DETECTING *LEPTOSPIRA* IN WATER:

EVALUATION OF A PROPOSED METHOD

A THESIS SUBMITTED TO THE GRADUATE DIVISION OF
THE UNIVERSITY OF HAWAI'I IN PARTIAL FULFILLMENT OF
THE REQUIREMENTS FOR THE DEGREE OF

MASTER OF SCIENCE

IN

NATURAL RESOURCES AND ENVIRONMENTAL MANAGEMENT

DECEMBER 2007

By
Ilima R. Hawkins

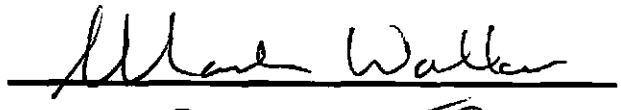
Thesis Committee:

Carl L. I. Evensen, Chairperson
Mark J. Walker
Dulal Borthakur

We certify that we have read this thesis and that, in our opinion, it is satisfactory in scope and quality as a thesis for the degree of Masters of Science in Natural Resources and Environmental Management.

THESIS COMMITTEE

Chairperson



Acknowledgements

I would like to acknowledge those who have provided support through the course of this project. First and foremost I thank Dr. Carl Evensen, my exceedingly patient advisor who has always been a source of excellent guidance. I have learned much from his good example. I also give special thanks to my other committee members Dr. Mark Walker and Dr. Dulal Borthakur, both of whom have been exceedingly generous and have unconditionally supported my academic development. I would also like to recognize Dr. Cathy Chan-Halbrendt for her continuous support, and Dr. John Yanaigda for his early assistance in my Master's program. A big thank you is also extended to Dr. David T. Webb, for providing imaging equipment.

A warm mahalo is extended to graduate students Adrienne Nishioka, Vanessa Fitz, Rudolph Fredua-Agyeman, Dan Adamski, Tai McClellan, and also to Dr. Kathy Chaston for support and advice. I'd also like to thank Susan Yamane and Zazza for their cheerful, patient help.

None of this would have been possible without the support of my best friend, Bradley Kam. His love and patience have always motivated me to do my best. I would also like to thank my Dad, Mom and my brother Ian for believing in my dreams.

This work is dedicated to Charlotte Hawkins, Georgette Buchner, and Aunty Thelma. Each in their own way has given me the strength to pursue my dreams. They are missed dearly.

Abstract

Leptospirosis is the most widespread zoonosis in the world, and its geographic distribution is spreading. The disease is caused by *Leptospira*, a pathogenic group of spirochetes transferred between a variety of maintenance and accidental hosts. Few management practices exist because little is known about leptospire ecology and there is no reliable method for environmental detection.

The goal of this work is to evaluate a two part methodology that isolates and detects pathogenic *Leptospira* in water. The first study uses suspensions of *Leptospira*, visually quantified in cells per milliliter, to evaluate the transmittance of different filters. The second study evaluates the sensitivity of a PCR detection method under real-world conditions. Results indicate a 0.2μm nitrocellulose filter is the optimal choice for filtration, and microbial DNA can be consistently recovered from used filter surfaces. Additional research will be necessary before this method can be used for field testing.

Table of Contents

List of Figures

List of Tables

CHAPTER 1
INTRODUCTION

Human *Leptospiral* infections were first recognized by Adolf Weil in 1816 and subsequently called "Weil's disease." Today the infection is known as Leptospirosis, and is recognized as the most widespread zoonotic disease in the world (Elliot, 1980; Levett, 1999). Human infection often results after physical contact with water, soils, or urine contaminated by infected animals (Levett, 2001). Although the disease is often perceived as common to urban slums in tropical third-world countries, hundreds of outbreaks have occurred in localities of various socioeconomic standing and climates. Those in Rome, Detroit Michigan, Springfield Illinois, Germany, India, American Samoa and Peru are only a few examples (WHO, 1999; Jansen *et.al.,* 2003; Kuriakose *et. al.*, 1997, ASEPA, 2003). Leptospirosis has recently been placed on the emerging infectious disease list by the Center for Disease Prevention and Control (CDC), but research and control efforts are hampered by an inability to detect *Leptospira interrogans* in environmental materials. The focus of this work is to propose and evaluate a testing procedure for environmental waters, and results will contribute to a methods development project funded by the Environmental Protection Agency's Regional Applied Research Effort (EPA RARE), and the U. S. Department of Agriculture's Cooperative State research, Extension and Education Service (USDA CREES).

Organism

Leptospirosis is caused by a pathogenic (disease-causing) genus of spirochetes called *Leptospira interrogans,* which is morphologically similar to the harmless *Leptospira biflexa* species complex (Faine *et al.*, 1992). *L. interrogans* and *L. biflexa*

have an aerobic metabolism, are capable of self-propelled motion, and possess a helical shape (Lester *et.al.*, 2004). *Biflexa* and *interrogans* share the same long and linear shape, ranging between 0.2-0.3 microns in diameter and 6-30 microns in length (Lester *et. al.*, 2004; Levett, 2001) (Figure 1.1). They are unique from other spirochetes in that they require fatty acids instead of carbohydrates for growth. *Leptospira* strains were historically differentiated based on antibody reactions but recent technological advancements have enabled more precise genetic techniques for identification and classification. *Leptospira* spirochetes may now be divided into 40 pathogenic species and subspecies, 41 subspecies of intermediary pathogenicity, and 13 harmless species (Ganoza *et al.*, 2006). A classification system is currently being developed based on this information.

Pathogenic leptospires survive well in the fresh waters, soils, and muds of both tropical and temperate climates (CDC, 1998). However, they are obligate parasites whose long-term survival requires the infection of a mammalian host (Braun *et.al.*, 1967; Hellstrom *et.al.*, 1978; Henry, Johnson *et. al.*, 1971; Henry, Johnson *et. al.*, 1978). Individual strains are often adapted for chronic infection of a specific host species; for instance *L. icterohaemorrhagiae* is well adapted to rat and mouse populations while *L. pomona* is most commonly found in cattle and bovines (WHO, 1999). Because of this adaptive trend it is sometimes possible to locate an infected host population by identifying the type of strain infecting a human.

Disease

L. interrogans cause infection by entering the bloodstream via mucosal membranes or through an open wound. Leptospirosis develops 2 days to 4 weeks after the initial infection and may consist of one or two phases with highly variable symptoms. The first phase is loosely characterized by mild flu-like symptoms that decline on their own. Approximately 90% of patients will only experience the first phase of disease, and because patients with mild symptoms often avoid seeking medical attention, hospital records are assumed to be conservative representations of the incidence of disease. Only 10% of those infected will develop the second and more serious phase of the disease which includes severe ailments such as tissue and organ infection, kidney damage, jaundice, liver failure, and occasionally death (Levett, 2001).

Numerous studies have shown the number of Leptospirosis outbreaks fluctuate seasonally, with higher instances of disease occurring during the warmer seasons in temperate regions and rainy seasons in tropical regions (Faine *et.al.*, 1992; Kuriakose *et al.*, 1997; Sarkar, Nascimento et al., 2002). The number of cases in the United States was formerly recorded by the Center for Disease Control and Prevention (CDC), but leptospirosis was removed from the list of reportable diseases late last century and was only monitored by some states (Walker, 2007). Recently the disease was re-added to the CDC's list of emerging infectious diseases, and monitoring has resumed.

Ecology and Transmission

Long-term hosts for *L. Interrogans* are known as maintenance hosts or reservoirs. They include a variety of mammals such as deer, foxes, muskrats, opossums, raccoons,

rodents, skunks, cattle, dogs, goats, horses, pigs, and sheep (Jansen *et.al.*, 2005). These animals are different from accidental hosts, like humans, because they are capable of maintaining a long-term *Leptospira*l infection while demonstrating little to no adverse symptoms (depending on the infecting strain). Chronic infection of reservoir hosts occurs in the renal tubules of the kidney, where optimal growth conditions exist. These conditions include an ambient temperature of 28 to 30°C, a pH of 5.2 to 7.7, plentiful moisture and minimal exposure to light (Standard Methods, 2005).

Because infection occurs in the kidneys, when the host urinates a small proportion of bacteria are released into the environment. *L. interrogans* have been documented to survive in the abiotic conditions for 3 to 5 days in moist soils, up to 10 days in fresh water, for 4 weeks in sterile tap water (pH 7), up to 3 days in aerated wastewater, and up to 12 to 14 hours in undiluted wastewaters (Standard Methods, 2005). However as mentioned before, leptospires that are in the environment must infect a new host to perpetuate the growth of a new population (Braun *et.al.*, 1967; Hellstrom *et.al.*, 1978; Henry, Johnson *et. al.*, 1971; Henry, Johnson *et. al.*, 1978).

While human infection may result from physical contact with infected animals or contaminated environmental surfaces, most cases are derived from physical contact with contaminated water (ASEPA, 2003; Bharti, Nally *et. al.*, 2003). Freshwater contamination results either from water contaminated urine from infected hosts, or leptospires may be washed from contaminated materials into water by precipitation and runoff. This may be the mechanism that underlies the high prevalence of disease occurrence in flooding zones (Morshed, Konishi et al., 1994).

Transmission to humans is thought to be regulated by four generalized factors, shown in Figure 1.2. Infected host populations are the root of the disease because they serve as the source of the pathogen, and the cause of environmental contamination. Once leptospires reach the abiotic environment, runoff generated from rainfall helps mobilize and transport the pathogen into freshwater supplies. Although precipitation levels vary depending on regional factors, runoff is also impacted by land use and land cover. The type of land development will significantly impact the level of runoff within a watershed. For instance, areas with urban development possess a large amount of impervious surfaces which generate faster and more voluminous runoff patterns. Natural or undeveloped areas typically have higher levels of infiltration and water retention, reducing the rate and volume of runoff. Land usage affects the types of host animals that may be present in an area. It also has local effects on hydrologic properties. Thus some impacts the global warming phenomena, thus some argue that land use change is the driving factor behind recent increases in human Leptospirosis (Wilcox *et.al.*, (2):2005; Wilcox *et.al.*, (10): 2005). Finally, the last step in the transmission of leptospirosis is the frequency with which humans come into contact with contaminated materials.

Since transmission depends on many site specific factors such as land use, host populations and host ecology, human behavior and precipitation patterns, control efforts must be targeted to face specific factors of local importance. A recent seroprevalance survey conducted in American Samoa identified pigs as the most significant reservoir host, and management techniques were subsequently targeted towards piggery sanitation (Winger & Aguilar, 2004).

The situation in Hawai'i is much different. Wild animals are the most likely sources of *Leptospira,* since the health of domestic animals and pets is generally protected by veterinary services in Hawaii. Limited data are available on the distribution and severity of naturally occurring infections in Hawaii's wild animals, and wildlife-based control efforts are not considered feasible. Additionally Hawai'i experts cannot agree whether rats, pigs, or mongooses, are the most significant contributors. This is because some of the strains infecting humans have symbioses with several long-term reservoir hosts, making it difficult to link a human infection to a single host species (WHO, 1999; Michigan DNR, 2006). Common strains in human infections include, in decreasing order of occurrence, *icterohaemorrhagiae*, *australis*, *ballum*, *bataiae*, *sejroe*, and *pomona* (Katz, *et al.*, 2002).

Since identifying and eradicating infected wild animals are not feasible, measures to control the occurrence of pathogenic spirochetes instead focus on public warning signs and other forms of education. Some practices advocated by the Hawaii Department of Public Health include avoiding swimming in stock ponds or slow-moving streams frequented by domestic and wild animals, and wearing protective gloves and boots for occupational activities like taro and rice farming.

Project Justification and Objectives

Leptospirosis presents more than just an issue of human health. The incidence of human infection may represent the successful establishment of an invasive species (or rather in this case, invasive family of bacteria), or the loss of obstacles blocking human transmission. While the exact cause is unknown, the introduction of *Leptospira* species

can be linked to increased human activity (Wilcox *et. al.*, (10): 2005). Alterations in the transmission cycle reflect changing patterns of community structure. Thus in a larger context the emergence of leptospirosis could reflect the rise of ecosystem degradation and the need for biological conservation. However the emergence of leptospirosis may only reflect increased medical awareness as a larger number of hospitals acquire the capability to test patients for the disease. Extensive environmental studies are needed to test hypotheses about the link between human leptospirosis and infected animal populations that may serve as reservoirs of the parasite.

Leptospirosis is also problematic for the agricultural industry. Livestock are easily infected and because they live in close confines, the infection is quick to spread. The worst of their symptoms include hemorrhaging, miscarriages and death, all of which generate negative economic consequences for farmers (ASIPMC, 2006). Although yearly vaccinations are a common practice, these are only specific to a few strains of *Leptospira* and leave animals open to infection by other strains. Control efforts such as implementing quarantines, antibiotic treatment, or animal destruction also cost money and lost revenue for farmers. Depending on the socioeconomic status of the farm, infected animals may go unidentified and the problem may be compounded by poor sanitation and inadequate control measures.

