DEVELOPMENT OF A FEASIBLE METHOD TO DETECT PATHOGENIC LEPTOSPIRA BACTERIA IN ENVIRONMENTAL WATERS

Roger S. Fujioka
and
Audrey Y. Asahina

WRRC Project Completion Report: WRRC-98-09

December 1998

Prepared for:

The Chamber of Commerce of Hawaii
1132 Bishop Street, Suite 200
Honolulu, Hawaii 96813

Account Number: 4-34678
Project Period: 1/1/97 to 6/30/98
Principal Investigator: Roger S. Fujioka

Water Resource Research Center
University of Hawaii at Manoa
2540 Dole Street
Honolulu, Hawaii 96822

I. INTRODUCTION

A. Leptospirosis: A Zoonotic Disease

Leptospirosis is a disease of man caused by a pathogenic bacteria belonging primarily to the species *Leptospira interrogans*. Since pathogenic *Leptospira spp.* are principally maintained in animal populations and transmitted to humans, leptospirosis is classified as a zoonotic disease. Furthermore, bacteria belonging to the genus *Leptospira* are commonly called spirochetes due its characteristic cork-screw shaped cell structure. It is this characteristic shape and cork-screw action which allows this bacteria to burrow and penetrate soft tissue or broken skin and cause infection in man and animals. Two to twelve days after infection *L. interrogans* can multiply in various internal organs resulting in a multiple array of disease symptoms. In humans, leptospirosis can manifest itself in two clinically distinct forms. The first is a self limiting disease characterized by the onset of flu-like symptoms which may include fever, chills, and muscle aches. However, if not treated in the early stages with antibiotics, leptospirosis may progress into a more serious illness called Weil's Disease whose symptoms include jaundice, hemorrhage, and renal failure. In such severe situations, death may occur in approximately 5 - 10% of all cases (Farr, 1994). In animals such as cattle, infection may lead to stillbirths, infertility and decreased milk production, resulting in great economic loss (Theirmann et al., 1984).

In some animals, such as rodents, *L. interrogans* can establish a long term infection with no apparent symptoms. In these animals, the kidneys are the primary target organ where the leptospira multiply and this results in the excretion of viable pathogenic leptospira via urine into the surrounding environment (soil, stream, mud). Infected animals (cattle, pigs, horses, sheep, mongoose, rodents) can thereby contaminate the environment used by humans and are believed to be the major source of human infection. Infection in man and animals proceeds by either coming into direct contact with the infected urine or indirectly via water or soil contaminated with the infected urine. A copy of the Department of Health, State of Hawaii's brochure on the transmission and prevention of this disease is included as Attachment One. Thus, a wide variety of animals and human appear to be susceptible to infection with *L. interrogans*. Since human to human transmission of leptospirosis is extremely rare, humans are considered as terminal or accidental hosts.

B. Leptospirosis Worldwide

Within the past several years there has been a steady increase of human leptospirosis cases worldwide which has resulted in the Centers for Disease Control and Prevention (CDC) to recently reclassify leptospirosis as an emerging infectious disease for man. Emerging and reemerging infectious diseases have been defined as those diseases which are currently increasing in incidence or threatening to increase in the near future. Changes in the environment brought about by human activities which affect the ecosystem and in the interaction of man with other living things have been identified as a major cause in the emergence of infectious diseases (Patz et al., 1996). Many newly emerging diseases in humans are actually long standing zoonoses of animal host species that surface in human populations after climatic, environmental, or ecosystem changes. Leptospirosis is a good example of an old zoonotic disease which is causing

more illnesses in man due to environmental changes and due to the greater interaction of human with animal habitats.

Although leptospirosis is more commonly associated with tropical climates, recent outbreaks have occurred in temperate regions during the warm and rainy seasons as well as in subtropical areas. Nationally, leptospirosis outbreaks have been reported after heavy rains and flooding in the state of Iowa (Fuortes et al., 1994). Internationally, the most recent leptospirosis outbreak occurred in 1995 in Nicaragua where 300-400 people contracted leptospirosis and at least 13 patients died from respiratory distress and pulmonary hemorrhage (MMWR, 1995). India has also observed a reemergence and a steady increase of this disease occurring within the past two decades in areas such as Tamilnadu, Kerala, and the Andamans. Although most outbreaks of leptospirosis have occurred in countries outside the United States, recent studies have described a newly described population at risk for the contraction of leptospirosis in the United states as inner-city residents (Vinetz et al., 1996) due to the ever increasing population of rodents in these areas.

