Motility. Sterile 0.22 μm membranes were placed on agar plates containing EM and EMJH media. Sterile rubber washers were then sealed to the membranes with sterile stopcock grease. Ten ml water samples were then placed in the center of the rings. The plates were incubated at 27° C until the water sample had evaporated. The rings and membranes were then removed and the plates inverted and incubated at 27° C. Checks were made at weekly intervals for leptospiral growth in the agar. This method was patterned after Smibert's decontamination method (71).

The method of Cox was used to attempt isolation of leptospires (11). Samples were collected in one liter volumes. The water was then filtered sequentially through Whatman #1 paper filters and 0.45 μm pore size membranes (Millipore). A drop of the filtrate was then placed on the center of the plate and observations made for leptospiral growth at the periphery of the bacterial growth after incubation

at 27° C.

Isolations were attempted through the use of the swinnex modifications, the polycarbonate cells and the 4 dram isolator vials described under equipment. Isolations were attempted by placing a water sample on the upper surface of the membrane and watching for the appearance of growth in the semisolid medium underneath the membrane, after incubation at 27° C. In some cases, the polycarbonate cells and the isolator vials were placed in air blown over a lamp to evaporate some of the sample volume.

Purification

Motility. Smibert plates were used as described (71). The medium was either EM or EMJH. Membrane filters of 0.22 μm pore size were placed on the agar surface and a rubber washer sealed in place. The sample was then placed in the washer and incubated at 27° C. After a period of 3-4 days, the washer and membrane were removed and the agar examined for leptospiral growth and contamination.

Agar wells were punched with a #4 cork bore (5 mm diameter). A 0.25 ml aliquot of a contaminated leptospiral culture was then placed in the well. The petri plates were then sealed and incubated at 27° C. They were then checked daily. Leptospiral growth was seen as translucent growth through the depth of the agar moving away from the site of inoculation. Agar plugs were removed at the leading edge of leptospiral growth and placed in semisolid EM or EMJH media. The semisolid

cultures were then allowed to grow up and were checked under a dark-field microscope for motile leptospires and contamination.

Purification was also accomplished using the swinnex modifications, the polycarbonate cells and the isolator vials. They were filled with semisolid leptospiral medium and fitted with 0.22 μm pore size membranes (Millipore). Contaminated leptospiral cultures were then placed above the membrane. After incubation, they were checked for macroscopic growth beneath the membrane in the semisolid medium.

Uni-Pore polycarbonate membranes (Bio-Rad Ca. #313-5209) were used on some of the isolator vials. They had a pore size of a maximum of 0.2 μm. They were glued on the smoothed end of the polycarbonate tube with MF cement (Millipore). The isolator was reassembled and autoclaved. After cooling, the isolator was filled with semisolid agar and a contaminated culture placed on the upper surface of the membrane. They were then incubated at 27° C and observations made for growth beneath the membrane. Combinations of double membranes were also used, including 0.22 μm (Millipore) and 0.2 μm (Bio-Rad), 0.45 μm (Millipore) and 0.2 μm (Bio-Rad), and 0.45 μm (Millipore) and 0.22 μm (Millipore). In all cases the larger pore size membrane was glued in place first and the smaller pore size last. The isolator vials were then treated as before and observations were made for growth upon inoculation and incubation.

Use of Drugs

Drugs tested and used in these studies are shown in Table 2. All the drugs on paper disks were Sensi-Disc (BBL, Cockeysville, MD). Neomycin was an ICN product (ICN Life Sciences Group, Cleveland, OH). Furazolidone was obtained from Eaton Laboratories (Eaton Laboratories, Division Morton-Norwich Products, Inc., Norwich, NY). The 5-fluorouracil was from Roche (Roche Laboratories, Nutley, NJ).

Based on previous literature the drugs neomycin (57), 5-fluorouracil (46), furazolidone (56), and combinations of these (10, 56) were added to leptospiral media to help reduce contamination of leptospiral cultures as an aid in isolation. Stock solutions of these three drugs were made up. A stock solution of neomycin sulfate was made by adding 151.5 mg of a 660 μg/mg assay to 500 ml of double distilled water, giving a final concentration of 200 μg/ml. This stock solution was then sterilized through a 0.22 μm pore size membrane. A stock solution of furazolidone was made by adding 200 mg Furoxone to one liter of double distilled water giving a final concentration of 200 μg/ml. This furazolidone stock solution was sterilized by autoclaving at 15 lb for 15 min. A stock solution of 5-fluorouracil was made by diluting a 10 ml volume containing 500 mg with 90 ml of sterile double distilled water giving a final concentration of 5 mg/ml. This solution was considered sterile. Neomycin and furazolidone were used at a concentration of 5 μg/ml in culture media. Fluorouracil was used at a concentration of

Table 2. Drugs Used And Their Activity

| Drug | Abv. | Conc. | Activity |
|---|---|---|---|
| Nalidixic acid | NA | 30 µg/disk | Potent inhibitor of DNA synthesis |
| Streptomycin | S | 10 µg/disk | Inhibition of polypeptide synthesis and misreading of the genetic message |
| Kanamycin | K | 30 µg/disk | Same as streptomycin |
| Neomycin | N | 200 µg/ml stock | Same as streptomycin |
| Thiosulfil | TH | 1.0 mg/disk | Sulfonamides bacterostatic acts as an antilog to paraaminobenzoic acid |
| Tetracyclines | Te | 30 µg/disk | Bacteriostatic effective inhibitors of phosphorylation and of protein synthesis |
| Colistin | Cl | 10 µg/disk | Disrupt the osmotic properties of membranes rich in phos-phatidylethanolamine |
| Nitrofurantoin | F/M | 100 µg/disk | Bacteriostatic and bactericidal mechanism not known |
| Furazolidone | F | 200 µg/ml | Same as nitrofurantoin |
| 5-Fluorouracil | Fu | 5 mg/ml | Acts as an analog of uracil affects nucleic acid synthesis |

100 µg/ml.

Further studies on drug efficacy in controlling contamination and in isolation of leptospires were necessary. A contaminated water isolate labelled A1 was used in this study. Fourteen screw cap tubes containing 5 ml of EM semisolid medium were inoculated with 0.5 ml of a contaminated A1 culture. Drugs were added to the tubes with two tubes without drugs for control. A nitrofurantoin paper disk with a concentration of 100 µg was added to tube one. A sulfamethizole paper disk with a concentration of 1.0 mg was added to tube two. A colistin paper disk with a concentration of 10 µg was added to tube three. Nalidixic acid paper disk with a concentration of 30 µg was added to tube four. A tetracycline paper disk with a concentration of 30 µg was added to tube five. A streptomycin paper disk with a concentration of 10 µg was added to tube six. A kanamycin paper disk with a concentration of 30 µg was added to tube number seven. Tube eight was brought to a concentration of 10 µg/ml furazolidone. Furazolidone and neomycin were added to tube nine with a concentration of 5 µg/ml per drug. Tube ten contained 100 µg/ml 5-fluorouracil, and 5 µg/ml neomycin and furazolidone. Neomycin was added to tube eleven to a concentration of 10 µg/ml. Tube twelve contained 200 µg/ml 5-fluorouracil. Tubes thirteen and fourteen contained EM medium with no drugs added.

Drug Plates. Solid agar plates were prepared by adding 7 ml of EM medium to 47 mm plastic petri plates (Millipore). Various drugs and

leptospiral cultures were then placed on the agar medium as diagrammed in Figures 4 and 5. Audries' River isolate was streaked on a quarter of plate 1. A trough was cut in the center of the agar parallel to the streak. This trough was filled with 0.1 ml of stock 5-fluorouracil. An F/M disk was then placed on the surface of the agar opposite the streak. Isolate TR Mun Son was inoculated into a trough on a quarter of plate 2. On the opposite quarter F/M and NA disks were placed. Plate 3 was inoculated in four slots with A1 isolate. A disk of TH was placed at the narrow end and a disk of F/M was placed at the large end of the slots. Isolate A8 was inoculated into two slots across the center of plate 4. On one side of the slots a K disk was placed with an S disk on the other side. Plate 5 had two slots through the center inoculated with A2 isolate. A slot on the outside running parallel to the other two slots was filled with 0.1 ml of stock furazolidone. Another slot on the opposite side of the plate running parallel was filled with 0.1 ml of stock 5-fluorouracil. Kakuchska isolate was inoculated in a slot in the agar of plate 6. On the opposite side of the plate a well was filled with 0.1 ml of stock 5-flourouracil. Two slots running across the center of plate 7 were inoculated with isolate F. A kanamycin disk was placed on one side of the slots and a C1 disk placed on the other side. Isolate C was inoculated into slots on plate 8. On one side of the slots an F/M disk was placed;on the other side a TH disk was placed. Isolate A12 was inoculated in a slot on the edge

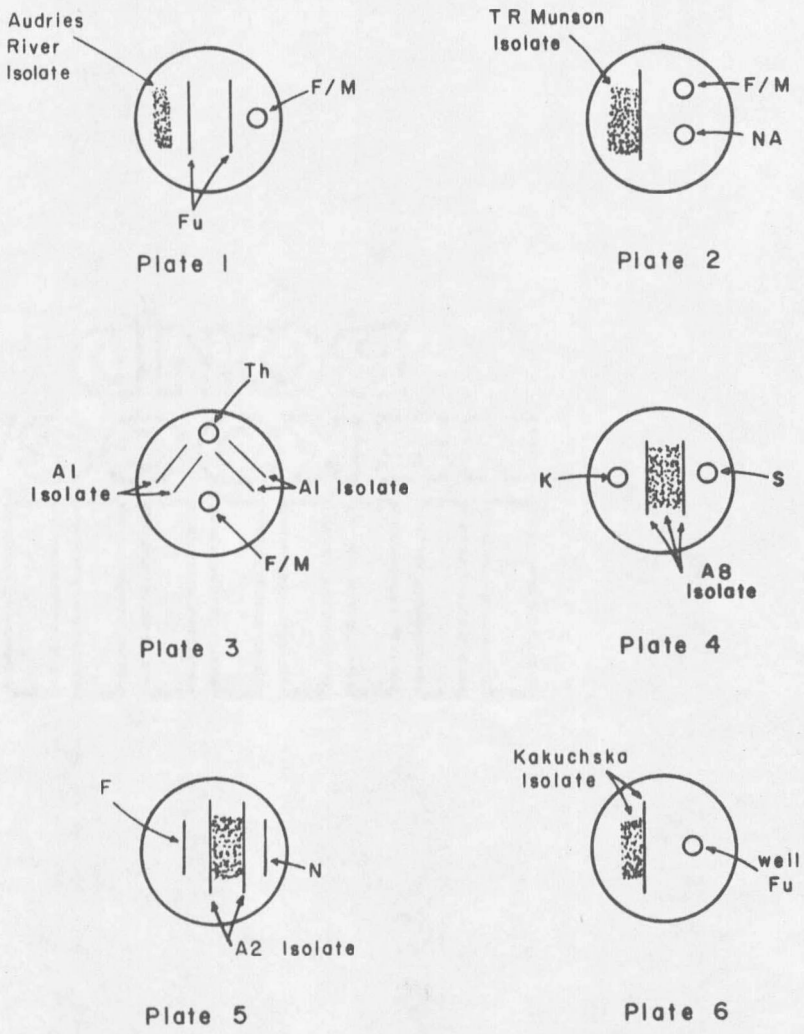



Figure 4. Drug Plate Configurations 1-6 for Study of Drug Efficacy in Purification. Stipling indicates surface streaking of the mixed culture. The straight lines indicate slits in the agar medium. The small circles represent paper drug disks.