A detection method is needed for pathogenic *Leptospira* in environmental samples. The availability of such a test would help to address both management and research issues, and provide information necessary to develop site-specific best management practices (BMPs). Results could also be used to improve farming practices, public awareness, and protection. Ideally the testing procedure would be quick and easy

to use, and robust in its results. I propose to examine the effectiveness of a presence or absence detection method for leptospires in water supplies. The objectives of this experimental work are to evaluate a two part environmental test consisting of a bacterial isolation method and an identification procedure. Each of the objectives will be evaluated independently, and the experiments will define the ideal conditions for the application of each procedure, as well as estimate the best level of performance to be expected. Since contact with contaminated water is a significant cause of human infection, this work will focus on leptospires in water samples. However some of the sampling approaches discussed here would also be applicable for environmental solids as well.

FIGURE 1.1 A Scanning Electron Micrograph of *Leptospira*

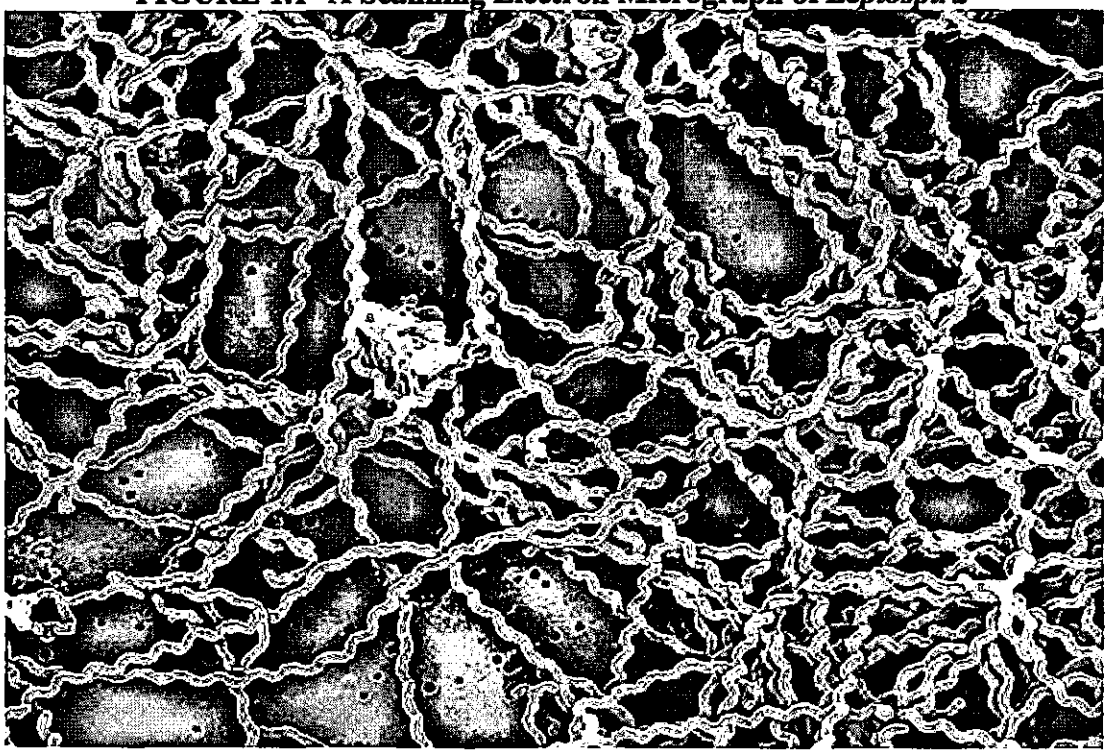


Figure 1.1: A scanning electron micrograph of *Leptospira* bacteria on 0.1 μm polycarbonate filter. Provider(s): CDC/NCID/HIP/Janice Carr.
Image source: http://www.buddycom.com/bacteria/nongram/Lepto138.jpg

FIGURE 1.2 Transmission of *Leptospira*

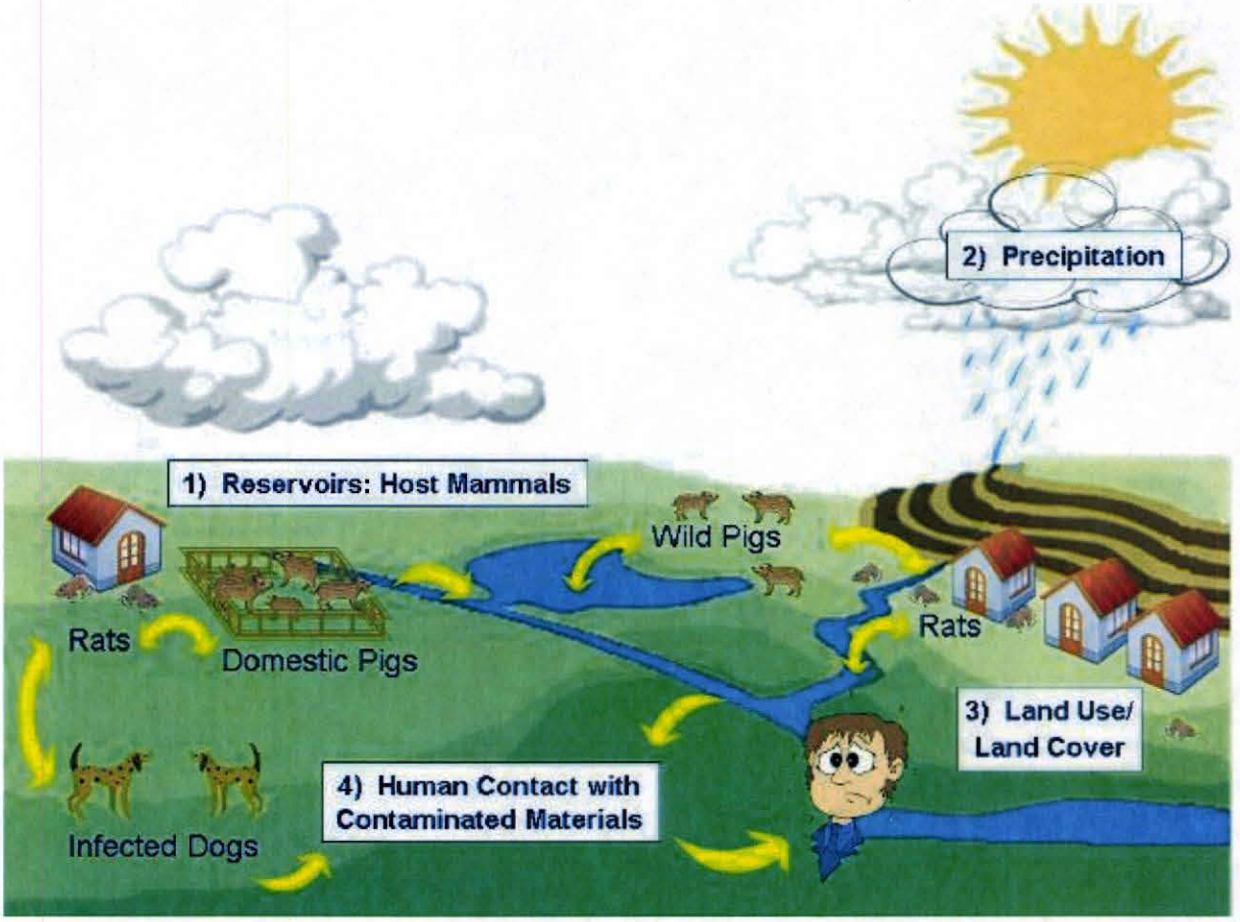


Figure 1.2: The image depicts factors regulating the transmission of *L. interrogans* to humans. 1) Reservoirs indicate the infected host populations, both domestic and wild. 2) Precipitation patterns and 3) land use/land cover both influence the level of bacterial mobilization into streams. 4) Human contact indicates the frequency with which humans come into contact with contaminated materials.

CHAPTER 2
COLLECTING *LEPTOSPIRA* FROM WATER: A FILTER EVALUATION

Introduction

Leptospirosis is the most widespread zoonosis in the world (Levett, 1999), and its geographic distribution is expanding (Winger, Aguilar *et. al,* 2004). The disease is caused by the pathogenic group of *Leptospira*, which affect many mammalian hosts. Approximately 10% of human infections develop into serious conditions that are potentially fatal (Levett, 2001). Few management practices have been developed to prevent transmission because little is known about leptospire ecology and no reliable method for environmental detection exists. Since a large proportion of human infection is associated with contaminated freshwater, it is assumed a promising step towards preventing transmission will be the development of a detection method for leptospires in water supplies.

The first challenge in detection will be to capture *Leptospira* present in water supplies. Leptospires are difficult to locate and collect because they are small (ranging from 0.2-0.3 microns in diameter and 6-30 microns in length), motile, and their spatial distribution is unknown (WHO, 1999). To increase the probability of collection, large volumes of water may be filtered to collect and concentrate bacteria on a membrane surface. Currently there are several standardized protocols that utilize this approach to enhance recovery of waterborne bacteria (Method 1622, EPA; MoBio UltraClean Water DNA Isolation). However only a limited amount of work has focused on filtering *Leptospira* and these studies used filters as a means to generate purified, dilute samples rather than a large-scale collection tool (Braun *et. al.*, 1967; Henry, Johnson *et. al,* 1978;

Wilson & Fujioka, 1995). While this aspect has been overlooked, filtration does offer the potential to be a useful tool for collection.

Currently little is known about the efficiency with which leptospires can be filtered from water supplies, but the way in which they are transmitted will likely be different than that of a more conventional, spherical microbe. The efficiency of filtration is largely determined by the diameter of the filter's pores and the hydrophobicity or hydrophilicity of the bacteria and filter materials. The appropriate pore size for *Leptospira* will likely be smaller than the conventional 0.45μm standard simply because leptospires are small in diameter and subsequently could pass through this level of selectivity. However, microbial-filter interactions are significantly influenced by the hydrophobicity both of the material and the outer membrane of the bacteria (Palaniappan, Ramanujam *et. al.*, 2007). Binding and adherence are greatly enhanced when both the bacteria and inert surface possess the same measure of hydrophobicity or hydrophilicity, but there exists a broad spectrum of bacteria with different degrees of hydrophilicity or hydrophobicity, so the same filter material will not be appropriate for every situation (Dahlback, Hermansson *et. al.*, 1981).

Bacterial hydrophobicity or hydrophilicity is largely determined by the matrix of surface proteins on the outer cell sheath, each of which contain numerous binding domains of either hydrophobic of hydrophilic natures. Collectively these binding domains determine the overall hydrophobicity or hydrophilicity of a bacterial surface. Several recent studies indicate the outer membrane of *Leptospira* consists of a relatively small number of proteins, and that the major leptospiral surface antigens present during host infection are hydrophobic lipopolysaccharides (LPS) (Cullen, Xiaoyi *et. al.*, 2005).

This suggests leptospires may exhibit a hydrophobic nature and experience higher retention when hydrophobic filters are used, but it should be noted the surfaceome of *Leptospira* is not well studied and LPS, a vital component in the infection process, has been hypothesized by some to be down-regulated while the spirochete is present in the abiotic environment. Thus it is unclear whether *Leptospira* are hydrophobic in nature.

The objective of this study is to evaluate how different pore diameters and hydrophobic and hydrophilic materials impact the efficiency of filtering quantified suspensions of *Leptospira*. The maximum efficiency with which leptospires can be collected was also determined. The specific membranes evaluated in this study are: 0.2μm Nitrocellulose, 0.22μm polyvinylidene fluoride (Durapore), 0.4μm polyvinylidene fluoride (Durapore), 0.4μm polycarbonate (Isopore), 0.45μm Nitrocellulose, 0.8μm glass fiber, and 40μm nylon mesh. Results quantify the observed performance of different filters and are used to select an appropriate filter for the collection of leptospires from environmental water. The broader application of this work is to evaluate the first half of a two-part environmental test for pathogenic *Leptospira*.

Methods

Media Preparation

Liquid and semi-solid media for *Leptospira* cultures were prepared according to the protocol given by Becton, Dickinson and company for Difco™ Ellinghausen and McCullough Medium as modified by Johnson and Harris (EMJH). A total of 2.3g of *Leptospira* medium base were dissolved in 900mL of sterile deionized water and autoclaved at 121°C for 15 minutes. Semi-solid media was prepared with 0.2% (wt/wt) Difco Agar Noble prior to autoclaving. After cooling, 100mL of Difco *Leptospira*

Enrichment Medium was added. To suppress the growth of other bacteria the selective agent 5-flurouracil (Acros Organics, cat. # 228440050) was added to a concentration of 200µl/mL.

Leptospira Suspensions

One of the most virulent strains of *Leptospira*, i*cterohaemorrhagiae*, is common to the general Pacific area and was selected for this research. A live culture of *Leptospira interrogans copenhageni icterohaemorrhagiae M-20* was obtained from the National Veterinarian Services Laboratory (NVSL) in Ames, Iowa, which is a leading center for research and control of animal diseases, including leptospirosis. The culture was stored in a dark cabinet at approximately 30°C. The culture was inoculated every 5 months in both EMJH broth and semisolid media. The appearance of a dinger's ring, a cloudy disc located approximately 0.5 – 2cm below the surface of semisolid media, was used as a visual indicator of growth. Culture quality was also visually affirmed using darkfield microscopy.