C. Leptospirosis in Hawaii

Leptospirosis is most often observed in tropical climates and this observation is supported by the fact that traditionally, the state of Hawaii reports more than 50% of all leptospirosis cases to the Centers for Disease Control and Prevention (CDC). This is of significance because the population of Hawaii is about one million people, making it one of the lowest in all 50 States. A recent five year survey (1992-1996) conducted in the State of Hawaii (Sasaki, 1997) has reported that there is as much as a 100-200 fold difference in the incidence of leptospirosis in Hawaii as compared to the United States. The average annual incidence in the United States as a whole is 0.05 cases per 100,000 individuals. This statistic is believed to be a significant underestimation of the actual incidence of leptospirosis. Furthermore, studies conducted in Hawaii reveal that active as opposed to passive surveillance for leptospirosis has resulted in a five-fold increase in the incidence of this disease (Sasaki et al., 1993). Thus, leptospirosis is believed to be one of the most under-reported diseases in the United States and throughout the world.

In recent years, leptospirosis in Hawaii has been most often associated with recreational exposures to freshwater. A recent publication (Katz et al., 1997) reported an outbreak among military personnel and visitors to the islands who engaged in wading or swimming in freshwater streams. Kayaking has also been reported to be a risk factor in some areas of the world (Shaw, 1992) which is of relevance for any individual engaging in this outdoor activity in Hawaii. Between the years 1992-1996, Kauai had the highest annual mean incidence rate of all the Hawaiian islands and Oahu the least. Incidences of leptospirosis are more often observed during the summer months (June - August), when more people participate in outdoor activities and enter environmental streams. However, people in occupations which involve working with animals (farmers) or environmental waters (taro farming) are at high risk for the contraction of this disease throughout the year.

II. IDENTIFICATION OF RESEARCH NEEDS AND PROJECT GOALS

A. Preventing the Transmission of Leptospirosis: Role of Environmental Waters

It is clear that the transmission of leptospirosis in humans occurs as a result of contact with environmental waters contaminated with leptospira bacteria. Thus, the first identified need in the development of an effective prevention and management program for this disease is to recognize the important role of environmental waters in the transmission of this disease. The second identified need is the recognition that current monitoring data cannot provide the necessary data to determine which streams are contaminated with pathogenic leptospira and which streams are not contaminated with pathogenic leptospira. This kind of information is required to effectively implement a program to prevent the transmission of leptospirosis.

The limitations in the current methods to reliably monitor environmental waters for the presence or absence of pathogenic leptospira are due to the following problems: 1) Using cultural methods, the growth characteristics of pathogenic and saprophytic leptospira are very similar and cannot be sufficiently differentiated. 2) The physiological and nutritional properties of pathogenic and saprophytic leptospira have not been adequately characterized and therefore for practical purposes all pathogenic leptospira are grouped into one species (*L. interrogans*) and all non-pathogenic and saprophytic leptospira are grouped into one species (*L. biflexa*). However, based on a sub-typing method using antisera, pathogenic leptospira can be differentiated into more than 200 serovars and saprophytes into more than 60 serovars. As a result, there are numerous recognized strains or varieties of pathogenic and saprophytic leptospira based on antigenic differences on the leptospira cells. 3) In environmental waters, saprophytic leptospira bacteria are always naturally present in high concentrations in all streams in Hawaii while pathogenic leptospira are believed to be present sporadically and at much lower concentrations. Thus, when culture method is applied to environmental waters, the growth of saprophytic leptospira consistently predominates and interferes with the recovery of pathogenic leptospira. 4) The culture medium for the growth of leptospira bacteria is not sufficiently selective and growth of leptospira bacteria is relatively slow as compared to other bacteria. As a result, prefiltration to remove most of the other bacteria is a necessary step in the culture of leptospira from environmental waters. However, this prefiltration step also removes some of leptospira from the sample. Moreover, the long incubation period required for the culture of leptospira prevents the useful application of the data to most environmental problems. 5) The traditional methods to identify pathogenic leptospira by serotyping after they have been cultured are slow, tedious and expensive because of the requirement for many different reagents.

In summary, reliable and feasible methods to routinely monitor environmental waters (streams) for presence of pathogenic leptospira are not available. An identified need is to develop feasible alternative methods which can reliably monitor environmental waters for the presence or absence of pathogenic leptospira bacteria.