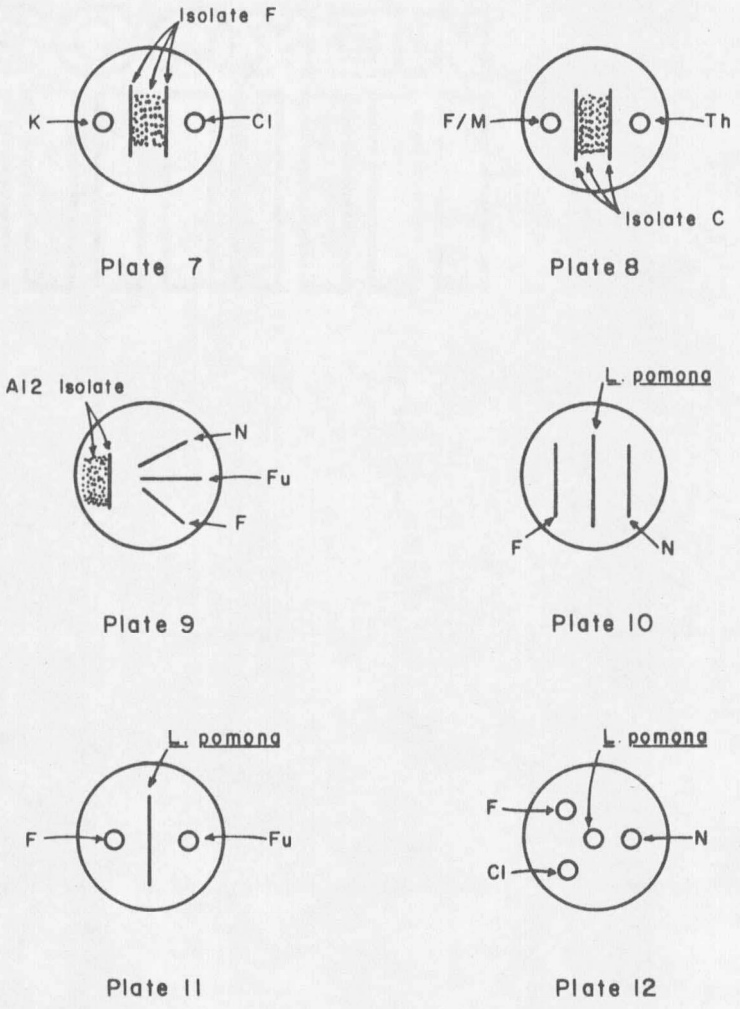



Figure 5. Drug Plate Configurations 7-12 for Study of Drug Efficacy in Purification. Stipling indicates surface streaking of the mixed culture. The straight lines indicate slits in the agar medium. The small circles represent paper drug disks or wells as in Plate 12.

of plate 9. Three slots running at angles to each other but converging at a direction toward the inoculated slot were respectfully filled with 0.1 ml of stock 5-fluorouracil, furazolidone and neomycin. Plate 10 had one center slot inoculated with *L. pomona* and slots on either side filled with 0.1 ml of stock neomycin and furazolidone. Plate 11 had a single slot across the center filled with 0.1 ml of stock furazolidone and 5-fluorouracil. Plate 12 contained a center well inoculated with *L. pomona* and three wells spaced evenly around the center well with 0.1 ml of furazolidone and neomycin in two and a disk of Cl in the other. These plates were incubated at 27° C and examined both macroscopically and microscopically for leptospiral growth and contamination. Agar plugs of apparently pure leptospiral growth were removed and placed in tubes of semisolid EM medium. These tubes were then incubated and checked for leptospiral growth and contamination.

Streak Plates. Streak plates using EM solid agar medium in glass petri plates were used to pick isolated leptospiral colonies. The leptospiral culture was streaked on solid EM medium of sufficient thickness to allow for extended incubation. The plates were then sealed, inverted and placed in a 27° C incubator. They were then checked daily for growth. When macroscopic growth was seen, a representative colony was observed under dark-field and if it appeared pure, the colony was transferred to semisolid EM medium.

Enumeration

Turbidity and Direct Count. Spectrophotometry and nephelometry were used to measure leptospiral concentration. Turbidity measurements were compared to direct count done with a Petroff-Hausser bacterial counting chamber.

Serotyping

Serology was performed on water isolates which were grown to high densities in liquid EM medium. Each isolate was then added in 0.05 ml amounts to 0.05 ml of a 1:25 dilution of each of the sera in a plastic-welled serology plate. This 1:50 sera dilution was incubated at room temperature for 1-1/2 hours. After incubation low power dark-field microscopy was used for examination. The isolates Ag22, A1, A2, A5, Ag23, A11, Ag21 and Kakuchska were tested with antisera. The isolates were tested against eight sera specific against *L. canicola, L. pomona, L. icterohaemorrhagiae, L. ballum, L. pyrogenes, L. grippotyphosa, L. bataviae* and *L. hardjo*. These sera represent eight different serogroups with the first seven being synonymous with the serogroup and *L. hardjo* representing the Habdomadis serogroup. An earlier trial had tested isolate C against sera specific for *L. canicola, L. pomona, L. icterohaemorrhagiae, L. grippotyphosa* and *L. hardjo*.

Enumeration by MPN of Natural Water Systems. Forty-eight isolator vials were filled with semisolid EM medium. Fluorouracil was

added to a concentration of 100 µg/ml. These isolators were then taken to four sites. A most probable number using three tubes and four dilutions was made at each site. Two ml of undilute water was added to each of three isolators. A 1:5 dilution was then made by adding 2 ml of water to 8 ml of phosphate buffer. Another 2 ml was added to three more isolators from the 1:5 dilution. This was continued until a final dilution of $10^{-2.1}$ had been made. After inoculating the MPN at each site, pH and temperature were taken. The isolator vials were then placed in a 27° C incubator and observed daily for leptospiral growth. Site A was at Hood Creek, a high mountain stream. Site B was at Buckskin Creek, a mountain drainage used for summer cattle range. Site C was in the open Gallatin Valley in the Hyalite Creek irrigation system. Site D was also in the Gallatin Valley along Mystic Creek in a combination suburb/agricultural area. The MPN was done in early September. An MF fecal coliform enumeration was also done at each site (2).

Survival Studies

Well Water Chamber Runs. *Leptospira hardjo* and *L. pomona* were grown up in liquid EMJH medium. They were then centrifuged at 5,000 rpm for 30 min and washed in autoclaved well water. They were then suspended in well water to final concentrations of $8.72 \times 10^7$ *L. hardjo* per ml and $7.48 \times 10^7$ *L. pomona* per ml as counted on a Petroff-Hausser bacteria counting chamber. Two sterile membrane diffusion chambers were then filled with 20 ml of the respective leptospiral suspensions.

The chambers were then placed in a 40 liter overflow tank filled with unchlorinated well water. Fresh well water was continually added at a rate of 5 liters per minute. The experimental apparatus and well water were the same as described by McFeters et al. (53). At time zero and thereafter at 2-day intervals, the chambers were sampled. Petroff-Hausser counts and absorbance readings at 420 nm were made.

Laboratory Survival Studies. Survival studies were done in a refrigerator set at 11° C. A plastic bucket was filled with stream water and placed in the refrigerator. Aeration was provided through two spargers supplied with air from an aquarium pump. Sterile membrane diffusion chambers were filled with washed suspensions of *L. pomona* and *L. patoc* and then placed in the bucket. The leptospiral cultures had been grown in EMJH liquid medium. They were spun at 5,000 rpm for 30 min and washed free of medium with sterile water from a small stream. The chambers were sampled daily or at two-day intervals and enumerations made with spectrophotometry, nephelometry and direct cell count.

Chamber Diffusion Experiments. A diffusion chamber with 0.1 µm and 0.45 µm pore size membranes in place as described in equipment was used in these diffusion experiments. The chamber was filled with 20 ml of distilled water and suspended in 2 liters of water from Keg Creek. The suspending water had a conductivity of 475 µmhos as measured on a Lab Line Lectro MHO meter. Fifty µg of dextran 500/ml (molecular weight 500,000) had been added to the 2 liters of suspending water. The total

carbon in the suspending water was then 87.5 µg/ml as measured on a Beckman Laboratory Carbonaceous Analyzer calibrated with 100 µg carbon/ml as glucose. At 15 min intervals samples were taken from the chamber and analyzed for carbon and conductivity.

A separate experiment was done on glucose diffusion. To 2 liters of distilled water, glucose was added to a final concentration of 0.2 mg/ml. A chamber was then suspended in the glucose water as in previous experiment. The glucose concentration was determined by the Glucostat, glucose-oxidase peroxidase system made by the Worthington Biochemical Corp. Samples were taken from the chamber every 30 min and analyzed for glucose concentration. Similar diffusion experiments had previously been done by McFeters and Stuart (54).

# CHAPTER 4

# RESULTS

## Media

Various media were used in these studies. They were tested first for their efficacy then for ease of preparation and sterilization. Some consideration was given to cost and availability of ingredients.

Ellinghausen and McCullough (EM) medium and the Johnson and Harris (EMJH) modification thereof were used in most of the studies. These media were the most dependable and consistently high leptospiral densities could be obtained. Ellinghausen and McCullough medium is clear and colorless. The Johnson and Harris modification is also clear, but it takes on a yellowish cast. Turbidity can be measured by nephelometry and to a lesser extent by spectrophotometry especially in the case of the colorless EM medium. A precipitant formed in EM medium when it was prepared as described by Ellinghausen and McCullough (25). The precipitation problem was eliminated when the modification as described in Materials and Methods was employed. This preparation modification did not appear to change the efficacy of the medium. The major drawbacks to EM and EMJH media are the length of time required for preparation and the filter sterilization requirement of the albumin portion. Filter sterilization was done using Millipore Sterifill Aseptic systems with 0.22 μm pore size membrane

filters (Millipore Cat #GSWP 04700). It was found that the filter sterilized portions of the media often suffered contamination which was reduced by double filtration through two sets of 0.22 μm membranes. The use of autoclaved water in media preparation also reduced contamination. Filter sterilization and media manipulations were performed in a bacteriological hood further reducing contamination problems.

The minimal essential media (MEM-BOH) described by Finn and Jones (45) did not perform as well as hoped. Initial growth and appearance of the Dinger ring was noted in semisolid media. Upon further incubation the Dinger ring generally became less distinct or disappeared altogether. Motile leptospires could be observed upon dark-field observation, however the high densities observed in EM and EMJH media were never obtained. The other minimal essential media (MEM-BT) described in the media section of Materials and Methods yielded leptospiral growth and lasting Dinger rings in semisolid form.

The serum-containing media both worked well. *Leptospira pomona*, *L. patoc* and *L. hardjo* were maintained on these media. Due to difficulties in obtaining serum and sterilization problems, the serum-containing media were of limited use.

Cox's serum-free media had the advantage of sterilization by autoclaving. High densities of leptospires could be grown using SM1 and SM2. These media deteriorated or were depleted rapidly leaving a culture with cellular debris and weakly motile cells. Dinger rings

formed quite rapidly with *L. pomona, L. patoc* and *L. hardjo* in semi-solid Cox's media, but became less distinct and often disappeared upon further incubation. Often the Dinger ring descended through the medium until it reached the bottom of the cultural vessel where it dispersed. Motile leptospires could still be observed throughout the medium upon dark-field examination. Some leptospires were isolated using SM1 semisolid medium. (See Figure 6.)

Isolation

Isolation attempts were unsuccessful using the filtration techniques as well as Smibert's and Cox's methods as seen in Table 3. A total of 129 samples were treated by one or more of these methods. A number of spiral organisms were isolated, but no leptospiral positive isolations were noted. The swinnex modifications and the polycarbonate cells were also unsuccessful in showing leptospiral isolates from the natural systems. Water samples spiked with cultures of *L. pomona* and *L. patoc* were used with the swinnex modifications and the polycarbonate cells. Recovery of leptospires from these spiked water samples was possible although less than 50% of the trials proved successful.

As seen in Table 4, the use of the isolator vials resulted in an overall isolation rate of 70% from 40 samples with an increasing isolation success in the later isolation attempts. Increased isolation success was associated with the use of 5-fluorouracil and EM semisolid medium. A 100 µg/ml concentration of 5-fluorouracil reduced background

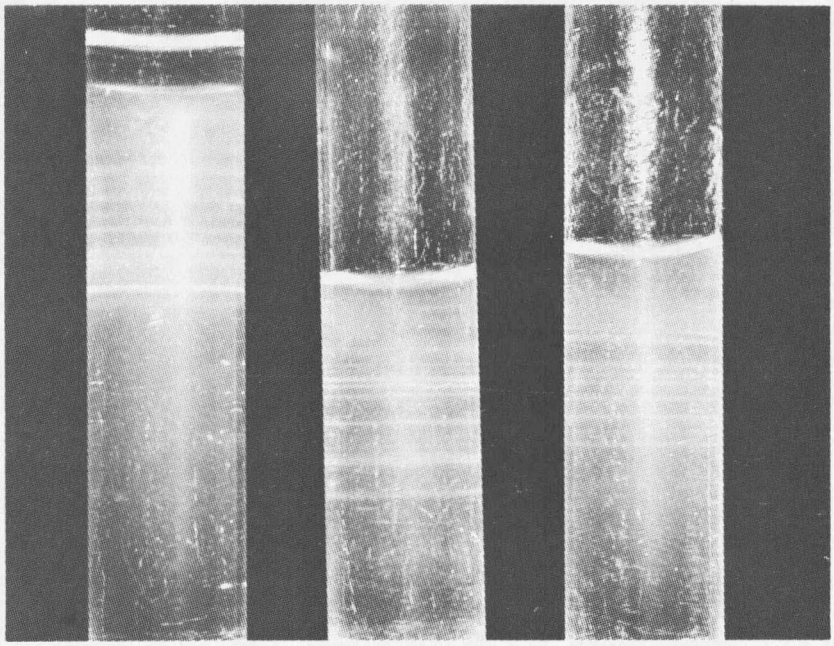


Figure 6. Culture Tubes of EM Semisolid Medium Showing the Dinger Ring Growth Phenomenon. The tube on the left is *Leptospira patoc*, a saprophyte. The center tube contains water isolated spiral organisms which develop growth similar to the leptospiral Dinger rings. The tube on the right is *Leptospira pomona*, a pathogen.