Simple Filtration

To compare how different filters retain leptospires, a starting suspension of *Leptospira* was generated by mixing 250µL of NVSL stock into 10mL of 0.01M phosphate buffered saline (PBS), at 0.2% wt-wt 5-flurouracil. The suspension was vortexed for 5 seconds, and cell density was estimated using a Petroff-Hauser counting chamber under 200 × magnifications on a Nikon Labphoto microscope (see Figure 2.1). Leptospires were identified as bright, linear objects that rotated around an axial point. Visual quantification was performed ten times to ensure adequate representation, and the

deviation of the estimated mean from the true population mean was calculated using equation A which is based off the Central Limit Theorem and is shown below:

(A): $$N \geq [z^2\theta^2] \div d^2$$

N is the number of times a suspension was visually quantified (N = 10), z is the acceptable value for 95% confidence from a standard normal distribution (z = 1.96), θ^2 is the variance of the population, which varied depending on the individual trials, and d is the maximum allowable deviation of the estimated population size from the size of the true population. The range of observed values for θ^2 was 0 – 58.8 (average being 3.57). Calculations with equation A indicate by using 10 repetitions of visual counts per suspension, the average observed cell concentration deviated from the true concentration by 0 - 4.75 cells/mL, which is acceptable for this study (see Appendix, Table A.1).

After quantifying the concentration of cells, the starting suspension was vacuum filtered at a force of -10 to -25kPa and the *Petroff-Hauser* chamber was used to quantify the concentration of leptospires in the filtered solution. Ten replicate counts were performed. Table 2.1 lists the different filters evaluated in this manner. Filtration efficiency was determined by calculating the percent transmission with equation B:

(B):

$$\frac{\textbf{Cell Concentration in Filtered Solution}}{\textbf{Cell Concentration in Starting Suspension}} \times 100 = \textbf{\% Transmittance}$$

Each filter was evaluated three times, and the resulting percent transmittance values were averaged. A two-way ANOVA was performed on the percent transmittance data for 0.2μm-0.4μm Nitrocellulose and Durapore filters.

Results

Filters with pore sizes equal to or smaller than 0.22μm retained the largest percentage of leptospires. The 0.22μm filter (hydrophilic polyvinylidene fluoride "Durapore") passed the smallest amount but, according to the 95% CI interval generated from the t statistic, the results were not significantly different from the performance of the 0.2μm Nitrocellulose membrane. All other filters demonstrated statistically different levels of transmission, and larger pores were found to transmit more leptospires than smaller ones. The only two varieties that did not follow this trend were the 0.45μm Nitrocellulose filter and the 0.8μm glass fiber filter. The 0.45μm Nitrocellulose was much more selective than either the 0.4μm Isopore or 0.4μm Durapore.

The 0.8μm glass fiber filter was originally found to transmit approximately 100.32% of leptospires into the filtrate. An independent trial was performed in which 10mL of pure distilled water was passed through a 0.8μm glass fiber filter, and the filtrate was quantified using the Petroff Hauser chamber (pre-filtration analysis demonstrated no fibrous material in the distilled water). Results demonstrated the glass filter generates an average of 22 small linear fibers (standard deviation of 4.6 artifacts) which resemble *Leptospira* under the microscope. These fibers are thought to have served as visual artifacts during the previous microscopy analysis and subsequently inflated the transmission levels obtained from the 0.8μm glass filter trials. Therefore, the glass fiber data were corrected by subtracting the average number of observed artifacts from each of the visual counts, and recalculating the transmittance levels. Only the corrected data are displayed in Figure 2.2. After the correction, the highest level of flow-through was observed with the 40μm Nylon mesh, which passed 62.97% of the bacteria.

A two-way ANOVA was performed by combining the 0.2μm Nitrocellulose and 0.22μm Durapore filters into a 0.2μm cohort, and the 0.4μm Durapore and 0.45μm Nitrocellulose filters into a 0.4μm cohort (see Table 2.3). Percent transmittance data for each of the three trials was transformed using an arcsine function, which is a common technique for analyzing percentage data. Results are located in Table 2.3, and data indicate both the pore size and hydrophobic or hydrophilic nature of the material significantly affect the number of spirochetes transmitted into the filtrate.

Discussion

The goal of this work was to identify the best filter for collecting leptospires from stream water. Since none of the filters retained 100% of leptospires in suspension, this method will always exhibit inefficiency. However, performance levels were maximized at 99.44% – 99.96% recovery with 0.2μm - 0.22μm filters, which is acceptable for the purposes of environmental sampling. Therefore based on the results obtained in this study, filtration is recommended for isolating leptospires from water supplies.

Both the pore diameter and filter material were found to impact the mechanics of the filtration process. A two-way ANOVA analysis demonstrated there is a significant relationship between the quantity of transmitted cells and pore diameter ($P = 0.001$) (see Figure 2.3), but as Figure 2.2 shows, the 0.45μm Nitrocellulose-based filter did not follow this trend and was in fact significantly more selective than either of its 0.4μm Isopore and Durapore counterparts. This phenomena may be explained by the fact that the ANOVA also demonstrated a significant relationship between the number of transmitted cells and the nature of the filter material ($P=0.000$), with hydrophobic

materials transmitting significantly fewer leptospires than hydrophilic materials (see Figure 2.3, Table 2.1). The hydrophobic 0.45μm Nitrocellulose-based filter transmitted fewer leptospires than either of the 0.4μm hydrophilic counterparts, suggesting retention of leptospires will be determined more by filter material than by pore size, if the diameter of the pores differs by $\leq 0.05\mu m$. The increased retention observed with hydrophobic materials also suggests that leptospires obtained from passaged cultures, or an abiotic non-living habitat, will possess an outer membrane which is predominantly hydrophobic in nature. Subsequently it is likely that leptospires in the environment will exhibit a hydrophobic nature, and collection efforts will be best enhanced by using a hydrophobic membrane.

Interestingly, results from the two-way ANOVA indicate the pore size and filter material (hydrophobicity and hydrophilicity) interact to generate significant differences in the level of leptospire transmittance ($P = 0.002$). Smaller pores are the major deciding factor in the overall selectivity of the membrane, and filters retain the most bacteria when pores are as small as or smaller than the microbes themselves. However, as the pore diameter increases transmission becomes increasingly dependant on adherence of bacteria to the filter surface, and subsequently the hydrophobic or hydrophilic nature of the material gains significance. This phenomenon may be best observed in Figure 2.2 where leptospire transmittance was statistically similar between hydrophobic and hydrophilic materials at the 0.2μm level, but was statistically different at the 0.4μm level. Even though pore diameters were slightly less significant than hydrophobicity ($P = 0.001$ vs. $P = 0.000$), these results are based upon lumping 0.2μm with 0.22μm, and 0.4μm with

0.45μm. It is believed that if these pore sizes had been exactly equal the level of significance would have been equal to that observed with the filter materials.

The results of this study indicate several factors important to *Leptospira* research. Firstly, smaller pores and hydrophobic materials significantly increase the collection abilities of a filter, thus the optimal choice for leptospire sampling in water is the 0.2μm Nitrocellulose filter. Additionally, the glass fiber filter was found to generate visual artifacts, so any quantitative microscopy work involving *Leptospira* should not utilize glass fiber filters unless a corrective factor is estimated and applied to visual count data to account for fibrous shedding. Finally, the observed increased retention by hydrophobic materials suggests the outer membrane of leptospires in the abiotic environment is hydrophobic.

Using filters with small pores and an increased affinity for microbes may generate problems such as clogging when water samples contain a large amount of suspended solids. In such cases a smaller volume of water may be processed, a filter with a larger pore may be used, or these techniques could be combined. Additionally, nested filtration may also be a useful collection technique. A 0.8μm glass fiber filter could selectively remove larger debris but transmit a significant proportion of leptospires to a smaller, more selective filter where they may be recovered.

As discussed in the introduction, there has also been interest in using filters to collect leptospires by selectively removing large debris while transmitting the bacteria into the filtrate. If this is a goal, the results of this study indicate a hydrophilic membrane with larger pores, such as the 0.45μm Isopore or 0.8μm glass fiber filters, would be ideal. Both transmit a reasonable amount of bacteria, potentially allowing the selective

purification of leptospires in solution. If the glass fiber filter is used, it should be noted that visual artifacts may be present in the filtrate, making the suspension difficult to quantify with a microscope. Culturing or molecular detection techniques would be preferred in these instances.

This study has demonstrated that both the pore size and filter material impact how well a filter collects *Leptospira*. A hydrophobic material with pores smaller than the standard 0.45μm used to collect waterborne bacteria is necessary to optimize retention. Although these results pertain to very controlled samples in a laboratory setting, they provide the necessary foundation to begin collecting leptospires from environmental waters with filtration. However once leptospires have been collected by filtration, their presence must still be detected. Future research will focus on developing a procedure to detect leptospires trapped on membrane surfaces.

FIGURE 2.1 Microscope View of the *Petroff-Hauser* Chamber

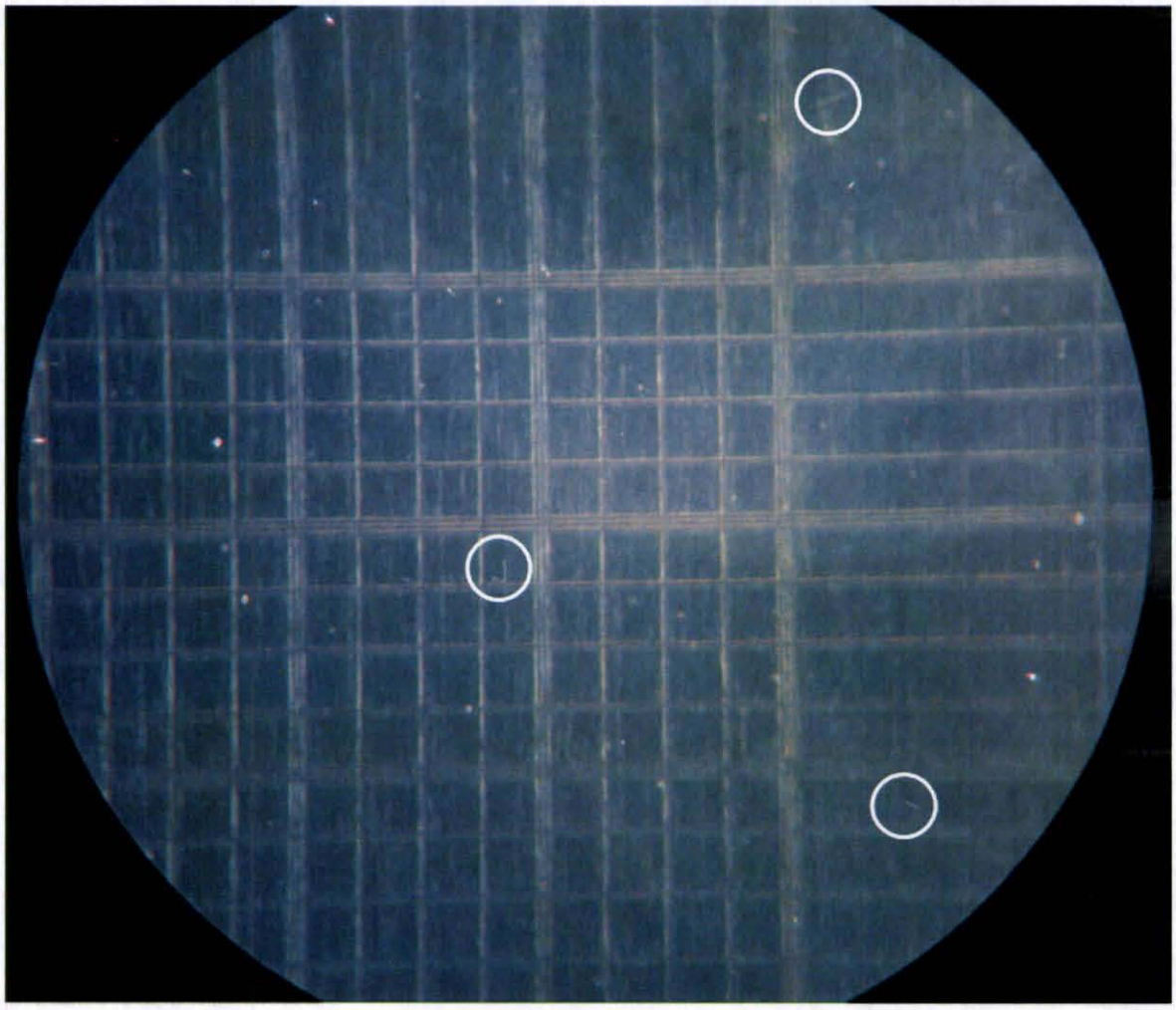


Figure 2.1: A view of the *Petroff-Hauser* counting chamber under darkfield microscopy. Three leptospires are encircled in white. Only leptospires that fall within the counting grid are counted for visual quantification purposes.