B. Project Goal and Objectives

The goal of this study was to develop feasible methods to monitor environmental waters (streams) for the presence or absence of pathogenic leptospira. When this goal is achieved, streams can be identified and classified as high risk, medium risk and low risk for the transmission of leptospirosis. Moreover, conditions such as rainy weather, rodent population, and correlating water quality parameters may be identified as risk factors in environmental transmission of leptospirosis. Based on these kinds of data, better management plans can be developed to control and prevent leptospirosis in humans. To attain the goal of this study, the following two objectives were pursued:

1. To evaluate the usefulness of adding an oxygen controlling enzyme system (Oxyrase) to leptospira growth medium for the purposes of increasing the growth rate of leptospira and to induce formation of surface colonies on agar medium.

2. To apply the Polymerase Chain Reaction (PCR) method as a feasible technique to detect and identify the pathogenic leptospira directly from environmental waters and to use this method to rapidly identify leptospira isolates recovered from streams.

III. EXPERIMENTAL DESIGN AND METHODOLOGY

A. Standard Culture Methods for Leptospira

The commercially available culture medium to propagate leptospira bacteria is called PLM-5 which is produced and sold by Intergen (Purchase, NY). This medium can be prepared as a liquid broth, semi-solid medium and as a solid agar medium. *Leptospira biflexa* was used as a representative saprophytic leptospira and *Leptospira interrogans* serovar *wolfii* was used as the representative pathogenic leptospira. Cultures of these leptospira were maintained in semi-solid PLM-5 media or PLM-5 liquid media and incubated at room temperature (24°C). In order to prepare the leptospira inoculum, approximately 1 ml of PLM-5 broth containing leptospira was used to inoculate a fresh tube of PLM-5 liquid medium. After 4 days of growth at 24°C, the active culture of leptospira was used as the inoculum for experiments.

B. Experimental Design in the Use of Oxyrase

There are two basic problems in the culture of leptospira bacteria. First, is the fact that other bacteria in the sample (stream water) will grow faster than leptospira bacteria and will prevent the growth and/or detection of leptospira in the culture medium used. For example, human blood samples that are inoculated into growth media must be incubated for up to a month or longer in order to allow for adequate time for the growth and detection of leptospira. The second problem is that leptospira bacteria prefer to grow under the surface (sub-surface) of either agar or semisolid growth medium. Thus, in a semi-solid agar medium prepared in a test tube,

growth is characterized by a concentrated growth of leptospira which forms a distinct band called Dingers Ring which is located a few centimeters below the surface of the growth medium. On a solid agar medium, colonies take a long time to grow and form as sub-surface colonies. Formation of subsurface colonies on agar medium makes it difficult to determine the concentrations of leptospira and to obtain pure cultures for further characterization. The observation that leptospira bacteria prefers to grow slightly below the surface of semi-solid and solid agar medium suggest that it prefers a microaerophilic or low oxygen environment for growth.

To overcome these two problems in the culturing of leptospira, the experimental approach was to supplement the growth medium with Oxyrase, since this enzyme product has been reported to increase the growth rate of many bacteria by controlling the oxygen levels in culture medium (Yu et al., 1991) (Tran, 1995). Oxyrase is an enzyme system derived from the membrane of the microorganism *Escherichia coli*. This product is a oxygen scavenger known for its unique ability to remove dissolved oxygen from aqueous, gaseous, and semi-solid environments. By varying the recommended concentrations of Oxyrase to be added to the growth medium, the percent of available oxygen present in the culture medium can be reduced. For example, to attain complete anaerobic conditions (100%), 10 mL of Oxyrase solution is the recommended concentration to be added to 100 mL of a bacteriological medium. To evaluate the effect of oxygen level on the growth of leptospira bacteria, we adjusted the amount of Oxyrase in the medium to attain levels equivalent to 5, 10 , 15, 20, 25, 50 and 100% anaerobic conditions. Thus, various concentrations (v/v) of Oxyrase were added aseptically to the PLM-5 semi-solid and agar base medium after the medium had been cooled to 50°C. To prevent the growth of contaminating microorganisms, 5-fluorouracil was also added to the culture medium at a final concentration of 100 ug/ml. The semi-solid PLM-5 medium was dispensed into sterile screw capped tubes while the PLM-5 agar medium was dispensed aseptically into OxyDishes which are specially designed plates to create anaerobic atmosphere in an agar plate when incubated under aerobic conditions. In summary, the experimental design of this phase of the study was to add various concentrations of Oxyrase to the leptospira growth medium and to determine the optimum level of oxygen which would allow the leptospira to grow as surface colonies and to also increase its growth rate.