Table 3. Results of Isolation Attempts From Natural Water and Recovery of Leptospires From Water Samples Spiked With *L. pomona*, using various methods

| Methods | Natural Water Samples Tested # | Leptospires Recovered From Natural Water % | Spiked Water[a] Samples Tested # | Spike Recovery % |
|---|---|---|---|---|
| Filtration | | | | |
| 1[b] | 24 | 0 | 5 | 0 |
| 2[c] | 5 | 0 | 3 | 0 |
| 3[d] | 15 | 0 | 10 | 10 |
| Smibert plate | 15 | 0 | 15 | 45 |
| Cox plate | 20 | 0 | 17 | 25 |
| Polycarbonate cell | 20 | 0 | 24 | 45 |
| Swinnex | | | | |
| 1[e] | 15 | 0 | 8 | 10 |
| 2[f] | 15 | 0 | 4 | 24 |
| Isolator vial | 40 | 70 | 50 | 100 |

[a]Spiked water samples gave a concentration of a minimum of $10^5$ *L. pomona* per ml.
[b]One liter sample volumes filtered through sequentially decreasing pore size membranes.
[c]Same as b with concentration done by centrifugation first.
[d]Pressure filtration of 10 ml sample.
[e]Swinnex modification 1.
[f]Swinnex modification 2

Table 4. Results of 40 Isolation Attempts Using the Isolator Vial And Some of the Water Conditions At The Sample Sites

| Sample | Date | Media | Cond.[a] umohos | Temp.[b] °C | Alk.[c] Meq/l | Lepto. Recovered | Drug[d] Used | Site* Description |
|---|---|---|---|---|---|---|---|---|
| A | 4/22 | EM[e] | 184 | 18 | 2.32 | no | no | 2/p |
| B | 4/22 | EMJH | 184 | 20 | 2.32 | no | no | 2/p |
| C | 4/22 | SM 1 | nd[f] | 27 | nd | yes | no | 3/p |
| D | 4/22 | SM 1 | nd | 15 | nd | no | no | 4/p |
| E | 4/22 | EM | 184 | 10 | 2.32 | yes | no | 2/p |
| F | 4/22 | EMJH | nd | 14 | nd | yes | no | 4/p |
| A1 | 5/18 | SM 1 | nd | 15 | nd | yes | no | 5/p |
| A2 | 5/18 | SM 1 | nd | 15 | nd | yes | no | 5/p |
| A3 | 5/18 | SM 1 | nd | 20 | nd | no | no | 1/p |
| A4 | 5/18 | SM 1 | nd | 20 | nd | yes | no | 1/p |
| A5 | 5/18 | SM 1 | 340 | 15 | 3.7 | yes | no | 6m/s |
| A6 | 5/18 | SM 1 | 340 | 17 | 3.7 | no | no | 6m/s |
| A7 | 5/18 | SM 1 | 340 | 22 | 3.7 | no | no | 6m/p |
| A8 | 5/18 | SM 1 | nd | 23 | nd | yes | no | 8/p |
| A9 | 5/18 | SM 1 | nd | 20 | nd | no | no | 8/p |
| A10 | 5/18 | SM 1 | 184 | 15 | 2.32 | no | no | 2/p |
| A11 | 5/18 | SM 1 | nd | 22 | nd | yes | no | 3/p |
| A12 | 5/18 | SM 1 | nd | 27 | nd | yes | no | 3/p |
| A13 | 5/18 | SM 1 | 184 | 17 | 2.32 | no | no | 2/p |
| A14 | 5/18 | SM 1 | 184 | 17 | 2.32 | yes | no | 2/p |
| Ag21 | 7/16 | EM | 354 | nd | 2.57 | yes | yes | 6v/p |
| Ag22 | 7/16 | EM | 186 | nd | 1.54 | yes | yes | 6v/p |
| Ag23 | 7/16 | EM | 382 | nd | 2.38 | yes | no | 6v/p |
| Ag28 | 7/16 | EM | 537 | nd | 4.24 | yes | no | 6v/s |
| History | 8/5 | EM | 270 | 6.3[g] | 2.93 | yes | yes | 6m/s |
| Logging | 8/5 | EM | 228 | 3.4[g] | 2.45 | yes | yes | 6m/s |
| Langahor | 8/5 | EM | 112 | 3.7[g] | 1.04 | no | no | 1/p |
| Fountain | 8/5 | EM | nd | 10 | nd | no | no | 9 |
| Keg | 4/25 | EM | 184 | 17 | 2.32 | yes | no | 2/p |
| Audries | 6/16 | EM | nd | 19 | nd | yes | yes | 6v/p |
| TRMunson | 6/16 | EM | 552 | 16 | 3.21 | yes | yes | 7/p |
| Kakuch/ska | 6/16 | EM | nd | 22 | nd | yes | yes | 4/p |

Table 4. Continued.

| Sample | Date | Media | Cond.[a] umohos | Temp.[b] °C | Alk.[c] Meq/l | Lepto. Recovered | Drug[d] Used | Site* Description |
|---|---|---|---|---|---|---|---|---|
| Bozeman 1 | 8/15 | EM | 271 | 13 | 3.05 | yes | no | 6v/s |
| Bozeman 2 | 8/15 | EM | 271 | 13 | 3.05 | yes | no | 6v/s |
| Bozeman 3 | 8/15 | EM | 271 | 13 | 3.05 | yes | no | 6v/s |
| Bozeman 4 | 8/15 | EM | 271 | 13 | 3.05 | yes | no | 6v/s |
| Black | 9/9 | EM | 184 | 13 | 2.32 | yes | yes | 2/p |
| Mystic | 9/9 | EM | 271 | 11 | 3.05 | yes | yes | 6m/s |
| Buck | 9/9 | EM | 377 | 12 | 4.02 | yes | yes | 6m/p |
| Hood | 9/9 | EM | 78 | 5 | .62 | no | yes | 6m/pr |

*Site description:

1 Springs or seeps
2 Irrigation canals
3 Drain ditches
4 Cattle watering
5 Ponds
6 Running streams
m mountain
v valley
7 Rivers
8 Bog
9 Drinking water
p Polluted
pr Pristine
s Slightly polluted

[a]Conductivity

[b]Temperature

[c]Alkalinity

[d]5-Fluorouracil

[e]See Table 1

[f]No data

[g]Average values

contamination, thus making it easier to make a microscopic search for leptospiral positive isolations.

Purification

The purification procedures which relied on a small membrane pore size of 0.22 μm or less were effective in separating leptospires from large diameter organisms such as *E. coli* and *Bacillus subtilis*. The isolations made from natural water systems contained rods, cocci and spirals capable of passing through these small pore size membranes, thus purification by single membranes was only partially successful.

Bio-Rad membranes with straight pore configuration versus the Millipore membranes with a compressed fiber matrix showed very little difference for purification purposes. Leptospires passed through the Bio-Rad membrane faster than through the Millipore membrane. This characteristic could possibly be used if the membrane were removed at a time when only the leptospires had passed and the spirals had not completely passed the membrane. This was not tried but it appeared to have potential as a method of purification.

Double membrane combinations of 0.22 μm (Millipore) and 0.2 μm (Bio-Rad) were very effective in separating the leptospires from spiral organisms and most of the rods and cocci as shown in Table 5. After a three-month incubation period, cultures of leptospires which were recovered from this double membrane system were free of spirals and rods, however, very small vibrios were seen. These vibrios had a diameter

Table 5. Comparison of Membranes and Membrane Combinations For Use In Isolation and Purification

| Membranes | Isolation Estimated Potential For Natural Samples | Purification of Mixed Cultures Passage Through Membranes | | | |
|---|---|---|---|---|---|
| | | Lepto | Spirals | Rods | Cocci |
| Millipore | | | | | |
| 0.45 µm | fair | 4[a] | 4 | 4 | 4 |
| 0.22 µm | good | 4 | 4 | 2 | 2 |
| Bio-Rad | | | | | |
| 0.2 µm | good | 4 | 4 | 2 | 2 |
| Combinations | | | | | |
| 0.22M-0.22M[b] | poor[d] | 3 | 2 | 0 | 0 |
| 0.22M-0.2B[c] | poor[d] | 3 | 0 | 0 | 0 |
| 0.45M-0.2B | good | 4 | 4 | 2 | 2 |
| 0.45M-0.22M | good | 4 | 4 | 2 | 2 |

[a]No passage 0-4 free passage apparent.

[b]Millipore compressed fiber matrix membrane.

[c]Bio-Rad straight pore configuration membrane.

[d]Prolonged incubation necessary.

of approximately 0.1 to 0.15 μm. They were the only contaminants apparent in these cultures. These vibrios could then be removed utilizing the agar well plates by picking the leading edge of leptospiral growth. An extended period of 15 days or more incubation time was required for the leptospires to pass through this double membrane system.

The other double membrane combinations shown in Table 5 gave the same results as the single membrane systems, thus they were not completely successful for purification.

Agar wells punched in agar medium and filled with contaminated water isolates were effective in separating the leptospires from nonmotile and slower moving bacteria. Often several leading edges of leptospiral and contaminant growth could be seen moving away from the site of inoculation. While the leading edge could be picked, some of the contaminants, especially the spiral organisms, would outdistance the leptospiral growth, making this technique unsatisfactory for purification until the spiral organisms were removed by other means.

The results of the drug study on isolate A1 are given in Table 6. Several drugs and drug combinations were shown to inhibit spiral growth. The drugs Ts, S, K, N and the combination of N, F and Fu inhibited the growth of these spirals, however the initial leptospiral growth was also restricted. Colistin appeared not to restrict the growth of the leptospires while inhibiting the spiral organisms. The

Table 6. The Results of the Drug Study on the Mixed Culture A1 in Semi-Solid EM Medium

| Tube | Drug | Leptospires | | Spirals | | Other Contaminants | |
|---|---|---|---|---|---|---|---|
| | | Day 3 | Day 20 | Day 3 | Day 20 | Day 3 | Day 20 |
| 1 | F/M[a] | 4[b] | 4 | 4 | 0 | 4 | 3 |
| 2 | TH | 4 | 4 | 4 | 0 | 4 | 3 |
| 3 | Cl | 4 | 4 | 0 | 0 | 4 | 3 |
| 4 | NA | 2 | 4 | 4 | + | 2 | + |
| 5 | Ts | 2 | +- | 0 | 0 | 2 | 4 |
| 6 | S | 2 | 4 | 0 | 0 | 2 | 4 |
| 7 | K | + | 4 | 0 | 0 | 2 | 4 |
| 8 | 2X F | 4 | 4 | 4 | 0 | 4 | 2 |
| 9 | N&F | 4 | 3 | + | 0 | + | 2 |
| 10 | N,F&Fu | + | 3 | 0 | 0 | 3 | 2 |
| 11 | 2X N | 3 | 4 | 0 | 0 | + | 2 |
| 12 | 2X Fu | 2 | + | 0 | +- | + | 0 |
| 13 | Control | 4 | 2 | 4 | 2 | 4 | 2 |
| 14 | Control | 4 | 2 | 4 | 2 | 4 | 2 |
| 15 | Cl&Fu | | 2 | | +- | | 0 |
| 16 | 2X N&F | | +- | | 0 | | +- |
| 17 | Cl&2X Fu | | 2 | | + | | 0 |

[a]See Table 2 for abbreviations.

[b]Rating scheme:
4 Too numerous to count.
3 300-400 organisms per field.
2 200-300 organisms per field.
+ 100-200 organisms per field
+- less than 100 organisms per field.
0 None seen.

rods, cocci and other contaminants were very resistant to most of the drugs except for Fu which appeared to inhibit them at a concentration of 200 µg/ml. At a concentration of 200 µg Fu/ml, the leptospires also were slightly restricted as evidenced by reduced leptospire density.