TABLE 2.1 Filter Characteristics

Filter (catalog number)	Type	Reference in Figure 2	Material	Pore Diameter
GE* Nitrocellulose-Mixed Esters of Cellulose Membrane (E02WP04700)	Hydrophobic membrane for water sampling	*0.2μm NC*	Nitrocellulose	0.20μm
Millipore Isopore Membrane filter (HTTP04700)	Hydrophilic	*0.4μm Isopore*	Polycarbonate	0.40μm
Fisher (09-719-555)	Hydrophobic membrane for water sampling	*0.45 μm NC*	Nitrocellulose	0.45μm
Millipore (AP1504700)	Hydrophilic prefilter for coarse debris removal	*0.8μm Glass*	Glass fiber	0.8μm
Small Parts Inc (CMN-0040)	Hydrophobic nylon mesh sheet	*40.0μm Nylon*	Nylon mesh	37μm
Millipore Durapore (GVWP04700)	Hydrophilic membrane for liquid purification	*0.22μm D.P.*	Polyvinylidene fluoride	0.20μm
Millipore Durapore (HVLP04700)	Hydrophilic membrane for liquid purification	*0.40μm D.P.*	Polyvinylidene fluoride	0.40μm

Table 2.1: Filter types tested to determine recovery efficiencies from suspension of pure culture of *Leptospira interrogans copenhageni icterohaemorrhagiae M-20.*

FIGURE 2.2 Transmittance of *Leptospira*

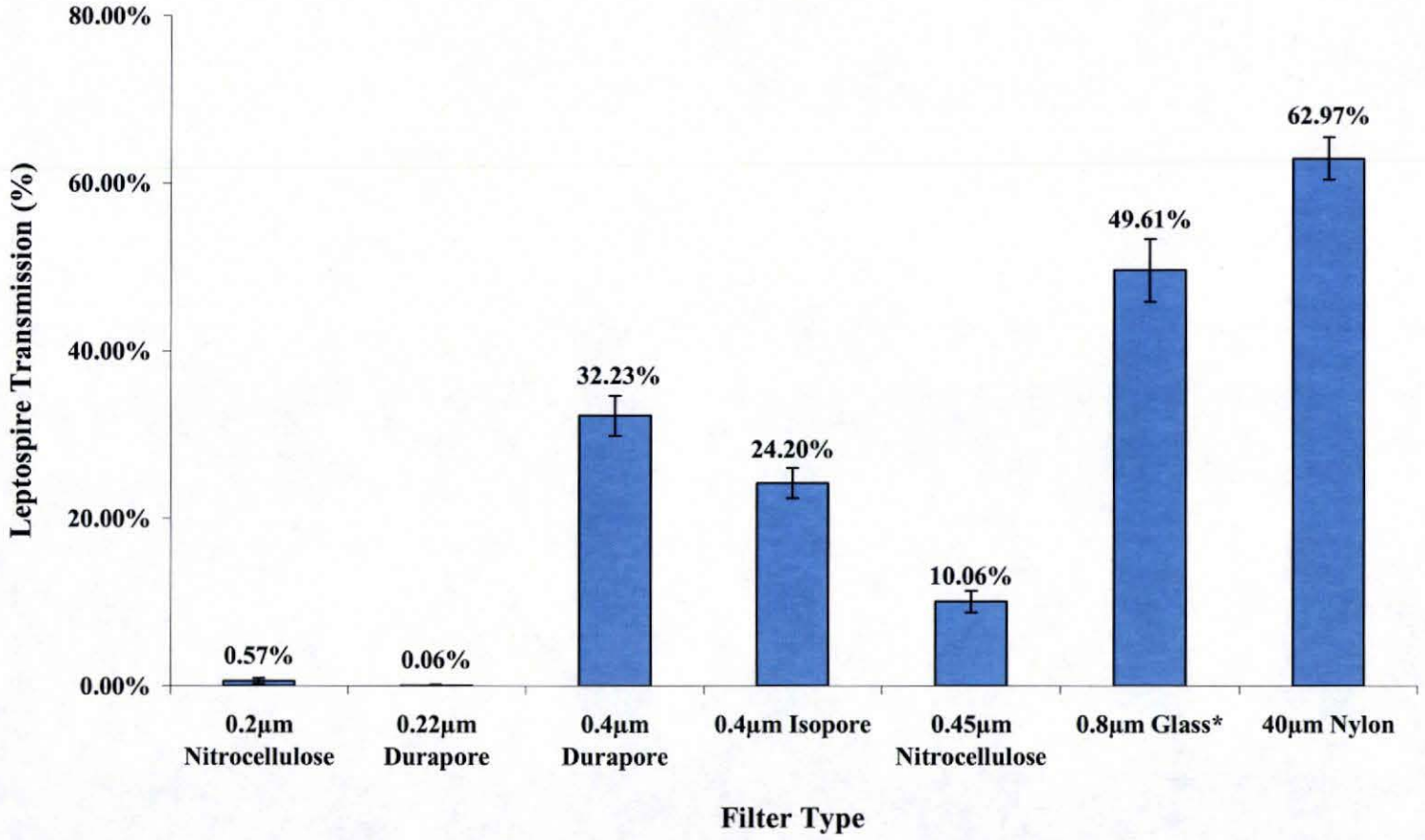


Figure 2.2: Data represent the quantity of *Leptospira* transmitted into the filtrate and are displayed as a percentage of the starting concentration. 95% confidence intervals were generated using the t statistic with 3 replicates performed for each individual filter type.

TABLE 2.2A Pore Size, Hydroscopic Material and % Transmittance

	0.2μm	0.45μm	Arcsine of 0.2	Arcsine of 0.4
Hydrophobic	A-0.57%	B-10.06%	0.64	1.27
Hydrophilic	A-0.06%	C-32.23%	0.13	1.39

TABLE 2.2B Two-way ANOVA
Arcsine Transmittance vs. Hydrophobicity, Pore Size

Source	DF	SS	MS	F	P
Material	1	0.10664	0.10664	7.51	0.025
Pore Size	1	2.66623	2.66623	187.8	0.000
Interaction	1	0.30406	0.30406	21.42	0.002
Error	8	0.11358	0.0142		
Total	11	3.1905			

S = 0.11912 R-Sq = 96.44% R-Sq(adj) = 95.11%

Table 2.2A: Tabular analysis compares 0.2 and 0.4μm pores with hydroscopic material and percent transmission values for each of the three replicate trials. The hydrophobic filter was Nitrocellulose, and the hydrophilic filter was Durapore. Values were transformed by taking the arcsine of the square root of % transmission values, and results are also displayed. Letters denote statistical similarities. Table 2.2B: A two-way ANOVA was performed on transformed values.

CHAPTER 3
IDENTIFYING *LEPTOSPIRA* IN WATER: EVALUATING A PCR METHOD

Introduction

Leptospirosis is common in a wide range of mammalian species, including humans. The disease occurs after infection by one of 90 genetically different serovars of the pathogenic *Leptospira interrogans* group (Ganoza *et al.*, 2006). Infection occurs when leptospires come into contact with an open cut or the mucosal membranes of the eyes, ears, or mouth. Most human infection results from physical contact with water, soils, or other environmental materials (Levett, 2001). Leptospirosis is often associated with seasonal flooding or heavy rain events especially in urban slums of the tropical third world. The disease manifests itself approximately 2 days to 4 weeks after initial infection, and often generates mild flu-like symptoms. In rare instances it causes severe ailments and fatality (Levett, 2001).

Since the majority of human infections result from physical contact with contaminated freshwater, there is much interest in monitoring contamination levels in water. While there is no standardized method for detecting *Leptospira* in water supplies, numerous research efforts have experimented with different technologies. Some approaches have used fluorescently labeled antibodies; some have focused on culturing techniques, and more recently polymerase chain reaction (PCR) (Henry, Johnson *et. al.*, 1971; Hodges, Ekdahl *et. al,* 1973; Rittenberg *et. al.*, 1958; Wilson & Fujioka, 1995; Haake, 2006; Vinetz *et. al.*, 2006). PCR is a very specific, genetic-based testing procedure with the potential to yield quantitative results. It was originally developed by Kary Mullis in 1986 and has come to be a common research tool for many biological fields (Russell, 2002). While researchers increasingly apply PCR to test environmental

samples for *Leptospira*, no standardized protocol has been accepted by any government agency for this purpose because the operational efficiency of this procedure has not been evaluated for this specific application.

Rigorous evaluation is necessary because studies attempting to detect *Leptospira* often assume negative results indicate the bacteria are not present when in fact negative results may actually reflect reaction inhibition due to an underlying problem with the methodology in which inhibitory substances are not effectively removed from DNA samples. Reaction inhibition may be total or partial and result in complete reaction failure, or reduced sensitivity of detection (Wilson, 1997). The reliability and sensitivity of environmental testing for pathogenic leptospires is currently unknown for leptospires. PCR first and foremost requires the successful extraction of *Leptospira* DNA from complex environmental samples. As Chapter 2 demonstrated, isolating *Leptospira* from large bodies of water is greatly enhanced using a filter with small pore diameters and a hydrophobic material, such as the 0.2μm Nitrocellulose filter. However, obtaining DNA from the bacteria trapped on these filters is challenging because DNA binds to hydrophobic materials.

Reliability also depends significantly on the purity of DNA samples. Environmental debris trapped with the bacteria on filter surfaces can contain inhibitory substances such as phenolic compounds, humic acids, and heavy metals (Wilson, 1997). These substances may affect reaction efficiency by inhibiting cell lysis, and by reducing amplification levels by degrading the DNA or interfering with polymerase activity (Wilson, 1997). A PCR-based test must also ensure the volume of recovered DNA is sufficiently small as to maximize the probability that even low levels of target DNA will

be present during amplification. Few if any of these challenges have been specifically addressed in studies which focus on detecting *Leptospira* in the abiotic environment, often because molecular kits oriented for blood, tissue, or media broth are frequently applied to complex environmental samples. Limited successes have been observed in field sampling but with the introduction of new kits and procedures for environmental samples, science is now better able to address the issues discussed above. Subsequently a PCR-based test may now be possible for detecting of pathogenic leptospires in the environment (Ganoza, Matthias *et. al.*, 2006).

The objective of this work is to determine the sensitivity of a PCR-based test for *Leptospira* with environmental samples. A DNA isolation procedure especially designed to collect microbial DNA from filter surfaces containing complex environmental properties is used to reduce the presence of inhibitory substances. Resulting samples of DNA are processed and run through PCR, and the relationship between the number of cells and the frequency of positive results is determined. Environmental properties that impact the reliability of the test will also be identified. I hypothesize that higher numbers of leptospires will generate more positive results, and that some properties of complex environmental samples will reduce the efficiency of the test. This work is a continuation of the previous study which evaluates a two-part environmental test for *Leptospira*.

Methods

To evaluate the testing procedure with environmental samples, stream water was collected from three watersheds with different land uses. Water samples were enriched with serial dilutions of *Leptospira icterohaemorrhagiae icterohaemorrhagiae M20* and

vacuum filtered. DNA was extracted from the filter surface, and the quantity and quality of DNA suspensions were evaluated. DNA samples were concentrated in smaller volumes and washed with ethanol before examination by PCR.

Stream Water Sampling

Streams were selected for sampling based on the results of the 1999-2001 O'ahu water quality study, published by the United States Geological Survey (USGS) National Water-Quality Assessment Program (NAWQA) (Anthony et. al, 2004). The goals of NAWQA are to conduct long-term, nationwide assessments of water resources in order to assess the spatial extent of water-quality conditions, characterize how water quality changes with time, and determine how human activities and natural factors affect water quality. Data from this study indicate O'ahu streams possess a wide variety of ecological and chemical properties, which is in large part due to the various land uses of the watersheds the streams drain. In several streams the levels of nutrients (nitrogen and phosphorus) and pest control chemicals (herbicides, pesticides and insecticides) were extremely high in agricultural and urban areas. In the NAWQA study frequent samples were taken from three streams, each draining an area with predominantly different land uses: Manoa stream (which drains a high-density urban area of Honolulu), Waikele stream (which drains in a mixed use area in central O'ahu), and Waihe'e stream (which runs through a heavily forested area of the Windward side). Since these streams drain areas of different land use and they have been routinely monitored for some time, they were chosen as the locations for the water sampling.

Stream water was sampled mid-morning at the USGS stations used for the NAWQA studies (see Figure 3.1). Manoa stream was sampled on July 2nd, 2007; Waihe'e stream on July 16th, 2007, and Waikele stream on July 30th, 2007. Manoa and Waikele were sampled during sunny days with under no precipitation but Waihe'e was sampled during a rainstorm, likely altering some of the water properties. Approximately 3 liters of water were carefully removed from a shallow, still area with care to minimize stream bed disturbance. Water samples were stored at approximately 4°C and characterized within 36 hours of the initial sampling. The amount of total suspended and dissolved solids were determined using protocols listed in the Standard Methods for the Examination of Water and Wastewater manual, 21st edition. Since the USGS website provides only limited access to monthly water quality data for these streams, a 250mL aliquot of each water sample was sent to the Agricultural Diagnostic Service Center (ADSC) at the University of Hawai'i for further characterization. Water samples were analyzed for pH, electrical conductance, total nitrogen, boron, calcium, copper, iron, potassium, magnesium, manganese, molybdenum, sodium, phosphorus, zinc, and various metals including arsenic, cadmium, chromium, nickel, lead, selenium, and vanadium.