C. Experimental Design in the Use of the Polymerase Chain Reaction (PCR)

The difficulty in using standard culturing and serological methods to detect and identify leptospira prompted us to seek a new method which can detect and identify leptospira rapidly and specifically. The Polymerase Chain Reaction (PCR) method is a relatively new method which fulfills these criteria by specifically directing the production of many copies of specific portions of the microorganisms chromosome which are commonly referred to as genes. Since the genes of each microorganisms are comprised of different sequences of DNA or RNA, the PCR method can be used to determine the presence of specific genes and in that way identify the presence of a specific microorganism. When performed correctly, that specific gene is identified and automatically amplified (copied). After a sufficient quantity of that gene is produced, it can be easily visualized as a characteristic band by gel electrophoresis techniques. The advantage of

this test is that only small quantities of genes are required. The PCR method is also rapid, sensitive, specific and does not necessarily require the initial isolation and culture of leptospira. The disadvantages of this test are that it is not quantitative and moreover this method is unable to distinguish between live and dead microorganisms.

The PCR method has been reported to detect leptospira in human samples (Merien, et al., 1992) (Bal et al., 1994) (Romero et al., 1998) (Brown et al., 1995) and in animal samples (Smith et al., 1994) (Gerritson et al., 1991). The use of PCR for the detection of leptospira from environmental samples has been limited to water inoculated in a controlled laboratory setting (Murgia et al., 1997). To date there has been no reported studies that can detect leptospira from environmental samples (i.e., stream water, soil, mud) using PCR. A serious problem in the use of PCR to detect microorganisms in environmental samples is the potential presence of natural compounds such as humic acids which can inhibit a necessary step in the PCR method.

D. Preparation of Environmental Samples for PCR

In previous studies we determined that saprophytic leptospira could be routinely recovered by culture methods from streams and sediment samples obtained from Manoa Stream. Thus, a Manoa Stream location near the University of Hawaii was used as the sampling site to obtain stream water samples to recover leptospira bacteria. After slight agitation of the stream bed silt, 1 liter of stream water was collected into a sterile plastic (Nalgene) bottle and transported to the laboratory for analysis. Initially, 500 mL of the collected sample were passed through a 0.45 um filter in order to removed larger debris (i.e., mud, leaves) and other microorganisms. Due to their burrowing capability, leptospira bacteria will penetrate these filters. Next, 100-200 mL of the filtrate containing the leptospira bacteria are passed through a swinnex filter containing a 0.1 um pore size membrane which retains most leptospira bacteria. This filter was then aseptically removed and placed into a 1.5 mL microcentrifuge tube and frozen until ready for processing.

E. DNA Extraction from Culture and Environmental Samples

To extract DNA from cultures of leptospira, the growth medium containing the leptospira was initially centrifuged at 30,000 rpm for 30 minutes in order to pellet the bacteria from the culture medium. The cells were then washed 3X with sterile 0.1 um filtered HPLC grade water to remove most of the residual culture media. The pellet was collected, dispersed and added to approximately 500 ul of a 10% Chelex 100 solution to remove PCR inhibitors. The leptospira/chelex mixture was then boiled for 30 minutes in hot water to lyse the bacterial cells and release the DNA. Each sample was then passed through a Centricon 100 (Perkin-Elmer) to concentrate any DNA present within the sample. The extracted DNA was then collected and stored for PCR processing.

A similar method was used to extract the DNA from the Manoa Stream sample. Briefly, the filter containing the concentrated sample of leptospira from Manoa Stream as described above was added to a microcentrifuge tube containing 500 ul of Chelex 100. The tube was then mixed (vortexed) to dislodge any leptospira caught in the filter and boiled for 30 minutes in a

water bath. The DNA containing fraction was passed through a Centricon 100 to concentrate the DNA from the solution and stored for PCR processing.

F. Selection of Primers for PCR

The PCR portion of this study utilized two sets of primers published in the literature for the detection of pathogenic and saprophytic leptospira. The primers used to detect pathogenic leptospira were designated G1 and G2 and were reported by Gravekamp et al. (1993) to be specific for the detection of only pathogenic leptospira. The primers used to detect saprophytic leptospira were designated SAPRO-1 and SAPRO-2 and were reported by Murgia et al., (1997) to specifically detect only saprophytic leptospira. These primers were prepared by the Biotechnology/Molecular Biology Instrumentation and Training Facility of the University of Hawaii.