Drug Plates. Examples of the growth and effect of the drugs in these plates are shown in Figure 7. The results of the drug plates set up as diagrammed in Figures 4 and 5 in Materials and Methods are given below. Audries' River sample grew confluently across plate 1. Microscopic examination showed contamination by spiral organisms and some rods and cocci. Plate 2 showed spiral organisms growing out ahead of the leptospires. They were seemingly attracted to the NA disk. After a longer incubation, both spirals and leptospires grew up to and under the drug disks. Isolate Al as well as spiral organisms grew confluently over plate 3 with no apparent inhibition by the drug disks. Growth on plate 4 was very slow and perpendicular to the line between the drug disks, however with prolonged incubation the isolate grew up to the drug disks. Spirals and leptospires grew confluently on plate 5 with no apparent inhibition by the drugs. The Kakuchska isolate was a very slow growing isolate, but it did eventually appear and grow across plate 6 after a confluent growth of spiral organisms had been established. Plate 7 showed growth of spirals and leptospires up to kanomycin, but only leptospires growing up to Colistin. A zone of spiral inhibition approximately 10 mm in diameter was seen around

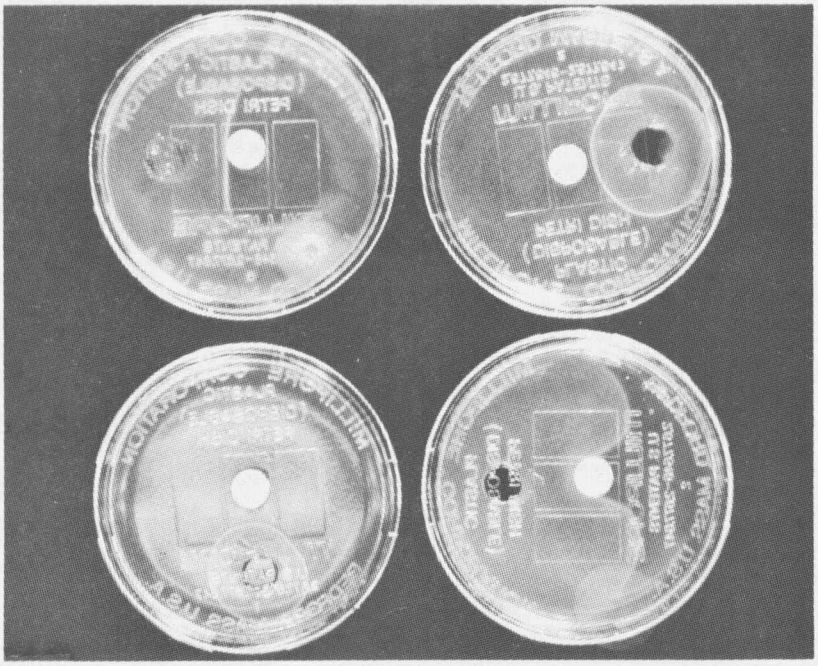


Figure 7. Examples of Drug Plates Used For Purification. Each plate has a 10 μg colistin drug disk on its surface. The upper left plate shows a zone of clearing around the drug disk and a growth front approaching the disk from the left. The zone of clearing represents spiral organism avoidance of the drug and the advancing edge is the slower moving leptospires. The lower right plate shows the leptospiral growth passing under and around the drug disk. The other two plates show leptospiral growth moving away from the site of inoculation.

the C1 disk. No inhibition was noted on plate 8 with the contaminants and leptospires growing evenly over the plate. Plate 9 never did show leptospiral growth, however spirals were found growing confluently over the plate. Plates 10, 11 and 12 were very slow in growing but eventual confluent growth was seen on all plates with no apparent zone of drug inhibition. The leptospiral cultures inoculated in wells and slits in the agar medium were found to appear macroscopically sooner than those streaked on the agar surface. Often areas which were streaked never showed leptospiral growth, but the streak areas were grown over by leptospiral growth through the agar from the slits and wells.

Streak Plates. Streak plates were useful for purification of some isolates. Individual colonies were picked from the streak plate and then placed in a tube of EM or EMJH semisolid media. After macroscopic growth was apparent in the semisolid medium the isolate was again streaked and an individual colony picked upon appearance of colonies on the streak plate. This method of purification was limited by the general observations made by Cox (11) for leptospiral growth on solid media. Other problems in streak plate purification were those of contamination by fungi and of surface and subsurface contamination by motile bacteria before leptospiral colonies could be observed and removed. The spiral organisms were found to be the main source of subsurface and surface contamination, thus, purification by streak plates was generally not successful in the presence of spiral organisms.

Electron Microscopy

Shadow cast and negative stain preparation of isolate C were made by Dr. Ken Lee for use on a transmission electron microscope. An example of one of the isolate C organisms with shadow casting is shown in Figure 8. The figure shows typical leptospiral morphology with the axial filaments (flagella) clearly visible.

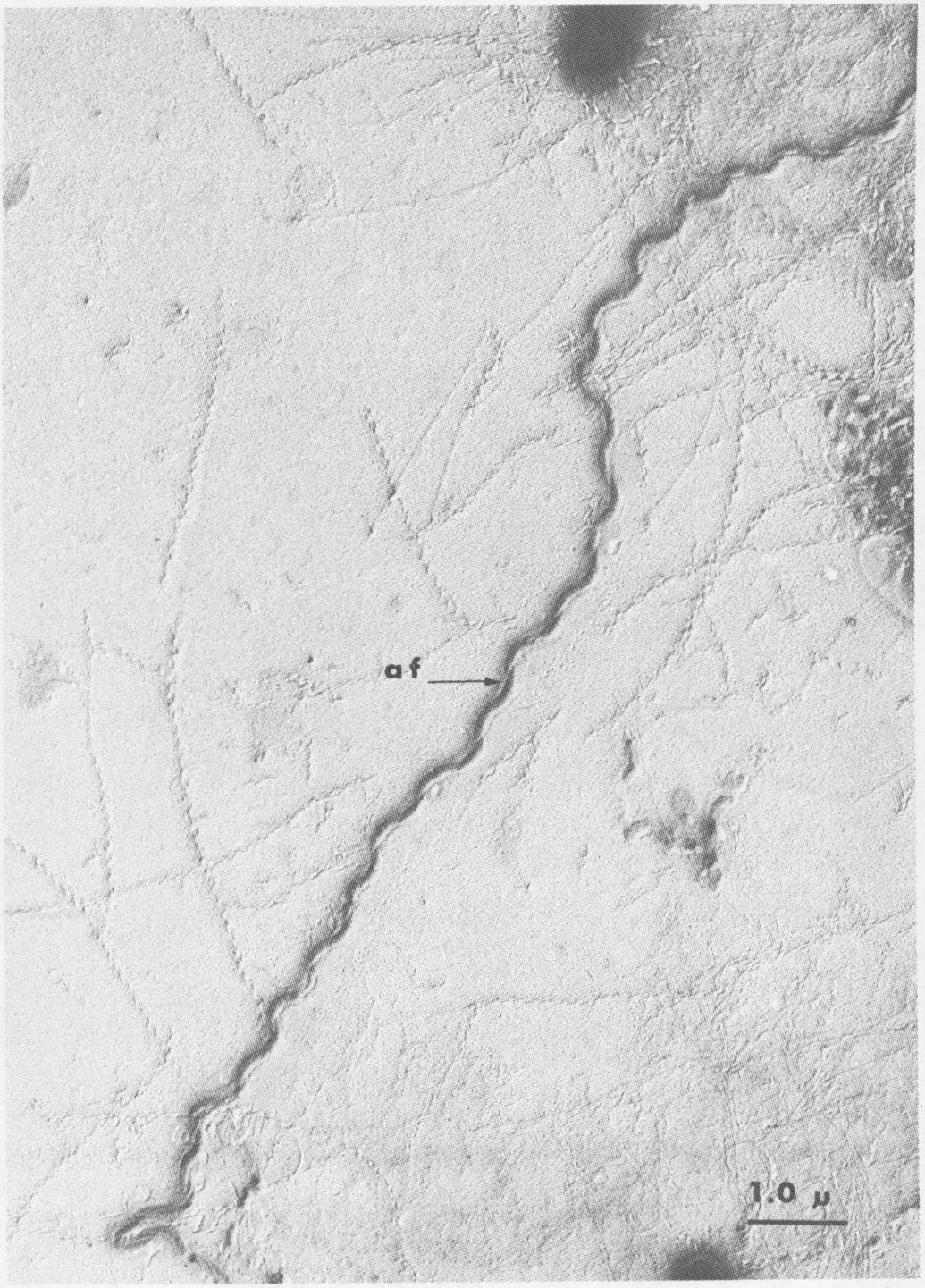
Serotyping

All the isolates, with the exception of Ag21 were negative for the eight sera. Isolate Ag21 showed agglutination with all the sera as well as a negative sera control, thus with no further testing done a nonspecific agglutination was the possible cause for Ag21 agglutination of all the sera as well as the negative control.

Enumeration

Turbidity and Direct Count. Turbidity measurements correlated well with direct counts made on a Petroff-Hausser bacterial counting chamber. Figure 9 shows nephelometry vs. direct cell count from the data of a survival study and Figure 10 shows absorbance. The linear regression coefficient is .97 which indicates a closeness of fit to a linear relation. Limitations are seen at organism concentrations below $10^6$/ml, with both the turbidity and direct count. At concentration below $10^6$ organisms/ml the turbidity was below the sensitivity of the nephelometer which was set with a standard of 20 nephelometry turbidity

Figure 8. Electron Micrograph of Water Isolate C. This shadow case preparation was done by Dr. Ken Lee and has a total magnification of 14,225. The axial filament (af) can be seen running along the cytoplasmic body.


af
1.0 μ

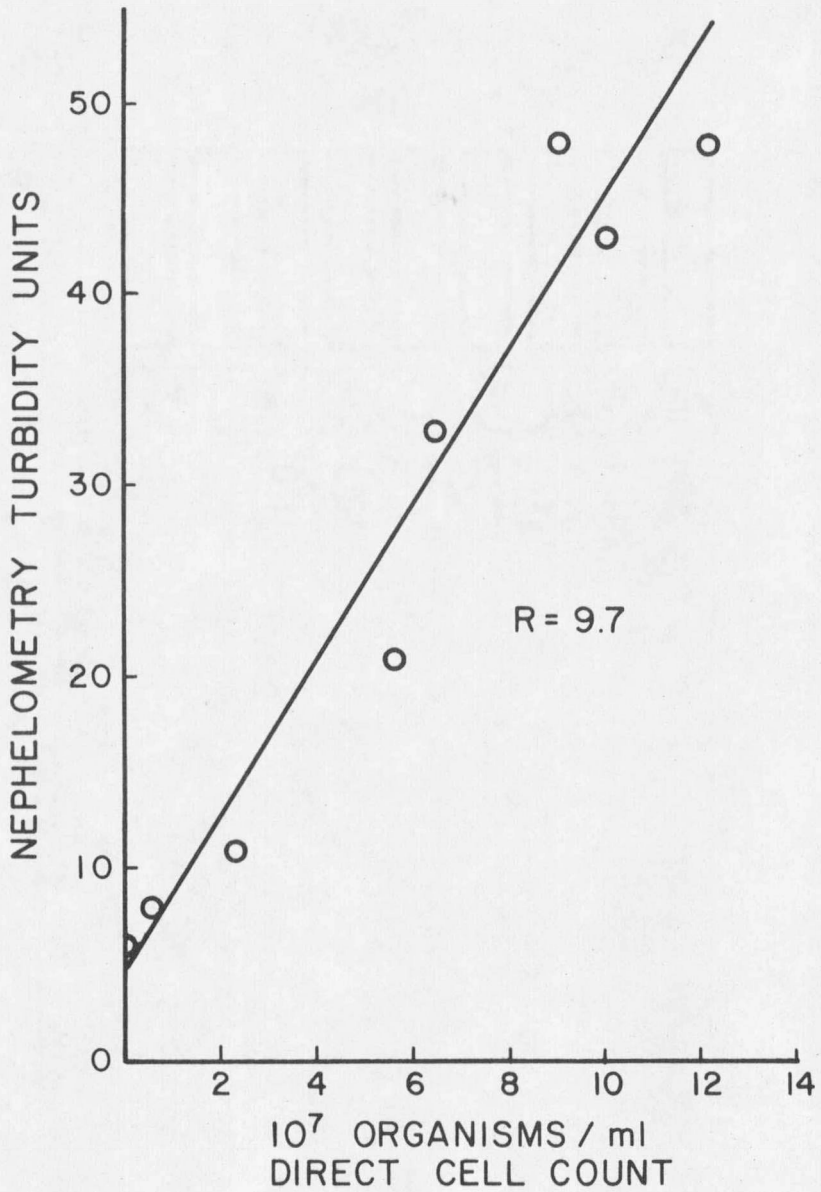



Figure 9. Comparison of Nephelometry and Direct Cell Count as Measures of Cell Densities in a Survival Study of *L. pomona*

