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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THOMAS DEAN ROBERTS

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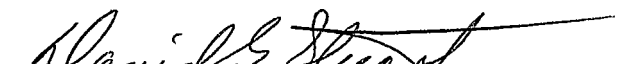
Approved:



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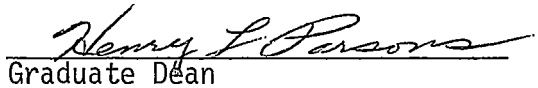

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ABSTRACT

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 antiμ. 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