G. Polymerase Chain Reaction (PCR) Procedure

To a 100 ul thin walled PCR reaction tube were added 2 ul of the leptospira DNA sample and mixed with the following buffer containing specified amounts of TRIS-HCl, KCl, $MgCl_2$, deoxynucleoside triphosphates (dNTPs), and *Taq* DNA polymerase and the selected primers. The samples were then placed into a thermal cycler (Model 9600, Perkin-Elmer) and programmed to allow for the amplification (copying) for the specific genes of *Leptospira*. The method used in the amplification with G1 and G2 primers were as follows: Initial denaturation of DNA at 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 0.5 minutes, and extension at 72°C for 1 minute. After the last cycle, extension was continued for a further 7 minutes at 72°C. The method used in the amplification of the SAPRO-1 and SAPRO-2 primers were as follows: Initial denaturation of DNA at 93°C for 3 minutes followed by 35 cycles of denaturation at 93°C for 1 minute, annealing at 63°C for 1.5 minutes, extension at 72°C for 2 minutes. After the last cycle, extension was continued for a further 10 minutes at 72°C.

IV. RESULTS AND CONCLUSIONS

A. Effect of Oxyrase on Characteristic Growth of Leptospira

As stated earlier, the two problems in the culturing of leptospira are the slow growth rate and the formation of sub-surface colonies on agar based media. To address these two problems, various concentrations of Oxyrase were initially added to PLM-5 semi-solid medium prepared in screw capped tubes and PLM-5 agar medium prepared in special plates to control the available oxygen in this agar medium. These media were inoculated first with saprophytic (*L. biflexa*) leptospira and then with pathogenic (*L. interrogans* serovar *wolfii*) leptospira and incubated under room temperature (25°C) and at 37°C.

The objective of the first set of experiments was to determine the effect of Oxyrase on the sub-surface position of the growth ring (Dinger's Ring) of saprophytic and pathogenic leptospira

in semi-solid medium prepared in screw-capped test tubes. Saprophytic and pathogenic leptospira were inoculated into separate semi-solid PLM-5 medium whose oxygen environment was adjusted to 0 (aerobic), 5, 10, 25, 50, and 100% anaerobic conditions by the addition of varying concentrations of Oxyrase. The inoculated cultures were then incubated at room temperature and at 37°C and observed for leptospiral growth. The results showed that an Oxyrase concentration equivalent to a 50% (5 mL Oxyrase/100 mL media) and 100% (10 mL Oxyrase/100 mL media) depletion of oxygen resulted in no growth of pathogenic and saprophytic leptospira clearly showing that the removal of too much oxygen is detrimental for the growth of leptospira. However, culture tubes which contained the equivalent of 0, 5, 10, 15, 25% anaerobic conditions allowed leptospira to grow and did not change the sub-surface growth characteristics (Dingers Ring) of this bacteria.

In summary the results of these experiments indicate that leptospira can grow under full oxygen conditions and under reduced oxygen conditions but not anaerobic and conditions in which most of the oxygen has been removed. The results support our hypothesis that leptospira bacteria actually prefer reduced oxygen conditions and should be classified as microaerophilic. Since the sub-surface growth position of leptospira did not change in response to varying oxygen concentrations, we conclude that reduced oxygen requirement is only one of the factors controlling the sub-surface growth of leptospira. We speculate that the other controlling factor is related to the characteristic burrowing action of leptospira cells. As a result, leptospira prefer to grow under sub-surface conditions rather than at the surface of the culture medium because the sub-surface environment provides a continuous solid or semi-solid matrix to satisfy the burrowing action of leptospira bacteria.

The objective of the second set of experiments was to determine the effect of Oxyrase on the growth of saprophytic and pathogenic leptospira when streaked onto the surface of PLM-5 agar medium. Thus, PLM-5 agar medium containing Oxyrase set at 0, 5, 10, 25% anaerobic conditions were prepared and streaked with a loopful of saprophytic and pathogenic leptospira. The results showed that the formation of subsurface colonies of leptospira bacteria on the agar medium occurred at the fastest rate when Oxyrase was added to the culture medium to attain 10% anaerobic conditions. Under these conditions, the time to form visible saprophytic leptospira colonies was reduced from >30 days without Oxyrase (100% aerobic conditions) to 4 days in a medium supplemented with Oxyrase to achieve a calculated 10% anaerobic condition. For pathogenic leptospira, the time for formation of colonies was reduced from >30 days in the absence of Oxyrase to 21 days in a medium supplemented with Oxyrase to achieve a calculated 10% anaerobic condition. Thus, the addition of Oxyrase to the agar medium was successful in increasing the growth rate of leptospira bacteria. However, the addition of Oxyrase to agar growth medium was not successful in creating an environment suitable for the formation of surface colonies of leptospira which would greatly improve on the methods to enumerate and to better characterize the leptospira isolates.

