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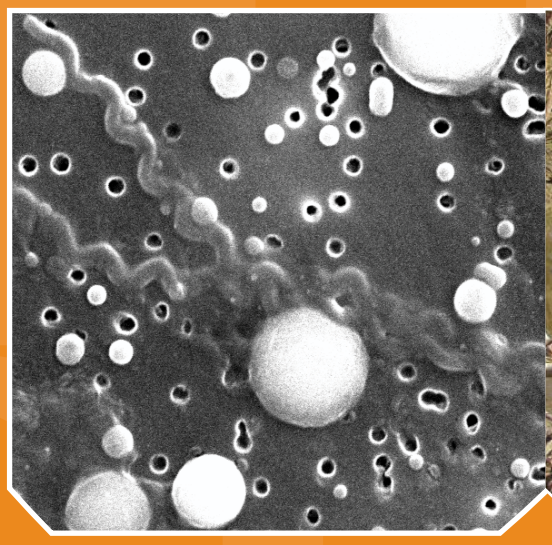
Method Development and Preliminary Applications for *Leptospira* Spirochetes in Water Samples



Office of Research and Development
National Homeland Security Research Center

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Method Development and Preliminary Applications for *Leptospira* Spirochetes in Water Samples

Prepared for:
National Homeland Security Research Center
U.S. Environmental Protection Agency

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Abstract

Leptospirosis is an increasingly important zoonosis that infects humans and animals through contact with contaminated water. The primary challenges for carrying out water sampling for *Leptospira* spirochetes include isolation, concentration, and quantitative detection of small numbers of target organisms in water.

The objectives of this work were to 1) develop a protocol to apply fluorescently labeled antibodies to water samples to detect virulent serovars of *Leptospira*, 2) develop a protocol to isolate *Leptospira* spirochetes from water samples, and 3) use these techniques to assess the occurrence of Leptospira in recreational waters in watersheds on Oahu and Kauai, in the Hawaiian Islands.

Fluorescently labeled antibodies caused agglutination in samples, which obscured individual spirochetes, so this approach was not pursued further. When nitrocellulose filters (0.45 μm pore diameter) were used to isolate spirochetes from stock suspensions, they retained approximately 90% of the spirochetes. Because these filters are commonly used for evaluating the presence and number of indicator organisms in water samples, it seemed appropriate to use them to concentrate spirochetes from natural waters. However, it may be equally useful to concentrate small-volume samples (50–100 ml) by centrifugation and to work directly with pelleted debris and polymerase chain reaction (PCR), thus avoiding inefficiencies associated with filtration. Finally, PCR was found to be potentially more useful than microscopy for environmental sampling, although this technique was qualitative rather than quantitative.

This report was submitted in fulfillment of grant #CR83271701 by Dr. Mark Walker under the partial sponsorship of the United States Environmental Protection Agency. This report covers a period from October 2005 to December 2006, and the work was completed as of May 2007.

Leptospirosis

Leptospirosis is considered a reemerging disease (Levett, 1999) that infects people who have contact with contaminated water, soil, or urine from infected animal hosts (Levett, 2001). The disease is commonly associated with flooding and is prevalent in flood-prone areas (Morshed, Konishi et al., 1994).

Leptospirosis is characterized by some researchers as the most common waterborne illness in the world (Bharti, Nally et al., 2003). Formerly, the number of cases in the United States was compiled by the Centers for Disease Control and Prevention (CDC), because leptospirosis was a reportable disease. However, leptospirosis was removed from the reportable disease list late in the last century, although it still is maintained as a reportable disease in many states, especially for veterinary cases.

Leptospirosis is caused by serovars of at least eight species of spirochetes (Slack, Symonds et al., 2006), which are the environmentally transmitted form of the pathogen. *Leptospira* spp. are further subdivided into serogroups and serovars, which are differentiated by commonly observed immunological reactions to different phenotypes within these categories. The classification arises in part from diagnostic techniques that use a library of spirochete stocks of different serogroups and serovars that react with blood serum from patients with suspected infection. Common pathogenic serogroups within *L. interrogans* include Canicola (associated with infected dogs) and Copenhageni strain M20 (also classified as Icterohaemorrhagiae and associated with infected rodents). The current taxonomic approach, developed primarily using immunology, is being supplemented by genotypic information. One of the difficulties in identifying spirochetes using the phenotypical approach is that antibodies in serum may not react exclusively with a single serovar. In fact, a single diagnostic test may indicate infection with more than one serovar.

Leptospira biflexa is also found in the environment but is nonpathogenic. Because *L. interrogans* and *L. biflexa* species are morphologically similar, they cannot be differentiated with microscopy. *Leptospira interogans* species spirochetes are helical and motile with dimensions of approximately 0.2–0.3 µm in diameter by 6–30 µm in length. Pathogenic leptospires belong to any of more than 200 known serovars, which are organized into at least 23 serogroups. Each serovar may be adapted to infect a particular reservoir host that sheds spirochetes primarily in urine (Levett, 2001). Common serogroups identified in patients with leptospirosis in Hawaii between 1974 and 1998 include (in descending order of prevalence) Icterohaemorrhagiae, Australis, Ballum, Bataiae, Sejroe, and Pomona (Katz, Ansdell et al., 2002).

The spirochetes survive well in fresh water, soil, and mud in tropical and temperate climates (CDC, 1998). One of the primary challenges for sampling water for spirochetes is isolation and concentration of these pathogens from large volumes of water. Research has focused on efficient techniques for isolating and detecting *Leptospira* spirochetes from bodily fluids and tissue samples (LeFebvre, Foley et al., 1985; LeFebvre, 1987; Faber, Crawford et al., 2000; Levett, 2001; Bunnell, Bushon et al., 2003). Although antibodies for serovars of *Leptospira interrogans* have been developed as clinical diagnostic and research tools, they have not been applied for water sample analysis. Culturing methods for *Leptospira* are also available, but the recommended incubation periods are exceedingly long (16–26 weeks) (Wilson and Fujioka, 1995).

Infection may result from contact with contaminated water or urine from infected animals, especially through skin abrasions and mucus membranes. Symptoms of illness range from mild febrile reactions to sometimes fatal disease. Leptospirosis is thought to be substantially under-reported, because symptoms are easily confused with those associated with common influenza, dengue fever, and other viral infections. Incidence of disease among humans has a marked association with seasonal weather trends. For example, the number of new cases in regions with endemic leptospirosis may increase during wet months (Kuriakose, Eapen et al., 1997; Sarkar, Nascimento et al., 2002). Researchers also have noted that incidence of leptospirosis in host animal populations coincides directly with seasonal fluctuations in rainfall (Shimizu, 1984; Miller, Wilson et al., 1991; Ward, 2002).

Brief History of This Regional Applied Research Effort

The U.S. Environmental Protection Agency's Regional Applied Research Effort (RARE) sponsored this project, which was funded in October 2005. The original project contract period was from September 2005 until August 2006. In November 2006, permission to continue the project until December 31, 2006, without additional funding was received.

The Quality Assurance Project Plan (QAPP) was submitted and revised in October 2005 and January 2006, respectively. The QAPP was approved by the U.S. Environmental Protection Agency's National Homeland Security Research Center in Cincinnati, OH, in January 2006.

The work took place at the University of Hawaii, Manoa, using laboratory space that the principal investigator prepared. Approval to operate at the University of Hawaii laboratory and equivalent space at the University of Nevada – Reno as Biosafety Level 2 (BSL-2) facilities was received in January 2006. Laboratory preparation at the University of Hawaii required extensive cleaning and removal and disposal of obsolete equipment, which took approximately two months (from the release of funds until January 2006). This was followed by inspection and installation of required safety equipment.