Culturing Leptospira

Liquid and semi-solid media for *Leptospira* cultures was prepared according to the protocol given by Becton, Dickinson and company for Difco™ Ellinghausen and McCullough Medium as modified by Johnson and Harris (EMJH). A total of 2.3g of *Leptospira* Medium Base was dissolved in 900mL of sterile deionized water and autoclaved at 121°C for 15 minutes. Semi-solid media was prepared with 0.2% (wt/wt)

Difco Agar Noble prior to autoclaving. After cooling, 100mL of Difco *Leptospira* Enrichment Medium was added. To suppress the growth of other bacteria the selective agent 5-flurouracil (Acros Organics, cat. # 228440050) was added to a final concentration of 200µl/mL.

One of the more virulent strains of *Leptospira* is *icterohaemorrhagiae*, which is common to Hawaii and the general Pacific area, and was selected for use in this research. A live culture of *Leptospira interrogans copenhageni icterohaemorrhagiae M-20* was obtained from the National Veterinarian Services Laboratory (NVSL) in Ames, Iowa, which is a leading center for research and control of animal diseases, including leptospirosis. The culture was stored in a dark cabinet at approximately 30°C. The culture was inoculated every 5 months in both EMJH broth and semisolid media. After 6 weeks of growth, the quality of a culture was visually affirmed using darkfield microscopy.

Generating Serial Dilutions of Leptospira

To prepare water samples with serial dilutions of *Leptospira* a dilute suspension of culture was generated. 100µL of liquid culture was diluted with 45mL of 0.01M phosphate buffered saline with 0.2% 5-flurouracil. The suspension was vortexed and cell density was visually assessed with a *Petroff-Hauser* counting chamber and Nikon Labphoto microscope under dark field, 200 × magnification. Leptospires were identified as bright, linear objects that flex and bend around an axial point. Ten repetitions of cell counts were performed for accuracy.

Once the concentration of cells in the suspension was determined, 50mL conical vials were filled with homogenized stream water. Aliquots of the diluted suspension were added to the stream water to yield the following serial dilution (numbers reflect the approximate total cells in 50mL of solution): 10^0, 10^2, 10^3, 10^4, and 10^5. Two blank controls were also taken from each stream. The lowest dilution was generated by stretching a pipette tip over an open flame, swiping the elongated tip under the microscope to view spirochete collection, and then dipping the contaminated tip into a 50mL water sample. Each serial dilution was replicated 5 times for each stream sample.

Isolating Leptospira DNA from Stream Water

To maximize the probability of detecting *Leptospira* in water, bacteria should be isolated from large volume samples. Previous work indicated a 0.2μm Nitrocellulose membrane retained approximately 99.43% of leptospires in 50mL of pure suspension. Thus a sterile 0.2μm Nitrocellulose was used to vacuum-filter each of the 50mL serial dilutions of stream water. A force of -10 to -25kPa was used for filtration. Filters were aseptically removed and total microbial DNA was extracted from the filter using the UltraClean Water DNA Isolation Kit from MoBio. A LabNet flat-top vortex adapter was used to perform flatbed vortexing. DNA was eluted in a final volume of 3mL of 10mM Tris. The quality and quantity of DNA was assessed using a NanoDrop-1000 spectrophotometer with v3.3.0 software. Although DNA concentrations were adequate to perform PCR, the 3mL volume was deemed too large to ensure trace amounts of target DNA would be included in a PCR reaction.

To reduce sample volume, DNA was concentrated using Bioline's co-precipitant pink protocol, with two 70% ethanol washes. Half the recommended volume of co-precipitant pink was used (3µl instead of 6µl) to ensure the concentration of co-precipitant in the end product was low. All 14,000 x rpm centrifugations of 50mL vials were performed in a Sorvall RC 5B Plus centrifuge, and 1.5 – 2mL tubes were centrifuged in a 5424 Eppendorf microcentrifuge. After the final wash, DNA was resuspended in 20µL of sterile water. Samples from Manoa stream underwent two series of precipitations. The first precipitation was performed following the protocol provided by MoBio, which does not include an ethanol wash. Following the MoBio precipitation, DNA was resuspended in 200µL of MoBio W5 buffer and precipitated again using Bioline's co-precipitant pink protocol.

Detecting Leptospiral DNA from Contaminated Filters

PCR was performed on the DNA samples to detect the presence of pathogenic leptospires in stream water. The gene for the gyrase subunit B was selected for identification because it is more evolutionarily diverged than 16s rDNA, a conventional marker (Slack *et al.*, 2006). Primers for gyrB are very specific to pathogenic leptospires and were never found to amplify non-pathogenic strains, helping to minimize the potential that genes in non-target organisms will be amplified. Gyrase B primer sequences are given below, and generate an expected product size of 502 base pairs:

2 Forward- TGAGCCAAGAAGAAACAAGCTACA

504 Reverse- MATGGTTCCRCTTTCCGAAGA

All polymerase chain reactions were performed using Promega's GoTaq Flexi PCR kit in a total volume of 50μL with the following reagent volumes: 31.75μL dH_2O, 4μL $MgCl^{2+}$, 10μL GoTaq buffer, 1μL of each primer, 1μL of dNTPs, 1μL of DNA, and 0.25μL Taq polymerase. Amplifications were performed with either an Applied Biosystems GeneAmp PCR System 2700 thermocycler or a MJ Research PTC-200 Peltier thermocycler, depending on availability. The thermocycling program was optimized to include an initial denaturation of 94°C for 5 minutes, 35 cycles of 94°C for 1 minute, an annealing temperature for 1 minute, and extension at 72°C for 1.5 minutes, after which a final extension at 72°C for 10 minutes was performed. 10μL of PCR products were run out on a 1.5% agarose gel at 94V, and then soaked in a 5μg/mL ethidium bromide solution for 15 minutes. A Kodak DC 290 camera was used in conjunction with Kodak 1D 3.5 software to analyze gels. Logistic regression was performed on successful results.

To determine whether impure DNA was the cause of unsuccessful results, one sample of the highest serial dilution was taken for each stream and purified using QIAGEN's QIAQUICK PCR Purification Kit. PCR was performed on these samples, and linear regression was used to identify the relationship between specific water quality characteristics and the probability of obtaining a positive result. Data are discussed below.

Results

Stream Water Quality

Stream water samples demonstrated unique characteristics and measurements are listed in Table 3.1. Waihe'e and Waikele streams had higher levels of cobalt, chromium

and nickel. Total dissolved solids were higher than suspended solids in all three streams. Manoa stream had the highest levels of total suspended solids, copper, dissolved nitrogen, and phosphorus. Total dissolved solids were relatively the same between the streams. Manoa stream had the lowest levels of arsenic, cobalt, nickel, and selenium. Waihe'e had the lowest amount of suspended solids and a slightly basic pH. Waikele stream had the highest electrical conductivity and highest levels of sodium, magnesium, iron, calcium, and manganese.

Water characterizations were also compared with the water quality standards set by the Hawai'i Department of Public Health (HDOH, 2004) (see Table 3.1). Values that exceeded recommended acute standards for all inland freshwater bodies were bolded. The amount of total nitrogen reported by ADSC exceeded HDOH's recommended limits for all three streams. Even in the wet season. Manoa had 2270μg/L of N, which was exceedingly high, and may be attributed to the high density urban land use which receives heavy applications of fertilizer. Waihe'e stream fell under the 600.00μg/L limit for total N, which DOH accepts as long as it remains at this level no more than 2% of the time (HDOH, 2004). Phosphorus levels also exceeded HDOH's recommendations for the dry season, and Manoa levels were so high they exceeded every phosphorus standard. The amount of selenium and nickel exceeded HDOH standards in all streams, and the amount of lead in Waihe'e was extremely high. Copper and chromium were very high in two of the three streams, but the levels of the other elements and metals were not problematic by HDOH standards.

DNA Recovery

After extracting DNA from filters used on the serial dilutions and blank controls, the quantity and purity of DNA obtained from the filters was determined using a Nanodrop 1000 spectrophotometer with version 3.3.0 software. Replicate values were averaged and displayed in Table 3.2. Absorbance of 260nm indicates the presence of DNA while absorbance at 280nm reflects the presence of proteins (Glasel, J. 1995). One estimate of DNA purity is the A_{260}/A_{280} ratio. Good-quality DNA will have a ratio of 1.7–2.0. Only 8 samples fell into this range (see Appendix). When the results from each trial were averaged, only two dilutions from Manoa stream fell within this range (see Table 3.2).

Additionally, strong absorbance around 230nm can indicate the presence of thiocyanates, chaotropic salts, phenols, or humic acid in the sample (Lubenhussen, 2004). The A_{260}/A_{230} ratio can help evaluate the level of carryover from the sample into purified DNA. As a guideline, clean DNA should have an A_{260}/A_{230} ratio greater than 1.5. None of the samples obtained from the MoBio extraction kit fell above 1.0 (see appendix and Table 3.2).

The average amount of DNA recovered from the extraction procedure was also graphed (see Figure 3.1). The amount of DNA recovered was not different between either the serial dilutions or between the three water types. This may indicate the amount of background DNA was not significantly increased by the leptospire additions. Alternatively, the extraction kit may have been limited in the amount of background DNA it could recover, and all samples met or exceeded this level.

The extraction procedure is sensitive to the presence of certain heavy metals but only iron has been officially recognized as an inhibitor (personal communication). While

DNA was collected consistently between all streams, sample quality may have been affected by the presence of some heavy metals.

Detecting Leptospiral DNA in the Sample

PCR products were electrophoresed on agarose gels and the results are displayed below in Figures 3.2 and Figure 3.3. Leptospiral DNA from the Manoa water samples was detected in at least 3 replicates of each dilution, and was not amplified in either negative control (see Figure 3.2A). The positive control yielded a double band, indicating the double bands observed in the serial dilutions likely do not reflect contamination during processing. Thus results indicate the testing methodology is able to detect pathogenic *Leptospira*l DNA, even at low levels. The frequency and total number of positive results are listed in Figure 3.2B, and binary logistic regression was performed on the data. The relationship between the number of spirochetes and the frequency of positive results was not significant. No *Leptospiral* DNA was detected in the Waihe'e and Waikele series (See Figure 3.3 A, B).

Three metals, Cr, Co, and Ni, were found to be present in low levels in Manoa, but at higher levels in the other two streams (identified from Table 3.1). The standard multiple regression analysis demonstrated each of the three metals had a significant impact on whether the spirochetes were detected (P for Co, Ni, and Cr = 0.000; see Table 3.3). Although the abundance of metals was always Cr > Ni > Co, the regression coefficients indicate the level of Ni was the most significant factor impacting test outputs, followed by Cr and then Co (see Table 3.3).

One of the highest dilutions from each stream was purified using a QIAQUICK kit, DNA was amplified using PCR, and results are shown in Figure 3.3C. None of the samples generated bands except the positive control, indicating DNA purity was not good enough to obtain results. The Manoa sample, which had worked prior to purifying, did not give a positive signal after the purification step.

Discussion:

The goal of this work was to evaluate the sensitivity of a *Leptospira* environmental test using natural water samples. Seeded stream samples and blank controls were processed using a multi-step, genetic-based detection method. Results indicate microbial DNA was recovered from all stream samples, and *Leptospira*l DNA was detected in the Manoa set even at low dilutions. Thus it is reasonable to conclude the method could be applied for stream sampling.

However while the Manoa test yielded data both the Waihe'e and Waikele analyses failed, indicating this testing procedure is not yet reliable enough for routine use in Pacific freshwater systems. Regression analysis demonstrated the operational efficiency could be affected by the presence of specific metals such as Ni, Co, and Cr. This would affirm a MoBio reference that the sensitivity of the DNA extraction kit to the presence of heavy metals (personal communication). If the amount of metals was the reason for the failed analyses, this procedure would be recommended only for areas with low levels of metals in freshwater. However, results from the regression analysis are not conclusive because linear regression only determines the strength of relationships between the input factors; it does not indicate whether an alternative problem existed. Since DNA was consistently obtained from all samples, and sample purity was poor for

all stream trials, the relationships between Ni, Cr, and Co and the frequency of positive results demonstrated by the regression analysis are probably coincidental.

Logistic regression on the Manoa samples did not demonstrate a relationship between the number of *Leptospira* cells and the number of positive signals. Theoretically this is possible because only one strand of target DNA is required to generate a positive signal, and the procedure does have the potential to be very sensitive. However, the logistic regression may not have demonstrated a relationship simply because more replicates were needed for each serial dilution. Due to budgeting and time constraints, this was not possible. More work is necessary to determine whether the number of replicates or test sensitivity influenced regression results.