In summary, the addition of Oxyrase to create 10% anaerobic conditions in solidified PLM-5 agar medium was successful in increasing the rate at which colonies of leptospira bacteria grew as subsurface colonies Thus, the addition of Oxyrase was successful in attaining

one of its objectives which was to reduce the time for the formation of colonies on agar media. These results also provide direct evidence that leptospira prefer to grow under reduced oxygen environment equivalent to microaerophilic conditions. These results support our original hypothesis that leptospira bacteria be classified as microaerophilic bacteria. In this regard the formation of sub-surface colonies is directly related to microaerophilic conditions which are provided by sub-surface conditions but not by surface conditions. However, we also believe that sub-surface conditions provide a continuous solid matrix which satisfies the burrowing growth characteristics of leptospira cells.

B. Use of PCR to Detect and Identify Pathogenic and Saprophytic Leptospira

Monitoring of environmental waters for pathogenic leptospira using culture method is not commonly done because culture methods are very slow, inefficient and unreliable. Moreover, the culture method is not sufficiently selective and this will allow both saprophytic as well as pathogenic leptospira to grow. Since saprophytes are commonly found in environmental water and in higher concentrations than pathogenic leptospira, culture methods generally recover only saprophytic leptospira from environmental samples. There is a recognized need to use alternative methods which can overcome the limitations of traditional culture methods to monitor environmental waters for the presence of pathogenic leptospira. The alternative method used in this study is the PCR method which has been reported to be able to rapidly and specifically detect saprophytic and pathogenic leptospira in clinical samples and from laboratory samples.

The objective of the first set of experiments was to evaluate a PCR method which was reported by Gravekamp et al (1993) to specifically detect pathogenic leptospira. Thus, the primers G1 and G2 as recommended by Gravekamp were obtained and the method applied to cultures of saprophytic and pathogenic leptospira. The results showed that positive reactions were observed for both saprophytic and nonpathogenic leptospira (*L. biflexa*), as well as pathogenic *L. interrogans*. These results differ from those reported by Gravekamp et al (1993) who reported that G1 and G2 primers selected for gene sequence products which were specific to pathogenic leptospira and not to saprophytic leptospira. Further studies are needed to determine why we were not able to confirm the results as published by Gravekamp et al (1993). In summary we have not yet established a reliable PCR method which can be used to identify pathogenic leptospira from saprophytic leptospira.

The second set of experiments was to evaluate the PCR method which was reported by Murgia et al (1997) to specifically detect saprophytic leptospira. Thus, the SAPRO-1 and SAPRO-2, primers recommended by Murgia were obtained and applied to cultures of saprophytic and pathogenic leptospira. The results showed that positive PCR products were obtained with the saprophytic leptospira cultures of *L. biflexa* and negative results were obtained when this method was applied to pathogenic *L. interrogans* serovar *copenhageni* cultures. These results were further substantiated by the data obtained when the SAPRO-1 and SAPRO-2 primers were used on filtered water samples obtained from Manoa Stream. The PCR results were negative when the concentrated Manoa Stream sample was used but was positive when this same sample was diluted 1:10. These results indicate the presence of natural inhibitors to the PCR method in environmental samples. Since saprophytes are the predominant leptospira in stream

water, it is safe to assume that the Manoa Stream sample contained saprophytic leptospira. In summary, we have confirmed that the PCR method as reported by Murgia can reliably and specifically detect and identify saprophytic leptospira from pathogenic leptospira. Thus, we have established a reliable PCR method which can be used to identify saprophytic leptospira from pathogenic leptospira.

V. PROJECT SUMMARY

Due to the reduced funding for this project, completion of the all the proposed objectives of this study was not realistic. However, this project gave us the opportunity to take incremental steps to fulfill the goal of this study which was to develop feasible methods to monitor environmental waters for the presence or absence of pathogenic leptospira bacteria. Two specific objectives were pursued to address this goal. The first objective was to evaluate the usefulness of a new enzyme system (Oxyrase) to increase the growth rate of leptospira bacteria and to determine whether oxygen level controlled the characteristic subsurface growth of leptospira. One accomplishment of this project was that we confirmed the results of Patel et al. (1995), that the Oxyrase system is a useful means to control oxygen concentrations and to control the growth of bacteria. In our study, we specifically determined that the addition of Oxyrase which created a 10% reduction in oxygen (microaerophilic conditions) resulted in increased growth rate of leptospira bacteria. Thus, one of the objective which was to develop a method to shorten the period for the growth of leptospira bacteria was achieved. These results also supported our proposed hypothesis that leptospira are microaerophilic and therefore prefer growth conditions with reduced concentrations of oxygen.