Although work commenced in October 2006, the early stages were hindered and delayed by lack of BSL-2 approval. Accordingly, the scope of work was revised in January 2006 to reflect this delay. In January 2006, we obtained a stock of a single serovar of *Leptospira* (*interrogans* serovar Copenhageni Icterohaemorrhagiae M-20) from the National Veterinary Services Laboratory, in Ames, Iowa. Work began with darkfield microscopy, polymerase chain reaction (PCR), and filtration trials to test strategies for isolating spirochetes from natural waters.

In early August 2006, the work initiated with RARE funds in Hawaii continued at the University of Nevada – Reno and University of Hawaii, Manoa, and was supplemented with funding provided by the U.S. Department of Agriculture's Cooperative State Research, Extension and Education Service (CSREES). The supplemental funding from CSREES has allowed expansion of the original RARE project, partly as an alternative for objective 1 (see below). This additional work is being carried out at the University of Hawaii by a graduate student (Ms. Ilima Hawkins) in the Natural Resources and Environmental Management Department in the College of Tropical Agriculture and Human Resources, under the direction of Dr. Carl Evensen.

The goal and objectives of experimental work, as amended in January 2006, are listed below. This final report discusses activities and outcomes associated with each objective.

Project Goal, Objectives, and Anticipated Outcomes

Project Goal: The project will develop a method to sample environmental waters and detect specific pathogenic serovars of *Leptospira*.

Project Objectives: The objectives that guide experimental approaches include the following:

(1) Develop a protocol to apply fluorescently labeled antibodies to water sample concentrates to detect virulent serovars of *Leptospira*.

(2) Develop a protocol to concentrate *Leptospira* spirochetes from water samples.

(3) Using these techniques, assess the occurrence of *Leptospira* in recreational waters in watersheds on Oahu and Kauai, in the Hawaiian Islands.

Anticipated Outcomes:

- an optimized protocol using an indirect antibody technique to identify serovars of *Leptospira*, beginning with Pomona serovar and progressing to homologous antigen and antibody provided by the National Veterinary Services Laboratory (NVSL), with a focus on the Copenhageni Icterohaemorrhagiae M-20 serovar;
- an expected analytic detection limit for isolating spirochetes; and
- an optimized and field-tested protocol for isolating *Leptospira* spirochetes from natural waters.

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Acronyms and Abbreviations

BSA	Bovine serum albumen
BSL-2	Biosafety Level 2
C	Celsius
Cat	Catalog
CDC	Centers for Disease Control and Prevention
cm	centimeter
CSREES	U.S. Department of Agriculture's Cooperative State Research, Education and Extension Service
DNA	Deoxyribonucleic acid
EMJH	Ellinghausen and McCullough culturing medium as modified by Johnson and Harris
F	Fahrenheit
FE-SEM	Field emission scanning electron microscope
FITC	Fluorescein isothiocyanate
g	gram
Hg	mercury
hr	hour
kV	kilovolt
LEP-FAC	Anti-*Leptospira* antibody labeled with FITC
LEP-020	Anti-*Leptospira* antibody, unlabeled
M	molar
mA	milliamp
mBa	millibar r
$MgCl_2$	magnesium chloride
ml	milliliter
µl	microliter
µm	micrometer
mm	millimeter
mM	millimolar
NVSL	National Veterinary Services Laboratory
PBS	phosphate buffered saline
PCR	polymerase chain reaction
QAPP	Quality Assurance Project Plan
RARE	Regional Applied Research Effort
sec	second
USEPA	United States Environmental Protection Agency
V	volt
wt	weight

Acknowledgements

The Groundwater Management Office of the U.S. Environmental Protection Agency's Region IX Office in San Francisco helped to design the initial proposal for the Regional Applied Research Effort (RARE) competition. Ms. Shannon Fitzgerald and Mr. Carl Goldstein were instrumental in supporting this effort. The University of Hawaii's College of Tropical Agriculture, Horticulture and Human Resources at Manoa provided laboratory space, office resources, and material support during the portion of the project that took place in Hawaii. In particular, Dr. Carl Evensen, chair of the Department of Natural Resources and Environmental Management, provided essential support, without which research could not have been conducted. Dr. Bruce Wilcox, director of the John A. Burns School of Medicine's Emerging and Infectious Disease program, provided support as well. Two graduate students at the University of Hawaii (Ms. Ilima Hawkins and Ms. Mayee Wong) worked diligently, patiently, and with great dedication on projects associated with this research effort. Dr. Frank Schaefer III, of the U.S. Environmental Protection Agency's National Homeland Security Research Center, in Cincinnati, OH, served as project officer for this project and provided guidance and very constructive suggestions throughout.

Chapter 1
Isolation of *Leptospira* Spirochetes Using Filtration Techniques

Hypothesis

The hypothesis that guided this part of the project was that we could effectively isolate spirochetes from natural waters using filtration, either with a nested approach (involving removal of debris and sediment with a coarse filter followed by a finer filter) or with a simple membrane filtration approach similar to that used to isolate indicator organisms from natural waters.

Methods and Materials

Suspensions of pure culture were prepared using liquid and semi-solid Ellinghausen and McCullough medium as modified by Johnson and Harris (EMJH) with *Leptospira* (*Leptospira interrogans* Copenhageni Icterohaemorrhagiae M-20) obtained from the National Veterinary Services Laboratory (NVSL) in Ames, Iowa. The semi-solid EMJH medium (DIFCO EMJH) was prepared with 0.2% noble agar (wt/wt) using triple-filtered (1.0/0.45/0.22 μm filters) distilled, deionized water and was supplemented with 200-μl/ml 5-fluorouracil (Acros Organics, Cat # 228440050) to suppress the growth of bacterial contaminants. Liquid EMJH was prepared as above but without noble agar. Inoculated EMJH was stored in the dark at 21 °C (70 °F) for 3–5 weeks (Levett, 2001). When cultures in the semi-solid medium began to present the characteristic cloudy, compressed layer of spirochetes approximately 1.5 cm below the surface (the Dinger's ring), an aliquot of the spirochetes was withdrawn from the ring and from the well-mixed liquid medium to be sure that spirochetes were present in both types of culture.

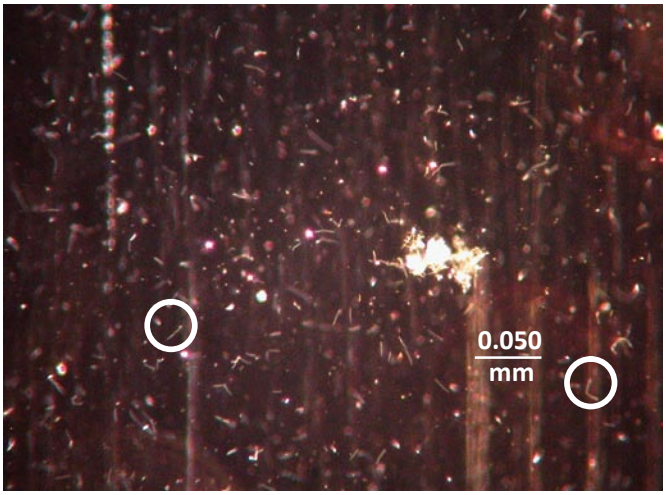

Figure 1. Petroff-Hausser bacterial counting chamber at 200×, with *Leptospira* spirochetes appearing as bright curvilinear objects (representative examples appear in white circles).