The reliability of this method is questionable. Almost all of the Manoa dilutions generated at least one negative result, and the method completely failed for the other two stream sets. Negative results must reflect the lack of target DNA in a sample or a problem with the chemical balance of the reaction. This may be due to the presence inhibitory substances like Ni, Cr, and Co, or it may be due to other unmonitored substances such as ion concentrations, polymerase inhibitors, or alternatively, the primer annealing temperature. PCR banding exhibited differences even when the same annealing temperature was used on different thermocyclers. The experiment was set up so that all samples contained leptospires, and the presence of DNA was verified in all samples, so the template must have been available in the PCR reaction mixture. Therefore the negative test results and positive detection of control DNA most likely reflect a problem with the quality of the sample. Many other studies using similar PCR methods have also experienced inconsistent success, suggesting the complex conditions

of environmental samples is a significant factor for this procedure, and the procedure must thoroughly address this issue to ensure the testing method is reliable. Too often positive results are emphasized while negative results are assumed to indicate a lack of target bacteria.

This study integrated well-tested protocols specific to environmental samples but found this method too unreliable for routine use. Although this method appears to be sensitive, much more work is required to improve its reliability and to complete the evaluations attempted here. This environmental test does not appear practical for rural, isolated areas, even if the reliability is improved. However more sophisticated and well funded laboratories may find it worthwhile to pursue this approach further.

FIGURE 3.1 Surface Water Gauging Stations

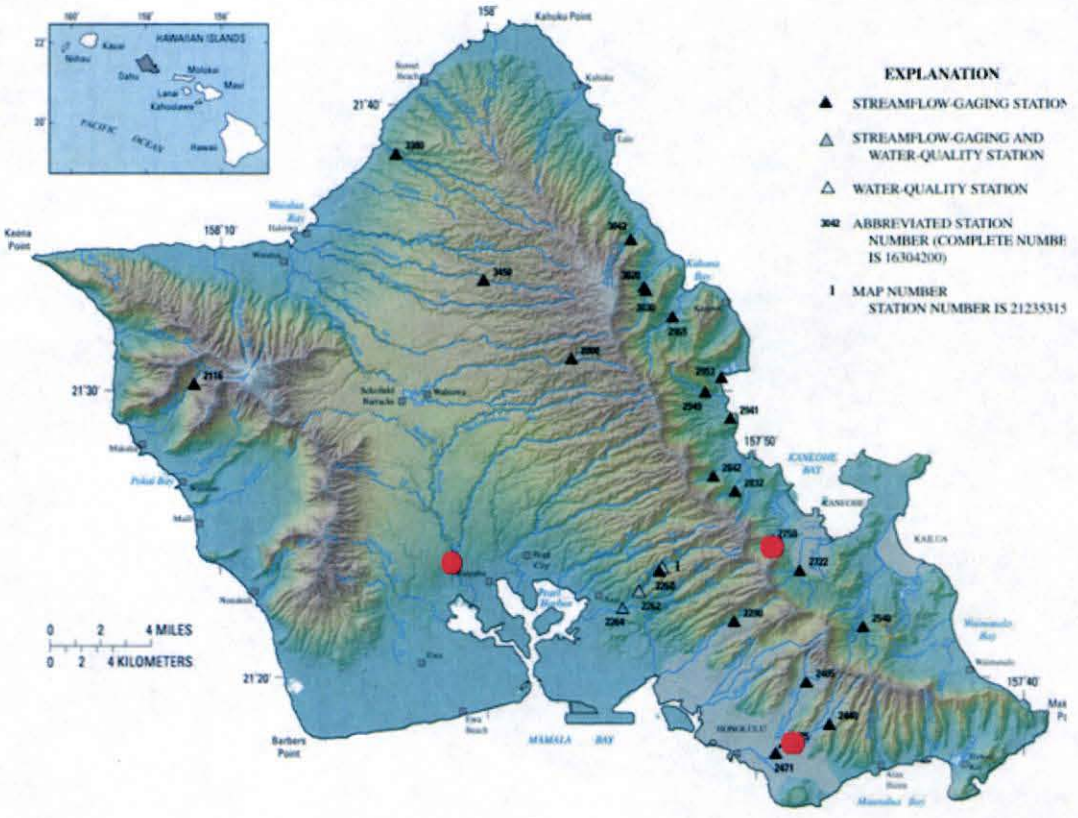


Figure 3.1: A map of USGS surface-water gauging stations on the island of O'ahu, in Hawai'i. Red circles mark the locations selected for the water sampling in this study.

TABLE 3.1 Water Quality Measurements

Characterizations	DOH Standards	Manoa	Waihe'e	Waikele
TSS (mg/L)	10	0.823	0.0767	0.423
TDS (mg/L)	-	0.0117	0.0217	0.0117
pH	-	7.6	**8**	7.6
EC mmhos/cm	-	0.205	0.19	0.54
*As (µg/L)	360	18	**137**	30
*B (µg/L)	-	50	23	100
*Ca (µg/L)	-	11120	9770	18740
*Cd (µg/L)	3+	1	**12**	0
*Co (µg/L)	-	4	22	10
*Cr (µg/L)	16	14	**53**	**30**
*Cu (µg/L)	**6**	**40**	**23**	0
*Fe (µg/L)	-	20	0.00	70
*K (µg/L)	-	2080	1640	2920
*Mg (µg/L)	-	10560	6370	18450
*Mn (µg/L)	-	0.00	0.00	10
*Pb (µg/L)	29	20	**147**	10
*Na (µg/L)	-	18120	13580	78490
*Ni (µg/L)	**5**	**9**	**33**	**20**
*Zn (µg/L)	22	20	2	20
*N (µg/L)	**800**	**2270**	**410**	**1510**
*Se (µg/L)	**20**	**27**	**196**	**30**
*P (µg/L)	**150**	**160**	**110**	**110**

Table 1: This table displays measurements of various water properties in Manoa, Waihe'e, and Waikele streams. Total suspended solids are abbreviated as TSS, total dissolved solids as TDS, and electrical conductivity as EC. The green cells represent non-metal elements, blue indicates metals, and pink denotes semi-conductive elements. * indicates values were provided by ADSC in µg/mL and were multiplied by 1000 to convert into µg/L, which are DOH standard units. Bolded values indicate the measurement exceeds the health standards set by the Hawai'i Department of Public Health.

TABLE 3.2 Average Quantity and Purity of Recovered DNA

Stream	Dilution	Total DNA (ng/µL)	$A_{260/280}$ (nm)	$A_{260/230}$ (nm)
Manoa	Control	4.135	10.315	0.065
	10^0	2.662	1.932	0.394
	10^2	2.87	2.912	0.348
	$*10^3$	3.1925	3.1925	0.28
	10^4	2.056	2.056	0.188
	$*10^5$	3.0175	3.0175	0.2675
Waihe'e	Control	3.59	-7.035	0.05
	10^0	4.492	3.594	0.06
	10^2	3.182	11.49	0.04
	10^3	2.6	0.106	0.048
	10^4	2.976	1.412	0.042
	10^5	3.87	4.654	0.056
Waikele	Control	3.06	7.345	0.07
	10^0	4.08	4.08	0.088
	10^2	2.966	6.65	0.064
	10^3	3.808	2.726	0.068
	10^4	4.002	8.266	0.052
	10^5	4.848	5.892	0.066

Table 3.2: The quantity and purity of DNA was estimated with a spectrophotometer. 260/280 absorbance ratios indicate the amount of protein in the sample, and 260/230 absorbance ratios indicate the level of salt and other impurities. Control values are the average of 2 trials, and dilution values are the average of 5 trials. * Indicates one of 5 values were omitted from the average calculation due to extreme deviation.

FIGURE 3.2 Average Total DNA Recovered

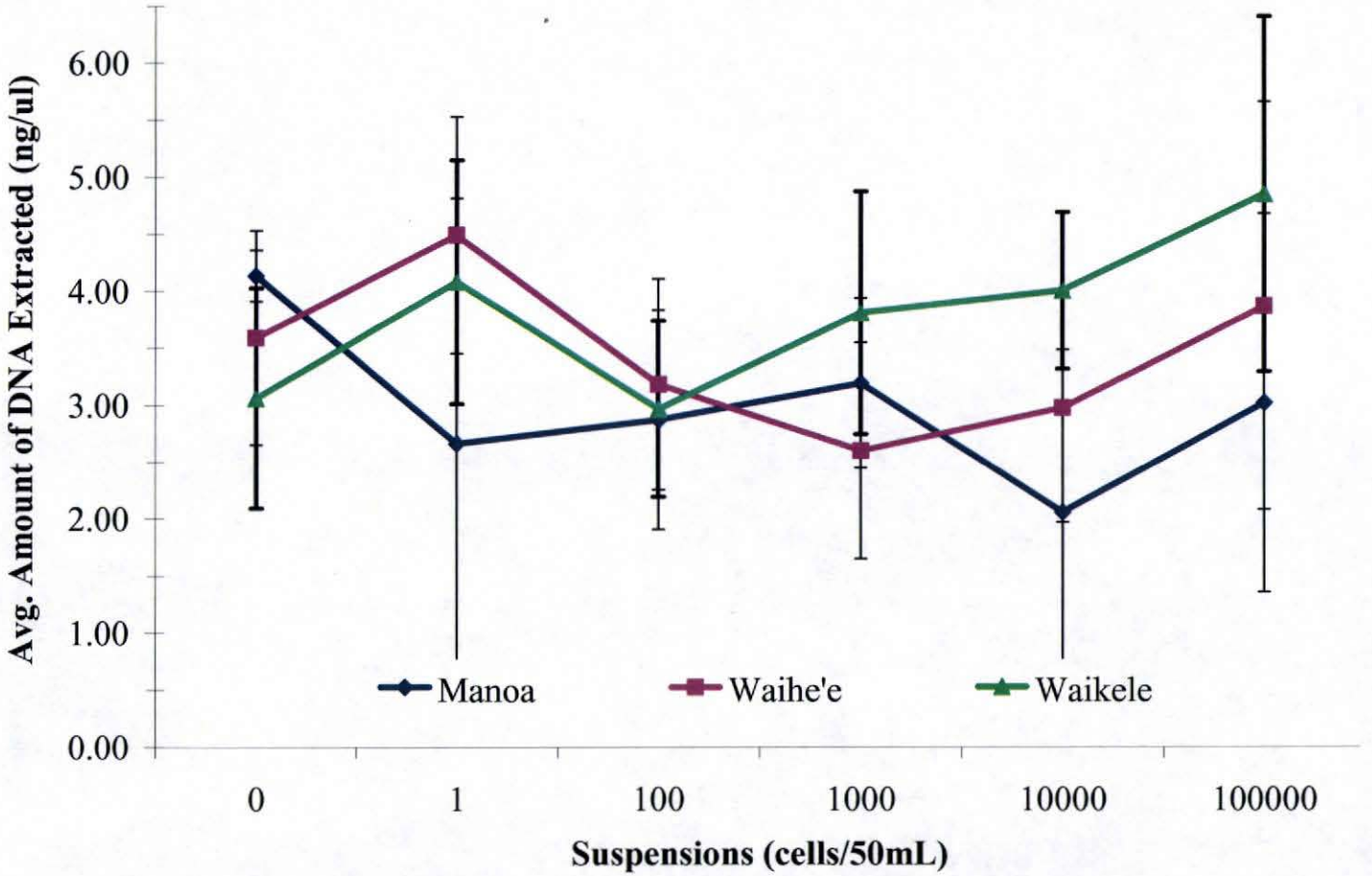


Figure 3.2: Average DNA concentrations from each dilution of the stream trials. Labels on the x-axis indicate serial dilutions with 0, ~ 1, 100, 1,000, 10,000, and 100,000 leptospires. 95% confidence intervals are shown.