Another application in the use of Oxyrase was to induce leptospira bacteria to form surface colonies instead of sub-surface colonies on agar medium. The desirability to induce the formation of surface colonies of leptospira is based on other bacterial methods which use surface colonies to substantially improve methods to enumerate the concentrations of leptospira and to further characterize this group of bacteria. Although Oxyrase did increase the growth rate for the formation of leptospira colonies, it did not induce the formation of surface colonies. Based on these results, we conclude that leptospira bacteria prefer to form sub-surface colonies because sub-surface environment in culture medium fulfills their two growth requirements: First, the reduced oxygen level which suits their microaerophilic growth requirements. Second, a continuos solid matrix which suits their need to burrow into a solid matrix as part of its growth cycle. These two conditions are present in the sub-surface of the agar but not at the surface of the agar. Based on this new understanding of leptospira growth requirements, we now plan to inoculate leptospira on a solid agar surface and then to cover the surface of the agar with a softer semi-solid medium to provide microaerophilic conditions and a solid matrix for the cells to grow. The colonies will form on a single plane at the surface of the solid agar. The soft top agar can then be poured or slipped off the solid agar exposing the solid agar with the colonies on a single plane. By this method we hope to achieve the desirable condition of being able to better enumerate the concentrations of leptospira, to separate colonies with different growth characteristics and to be able to recover these colonies in a purified form for further analysis.

The second objective of this project was to apply the polymerase chain reaction method in the development of a more feasible technique to detect and identify the pathogenic and saprophytic leptospira directly in environmental waters and to rapidly identify any leptospira which had been isolated from environmental waters. The development of a PCR method to specifically identify saprophytic leptospira was achieved. This capability will greatly improve our ability to identify the many environmental and animal isolates of leptospira bacteria. However, we encountered two problems. The first is the presence of inhibitors of the PCR method which are present in stream samples. Therefore, all natural stream samples must be tested for the presence of inhibitors to verify negative results. Moreover, we have incorporated the use of methods to remove these inhibitors. The second problem is that the reported PCR method to specifically identify pathogenic leptospira was limited in its application because the method cross reacted with some saprophytic leptospira as well. Thus, additional studies must be completed to establish this method to specifically identify only pathogenic leptospira. This capability is needed to ensure that we can identify whether saprophytic or pathogenic leptospira are present in any sample, quickly and reliably. As a result, we will further evaluate other PCR methods and primers which have been reported to specifically identify pathogenic leptospira.

In conclusion, by completing this project we achieved the basic objective of establishing the PCR methodology in our laboratory as a rapid and feasible method to detect and identify leptospira. We have also improved the culturing method for leptospira bacteria by using the Oxyrase system. By integrating the use of improved culture methods and applying new molecular methods, we have increased the capabilities of our laboratory. Our laboratory is now in a better position to seek external funding to complete all of the goals of this study and to assist health agencies in the prevention of leptospirosis in Hawaii and throughout the world.

ACKNOWLEDGMENTS

The University of Hawaii Water Resources Research Center would like to thank the Chamber of Commerce of Hawaii for funding this project and providing the catalyst for further research concerning leptospirosis, a disease of great importance to the people of Hawaii and now considered an emerging new disease throughout the world.