The spirochete suspension density was determined using a Petroff-Hausser counting chamber, observed at magnification of 200× on a Nikon Labophot microscope equipped for darkfield microscopy (Figure 1). Spirochetes were bright curvilinear objects, approximately 20 μm long, often flexing or spinning along their long axes in suspension. Ten replicate counts were averaged to determine each suspension density.

Fifty ml experimental suspensions were prepared containing approximately 1.5×10^6 spirochetes per ml in autoclaved, filtered 0.01 M phosphate buffered saline (PBS) solution. Filtration trials used 30 ml of the suspension, with several filters (see Table 1) mounted in a 47-mm filter holder (Osmonics, model #PFC0004703), with a vacuum of 5 inches of Hg to draw the sample into a 50-ml tube. The number of spirochetes retained on the filter was estimated by comparing the average of ten replicate 10-μl aliquots of filtrate with the numbers of spirochetes present in the stock suspensions.

The filter materials evaluated (Table 1) included nitrocellulose (0.22- and 0.45-μm pore diameters), polyvinylidene fluoride (Durapore 0.22 μm and 0.40 μm pore diameters), glass fiber (1.0 μm), and nylon mesh (37 μm). Fisher (Cat # 09-719-555) nitrocellulose (0.45 μm) and Millipore Durapore polyvinylidene fluoride filters (0.22 μm) were examined by scanning electron microscopy to verify that spirochetes were present following filtration. Specimens were prepared by passing 0.200-ml aliquots from undiluted liquid EMJH cultures through filters at low vacuum (~ 5 inches Hg), followed by 200 μl of fixative (Karnovsky's Fixative, Electron Microscopy Services Cat # 15720, prepared as 16% paraformaldehyde, 50% electron microscopy grade glutaradehyde, 0.2 M sodium phosphate buffer, with distilled water, per manufacturer's instructions) to preserve organism structure. The fixative was added with vacuum off. After 20 minutes, excess fixative was drawn through the filter to waste with vacuum. The specimens were vacuum freeze-dried (- 0.133 mBar, - 40 °C, with a Labconco Freeze Dry System – Freezone 18) mounted on a 1.6-cm diameter carbon stage on a bed of dessicant (anhydrous calcium sulfate – Drierite©) for 24 hrs. A multimolecular platinum layer was applied to the filters by sputter-coating using an EMITECH model K575x Turbo Sputter Coater with 30-mm platinum target, sputter cycle of 20 seconds, under ultrahigh purity argon gas at 85 mA, in a vacuum of at least 10^{-5} mBar. The samples were examined using a Hitachi Field Emission Scanning Electron Microscope (FE-SEM) model S-4700 type II, operated at a voltage of 10 kV.

Filter (Catalog number)	Type	Material	Pore Diameter
GE* Nitrocellulose-Mixed Esters of Cellulose Membrane (E02WP04700)	Hydrophobic membrane for water sampling	Nitrocellulose	0.22 μm
Millipore Durapore Membrane filter (HTTP04700)	Hydrophobic	Polyvinylidene fluoride	0.40 μm
Fisher (09-719-555)	Hydrophobic membrane for water sampling	Nitrocellulose	0.45 μm
Millipore (AP1504700)	Hydrophilic prefilter for coarse debris removal	Glass fiber	1.0 μm
Small Parts Inc (CMN-0040)	Hydrophobic nylon mesh sheet	Nylon mesh	37 μm
Millipore Durapore (GVWP)	Hydrophobic membrane for liquid purification	Polyvinylidene fluoride	0.22 μm
Millipore Isopore (HTTP)	Hydrophilic membrane for filtration of biological liquids	Polycarbonate	0.40 μm

* http://www.osmolabstore.com/OsmoLabPage.dll?BuildPage&1&1&327

Table 1. Filter types tested to determine recovery efficiencies from suspensions of pure culture of *Leptospira interrogans* Copenhageni Icterohaemorrhagiae M-20

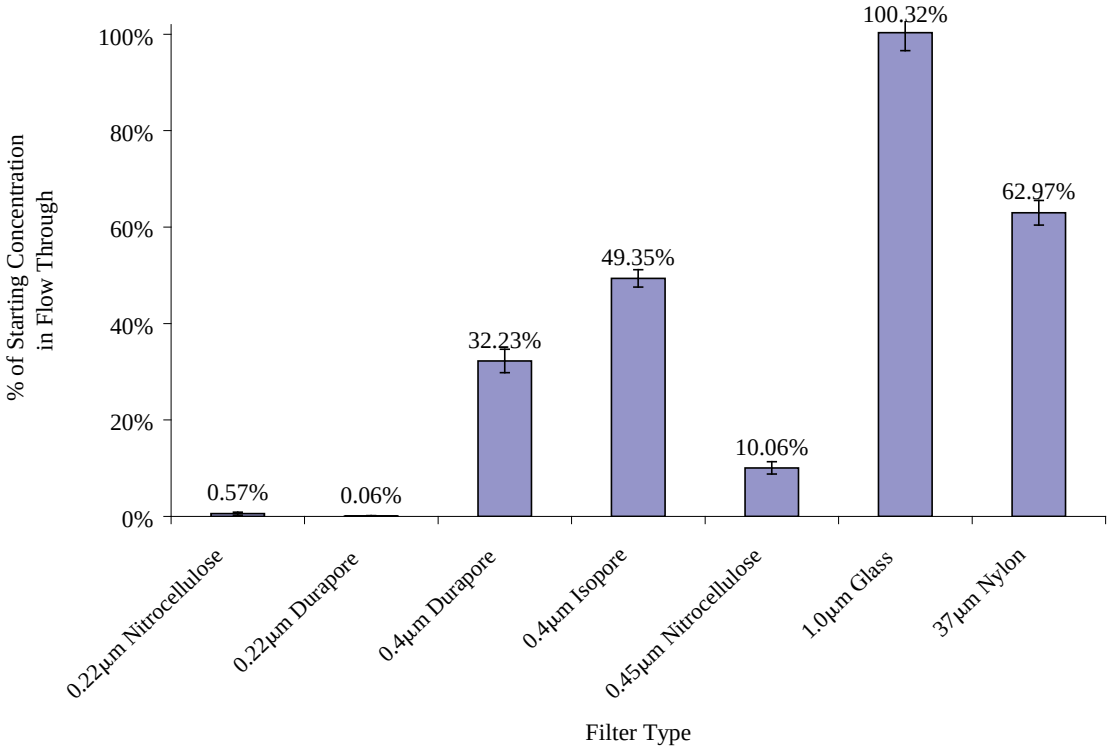

Figure 2. Average percent of starting numbers of spirochetes in stock suspensions that were found in filtrate, with 95% confidence intervals displayed.

Outcomes

Filtration Trials: The filtration results are displayed in Figure 2. These results suggest several important aspects of using filters to isolate spirochetes from environmental samples. First, in order to isolate nearly 100% of spirochetes from sampled volumes, the optimal pore diameter should be less than 0.45 μm [a standard pore size used to detect indicator organisms in 100 ml of water (Clesceri, Greenberg et al., 1998)]. Second, the results suggest that the filter material itself may affect recovery rates. For example, with pore diameters of 0.4 (hydrophilic polyvinylidene fluoride Durapore filters) and 0.45 μm (Fisher nitrocellulose filters), flow through recovery rates varied from <32% to <10%, respectively. This effect is also seen in the results from glass fiber filters and nylon mesh filters. The results from trials with glass fiber filters may be biased in part, because glass fiber filters are very similar in appearance to spirochetes, which likely led to false positive results. Given the difficulties of counting spirochetes microscopically, this suggests that glass fiber filters would be unsuitable as

prefilters for natural water samples, unless the analytical endpoint focuses on detection of DNA rather than the physical form of the parasite. This is because, using microscopy, glass fibers could be mistakenly identified as spirochetes. However, the similar morphology would not interfere with techniques that rely on detection of specific DNA sequences.