FIGURE 3.3A Manoa

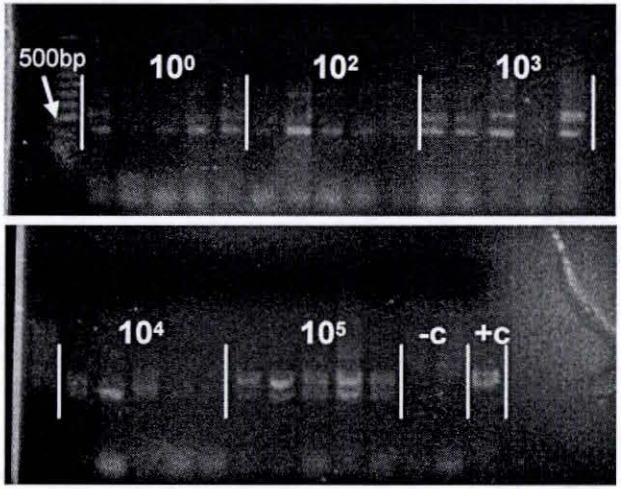


FIGURE 3.3B Tally of Positive Signals

	10^0	10^2	10^3	10^4	10^5	- C
Positive Results	3	3	4	3	5	0
Frequency	0.6	0.6	0.8	0.6	1.0	0

***Binary logistic regression:** G = 3.375, DF = 1, P = 0.066

Fig. 3.3A: PCR results from the Manoa stream samples. Serial dilutions are labeled and replicates are outlined with white bars. There were 2 negative controls and 1 positive control. **Fig. 3.3B:** Tabulated results from the Manoa PCRs,

FIGURE 3.4A Waihe'e

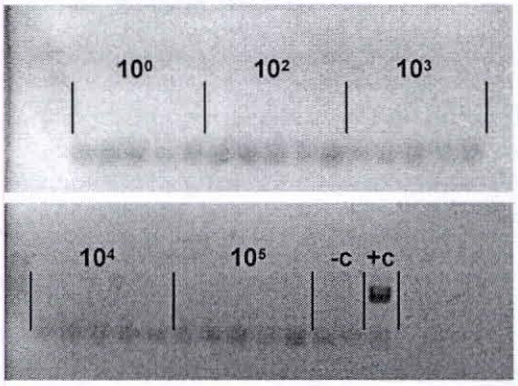


FIGURE 3.4B Waikele

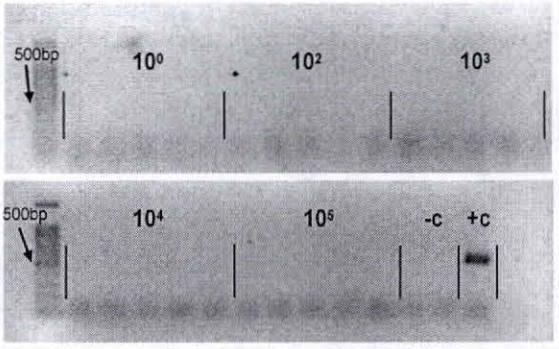


FIGURE 3.4C Purification

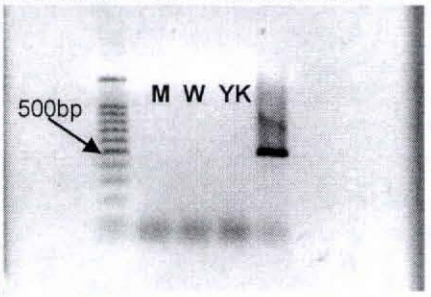


Fig. 3.4A: PCR results from Waihe'e stream. Lane marker was added but did not fluoresce brightly. The positive control exhibits a dark blurry band. **Fig. 3.4B:** PCR results from Waikele stream. The positive control exhibits a crisp band. Lane marker is blurry. **Fig. 3.4C:** PCR results: a single sample of the 10^5 dilution was purified from each stream. Abbreviations include M = Manoa, W = Waihe'e, and YK = Waikele.

TABLE 3.3 Relationships between Detection and Water Quality

Predictor	Coef	SE Coef	T	P
Co	-0.51852	0.04287	-12.10	0.000
Ni	-0.96296	0.09657	-9.97	0.000
Cr	0.81481	0.07631	10.68	0.000

*Regression equation: $y = -0.519\ Co - 0.963\ Ni + 0.815\ Cr$

Table 3.3: Linear regression was used to correlate three water quality measurements with the efficiency of the test results. An equation of the relationship between the three factors and the probability of obtaining a positive result is shown below the tabular output.

CHAPTER 4
PROJECT SUMMARY

The goal of this project was to develop an environmental test for pathogenic *Leptospira* in water. Research was conducted over a two-year period from December, 2005 through August, 2007. The filtration studies were funded by the United States Environmental Protection Agency (EPA), and the genetic analysis was funded by the United States Department of Agriculture's Cooperative State Research, Education, and Extension Service (USDA CSREES). These works are original investigations that are intended to provide a foundation for additional methods development and field testing. It is hoped that agencies interested in detecting and/or monitoring *Leptospira* in water supplies will find this data to be a useful starting point.

The proposed environmental test consists of a two-part method which isolates bacteria from environmental samples and then uses a genetic test to identify the presence of pathogenic *Leptospira*. This work was divided into separate studies that evaluated the performance of different filters in collecting *Leptospira*, and the sensitivity of detection under real-world conditions. Results indicate that filtration is highly efficient in collecting leptospires, and that PCR-based detection has the potential to be both specific and sensitive. However, further research is necessary before the limits and sensitivity of the procedure can be well-defined.

The first study examined how various filters performed with liquid suspensions of *Leptospira*. The 0.2μm nitrocellulose and 0.22μm Durapore membranes gave the highest level of bacteria retention, allowing only 0.06% and 0.56% of the respective starting concentrations through. The 0.2μm nitrocellulose membrane was selected as the best choice for the environmental test because a MoBio company representative indicated

nitrocellulose is more compatible with the MoBio UltraClean Water DNA Isolation kit, used in the second study.

Interestingly, Chapter 2 results show that 0.4 -0.45μm filters passed 10.06 – 49.35% of bacteria in solution, challenging a general assumption that 0.45 microns is small enough to retain all single-celled organisms. Due to the observed retention levels it was determined a smaller pore would be necessary to maximize the collection of leptospires. The reason for this anomaly could be attributed to the fact that spirochetes are linear in shape and inherently flexible, which may enable them to thread through a filter pore. Even 0.2μm pores demonstrated low levels of flow through, so an outside collaborative investigation using scanning electron microscopy was performed. Results demonstrated the filter surface inherently possesses a small amount of overlapping holes which generates a larger-than normal "super-pore", big enough to pass a leptospire. Although very selective filters will still pass a small proportion of bacteria, results indicate bacterial retention can be approximately maintained around 99.43% so this phenomenon is not of significant concern for the goals of this project.

Filtration was originally chosen as the means to collect and concentrate leptospires because it is commonly used in standardized water-borne bacteria tests. In particular, e-coli and fecal coliforms are routinely monitored with methodologies that use filtration. Even so, the practicality of using filtration for leptospire testing has been questioned by some, largely because filtration could be restricted by high levels of total suspended solids in water supplies. The level of total suspended solids depends on the site specific characteristics such as land use, climate and hydrologic conditions, and is highest during storm events. Under certain conditions the amount of suspended

particulate matter could become so high that large-volume water samples would clog the filter and prevent testing. However, clogging was never observed during this investigation, and the quantity of DNA recovered was consistent for all samples (see 3.1.2). Additionally, TSS concentrations were not above 1.0 mg/L, even though Waihe'e stream water was collected during a heavy rain, high flow event. Thus potential problems with high TSS during large hydrologic events may not be a problem in conservation lands similar to the Waihe'e drainage basin.

The significance of TSS levels during testing is largely determined by the goals of specific field samplings. For instance, some agencies may opt to perform routine water testing where TSS levels are predictable and enable the samples to be processed; in these instances locations such as water gauging stations, swimming holes, or areas of point source pollution would be adequate for study. Other groups may prefer to test during specific high-flow events when TSS levels are extremely high and could prove more challenging to the methodology. If this is the case, it is recommended that the method's simple filtration be changed either to nested filtration, which provides graduated selectivity, or to another process. One alternative is that samples may be centrifuged to compact the particulate matter, excess water could then be siphoned off, and microbial DNA could be recovered from the solid material using MoBio's UltraClean Soil DNA Isolation Kit, cat # 12800-50. However, in most instances MoBio's UltraClean Water DNA Isolation Kit will be adequate for water sampling.

The second investigation attempted to define the limits of genetic testing on filters through which *Leptospira* spiked samples had been passed. DNA concentrations recovered from used filters was statistically similar for all samples, suggesting the

UltraClean Water DNA Isolation Kit performs consistently under the various water and microbial conditions measured. However, the volume and impurities of the resulting DNA did not make PCR immediately appropriate, so samples were concentrated and washed using Bioline's Co-Precipitant Pink protocol. The use of the co-precipitant aided in visualizing the DNA but prevented spectrophotometer analysis, so the purity and concentration of the samples after concentration was unknown. The PCR analysis only had amplicons for the Manoa samples, but all the positive controls were amplified which suggests the failures are attributable to the sample quality. Interestingly, the Manoa set was concentrated twice, once with the procedure given by MoBio's UltraClean Water DNA Isolation kit and then by Bioline's protocol. Initial concentrations using the MoBio protocol demonstrated no visible DNA pellet, and the DNA was resuspended in a buffer not suitable for PCR. So the Bioline procedure was adopted because it helps visualize smaller DNA pellets, and produces PCR-ready DNA. Given the results observed here, Bioline's co-precipitant pink is not recommended as the sole method for prepping DNA for PCR.

After the large number of failed detection attempts, some DNA samples were purified using QIAGEN's QIAQUICK PCR Purification kit and tested again for the presence of *Leptospira*l DNA. The method failed again, except for the positive control. Additional research is recommended to identify a method that will adequately prep the DNA for PCR. Several options include using MoBio's general protocol provided with the UltraClean kit or MoBio DNA cleanup kits such as the PowerClean DNA Cleanup Kit (catalogue # 12877-50), UltraClean PCR Purification kit (catalogue # 12500-50).

The objective of this second study was to identify the sensitivity of genetic-based detection, which was defined as the probability of obtaining a false negative result due to a low number of leptospires in the starting sample. While only a single strand of target DNA is needed for PCR to produce amplicons, sample processing methods contain numerous steps during which small amounts of DNA can be lost. This loss may become significant for samples with low levels of target bacteria simply because smaller amounts of DNA are more likely to be lost. Other studies have been successful in quantitatively measuring this sensitivity with calibration curves (Walker, 1998), thus it was hypothesized that the same phenomenon would be observed here: smaller dilutions would have higher probabilities of giving false-negative results. If true, this would be of significant importance because this procedure is aimed at detecting the presence of a microbe sparsely scattered throughout a water supply. The results of the logistic regression on the Manoa sample set indicate the number of cells in the sample have nothing to do with the frequency of detection. Since the results conflict with a demonstrated phenomenon, the number of replicates used in the analysis was likely too small to give an accurate representation.

General results from both investigations demonstrate this procedure has the potential to become reliable and sensitive. The UltraClean Water kit is recommended for field testing in Hawai'i and other areas of similar hydrology. Additional work is required to determine the best method of prepping DNA samples for PCR. However, it is predicted that PCR will be a sensitive and specific method of detection once DNA quality is improved. Unfortunately, PCR is both technically sophisticated and time consuming. The initial goals of this project were to develop a rapid, cheap, easy-to-use environmental

test, and PCR does not fulfill this objective. Because of the growing acceptance PCR is receiving in various scientific fields, it is hoped this method will be useful to a variety of agencies once the issues of DNA purity are resolved.

Should this procedure be optimized it would provide the opportunity to address a variety of agency and research needs. Having the capability to identify areas contaminated with pathogenic leptospires would enable field testing that could determine where leptospires frequently occur within a freshwater body. Once sampling strategies are devised, this methodology can be implemented to identify and monitor contamination. Routine testing will help in the development of public protection strategies and land management strategies that minimize pathogen inputs. For instance, in Manoa valley this test may help determine point-source areas of pollution that correspond to the habitat of a specific host population, such as rats or pigs. In American Samoa, this test may help identify piggeries discharging contaminated effluent into streams. This would enable specific improvements in animal waste management strategies and result in cleaner, safer streams for the territory.

Having the capability to very specifically detect leptospires may also help address research interests. The number of and diversity level within *Leptospira* serovar populations could be identified, and if a real-time PCR protocol was calibrated, the quantity of bacteria could also be estimated. This would provide more insight into whether any trends exist for the quantity and spatial distribution of the bacteria. Although this project did not fully optimize the methodology, there is much incentive to pursue the research further. It is hoped future research will continue this work and succeed in developing a sensitive, reliable procedure.

Appendix A

TABLE A.1 Accuracy of Visual Quantification as a Function of Sample Size

	D (Deviation of Observed, True Mean)		
N	Low	Average	High
1	0	9.14	15.03
2	0	6.46	10.63
3	0	5.28	8.68
4	0	4.57	7.52
5	0	4.09	6.72
6	0	3.73	6.14
7	0	3.45	5.68
8	0	3.23	5.32
9	0	3.05	5.01
10	0	2.89	4.75
11	0	2.76	4.53
12	0	2.64	4.34
13	0	2.54	4.17
14	0	2.44	4.02
15	0	2.36	3.88
16	0	2.29	3.76
17	0	2.22	3.65
18	0	2.15	3.54
19	0	2.10	3.45
20	0	2.04	3.36

Table A.1: The variance of the observed population means from the true population means were calculated for each of the filter trials (10 counts each). The smallest, average, and largest variance values were 0, 21.75, and 86.00. These values were used to calculate the deviation of the observed population mean from the true pop. mean, for a range of sample sizes using Equation A. Data indicate the range of accuracy to be expected using the Petroff Hauser visual quantification procedure depending on the sampling size chosen.