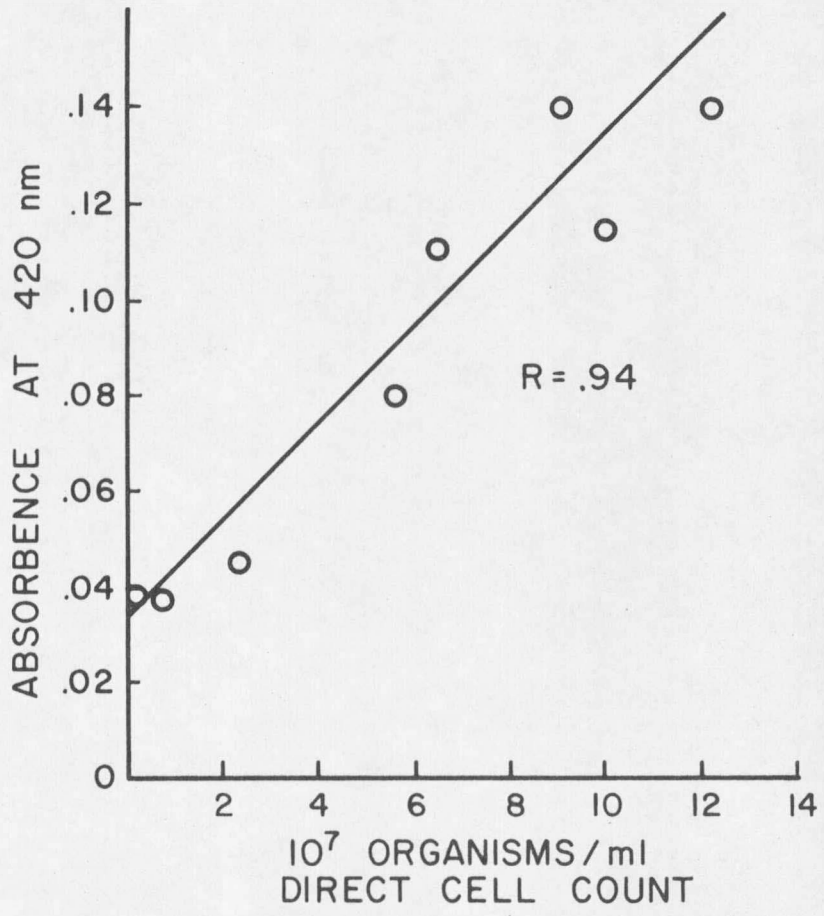



Figure 10. Comparison of Direct Cell Count and Absorbance at 420 nm as Measures of Cell Densities in a Survival Study of *L. pomona*

units. Ellinghausen also used nephelometry for estimation of leptospiral concentration. His data likewise show that concentrations of $10^6$ organisms/ml or more were required before turbidity could be measured (23,27). Larson et al. and Ellinghausen et al. used the Petroff-Hausser bacterial counting chambers to do direct counts. They found that the chambers were only useful above $10^6$ organisms/ml which amount to 1 organisms per counting square (25, 51). Therefore, other methods of enumeration must be utilized for organsm concentration below $10^6$/ml.

Chamber Diffusion Experiments

The diffusion of glucose, inorganic ions and total carbon through the double layer membranes of 0.45 μm and 0.1 μm pore size was similar to that shown by McFeters and Stuart with their single membrane of 0.45 μm pore size. Figure 11 and 12 show the diffusion rates obtained with the double membranes as compared to the diffusion rates obtained by McFeters and Stuart (54).

Survival

The leptospiral response of exposure to well water and to a laboratory water environment are shown in Figure 13. These survival curves based on turbidity and direct cell counts indicate a minimum survival time of 8 days. It was felt that the double membrane system provided a reasonable exposure to the suspending fluid while containing

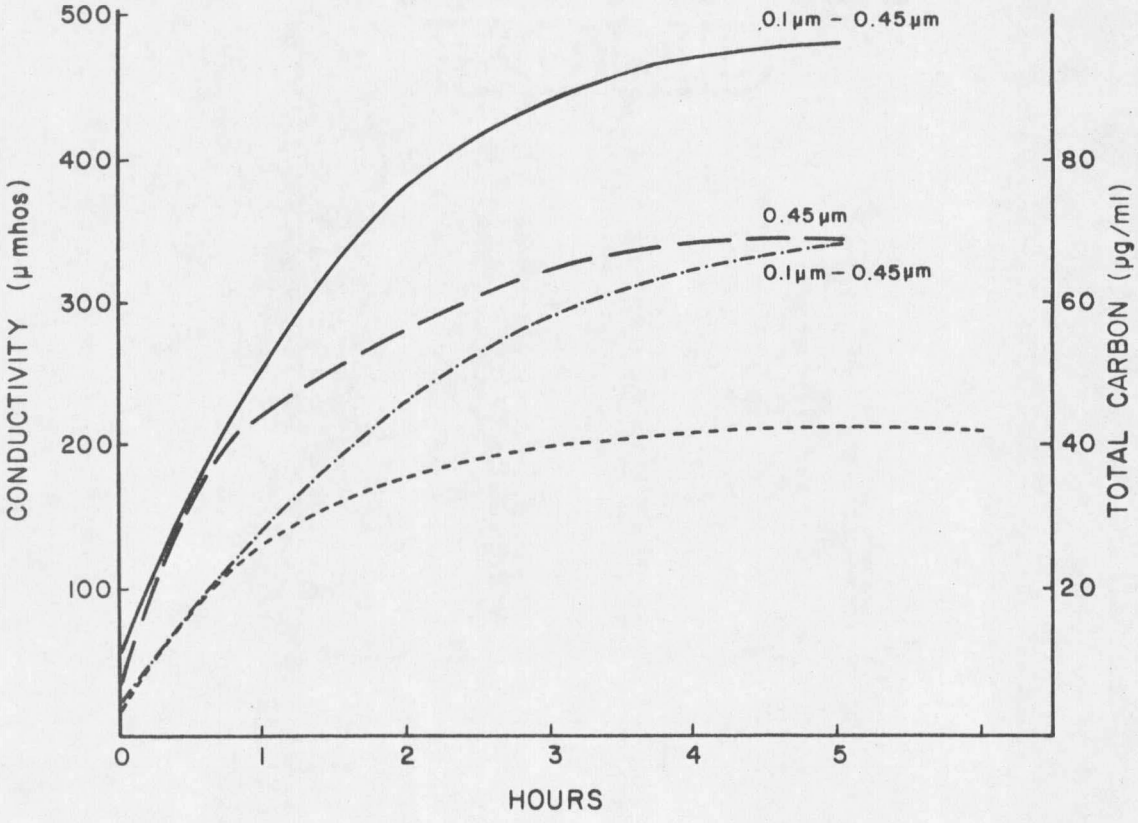



Figure 11. Comparative Diffussion of Carbon-Containing Compounds Across a 0.45 µm Membrane (— — —), a Dialysis Tubing Sac (-----) (54) and a 0.1-0.45 µm Double Membrane Layer (·-·-·). Ion diffusion across a 0.1-0.45 µm double membrane layer (————) as measured by conductivity.

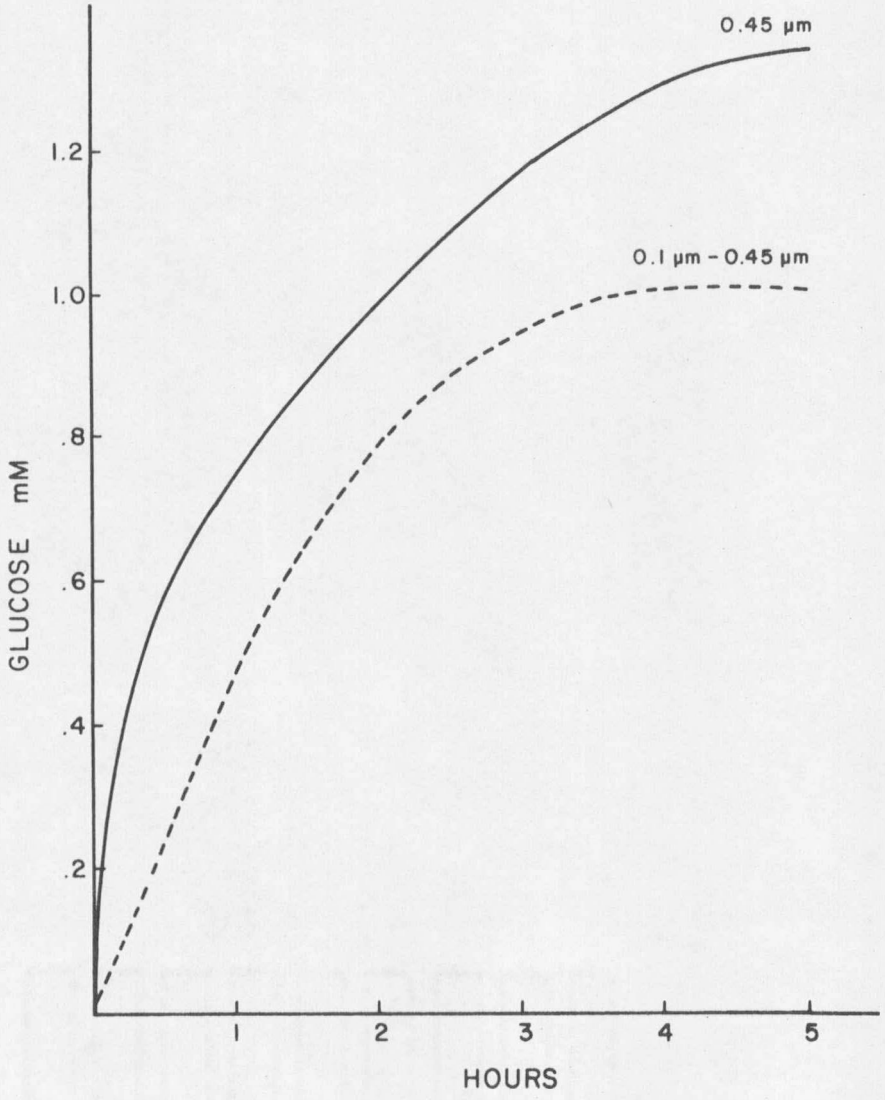



Figure 12. Comparison of Glucose Diffusion Across a 0.45 µm Membrane (54) and a 0.1-0.45 µm Double Membrane Layer

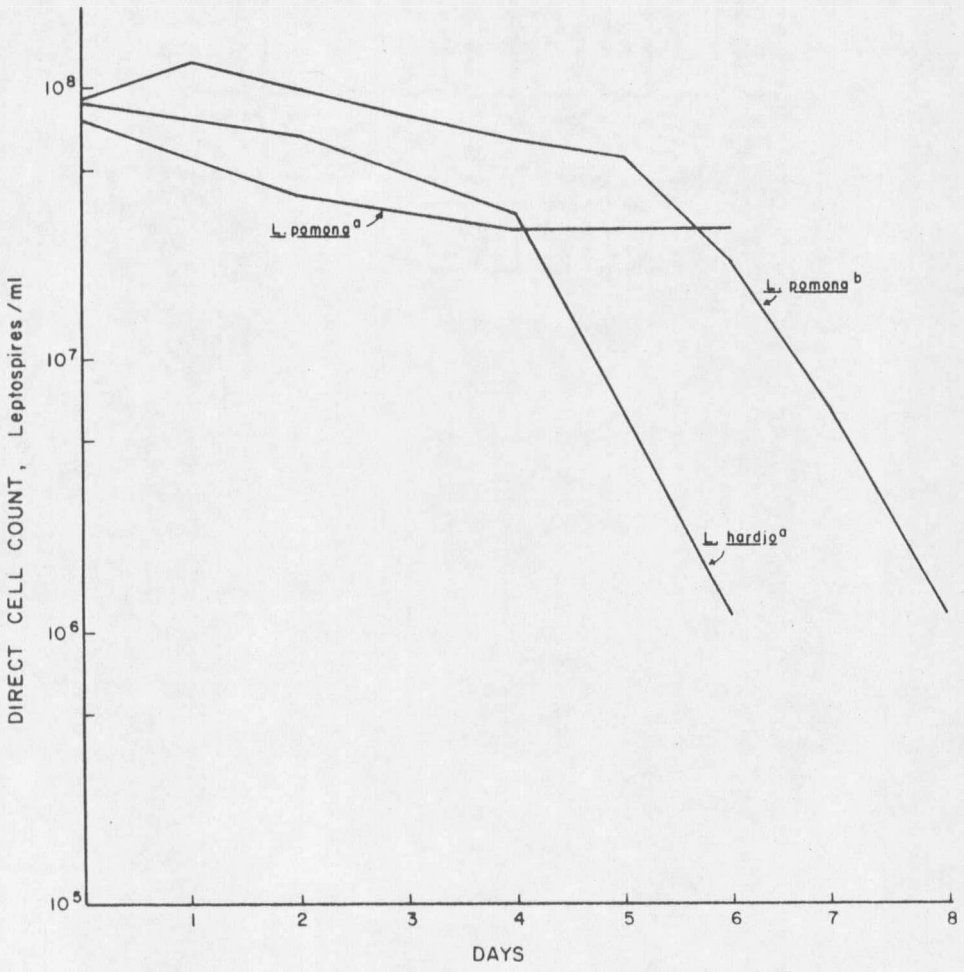



Figure 13. Survival of *L. pomona* and *L. hardjo* in Continuous Flowing Well Water[a] and a Laboratory Water Environment[b] as Measured by Direct Cell Count

the leptospires. The diffusion rates of carbon, inorganic ions and glucose are shown to have reached equilibrium within approximately 6 hours as seen in Figures 11 and 12. At the rate of die-off shown in these experiments, a half-time of 39 hours for *L. pomona* in the laboratory and 35.4 hours for *L. hardjo* in the well water was determined graphically from Figure 13. Assuming this die-off remains stable until die-off is complete, a theoretical survival of 26 days could be expected when the starting population is $10^8$ leptospires per ml as in the laboratory experiments.