For environmental samples, in order to sample volumes of water that are at least comparable to those used for detecting indicator organisms, it may be best to prefilter the sample using either a glass fiber filter or a nylon mesh filter to remove large pieces of debris prior to working with the 0.22-µm pore diameter filters. It may be possible to use 0.45-µm pore diameter filters (especially the nitrocellulose filters) to isolate spirochetes from environmental samples, given that approximately 90% of spirochetes appear to be isolated on or within the filters. This suggests that a polymerase chain reaction (PCR) method could be adapted for detecting the spirochetes directly on the filter itself, rather than concentrating them from a large-volume filtrate after coarse filtration.

Scanning Electron Microscopy Results: Scanning electron microscopy results (Figures 3, 4, 5) support, in part, the results presented in Figure 2. In Figure 3, spirochetes are visible on the 0.22-µm pore diameter filter (Durapore® 0.22 polyvinylidene fluoride filters). The image of the filter suggests that a small number of pores are spaced closely enough to slightly overlap, such that the resulting pore diameter could be approximately equal to the diameter of a spirochete. As a consequence, a pressure gradient across the membrane could force spirochetes through these large pores, leading to passage through filters that should retain them. In fact, a small proportion of spirochetes passed through filters that were expected to completely retain spirochetes under the experimental conditions [the 0.22-µm pore diameter nitrocellulose and 0.22-µm Durapore® filters (Figure 2)].

The results of FE-SEM trials with 0.45-µm pore diameter nitrocellulose filters are more difficult to interpret than those with the 0.22-µm pore diameter nitrocellulose and Durapore filters. After four trials, no spirochetes were detected on any part of the filter. The electron micrograph of the filter surface has four important discernible features. First, the pores are irregularly shaped and pore diameter varies widely within the matrix. Second, the resulting pores have a clear third dimension, or depth, that cannot be captured well by electron microscopy because of the narrow field of focus. Third, pore geometry and orientation vary considerably with depth of the filter, such that pores are irregular in radius and orientation, and inconsistent and often tortuous through the fiber matrix. Fourth, the filter matrix is a composite of homogeneous fragments of nitrocellulose, each of which is larger in diameter than spirochetes.

The first characteristic of these filters is important with respect to expected retention of spirochetes. The pore diameter of this type of filter is determined by retention of *Serratia marcescens*, a rod-shaped organism that has a size range of 0.5–0.8 µm in diameter by 0.9–2.0 µm in length. Product certification for pore size is based on overall retention of the *S. marcescens*, rather than direct examination of the filter surface.

Filter performance could be determined by more than one process, including hydrophobic bonding and mechanical retention. The tests performed on the filter by the manufacturer do not differentiate between the mechanisms of retention. Accordingly, it is possible that even though pore size appears to be highly variable, in some cases larger than the 0.45 µm specified for these filters, an additional factor related to sorption, such as hydrophobic binding, could retain spirochetes on or within the filter. The second factor, depth of the filter, suggests that spirochetes that could not be found on the surface of the filter were retained out of the field of focus within the filter itself. Third, the variation of pore geometry and orientation may also enhance retention, because spirochetes may be forced into contact with the filter material due to the tortuosity of flow paths. This could enhance the likelihood of contact with the filter matrix, which would increase the opportunities for sorption. Finally, the actual surface area of the filters is much larger than the 958-mm^2 surface presented in the filter holder. This would enhance the likelihood of sorption, if hydrophobic binding occurs.

Overall, although filtration can be used to isolate spirochetes from water samples, it is unclear whether this is a useful intermediate step with respect to detection. Although a large proportion of spirochetes can be retained by filters with a pore diameter commonly used to isolate indicator organisms from water samples (0.45-µm pore diameter), the filters must be processed to recover spirochetes. Given that sample volumes are likely to be small (100 ml or less if waters have significant suspended sediment content) and that under ideal conditions a maximum of 90% of spirochetes in liquid filtered by a 0.45-µm pore diameter filter will be retained, additional inefficiencies will be introduced during filter processing. This suggests that rather than relying on processing techniques to obtain spirochetes from filters, it may be useful to apply a detection technique to the filter itself. In order to increase efficiency of isolating spirochetes from liquid, it may be appropriate to work with filters that have a smaller pore diameter (e.g., the polyvinylidene fluoride filters with 0.22-µm pore diameter). Although microscopy could be useful for this, PCR may be more appropriate because of difficulties associated with agglutination (discussed below) and the presence of debris that may obscure spirochetes.

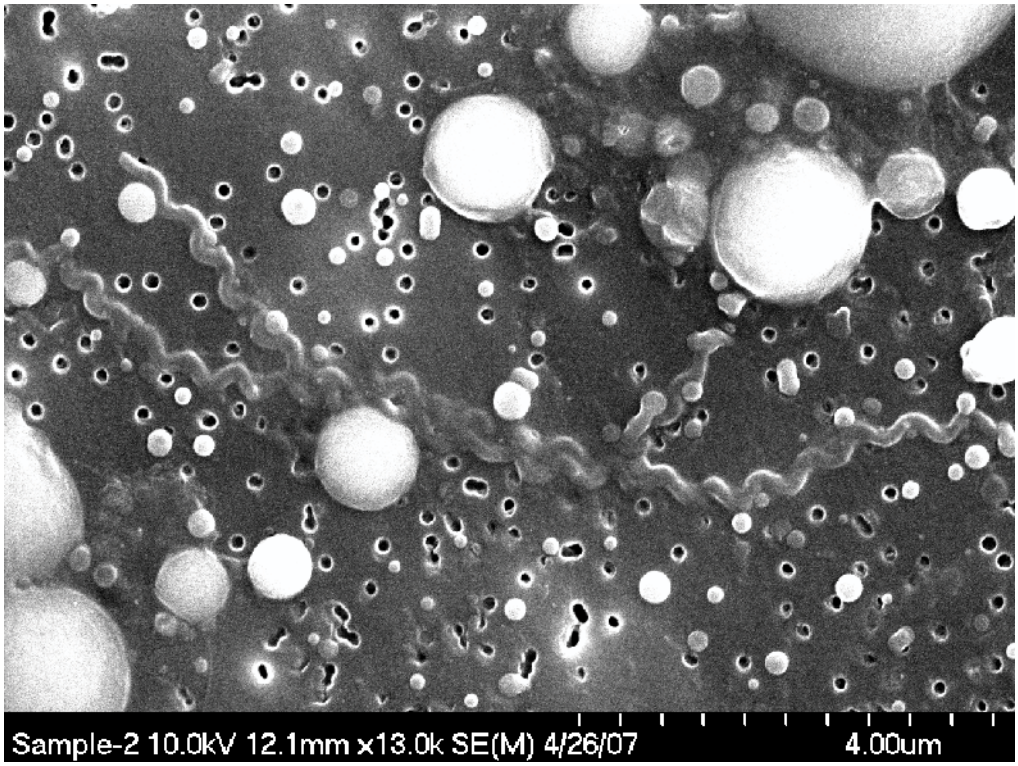

Figure 3. *Leptospira* spirochetes on the surface of a 0.22-μm pore diameter filter (isopore polycarbonate membrane). Arrows indicate overlapping pores that could be large enough to allow passage of spirochetes under vacuum.