Appendix B

TABLE A.2 Quantity and Purity of Recovered DNA

MANOA STREAM				WAIHE'E STREAM				WAIKELE STREAM			
Dilution	**Total DNA (ng/µL)**	**A_{260} / 280 (nm)**	**A_{260} / 230 (nm)**	**Dilution**	**Total DNA (ng/µL)**	**A_{260} / 280 (nm)**	**A_{260} / 230 (nm)**	**Dilution**	**Total DNA (ng/µL)**	**A_{260} / 280 (nm)**	**A_{260} / 230 (nm)**
C - 1	4.02	9.47	0.06	C - 1	3.84	2.83	0.07	C - 1	3.84	2.83	0.07
C - 2	4.25	11.16	0.07	C - 2	2.28	11.86	0.07	C -2	2.28	11.86	0.07
10^0	6.66	1.55	0.67	10^0	3.06	5.45	0.12	10^0	3.06	5.45	0.12
10^0	0.93	2.26	0.23	10^0	4.43	3.76	0.07	10^0	4.43	3.76	0.07
10^0	3.41	1.89	0.39	10^0	4.92	7.96	0.05	10^0	4.92	7.96	0.05
10^0	1.06	2.15	0.36	10^0	5.42	3.01	0.08	10^0	5.42	3.01	0.08
10^0	1.25	1.81	0.32	10^0	2.57	2.23	0.12	10^0	2.57	2.23	0.12
10^2	2.18	1.88	0.23	10^2	1.81	1.43	0.06	10^2	1.81	1.43	0.06
10^2	4.03	5.88	0.16	10^2	3.24	2.56	0.06	10^2	3.24	2.56	0.06
10^2	1.86	1.63	0.5	10^2	3.91	1.82	0.07	10^2	3.91	1.82	0.07
10^2	4.1	2.67	0.4	10^2	3.57	2.31	0.07	10^2	3.57	2.31	0.07
10^2	2.18	2.5	0.45	10^2	2.3	25.13	0.06	10^2	2.3	25.13	0.06
10^3	2.43	2.23	0.42	10^3	5.08	1.68	0.08	10^3	5.08	1.68	0.08
10^3	2.66	3.24	0.25	10^3	4.82	2.75	0.07	10^3	4.82	2.75	0.07
10^3	3.71	3.48	0.16	10^3	2.38	2.95	0.07	10^3	2.38	2.95	0.07
$*10^3$	93.53	1.86	0.57	10^3	2.73	2.79	0.05	10^3	2.73	2.79	0.05
10^3	3.97	2.46	0.29	10^3	4.03	3.46	0.07	10^3	4.03	3.46	0.07
10^4	1.78	2.46	0.27	10^4	4.22	14.71	0.05	10^4	4.22	14.71	0.05
10^4	4.54	2.39	0.23	10^4	3.99	5.1	0.06	10^4	3.99	5.1	0.06
10^4	0.67	-1.1	0.12	10^4	3.97	2.44	0.06	10^4	3.97	2.44	0.06
10^4	2.66	1.94	0.11	10^4	5.01	3.47	0.05	10^4	5.01	3.47	0.05
10^4	0.63	0.81	0.21	10^4	2.82	15.61	0.04	10^4	2.82	15.61	0.04
10^5	0.49	-0.77	0.12	10^5	7.31	2.18	0.11	10^5	7.31	2.18	0.11
10^5	3.91	1.71	0.47	10^5	3.63	2.03	0.07	10^5	3.63	2.03	0.07
10^5	4.03	2.6	0.35	10^5	6.12	6.12	0.05	10^5	6.12	6.12	0.05
$*10^5$	49.1	2.06	0.49	10^5	3.96	7.22	0.06	10^5	3.96	7.22	0.06
10^5	3.64	-2.4	0.13	10^5	3.22	11.91	0.04	10^5	3.22	11.91	0.04

Table A.2 Spectrophotometer readings for each of the DNA samples.

REFERENCES

1. Anthony, Stephen S.; Hunt, Charles D. Jr.; Brasher, Anne M.D.; Miller, Lisa D.; Tomlinson, Michael S. 2004. *Water Quality on the Island of Oahu, Hawaii, 1999-2001*:Reston, Va., U.S. Geological Survey Circular 1239:37.

2. ASEPA: Winger, Kirk: Aguilar, Judith; Besser, Richard; Rosenstein, Nancy; lark, Thomas. December 2003. *Leptospirosis: A Seroprevalance Survey on American Samoa.* Department of Health and Human Services, Centers for Disease Control.

3. Bharti, A.R.; Nally, J.E.; Ricaldi, J.N.; Matthias, M.A.; Diaz, M. M.; Lovett, M. A.; Levett, P. N.; Gilman, R. H.; Willig, M. R.; Gotuzzo, E.; Vinetz, J. M. 2003. *Leptospirosis: A Zoonotic Disease of Global Importance.* Lancet Infectious Diseases, 3(12):757-771.

4. Braun, John L.; Diesch, Stanley L.; McCulloch, William F. July 18, 1967. *A Method for Isolating Leptospires from Natural Surface Waters.* Canadian Journal of Microbiology, 14:1011-1012.

5. CDC (Centers for Disease Control and Prevention). 1998. *Update: Leptospirosis and Unexplained Acute Febrile Illness Among Athletes Participating in Triathlons-Illinois and Wisconsin, 1998* (Reprinted from MMWR vol 47,pg 673-676, 1998). Journal of the American Medical Association, 280(17):1474-1475.

6. Cullen, Paul A.; Xu, Xiaoyi, Matsunaga, James; Sanchez, Yolanda; Ko, Albert I.; Haake, David A.; Adler, Ben. 2005. *Surfaceome of* Leptospira *spp.* Infection and Immunity. 73(8):4853-4863.

7. Dahlback, Bjorn; Hermansson, Malte; Kjelleberg, Staffan; Norkrans, Birgitta. 1981. *The Hydrophobicity of Bacteria – An Important Factor in Their Initial Adhesion at the Air-Water Interface.* Microbiology. 128:267-270.

8. Elliott, S.H. 1980. *Discussion and Clinical Diagnosis of Leptospirosis.* Journal of American Medical Technology, 42:37-44.

9. Hawaii Department of Public Health. *Hawaii Administrative Rules: Title 11, Department of Health. Chapter 54: Water Quality Standards.* August 31, 2004. Online source: http://www.hawaii.gov/health/about/rules/11-54.pdf

10. Faine, S. Ballows, A; Truper, H.G.; Dworkin, M.; Harder, W; Schleifer, K.H. 1992. *The Genus* Leptospira Interrogans: *The Prokaryotes Volume IV.* Springer-Verlag, New York, N.Y.

11. Ganoza, Christian A.; Matthias, Michael A.; Collins-Richards, Devon; Brouwer, Kimberly C.; Cunningham, Calaveras B.; Segura, Eddy R.; Gilman, Robert H.; Gotuzzo, Eduardo; Vinetz, Joseph M. August 22, 2006. *Determining Risk for Severe Leptospirosis by Molecular Analysis of Environmental Surface Waters for Pathogenic Leptospira.* PLoS Medicine, 3(8):1329-1340.

12. Haake, David D. August, 2006. *Molecular Epidemiology of Leptospirosis in the Amazon.* PLoS Medicine, 3(8):1214-1215.

13. Hellstrom, J.S.; Marshall, R.. 1978. *Survival of Leptospira*-Interrogans-Serovar-Pomona *in an Acidic Soil under Simulated New-Zealand Field Conditions.* Research in Veterinary Science, 25(1):29-33.

14. Henry, Ronald A.; Johnson, Russel C.; Bohlool, B. B.; Schmidt, E. L. May 1971. *Detection of Leptospira in Soil and Water by Immunofluorescence Staining.* Applied Microbiology, 21:953-956.

15. Henry, R. A.; Johnson, R. C. March 1978. *Distribution of the Genus Leptospira in Soil and Water.* Applied and Environmental Microbiology, 35:492-499.

16. Hodges, R. T.; Ekdahl, M. O. 1973. *Use of a Fluorescent Antibody Technique for the Serological Differentiation of Leptospiral Serotypes n Cultures and in Bovine Urine.* New Zealand Veterinary Journal, 21:109-116.

17. Jansen, Andreas; Schoenberg, Irene; Frank, Christina; Alpers, Katharina; Schneider, Thomas; Stark, Klaus. 2005. *Leptospirosis in Germany, 1962-2003.* Emerging Infectious Diseases. Vol. 12(7):1048-1054.

18. Katz, A. R.; Ansdell, V. E.; Effler, P. V.; Middleton, C. R.; Sasaki, D. M. 2002. *Leptospirosis in Hawaii, 1974-1998: Epidemiologic Analysis of 353 Laboratory Confirmed Cases.* American Journal of Tropical Medicine and Hygiene, 66(1):61-70.

19. Kuriakose, M. C.; Eapen, C. K.; Paul, R. 1997. *Leptospirosis in Kolenchery, Kerala, India: Epidemiology, Prevalent Local Serogroups and Serovars and a New Serovar.* European Journal of Epidemiology, 13(6):691-697.

20. Lester, John W.; LeFebvre, Rance B. *Detection of Leptospira* Interrogans. Methods in Molecular Biology. vol216:193-200.

21. Levett, P. N. 1999. *Leptospirosis: Re-Emerging or Re-Discovered Disease?* Journal of Medical Microbiology, 48(5):417-418.

22. Levett, P. N. 2001. Leptospirosis. Clinical Microbiology Review. 12:296-326.

23. Luebbehusen, Hillary. June 2004. http://www.bcm.edu/mcfweb/?PMID=3100.

24. Michigan Department of Natural Resources. 2005. http://www.michigan.gov/dnr/0,1607,7-153-10370_12150_12220-26943--,00.html

25. Morshed, M. G.; Konishi, H.; Terada, Y.; Arimitsu, Y.; Nakazawa, T. 1994. *Seroprevalence of Leptospirosis in a Rural Flood Prone District of Bangladesh.* Epidemiology and Infection, 112(3):527-531.
26. Palaniappan, Raghavan U.M.; Ramanujam, Subbupoongothai; Chang, Yung-Fu. 2007. *Leptospirosis: Pathogenesis, Immunity, and Diagnosis.* Current Opinion in Infectious Diseases. 20:284-292.

27. Russel, Peter J. 2001. i*Genetics,* Benjamin Cummings, San Francisco, CA. pp.3.

28. Rittenberg, Marvin B.; Linscott, William D.; Ball, Meridian Greene. July 21, 1958. *Simple Method for Separating Leptospirae from Contaminating Microorganisms.* Journal of Bacteriology, 76:330-331.

29. Glasel J. 1995. *Validity of nucleic acid purities monitored by 260nm/280nm absorbance ratios.* BioTechniques, 18: 62-63.

30. Sarkar, U.; Nascimento, S. F.; Barbosa, R.; Martins, R.; Nuevo, H.; Kalafanos, I.; Grunstein, I.; Flannery, B.; Dias, J.; Riley, L. W.; Reis, M.G.; Ko, A. I. 2002. *Population-Based Case-Control Investigation of Risk Factors for Leptospirosis During an Urban Epidemic.* American Journal of Tropical Medicine and Hygiene, 66(5):605-610.

31. Slack, Andrew T.; Symonds, Meegan L.; Dohnt, Michael F.; Smythe, Lee D. October 2006. *Identification of Pathogenic Leptospira Species by Conventional or Real-Time PCR and Sequencing of the DNA Gyrase Subunit B Encoding Gene.* BMC Microbiology, 6:95-105.

32. Standard Methods for the Examination of Water and Wastewater. 21st Edition. 2005. American Public Health Association; American Water Works Association; Water Environment Federation. Baltimore, Maryland. pp.2:55-2:58; 9:125-9:127.

33. United States Environmental Protection Agency. April 2001. *Method 1622:* Cryptosporidium *in Water by Filtration/IMS/FA.* Washington D.C., Office of Water.

34. Walker, M. 2007. *Method Development and Preliminary Applications for Leptospira Spirochetes in Water Samples.* Unpublished EPA Report.

35. Walker, Mark J.; Montemagno, Carlo; Bryant, Juliet C.; Ghiorse, William C. 1998. *Method Detection Limits of PCR and Immunofluorescence Assay for* Cryptosporidium parvum *in Soil.* Applied and Environmental Microbiology. 64(6):2281-2283.

36. Wilcox, B.A., Colwell, R.R. *Emerging and Re-Emerging Infectious Diseases: Biocomplexity as an Interdisciplinary Paradigm.* Eco- Health. vol.2(2005):244-257.

37. Wilcox, B.A.; Gubler, D.J. *Disease Ecology and the Global Emergence of Zoonotic Pathogens.* Environmental Health and Preventive Medicine. 10:263-272.

38. Wilson, R.; Fujioka, R. 1995. *Development of a Method to Selectively Isolate Pathogenic Leptospira from Environmental Samples.* Water Science and Technology, 31(5-6):275-282.

34. Winger, Kirk: Aguilar, Judith; Besser, Richard; Rosenstein, Nancy; Clark, Thomas. December 2004. *Leptospirosis: A Seroprevalance Survey on American Samoa.* Department of Health and Human Services, Centers for Disease Control.

39. World Health Organization. 1999. Leptospirosis Worldwide. Weekly Epidemiologic Record, 74:237-242.