Dark-field observations of the leptospires during direct cell counts showed a range of 30 to 60 percent motility per counting square. A maximum viability number is represented by the total number of cells counted and a reasonable minimum viability is represented by 25 percent of the total count based on 30-60% motility which falls below the lowest motility range observed, thus a minimum and maximum viability range may be noted as indicated by the cross-hatching in Figures 14 and 15.

MPN Enumeration From Natural Water

The results of the enumeration of leptospires from four natural water sites by MPN procedures is given in Table 7. These results show a correlation of leptospiral numbers to fecal coliforms and temperature. The first three sites showed fecal coliform counts in the same range, thus the failure to recover leptospires at Hood Creek is possibly due

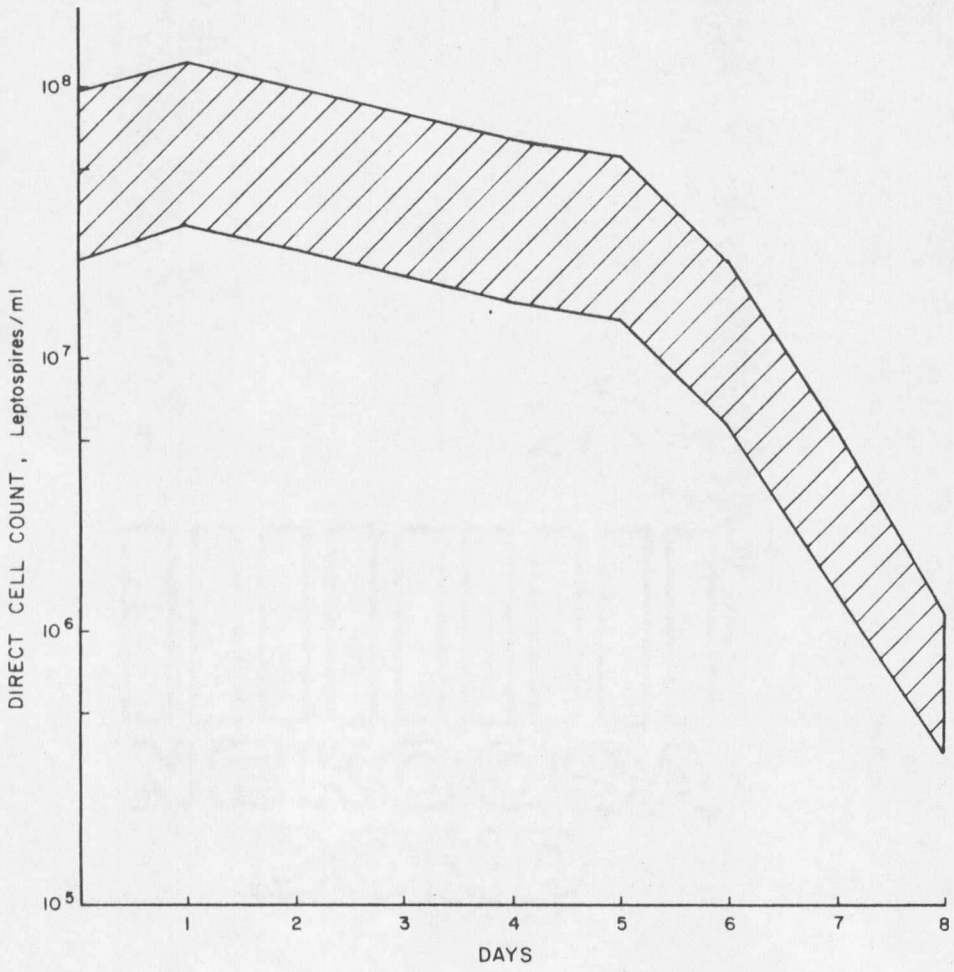



Figure 14. Survival of *L. pomona* in a Laboratory Water Environment With a Maximum and Minimum Viability Range Based on Direct Cell Counts and the Percentage of Motile Cells in the Count

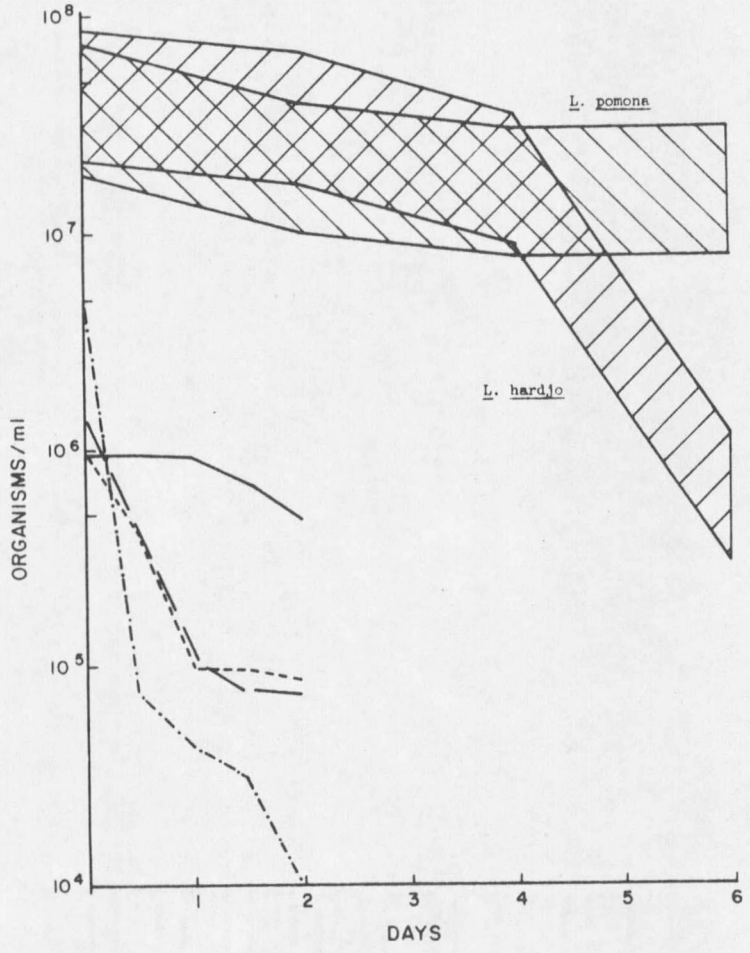



Figure 15. Comparison of *L. hardjo* and *L. pomona* Survival to *Salmonella enteritidis* ser. paratyphi B (·-·-), *Vibrio cholerae* (— — —), *Salmonella enteritidis* ser. paratyphi D (————), and *Salmonella typhi* (-----) Survival (53) in Continuous Flowing Well Water. The *L. pomona* (\\\) and *L. hardjo* (///) areas represent the maximum and minimum viability range based on direct cell count and motile cell percentage of the direct count.

Table 7. Results of a Most Probable Number (MPN) Enumeration of Leptospires at Four Natural Water Sites

| Site | Temp. C° | pH | Fecal Coliforms Per 100 ml | Leptospire MPN Data | | | | MPN per 100 ml | 95% Conf. |
|---|---|---|---|---|---|---|---|---|---|
| | | | | 0 | .2 | .04 | .008 | | |
| Hood | 5.0 | 7.3 | 172 | 0/3 | 0/3 | 0/3 | 0/3 | <1.8 | (0-3) |
| Mystic | 11.5 | 7.2 | 232 | 2/3 | 0/3 | 0/3 | 0/3 | 5 | (1-36) |
| Buck | 12.5 | 7.0 | 152 | 3/3 | 0/3 | 0/3 | 0/3 | 11 | (4-120) |
| Black | 13.0 | 7.2 | 2080 | 3/3 | 1/3 | 0/3 | 0/3 | 23 | (7-210) |

to the cold and pristine water. The high fecal coliforms at Black correlate with the numbers of leptospires recovered.

# CHAPTER 5

# DISCUSSION

Transmission through water systems is a major route of leptospirosis spread.

Thus survival of leptospires in water, especially the pathogenic serovars, is an important consideration in the epidemiology and control of leptospirosis. The effect of pH, temperature, moisture and types of suspending fluids have been examined by various researchers. Okazaki and Ringen found that survival in a pH range of 6.0-8.4 was possible, but above or below these ranges the leptospires were rapidly rendered nonmotile. At freezing temperatures, pH did not appear to affect survival, however, at temperatures of 7-10° C a lower pH was conducive to longer survival than at temperatures of 20-26° C. The opposite pH effect was noted at a higher pH with a longer survival at 20-26° C and decreased survival at the lower temperatures of 7-10° C. Moisture was found to be very important with survival of 30 min in a dry soil and 3 days in a moist soil. A super saturated soil showed survival up to 193 days. Thus Okazaki and Ringen considered a temperature range of 7-26° C and a pH range of 6.0-8.4 to be necessary for survival of *Leptospira pomona* in the environment (59). Diesch found that *Leptospira pomona* survived for a period of 6 days when placed in selas candles and suspended in manure. Survival of 61 days was noted in the manure of an oxidation ditch, 5 days in ditch effluent and 4 days in sludge (16).

Crawford et al. found that *l. pomona* survived for 13 days but not more than 20 days in natural water under laboratory conditions (13). Studies by Chang et al. of *L. icterohaemorrhagiae* showed survival of 8-9 days at 5-6° C, for 5-6 days at 25-27° C and for 3-4 days at 31-32° C in river water. Survival in domestic sewage was 12-14 hours but increased to 2-3 days when aerated (8). Ryu and LIU found that leptospires (*L. australis H* and *L. semarang*) lost their viability above 40° C and were able to maintain viability at 0-30° C (65).

Survival conditions with a temperature range of 7-26° C and a pH range of 6.0-8.4 are shown to correspond with the incidence of leptospirosis (30,31,47). Thus in late summer when water temperature is high and water levels low (resulting in less dilution) an increase in leptospirosis cases is seen.

These survival studies utilized four procedures to determine survival and/or viability of the leptospires. Susceptible hosts such as hamsters, guinea pigs and weanling mice (Webster Swiss) (13,32,59) were used to recover and to check for virulence of leptospires from various environmental models. Leptospiral motility as seen under dark-field was also used as an indication of survival in various testing systems (8,13,16). Some attempts were made to enumerate through the use of direct microscopic counts (8). Cultural attempts were also made in leptospiral culture media to show survival in test systems (13, 17,65).

A system of enumeration remains to be worked out for survival of leptospires in various environmental conditions. The results obtained in our well water and laboratory survival studies were obtained by direct cell counts, with attempts to estimate viability by observation of motility. An assumption of viability based on motility seems reasonable, however as shown by Okazaki and Ringen (59) viability demonstrated by culturing and by motility did not always correspond. These measures of viability were reasonably close in some cases such as survival in supersaturated soil being positive for 193 days based on motility and for 183 days based on culturing. However, in simulated natural conditions, leptospiral motility showed viability at 14 days, while culturing indicated survival only up to a maximum of 6 days. Using the assumption of motility representing viability, a survival curve for a period of time can be generated. The numbers of cells must remain high for counts to be made with counting chambers. A concentration of 10,000-20,000 leptospires per ml is probably the minimum number yielding detection of even one cell on high-power dark-field (81), and a concentration of at least $10^6$ cells/ml is needed before accurate counts can be made (51). The other method used for obtaining density in these survival studies was turbidity as measured by spectrophotometry and nephelometry.