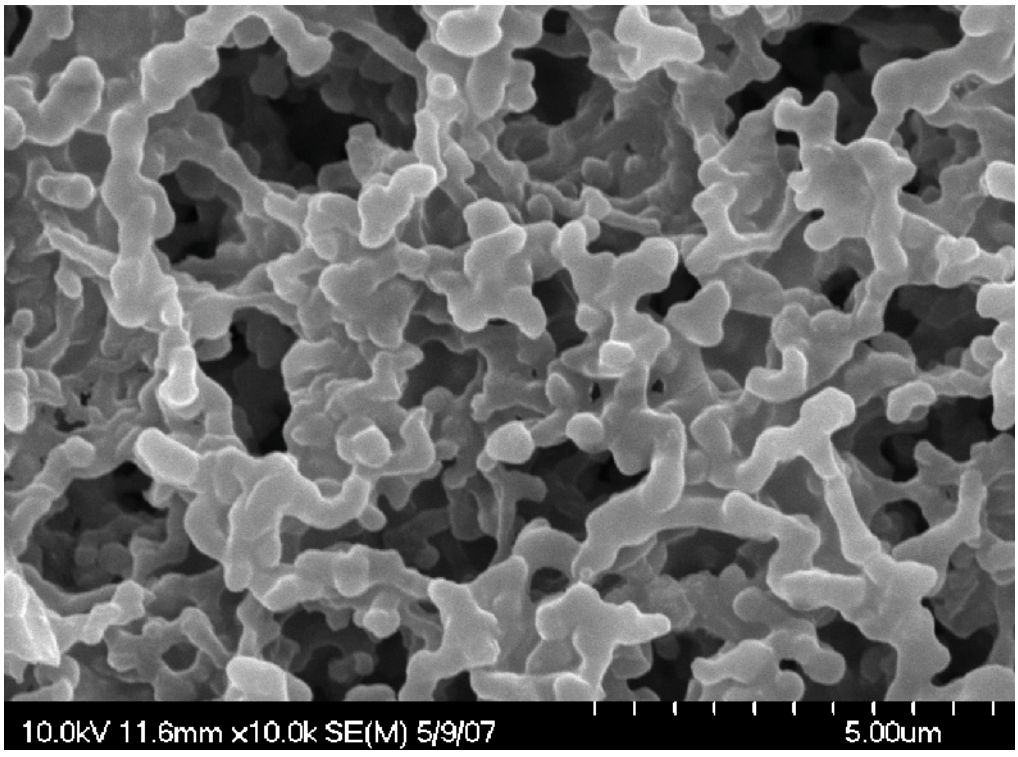

Figure 4. Surface of a 0.45-μm pore diameter filter (Fisher Cat # 09-719-555, 47 mm diameter).

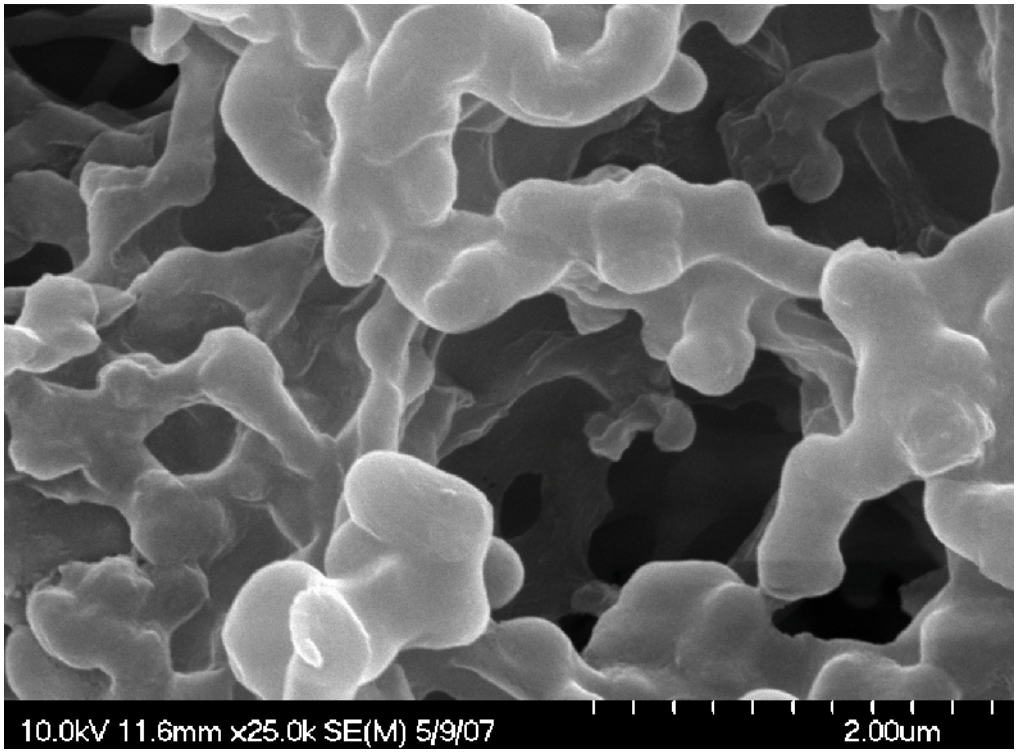

Figure 5. Surface of a 0.45-μm pore diameter filter (Fisher Cat # 09-719-555, 47 mm diameter) in greater detail than in Figure 4, demonstrating variation in pore diameters and potential interferences due to filter matrix materials.

Chapter 2
Application of FITC-Labeled Antibodies for *Leptospira* Spirochete Detection

Hypothesis

The hypothesis that guided this portion of the experimental work focused on application of antibodies to specific serovars of *Leptospira*. The antibodies were labeled either directly with fluoroscein isothiocyanate (FITC) or used with a secondary fluorescently labeled antibody that reacted with the primary anti-*Leptospira* antibody.

Methods and Materials

The purpose of this experimental work was to enhance the visibility of spirochetes with a labeled antibody that would be species and serovar specific and could be applied using a simple protocol. The approach was to use the specificity of the antibody binding to exclude nonpathogenic, saprophytic spirochetes that could be observed using darkfield microscopy, but could not otherwise be distinguished from pathogenic spirochetes. It was thought that epifluorescent microscopy with high-resolution optics would make it possible to overcome the interferences of background debris that obscure spirochetes using darkfield microscopy. A key element of this approach involved use of 40× and 60× objectives, which would increase magnification available using darkfield microscopy (a maximum of 200×, using a 20× objective and 10× eyepiece).

The NVSL *Leptospira* Copenhageni Icterohaemorrhagiae M-20 cultured organisms were tested with two homologous antibodies (either FITC-labeled or unlabeled, with a complementary goat-anti-mouse antibody provided by Invitrogen (Spectra–Alexa Fluor 488 goat-dervied anti-mouse IgG antibody). An antibody application and rinsing protocol was adapted from the procedure developed for immunofluorescent staining of *Cryptosporidium* oocysts in water samples (USEPA, 2001). The specifics of the antibody staining protocol are detailed below:

- Dilutions of LEP-FAC antibody (LEP-FAC is a FITC-labeled mouse-derived anti-*Leptospira* Copenhageni Icterohaemorrhagiae M-20 antibody available from NVSL) were prepared using filtered, sterilized (2 fiberglass Millipore Cat # APFD 04700 prefilters), followed by a 0.45-µm nitrocellulose filter (Fisher Cat # 09-719-555), followed by a 0.22 polyvinylidene fluoride filter (Fisher Cat # 09-719-2B) 0.01-M PBS solution containing 1% bovine serum albumen (BSA) (wt/wt) to avoid nonspecific binding. In addition, the PBS also contained 0.2% (wt/wt) 5-fluorouracil added to suppress microbial contamination.
- 0.010-ml aliquots of diluted LEP-FAC were added to 0.100 ml of prepared stock suspensions in 1.5-ml snap-cap microcentrifuge tubes and incubated at 21 °C (70 °F) for 30 minutes in the dark.
- For trials with the unlabeled primary antibody (LEP-020) and complementary Spectra–Alexa Fluor 488 antibody, the same diluent and incubation protocol [21°C (70 °F) for 30 minutes in the dark] were used, with an intermediate rinse and centrifugation step to remove excess primary antibody. The rinse took place by adding 1.4 ml of 0.01 M PBS to the 0.1 ml of suspension used for application of the unlabeled primary antibody, followed by light vortexing (10 sec), and centrifugation at 6,000×g for 3 minutes to precipitate spirochetes. Aspiration reduced the volume to 0.1 ml. Trials were carried out with dilutions of secondary antibody including 1:100 and 1:200, volume Spectra–Alexa Fluor 488 antibody:volume 0.01 M PBS with 1% wt/wt BSA as diluent. A total volume of 0.01 ml of diluted secondary antibody was added to stock suspensions, which was incubated at 21 °C (70 °F) for 30 minutes in the dark.
- 1,000 ml of PBS with 2% wt/wt DABCO (1,4-diazabicyclo[2.2.2]octane, Sigma Chemical Co. Cat # D-2522) was added to reduce fluorescence quenching and mixed by light vortexing (10 sec.).
- Spirochetes were precipitated by centrifugation (6,000×g) for 3 minutes.
- The supernatant was removed by aspiration to 0.1 ml.
- The pellet was examined with fluorescent and darkfield microscopy at 200× magnification for the presence of spirochetes.

Outcomes

Immunofluorescent staining experiments with *Leptospira* serovar Pomona began in October 2005, using a multivalent mouse-derived antibody provided by Dr. Rance LeFebvre (University of California – Davis, School of Veterinary Medicine). Working with *Leptospira* Pomona and an anti-mouse labeled antibody coupled with the secondary antibody (Spectra–Alexa Fluor 488), initial promising results were obtained; however, clumps of spirochetes were clearly visible at 600× magnification.

During these limited trials, the National Veterinary Services Laboratory (NVSL: Ames, Iowa) was contacted to discuss obtaining a serovar that could be produced in large quantities, as a standard, along with complementary antibodies. The NVSL maintains serovar *Leptospira* Copenhageni

Icterohaemorrhagiae M-20 and two mouse-derived antibodies, one with a FITC-label and one without. The antibodies are for experimental and diagnostic applications, primarily for use with blood from infected animals from which clotting factors have been removed. Clinical diagnostic work is based on an immunoreaction [the microscopic agglutination test (Cumberland, Everard et al., 1999)] that relies on using a standard suite of *Leptospira* serovars and antibodies isolated from serum obtained from infected hosts. The NVSL's stocks are primarily applied as positive controls for diagnosis. The diagnostic technique relies on agglutination of spirochetes with surface proteins and carbohydrates specific to individual serovars. The presence of spirochetes is inferred from visible agglutination on darkfield microscopic examination of individual reaction wells.

Single-stage antibodies – LEP-FAC: Initial results with a 1:20 dilution led to agglutination of spirochetes. In addition, the sample, which consisted only of spirochetes that had been washed and resuspended in distilled water, contained small fluorescing particles that were filamentous, though too wide and too long to be mistaken for spirochetes. However, these filamentous particles fluoresced so brightly in some microscopic fields that the spirochetes were obscured.

Attempts were made to dilute the antibody to reduce agglutination (1:10, 1:20, 1:30, 1:40, 1:60). At a 1:60 dilution, the spirochetes were no longer agglutinated, but they also were not labeled sufficiently to be visible by epifluorescence microscopy.

Two-stage antibodies – LEP-020 and Spectra–Alexa Fluor 488: The two-stage antibody approach yielded results similar to those obtained from the single-stage antibody approach. Spirochetes were agglutinated and not easily distinguished from other types of autofluorescing debris present in the sample. As with application of single-stage antibodies alone, we could not identify a dilution that avoided agglutination but also clearly labeled spirochetes.

Application of antibodies to filters: Another approach was to use a nitrocellulose filter (Fisher Cat # 09-719-555, 47 mm diameter) to isolate spirochetes and then to apply the antibody and fluorescence preservative solutions to the filter directly rather than rely on centrifugation, mixing by vortexing, and application of antibodies to the concentrate. We sought an optimal dilution for application of labeled and unlabeled primary antibodies and found significant background fluorescence. The background fluorescence obscured the spirochetes, which could also not be seen with darkfield microscopy because of the filter matrix.

Assessment of antibody use for identifying pathogenic spirochetes in samples: Based on the two types of trials (application of antibodies with and without fluorescent labels to small-volume suspensions of spirochetes and application of antibodies to spirochetes isolated on filters), it was apparent that epifluorescence microscopy will not be a feasible way to detect spirochetes in environmental samples. Microscopy is limited in several ways including:

- Agglutination
- Presence of interfering debris
- Lack of resolution with epifluorescent light sources, leading to uncertainties in identification

Agglutination produces large masses of spirochetes that cannot be distinguished from the autofluorescing debris in samples. Although the antibodies bound to spirochetes and fluoresced brightly, they also caused agglutination, which was the primary purpose for which these reagents were developed. Consequently, it is unlikely that application of antibodies would be useful for environmental samples, especially because of autofluorescing particles in samples that closely resemble agglutinated spirochetes.

Autofluorescing particles also interfere with visualizing labeled spirochetes. Even when the individual spirochetes were visible under darkfield microscopy, fluorescing background debris obscured or blocked them. Given that the sample matrix was extremely simple (pure cultures with triple-filtered diluent), it is likely that background interferences would be increased in natural water samples, which would magnify the difficulty of finding and identifying labeled spirochetes.

Although epifluorescence microscopy offers the benefit of excluding nonfluorescing particles from observed specimens, it does not offer the same level of resolution obtained by high-contrast darkfield microscopy, even with high magnification objectives (>20×). Epifluorescence does not appear to have the contrast and resolution needed to distinguish spirochetes from other small, fluorescing, linear objects. Samples observed through the microscope were unsatisfactory as well, because very few details, such as the tightly wound coils, were visible with the limited light source available when epifluorescence was applied. In addition, the spirochetes were no longer motile, as they often are when using darkfield microscopy. This adds uncertainty about specificity, because other linear autofluorescing particles could be mistakenly identified as spirochetes under epifluorescence alone.

Chapter 3
Preliminary Field Application – Use of PCR for Detection of *Leptospira* Spirochetes in Natural Water Samples

Hypothesis

PCR could be a very sensitive, qualitative technique to determine whether spirochetes are present in natural waters. It could be adapted for use with spirochetes retained on filter surfaces or used directly with concentrates from natural waters developed with centrifugation.

Methods and Materials

Preliminary sampling of natural waters to detect pathogenic spirochetes: Natural water samples (50 ml) were collected from Manoa Stream in Honolulu on Oahu Island, Hawaii, (Figure 6) using a modified version of method 9222-D (Clesceri, Greenberg et al., 1998). These were transported on ice and centrifuged upon arrival at 1500×g for 15 minutes. The resulting pellet (~1 ml) was vortexed to resuspend the solids and transferred to 1.5-ml microcentrifuge tubes. We carried out DNA extractions (as described below) directly with the pellet and residual liquid.