REFERENCES

1. Bal, E.A., Gravekamp, D., Hartskeerl, R.A., DeMeza-Brester, J., Korver, H., and W.J. Terpstra. 1994. Detection of Leptospires in Urine by PCR for Early Diagnosis of Leptospirosis. J. Clin. Microbiol. 32(8):1894 - 1899.
2. Brown, P., Gravekamp, D., Carrington, D., Van de Kemp., H., Hartskeerl, R., Edwards D., Everard, C., Terpstra, W., and P. Levett. 1995. Evaluation of the Polymerase Chain Reaction for Early Diagnosis of Leptospirosis. J. Med. Micr. 43:110 - 114.
3. Farr, R.W. 1994. Leptospirosis. Clin. Infect. Dis. 21:1-8.
4. Fuortes, L. 1994. Leptospirosis: A Consequence of the Iowa Flood, Iowa Med., 84 (10):449-450.
5. Gerritesen, J.J., Olyhoek, T., Smits, M.A., and B.A. Bokhout. 1991. Sample Preparation Method for Polymerase Chain Reaction -Based Semiquantitative Detection of *Leptospira interrogans* Serovar *Hardjo* Subtype *Hardjobovis* in Bovine Urine. J. Clin. Microbiol. 29(12):2805 - 2808.
6. Gravekamp, C., Van De Kemp, H., Franzen, M., Carrington, D., Schoone, G.J., Van Eys, G.J.J.M., Everard, C.O.R., Hartskeerl, R.A., and W.J. Terpstra. 1993. Detection of Seven Species of Pathogenic Leptospires by PCR Using Two Sets of Primers. J. Gen. Microbiol. 139:1691-1700.
7. Katz, A.R., Sasaki, D.M., Mumm, A.H., Escamilla, J., Middleton, C.R., and S.E. Romeo. 1997. Leptospirosis on Oahu: An Outbreak Among Military Personnel Associated With Recreational Exposure. Mil. Med. Feb;162(2):101-104.
8. Merien, F., Amouriaux, P., Perolat, P., Baranton, G., and I. Saint Girons. 1992. Polymerase Chain Reaction for Detection of *Leptospira spp.* in Clinical Samples. J. Clin. Micr. 30(9):2219 - 2224.
9. MMWR. 1995. Outbreak of Acute Febrile Illness and Pulmonary Hemorrhage - Nicaragua, 1995. November 10, 1995. 44 (44):841-842.
10. Mugia, R., Riquelme, N., Baranton, B., and M. Cinco. 1997. Oligonucleotides Specific for Pathogenic and Saprophytic Leptospira Occurring in Water. FEMS Micr. Let. 148:27 - 34.
11. Patel, J.R. and L.R. Beuchat. 1995. Enrichment in Fraser broth supplimented with catalase or Oxyrase, combined with the microcolony immunoblot technique, for detecting heat-injured *Listeria monocytogenes* in foods. Int. J. Food Micr. 26:165 - 176.
12. Patz, J., Eptstein, P., Burke, T., and J. Balbus. 1996. Global Climate Change and Emerging Infectious Diseases. JAMA. 275 (3):217-223.
13. Romero, E., Billerbeck, A.E., Lando, V., Camargo, E.D., Souza, C.C., and P.H. Yasuda. 1998. Detection of Leptospira DNA in Patients with Aseptic Meningitis by PCR. J. Clin. Micr. 36(5):1453-1455.
14. Sasaki, D.M., Pang, L., Minette, H.P., Wakida, C.K., Fujimoto, W.J., Manea, S.J., Kunioka, R., and C.M. Middleton. 1993. Active Surveillance and Risk Factors for Leptospirosis in Hawaii. Am. J. Trop Med Hyg. 48 (1):35-43.

15. Sasaki, D. 1997. Review of Surveillance, Prevention and the Epidemiology of Leptospirosis in Hawaii: 1992 - 1996. Leptospirosis a Re-Emerging Disease. Symposium for the Annual Meeting of the American Society of Tropical Medicine and Hygeine. Orlando, Florida.
16. Shaw, R.D. 1992. Kayaking as a Risk Factor for Leptospirosis. Miss. Med. June 89(6):354 - 357.
17. Smith, C.R., Ketterer, P.J., McGowan, M.R., and B.G. Corney. 1994. A review of laboratory techniques and their use in the diagnosis of *Leptospira interrogans* serovar *hardjo* infection in cattle. Aust. Vet J. 71:290 - 294.
18. Theirmann, A.B. 1984. Leptospirosis: Current Development and Trends. J. Am. Vet. Med. Assoc. 184:722-725
19. Tran, T.T. 1995. Evaluation of Oxyrase enrichment methods for isolation of *Campylobacter jejuni* from inoculated foods. Let. Appl. Micr. 21:345-347.
20. Vinetz, J.M., Glass, G.E., Flexner, C.E., Mueller, P., and D.C. Kaslow. 1996. Sporadic Urban Leptospirosis. Ann. Int. Med. 125(10):794-798.
21. Yu, L.S.L. and D.Y.C. Fung. 1991. Effect of Oxyrase Enzyme on *Listeria monocytogenes* and other facultative anaerobes. J. Food Safe. 11:163 - 175.