Direct cell counts made with a Petroff-Hausser bacteria counting chamber correlated well with turbidity by nephelometry and spectrometry

as seen in Figures 9 and 10 in Results. As with direct cell counts, turbidity measurements are limited by the need for high bacterial densities and lack the ability to differentiate between viable and nonviable cells. Turbidity measurements become useful for observing increasing population growth and can provide accurate densities provided the organisms do not grow in clumps or chains and do not absorb light (27,48).

With the limits of directing cell counts and turbidity in mind, we can obtain some information on possible survival of leptospires over a period of time as compared to water-borne enteric pathogens in the same water system. Using data obtained by McFeters et al. on enteric pathogen survival in well water (53) and comparing it to leptospiral data obtained in the same system, it can be seen that the leptospires remain at higher densities where the enteric pathogen concentrations fall off quite rapidly, as shown in Figure 15 in Results.

The difference in survival between *L. hardjo* and *L. pomona* may be due to the greater sensitivity of *L. hardjo* to this well water environment resulting in a decreased survival after four days. The Hebdomadis serogroup, which includes *L. hardjo*, has been known to be more difficult to cultivate *in vitro* (1). This apparent fastidious expression upon *in vitro* cultivation may also be reflected in decreased survival as compared to *L. pomona*, a more easily cultivatable leptospire.

The survival studies of the enteric pathogens were begun at lower concentrations. The lower concentrations were used to help prevent cryptic growth from high nutrient concentration obtained from die-off of a portion of the starting population. Lower cell numbers of those organisms can be enumerated with plate culturing and membrane filtration techniques. Thus the problems encountered with enumeration of leptospires with turbidity and direct count are avoided.

A problem which further complicated the well-water survival studies was clumping, making direct counting difficult and changing the light scattering patterns in the turbidity measurements. This clumping phenomenon has been described in certain liquid media (81,82). It was suggested that this clumping may be a survival response giving the leptospires some protection and/or advantage in the hostile well water environment. This clumping may be the result of natural chemical constituents causing flocculation of the leptospires. Clumping was not noted in any of the other survival experiments.

The laboratory survival experiments show results similar to those in the well water as seen in Figure 13 in Results. The starting concentrations of the laboratory experiments were higher in an attempt to follow the die-off in the diffusion chambers over a longer period of time.

Due to the limitations of turbidity and direct count as enumeration techniques for leptospires, other methods of enumeration were

sought. An enumeration technique which showed accurate leptospiral viability and which could be used at low concentrations was needed to follow the survival over a longer period of time. Enumeration by culture techniques has as its major problems long incubation times and difficulty in showing growth with small inoculation sizes. Long incubation time leads to problems of media drying and increased contamination. Enumeration by an MPN technique appears to be the best method for use on low concentrations and to provide information on viability. Most probable number methods rely on the ability to detect growth or the lack of growth in a dilution series (2). Methods of earlier detection were explored. The MPN for fecal coliforms relies on the production of acid and gas from lactose at 44.5° C after an incubation time of 24 hrs (2). This reaction is enzyme mediated, therefore it seemed reasonable to look for an enzyme or enzyme product produced by actively growing leptospires. Two enzymes were studied. Ellinghausen and Sanvik described tributyrinase activity and a method for demonstrating soluble tributyrinase (24). It was thought that tributyrin could be added to a semisolid medium which was inoculated as an MPN. Upon growth, the leptospires would produce the lipase tributyrinase and clear the tributyrin from the media long before the leptospires themselved could be detected. Tributyrin was added to EMJH semisolid medium at a concentration of 2 µl/ml. This produced a cloudy appearance. Upon addition of one ml of a $10^7$ *L. pomona*/ml culture and

incubation at 27° C, the cloudy appearance began to disappear. Upon further incubation the cloudy appearance disappeared. Motile leptospires were noted with dark-field microscopy. Further incubation resulted in a reappearance of the cloudy material rendering the method ambiguous.

Some bacteria such as the genus *Staphylococcus* produce a nuclease which is an extracellular enzyme capable of hydrolyzing DNA and RNA (14). Methods for detecting this enzyme have been worked out (41). DNase test agar was made up and placed in glass petri plates. Drops of *L. pomona*, *L. patoc* and *L. hardjo* were then placed on the plate. Small zones of clearing could seen at the periphery of the drops upon the addition of a cold 25% trichloroacetic acid. However upon prolonged incubation the zones did not increase in size. This enzyme was abandoned as a possible candidate for rapid MPN detection. It is of interest that a zone of clearing was seen. This may indicate the possibility of a exocellular nuclease being present with a low activity and/or concentration. A contaminated system may have been the cause of the clear zone with a nuclease being produced by the contaminant.

The detection of an enzyme or enzyme product may be a possible answer for early growth detection in leptospiral MPN's. However, care must be taken to either find a unique enzyme system or pure culture work must be done to prevent contamination by enzymes from the con-

taminating bacteria. Tributyrinase activity was noted with spiral organisms presumably belonging to the genus *Spirillium*. DNase activity has been shown for *Bacillus*, *Streptomyces*, *Serratia*, *Proteus* and *Staphlococcus* (41). This illustrates the need for a pure culture system or a unique enzyme or enzyme product as a method of early MPN detection.

The detection of a specific cell component may be advantageous in enumeration. Living organisms have a constant concentration of the energy transfer molecule adenosine triphosphate (ATP) (39). A method of measuring this concentration has been developed using a luciferin-luciferase system with a luminescence Biometer (38). This technique was applied to a culture of *L. pomona* grown in liquid EMJH medium. A serial dilution was made with an initial cell concentration obtained by direct counting with a Petroff-Hausser chamber. Adenosine triphosphate was extracted and three replicates at dilutions of $10^{-1}$, $10^{-3}$ and $10^{-4}$ were read on a duPont luminescence Biometer. The results obtained in this single experiment did not show correlation between direct cell counts, serial dilutions or ATP concentrations.

At that time no effort was made to repeat this experiment to find possible problems and to obtain correlating data. This method remains as a promising possibility for leptospiral enumeration. The necessity for pure cultures and the high cost involved are possible limiting factors.

Early detection of an MPN or leptospiral viability in culture may be possible through the use of microscopic observations. Postgate et al. has suggested a method for observing bacterial viability by slide culture (61) The use of microslides (rectangular glass capillaries) as counting chambers and to provide an environment conducive to faster leptospiral growth may have applications for detection and enumeration of leptospires (50).

Macroscopic observations may be indicative of leptospiral growth, however in cases where culture purity is in question such as in *in situ* survival studies, care must be taken in interpreting apparent leptospiral growth. Several observation methods may be employed to facilitate readings of MPN or viable growth. Nephelometry has possibilities as an early detection method in liquid culturing. Growth in semisolid may be shown by Dinger ring formation, however other organisms have been shown to produce the Dinger ring growth phenomenon. The small spiral organisms which are continual contaminants in membrane isolation techniques show a ring growth which closely resembles leptospiral Dinger rings (7). Other researchers have shown a similar growth phenomenon in semisolid medium with other bacteria (15). Leptospiral growth in solid agar medium usually forms opaque growth descending throughout the depth of the agar. Various forms of leptospiral growth in solid agar have been noted including small opaque colonies and translucent colonies with a larger diameter and

veil-like appearance (12,73,78). Spiral contaminants often produce veil-like colonies descending through the depth of the agar. Canale-Parola described these spiral colonies as subsurface, spreading, semi-transparent, unpigmented colonies which were round or nearly so (7).

Various types of media have been used for culturing leptospires. Several types were used in this study (Table 1, Material and Methods) with the objectives of providing a medium capable of promoting rapid growth from small inoculum sizes and high leptospiral densities in a short time span. The first type of medium utilized was a tween-albumin medium described by Ellinghausen and McCullough (EM) (25). This media had been formulated as a substitute for serum-requiring media. The fatty acids found in the tween complex were found to be less toxic yet still available for leptospiral utilization as an energy source, The previous attempts at a serum-free media had incorporated free long-chain fatty acids such as oleic. It was felt that leptospires grown in these media with free long-chain fatty acids developed a resistance to the lytic activity of fatty acids. There was also concern about possible loss of virulence in these media employing free long-chain fatty acids (11,21,68,72). The initial use of EM medium proved unsuccessful. Some leptospiral growth was obtained with *L. pomona*, however, the cultures never did obtain high densities and they appeared to die at a rapid rate. A maintenance medium was sought to prevent loss of the experimental cultures after the poor growth results with

EM medium. Rabbit serum and fetal calf serum was obtained from Dr. Malcolm H. Smith at the Veterinary Science Research labs. Utilizing these sera, Fletcher's and Stuart's semisolid media were made as described (29,76). These media were successful as maintenance media. A modification of EM as described by Johnson and Harris (EMJH) (45) was then made which showed success at cultivation of *L. pomona*, *L. hardjo* and *L. patoc*. EMJH media was then used routinely for leptospiral growth with the only major problem being that of contamination. The need for faster culture growth and to overcome the problem of slow growth with a small inoculum size prompted the use of a minimum essential medium described by Finn and Jones (28). They ran an evaluation of media based on temperature, inoculum and cost and found that a medium (MEM-BOH) incorporating minimum essential medium oleic acid and bovine serum albumin fraction five was the least costly per cell concentrations produced and that higher cell concentrations could be obtained in a shorter time. This medium did not produce well when made as described by Finn and Jones. Several modifications were then tried which included the substitution of tween-80 for the sodium oleate and increasing the albumin supplement. These modifications also proved unsuccessful. A further minimum essential medium was then tried with its formulation based on leptospiral metabolic requirements and comparison of the formulae of various leptospiral media. Ammonium chloride was used as the nitrogen source, tween-80 as the source of fatty acids

(in particular, bound oleic) for energy production, bovine serum albumin (fraction five) as a serum substitute and vitamin B12 as a growth supplement (21,43). Leptospires do not require any amino acids, however it was thought that the amino acids as well as the other nutrients found in Dulbecco's modification of minimum essential media would promote growth at lower cell densities. This minimum essential medium formulation (MEM-BT) did promote the growth of leptospires, although no advantage over EMJH medium was noted. In further attempts to promote growth Em medium was again tried. All the previously made stock solutions were discarded and fresh ones made. A modification in preparation as described in Materials and Methods was utilized to eliminate the precipitation problem which had been encountered. The efficacy of this newly-modified EM medium was found to be very good and EM became the culturing media of choice. The initial reasons for the failure of EM medium to produce expected growth remain unknown, but blame can possibly be placed on one or all of the stock solutions and perhaps a contaminant in the preparatory water.

At one time during these studies, the supply of bovine serum albumin was low. A substitute medium was needed. The media described by Cox (SM1) (SM2) (11) were made and found to be a good growth media, however upon any prolonged incubation rapid death of the leptospires was noted.

The failure of leptospires to grow or the prolonged incubation

needed to obtain growth with small inoculum sizes has been a continual problem in leptospiral culturing (44,81). A 1:10 inoculum size is generally recommended for culture transfer and growth for experimental purposes as an attempt to bypass the small inoculum problem (77,81). Attempts to overcome the difficulty of small inucolum sizes were made in these studies for the purpose of enumeration through MPN techniques which require small inoculum sizes and demand potential growth from each individual cell placed in the medium. Toxic substances may be present in the culturing media. The water used for media preparation is the most probable source for chemical contaminants such as heavy metals, pesticides and herbicides. The use of distilled water or water produced from activated carbon, ion-exchange may prove to be less toxic, however the purity of these waters may be in question. Studies in these laboratories indicate that water obtained from a Milli-Q activated carbon, ion-exchange system may contain toxic materials and retard growth of bacteria when media are prepared utilizing this water.

Early researchers found that attempts to cultivate leptospires on sterile human feces failed until the feces had first been inoculated with *Bacillus coli (E. coli)*. Thereafter, leptospiral growth was noted (20). Based on this observation, media conditioning was attempted by growing *E. coli* and/or leptospires in liquid EMJH or EM. After a short period of incubation, the medium was centrifuged and then refilter sterilized. This medium was then reinoculated with leptospires and

the rate of growth was observed as compared to untreated medium. The treated medium did support growth of leptospires, however no medium conditioning advantage was noted as compared to untreated medium. Johnson et al. found that the addition of sodium pyruvate (100 μg/ml) was effective in promoting growth with small inocula and in nutritionally fastidious serotypes (44).