Sensitivity: We evaluated the sensitivity of the PCR using Leptospira Copenhageni Icterohaemorrhagiae M-20 in serially diluted suspensions. This step relied on the same diluent as that applied for experiments described in Chapter 2. DNA from pellets was then extracted using a DNeasy® Blood and Tissue Kit (Qiagen, Inc., Valencia, CA., Cat # 69506) and amplified by PCR. Controls consisted of a negative control and a positive control drawn from stocks provided by the University of Hawaii. Aliquots of samples also were cultured using semi-solid and liquid preparations of EMJH medium.

DNA extraction and amplification by PCR: DNA was extracted from liquid or liquid/sediment mixtures using the DNeasy® Blood and Tissue Kit. For each 52-µl reaction, the PCR reaction used the following:

distilled H_20	27.5 µl
$MgCl_2$	5 µl
Buffer	15 µl
G1 primer 1	1 µl
G2 primer 2	1 µl
Taq	0.5 µl
DNA sample	1 µl

Samples were processed in a GeneAmp 9700 Thermacycler (Applied Biosystems, Foster City, CA.) at 94 °C for 5 minutes, followed by 30 cycles at the following temperatures:

94 °C for 1 minute
51.7 °C 1 minute
72 °C 1.5 minutes
Final extension at 72 ºC for 10 minutes

The reactions used G1 and G2 primers (as described by Gravekamp et al., 1993), G1 Sequence: CTG AAT CGC TGT ATA AAA GT; G2 Sequence: GGA AAA CAA ATG GTC GGA AG, and positives were verified by amplification of a 16S rRNA region by an external laboratory (Vinetz, J., U.C. San Diego), followed by sequence analysis (Matthias, Diaz et al., 2005). Amplicons were separated by an electrical potential of 90 V applied for 1.5 hours to a 1% agarose gel (developed in a 40-mM tris acetate buffer).

After immersing the gel in an ethidium bromide solution for 10–20 minutes, the position of fluorescing bands was evaluated using imaging software.

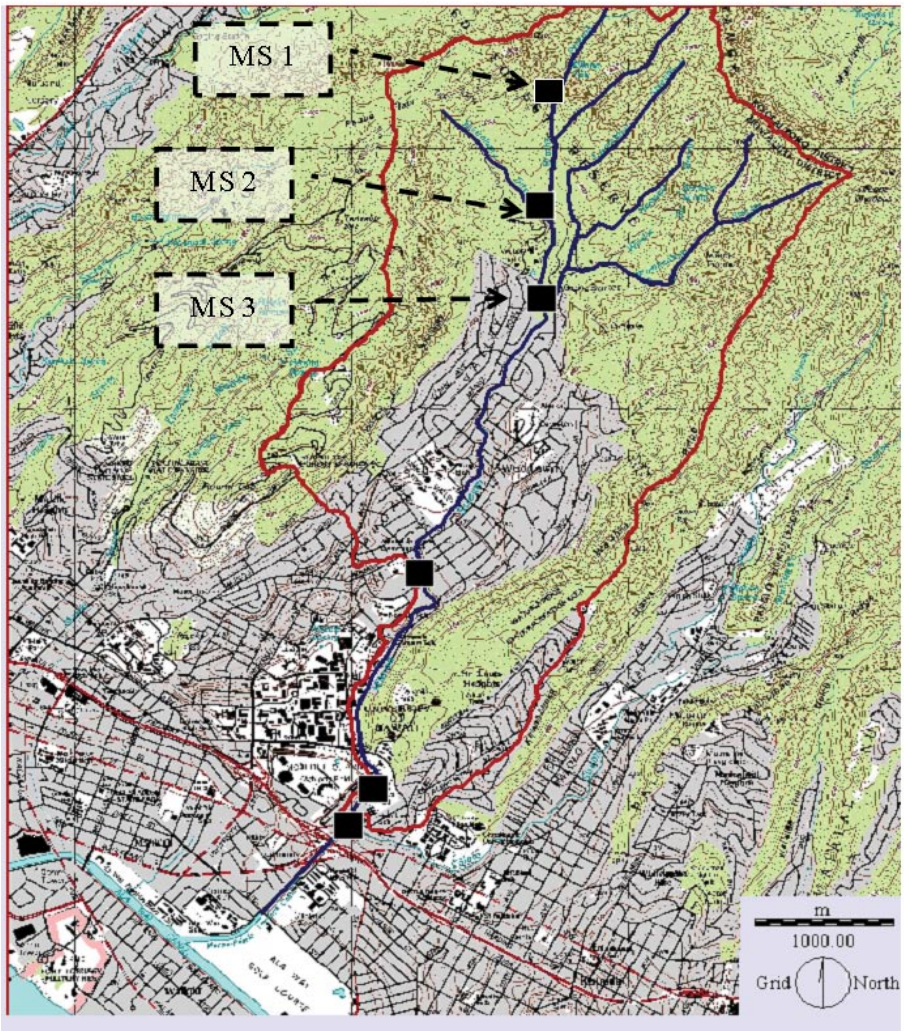

Figure 6. Sampling locations on Manoa Stream, Honolulu, HI. Labeled locations correspond with results presented in Figure 7, for PCR followed by amplicon sequencing. MS 1, Manoa Sample 1; MS 2, Manoa Sample 2; MS 3, Manoa Sample 3.

Outcomes

Field samples: Amplicon from one sample (MS 2), collected at the base of Manoa Falls, appeared to have DNA from a pathogenic serovar of *Leptospira*, when compared with signals from controls in the electrophoresis gels. After further amplification, we submitted the sample to the University of Hawaii's Department of Microbiology Gene Sequencing Facility. The results indicated that serovar Icterohaemorrhagiae was likely present in the original sample. Of eleven samples collected from Manoa Stream, three samples were found to be positive (Figure 7). To further verify the results, a laboratory maintained at University of California San Diego by Dr. Joseph Vinetz analyzed amplicons from all samples with extracted DNA from a blind reference strain. Dr. Vinetz's laboratory identified spirochetes in three samples (MS 1, MS 2, MS 3) that included two pathogenic (MS 1, MS 2) and one nonpathogenic form (MS 3). These were identified by sequence analysis as *L. alexanderi* (MS 1), *L. borgpetersenii* (MS 2), and *L. biflexa* (MS 3), as was a blind reference strain (*L. borgpeterseni*).

Sensitivity: Serial 1:10 dilutions of a passaged NVSL stock were prepared, beginning with approximately 915,000 spirochetes in 1 ml of suspension and progressing to an estimated 9 spirochetes in 1 ml. Unknown triplicate replicates of these dilutions along with blanks were prepared. Ms. Mayee Wong of the John A. Burns School of Medicine used G1 and G2 primers (see PCR protocol above for further information about primers) and performed the PCR to evaluate the sensitivity of this assay. The results are depicted in Figure 8.

The results indicate that blank samples were contaminated at some stage in the process. In tracing our own procedures and discussing this with Ms. Wong, the contamination likely occurred during sample processing for the PCR rather than in our laboratory. All blanks were positive, which renders the rest of the results suspect. However, when very few spirochetes were present (in sample A) results were only partially positive, which suggests that the threshold for detection may be less than 9 spirochetes. When this experiment is repeated, these trials will be carried out with dilutions beginning with lower numbers per ml (possibly 10^3) and progressing in 50% dilutions.

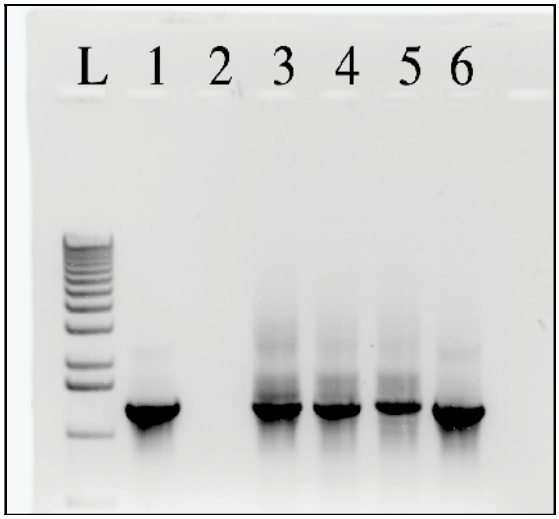

Figure 7. PCR amplification of leptospires from stream sampling in Hawaii, using primer pair G1 / G2 (Gravekamp, et al., 1993). From left to right: lane L) size ladder; 1) positive control; 2) negative control (water); 3) Manoa Stream sample MS-1; 4) MS-2; 5) MS-3; 6) reference sample from Hawaii, *L. borgpeterseni*.

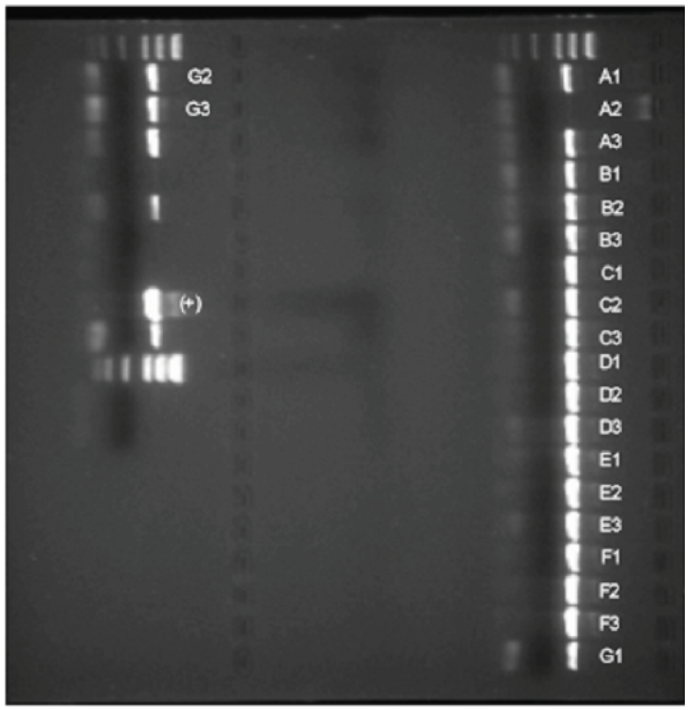

Figure 8. Results of PCR of replicate suspensions of spirochetes, serovar Copenhageni Icterohemorrhagiae M-20. The labels indicate sample dilutions (A-G) and replicate number (1-3). Dilutions are ten-fold beginning with F (915,000/ml) through E, D, C, B and A (9/ml). Sample G is a blank.

Chapter 4
Recommendations for Further Work

Several aspects of the research discussed in Chapters 1–3 could be explored further to develop a sampling method with well-characterized sensitivity that could be applied for field application. These include using filters to isolate spirochetes from natural waters, with the ultimate goal of applying PCR to qualitatively determine whether pathogenic spirochetes have been isolated from water samples, working directly with debris pellets developed by centrifugation to apply PCR (without filtering), and evaluating the application of either method to natural waters. Each is discussed below.

Isolation using filtration

The results of trials indicate that a substantial proportion of spirochetes in pure suspensions can be isolated from water with a 0.45-µm pore diameter nitrocellulose filter. The filter is readily available and is commonly applied to determine whether indicator organisms, including *E. coli* and, more generally, fecal coliform, are present in water samples. One of the concerns about field application of this method is whether interfering debris and sediment will limit the volume sampled to less than 50 ml. In several trials with soil/water slurries (~1 g soil/100 ml water, representing ~10 mg/l total suspended solids, work not discussed in the report), filters clogged and failed before relatively small volumes (<10 ml) could be processed. It is unclear whether such a concentration of total suspended solids will be often equaled or exceeded in natural waters. However, during high-flow events, especially in erosion prone watersheds with unstable, steep headwater areas, it is possible that total suspended solid concentrations could reach and exceed this level. Given the link between flooding and outbreaks of leptospirosis, such events may be important to sample. However, it may be most efficient to concentrate sediment and spirochetes in samples collected from such events directly by centrifugation of volumes of 50–250 ml. In either case, whether the sample is concentrated on a filter or pelleted by centrifugation, the concentrate will be a compact sample that likely can be transported without significant loss during shipping, especially if genomic DNA from pathogenic spirochetes is the analytic target rather than direct examination of the filter using microscopy.

One promising technique that could be explored further is use of a water DNA isolation kit (for example, the UltraClean™ water kit, Cat # 14800-10, MoBio Laboratories, Inc.) These kits are designed for extracting microbial DNA from filters used to isolate microorganisms from water samples and have been in use for several years but have not been applied for use with *Leptospira* spirochetes. When coupled with PCR and pathogen-specific primers, as described by Smythe et al. (2002), the combination of filtration to concentrate spirochetes and qualitative or quantitative PCR for detection could yield a process that provides field researchers with a technique to collect a highly portable set of samples for transport and analysis in laboratories equipped to perform either type of analysis.

Application of PCR

The preliminary applications of PCR described in this report were successful in determining that pathogenic and nonpathogenic spirochetes were present in water samples concentrated by centrifugation, though the determinations were qualitative. It is unlikely that such a procedure (preliminary amplification of DNA, followed by reamplification and genetic sequencing of the amplicon) would be practical for field use because of the expense and need for specialized equipment and training in two laboratories.

In terms of sensitivity and the potential for application, PCR appears more practical than microscopy for determining the presence or absence of pathogenic spirochetes in concentrates from natural waters. It can also be applied for quantitative and very specific real-time sampling (Slack, Symonds et al., 2006). In fact, Ganoza et al. (2006) recently reported results from an extensive survey of water quality in Peru using PCR as a quantitative method of detection. The results indicated the presence of pathogenic spirochetes in samples from three different environments, in concentrations ranging from 2 to 17, 147 pathogenic spirochetes/ml in positive samples. However, it was unclear whether the authors determined a lower limit of detection for the method that they applied, or how the lower limit of detection might change in the presence of chemical and biological interferences with the PCR reaction. This is an important step that should be completed, especially if surveys relying on PCR are conducted to determine risk associated with contact with or consumption of specific water sources.

Field applications

In addition to establishing a laboratory limit of detection for a sampling protocol, it will be important to carry out field trials with natural waters to quantify expected limits of detection in the presence of naturally occurring chemical and biological compounds. This includes humic acids and sediments, both of which may be important in tropical surface waters. This could be done with seeding and direct use of DNA extracts from standardized suspensions of spirochetes and serial dilutions in natural waters.

With a method limit of detection estimated for field application, it will also be important to publish a complete protocol that can be included as a standard reference. The current standard method for collecting and processing samples for *Leptospira* spirochetes [method 9260 I (Clesceri,

Greenberg et al., 1998)] specifies culturing and animal inoculation as potential analytic endpoints, with a clear statement that successful cultures may contain a mixture of saprophytic and pathogenic spirochetes, as well as other microbial contaminants. It does not provide expected limits of detection for either field or laboratory methods. A complete description of a field method that describes application of filters, or centrifugation, to isolate and concentrate spirochetes, followed by a filter processing, DNA extraction protocol, and details of the PCR, would be useful, especially if accompanied by guidelines about expected sensitivity and specificity of the entire field and laboratory protocol.

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