Leptospires appear to be microaerophilic since they form growth rings at 3-10 mm below the surface in tubes of semisolid or solid medium. These rings (see Figure 6) are thought to form based on nutritional response to oxygen partial pressure and as a response to metabolic waste products diffusing away toward the bottom of the tube, thus a narrow band of growth forms with a possible upper limit being defined by oxygen partial pressure or $CO_2$ partial pressure and the lower limit by toxic metabolic or medium substances diffusing away from the growth mass in the Dinger ring. Often multiple rings may occur with as many as 20 discrete rings forming as a function of incubation time. Prolonged incubation results in increased ring formation. Several explanations for these multiple rings may be made. The nutrient response may be different for various strains, thus these individual strains may seek their own levels based on nutrient gradients. This idea corresponds with the increased ring formation found after prolonged incubation indicating that individual strains may show up as discrete Dinger rings after the appropriate incubation allowing

growth dense enough to be observed macroscopically as a ring. Another possible explanation for these multiple growth rings may be migration of leptospires to seek new nutrient sources. The prolonged incubation may allow diffusion of gas to an increasing depth pushing the oxygen sensitive organisms to increasing depth, but leaving behind those leptospires which have developed an "oxygen tolerance."

Various cultural observations were made which may be explained by one or more of the above discussions. The use of SM1 or SM2 semi-solid medium was abandoned because the Dinger ring which formed rapidly began to descend through the medium until it reached the bottom where it dispersed. This may indicate that the leptospires migrated through the medium in response to nutrient depletion and, upon reaching the bottom of the tube, all the nutrients were gone, thus the growth ring dispersed. The Dinger rings remained visible in a more complete medium such a EMJH or EM, which may indicate a better nutrient source.

In solid medium, several growth fronts could be seen for leptospires inoculated in wells cut in the agar. These growth fronts could possibly be identified as strain differences responding to nutrient gradients and displaying varying degrees of motility.

The apparent microaerophilic nature of leptospires was explored as a means to decrease the growth time and to promote growth from small inoculum sizes. The atmospheres above the leptospiral cultures were manipulated by evacuation and then replenishing with various gas

mixtures with the volumes added controlled by manometric measurements. Several researchers have explored the effect of $CO_2$ and oxygen upon the growth of leptospires (23,85).

Various atmospheres were attempted in these studies including varying percentages of $CO_2$, oxygen, nitrogen and decreased partial pressures through varying evacuation pressures. These studies did show response of leptospiral growth to various atmospheres. However, due to impure gas mixtures and other experimental problems, no definitive data were obtained. These limited studies indicated that changes in oxygen partial pressure may be beneficial in growth promotion, although more research is needed.

The isolation of leptospires was successful in a variety of environments. Most of the isolations were made in free running water or standing water, however limited isolation attempts in moist soil also proved positive for leptospires. Other researchers have detected leptospires in soils and have speculated on the role soil plays in survival and transmittance (4,37,42,59). Isolation was possible in water over a wide temperature range (3.5°-27° C), a wide quality of water (pristine to polluted) and from stagnant as well as running water, thus the ability to detect leptospires in moist soils as well as these diverse water conditions indicates a wide adaption and diversity in the environment.

The enumeration study (Table 7, Results) based on most probable

numbers at various sites ranging from cold (5.0° C), "pristine" waters to warmer (13° C), "polluted" waters does indicate variation in population densities with variation in temperature and water quality. Hood Creek, a high mountain site with cold water (5° C) draining natural and undisturbed land, failed to show leptospires. A high fecal coliform count was noted at this site which might indicate pollution, however, based on previous fecal coliform counts and the general appearance of the sample site, no obvious pollution of pollution source could be detected. The high fecal coliform count may have been the result of wild animal activity in the area. The next site, Buckskin Creek, also a high mountain stream, gave positive leptospiral isolations with an MPN of 11 per 100 ml. Buckskin drains a larger area, with greater land use including intensive summer cattle grazing and some human activity. As noted from Table 7, the temperature was also higher than that of Hood (12.5° C). The positive isolation of leptospires at Buckskin may be expected based on the increase in temperature, the possibility of available nutrients and the susceptible host animals providing a possible reservoir. The Mystic site, which was in the open valley floor in a multiple land-use area, also showed positive leptospiral isolation with an MPN of 1.8. Mystic was considered to be only slightly polluted and had a larger water volume and faster flow than Buckskin. These characteristics would indicate the possibility of a leptospiral population, but because of lesser pollution and larger volume and faster

flow, fewer leptospires might be expected as compared to Buckskin. The fourth site was situated in the irrigation system branching off Hyalite Creek. There was a herd of cattle bedded and grazing at the sample site. Cattle were seen standing and drinking in the water at the sample site and heavy concentrations of manure were on the bank as well as in the irrigation canal. The high fecal coliform counts (2080/100 ml) reflect the herd pollution. The polluted water as well as the higher temperatures would suggest higher probability of finding large leptospiral populations. The leptospiral MPN is the same as Buckskin, however, the additional positive tube at $10^{-0.7}$ dilution indicates a larger number of leptospires at this site.

Based on this study, pollution and temperature seem to correlate with probable leptospiral densities. More work is needed to obtain definitive information in regard to water quality, temperature and leptospiral densities. This type of information will contribute to the understanding of survival.

The leptospires which have been isolated appear to belong to the "Biflexa Complex" based on the limited serology and the isolation environments.

A method using membrane filters was chosen for isolation attempts. Previous researchers were successful in isolating leptospires by filtering surface water samples through membranes of 0.45 µm pore size (6,63,79). Based on attempts at filtering water samples and

water samples spiked with leptospires, it was felt that better isolation might be achieved if the leptospires were allowed to pass through the membranes in what might be described as a chemotactic response. Smibert described a technique using membranes and solid agar medium for separating leptospires from a contaminating bacteria by taking advantage of chemotaxis and the physical size and shape of the leptospires (71). Their extreme motility and their thin thread-like morphology allowed them to swim through pore sizes too small for contaminating bacteria to pass, thus purification was effected. Leptospiral growth on semisolid media has been shown to be better than on solid media (21,81). Based on this knowledge, a method permitting utilization of Smibert's decontamination procedure with movement into semisolid instead of solid medium was sought. The two swinnex modifications and the polycarbonate cell as described in Materials and Methods served as prototypes for the isolator vial, which gave the best results in isolation attempts.

Isolation using the isolator vial was first accomplished with EMJH and SM1 media, however better results were seen when EM medium was used and 5-Fu was added to a 100 µg/ml concentration. A 2 ml water sample was placed in the isolation chamber. The isolator vial was then incubated at 27° C with evaporation of the water sample being accelerated to help concentrate the leptospires. Later on, it was felt that evaporation was not necessary. The isolation success of these isolator

vials was probably based on several factors. Sampling could be made at the sampling site with immediate transfer of the sample to the isolation chamber where it was exposed to a nutrient environment. The medium level was kept as low as possible with a miniscus forming at the medium membrane interface. This was done to promote a natural gravity flow of sample through the membrane thus concentrating the organisms in the vicinity of the membrane. It was felt that the diffusion of 5-fluorouracil upward through the membrane reduced the susceptible bacterial population, thus reducing the problem of antagonistic interactions among the mixed population which had been noted by other researchers (8). The mixed population which was capable of passing the membrane appeared not to influence the leptospiral growth rate or population size. The immediate exposure to a nutrient environment, reduction in the antagonistic population and the natural concentrating effect were felt to be positive factors facilitating isolation success with the use of the isolator vials.

An observation of high coliform bacteria counts in an unpolluted pristine stream prompted laboratory studies on the cause of these high coliform counts. The supernatant from an exenic *Chlorella* algal culture was used as a growth medium for various coliform bacteria. *Chlorella* mats were found to be associated with the sample sites having high coliform counts (52). This same *Chlorella* supernatant was tested with *L. hardjo, L. pomona* and various water isolators. The leptospires

were found to be surviving at low densities over a two-month period in the supernatant after three passages into fresh algae supernatant. The inoculum after the third passage was considered to be free of any leptospiral medium from the initial inoculation, thus the algae supernatant was found to be non-toxic and perhaps providing nutrients for limited leptospiral growth.

The survival of pathogenic leptospires in soil (37,59), in water (4,8,66) in animal wastes (16,17) and in algae supernatant indicates an adaptability to *in vitro* survival. This *in vitro* survival expands the epidemiological complexity. The harboring of leptospires in soil and water has been indicated by increased leptospiral case incidence during flooding and heavy rains (4,42). The transmission of leptospires is probably accomplished through exposure to contaminated water, however the leaching of leptospires from the soil and wastes as well as the survival in water and continual contamination by animal reservoirs provide potential continuous leptospiral population in the water.

# CHAPTER 6

## SUMMARY

Leptospiral cultural media and methods were examined in an attempt to promote leptospiral growth from a small inoculum size and to obtain high cell densities in a short time period. Three basic types of media including serum albumin-tween, minimum essential medium, and whole serum medium were used. Ellinghausen and McCullough's albumin-tween medium was found to give the best overall results for rapid growth and culture maintenance, thus, this medium became the medium of choice.

Limited studies were made to find methods for early detection and faster leptospiral growth for the purpose of enumeration through the use of most probable numbers techniques. These methods included possible enzyme assays, atmosphere manipulations and media conditioning. None of these methods were used, although they may deserve further investigation.

Leptospiral survival was studied at two sites using membrane side wall diffusion chambers. Survival of *L. hardjo* and *L. pomona* were studies in a continuous well water flow with a stable temperature of 11° C. A minimum survival of 6 days was noted for both *L. hardjo* and *L. pomona*. A die-off half-time was obtained graphically for *L. hardjo* and found to be 35.4 hours. This gives a projected theoretical survival of 23.6 days with the initial experimental concentration of $10^8$ cells/ml and with stable die-off being assumed. Previous studies

had been performed with indicator bacteria and some pathogenic bacteria in this well water system. Average die-off half-times for coliform and enterococci indicator bacteria were found to be 17.0 and 22.0 hours, respectively. The pathogenic bacteria *Shigella flexneri* and *Salmonella enteritidis* ser. paratyphi D were found to have die-off half-times of 26.8 and 19.2 hours, respectively (53). Thus, *Leptospira hardjo* appeared to have a longer survival potential than both indicator and enteric pathogenic bacteria tested.

*Leptospira pomona* survival was studied in refrigerated stream water held at 11° C in the laboratory. A minimum survival of 8 days was noted. A graphic die-off half-time of 39 hours was calculated giving a theoretical survival of 26 days with a starting concentration of $10^8$ cells/ml and stable die-off being assumed.

Leptospiral isolation was attempted using various membrane filter systems. Techniques which forced the samples through the membranes were found to be less effective than a system which allowed the leptospires to pass through the membrane by natural motility in a chemotactic response. Membranes of 0.22 μm pore size were found to give the best isolation results based on recovery and fewer contaminants. A device made from a 4 dram vial and 1/2" polycarbonate tubing was used to allow leptospires in a sample to move across a membrane into semisolid culture medium. It was found that the addition of 100 μg/ml 5-fluorouracil to the semisolid isolation medium helped control

background contaminants, while not affecting the leptospiral growth and recovery. Seventy percent recovery from natural water was achieved through the use of this isolation device.

Purification of these isolated leptospires was found to be necessary, because of the large motile population capable of moving across the membrane. A purification system which relied on leptospiral motility and drug usage was found to be satisfactory for removing the major problem contaminants which were found to be small spiral organisms presumably belonging to the *Spirillaceae*. The drug found to be most effective against these spiral organisms was colistin. Inoculation of a solid agar plate in a well or slit was found to promote faster establishment of growth. The drug was placed a distance from the inoculation site and purification effected by the motile leptospires producing a growth front moving toward the drug with the spirals avoiding the drug. Further purification was done with streak plate methods and membrane passage.

Some of the isolated leptospires were tested serologically with a limited battery of specific antisera. None of these isolates were found to react with any of the sera with the exception of isolate Ag 21 which appeared to produce a nonspecific agglutination with all the sera including leptospiral negative sera.

Leptospiral recovery was possible from moist soils, rapidly running streams, deep flowing rivers, ponds, bogs and cattle watering

troughs. Enumeration of leptospires at four natural water sites showed that the relative numbers of leptospires varied with pollution and temperature. Sites with high pollution and temperature (13° C) showed greater leptospiral numbers than those of lower pollution and temperature (5° C) with "pristine" sites showing no leptospiral recovery.

The ability to recover leptospires from a variety of natural environments reflects a possible wide distribution. Survival in various natural environments such as soil, water and wastes as well as the ability of *L. hardjo*, *L. pomona* and water isolates to reproduce in the supernatant of an exenic Chlorella culture in the laboratory gives credence to this wide distribution. The possible harboring of pathogenic leptospires in these various environments may assure a continuous infectivity of surface waters.

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