Detecting *Leptospira* in Water: Preliminary Results from a Regional Collaboration

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ABSTRACT

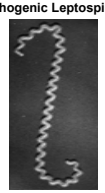
The United States Centers for Disease Control has re-added Leptospirosis to the list of emerging infectious diseases. Leptospirosis is caused by one of many strains of infectious Leptospira *bacteria, and can be fatal if left untreated. The increased frequency of this disease is due to a complex transmission cycle with many site-specific regulatory factors. This makes localized environmental detection a necessary step in the development of adequate control measures. Hospital data and seroprevalance surveys indicate more than 70% of human infections result from physical contact with contaminated freshwater supplies. The objective of this project is to develop and evaluate a freshwater test for* Leptospira *that could be useful for routine monitoring of freshwater streams. Preliminary results indicate a 0.2µm nitrocellulose filter would be the optimal choice for* Leptospira collection*. They also indicate* Leptospira *DNA can be extracted from* Leptospira *trapped on a nitrocellulose filter, and that this DNA can be identified using a polymerase chain reaction (PCR) and gel electrophoresis. Research is ongoing and is currently evaluating the sensitivity of this methodology in three distinct Hawaiian streams.*

INTRODUCTION

The Disease: Leptospirosis

- Common in the tropics, also found in temperate regions.[1,2]
- The U.S. reports approximately 40 to 120 cases/year.[3]
- Most cases result from physical contact with contaminated freshwater.[2,4,5]
- Disease manifests 2 days to 4 weeks after the initial infection.[1]
- Often runs a biphasic course with highly variable symptoms:
  - Phase 1: vague flu-like symptoms, often declines without treatment.[1,5]
  - Phase 2: develops in 10% of those infected; Symptoms include organ damage and death.[1]
- Treatment involves a course of strong antibiotics.[5]

A pathogenic Leptospire[9]



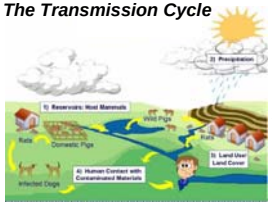
The Organism: Leptospira

Aerobic, helical, motile bacteria requiring fatty acids for growth.[3,5]

- Range from 0.2-0.3 microns in diameter and 6-30 microns in length.

Carrier species:[4] Pigs, rodents, dogs, cattle, goats, sheep, horses, deer, foxes, muskrats, opossums, raccoons.

The Transmission Cycle



Pathogenic *Leptospira* reproduce in a mammalian host[2,4]

- They colonize in the renal tubules of the kidney
- A small proportion are released during urination
- Humans contact materials contaminated with this urine[2,4]

The incidence of disease depends on:[7]

- The number and spatial arrangement of infected carriers
- Human contact with contaminated materials and animals
- Runoff and sanitation levels

Project Objective: Environmental Detection

Effective management requires environmental testing, but no standardized protocol exists[1,3,6,8].

Objective: To design and evaluate a water test for *Leptospira*.

The water test involves:

1) Filtering *Leptospira* out of environmental waters
2) Collecting microbial DNA off the used filter
3) Testing the DNA sample for harmful *Leptospira* \using the polymerase chain reaction (PCR)

American Samoa: Effluent Drainage[10]

Hawai'i Warning Sign[11]

METHODS

Selecting an Appropriate Filter

The following filters were evaluated using pure suspensions of *Leptospira icterohaemorrhagiae icterohaemorrhagiae M20*: 0.8 µm glass, 0.45 µm Durapore (polyvinylidene fluoride), 0.4µm nitrocellulose, 0.22µm Durapore, 0.2µm nitrocellulose, 40um nylon mesh.

1) 250µL of *Leptospira* culture was mixed in 10mL of 0.01M Phosphate Buffered Saline.
2) Cell density was assessed with a Petroff Hausser counting chamber in darkfield microscopy with 10 repetitions.
3) The suspension was vacuum filtered at 10 to 25 kPa using each of the filters listed above.
4) The concentration of cells in the filtered solution was assessed using the procedure from step 2.
5) Each filter was evaluated three times, and average cell counts were determined for each filter type.
6) Averages were used to calculate the percent flow-through of each filter using the following formula:

$$\frac{\text{Bacteria in Filtered Suspension}}{\text{Bacteria in Starting Suspension}} \times 100 = \%\ \text{Flow Through}$$

Obtaining and Identifying Leptospira DNA

To establish whether DNA can be collected and properly identified from used filters, two filter types were evaluated: 0.45 µm nitrocellulose & 40 µm nylon mesh. These pore sizes are less selective and less likely to give a positive result.

1) 1mL of live culture was vacuum filtered at 10 to 25 kPa. Triplicate repetitions were performed for each filter.
2) The used portion of the filter was cut out and used for step 3.
3) DNA was extracted off the filter using QIAGEN's DNeasy Blood and Tissue DNA Extraction kit, tissue protocol.
4) DNA quantity and quality were verified with a Nanodrop 1000 Spectrophotometer.
5) PCR was performed using: 1µl DNA sample, 1µl of primers (gyrase B, Slack et.al.. 2005), and Promega GoTaq Flexi PCR reaction mix (recipe upon request).

RESULTS

Filtration

Figure 1

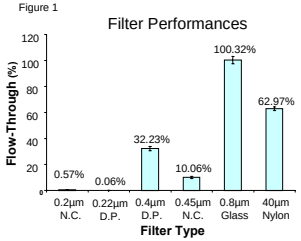


Figure 1: *95% confidence intervals are also shown. Abbreviations include: "N.C." for nitrocellulose and "D.P." for Durapore by Whatman.*

- **Smaller pore sizes passed lower proportions of *Leptospira*.**
- **The 0.8 µm glass filter was found to generate a large amount of visual artifacts.**
- **ANOVA results: 50% of variability is due to filter- specific characteristics like pore size and membrane material (R2 ≈ 50%).**
- **0.2µm and 0.45 nitrocellulose were selected for further analyses.**

DNA Detection

Gel Electrophoresis: PCR Results

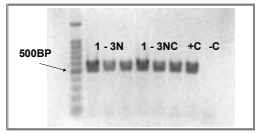


Figure 2: *1 - 3N refers to the three trials of 40 µm nylon mesh filters, 1 - 3NC refers to three trials on 0.4µm nitrocellulose filters, +C refers to the positive control, & – C refers to the negative control.*

- **An average of 1.65 ng/ul of DNA was obtained from the 40µm nylon filter.**
- **An average of 4.02 ng/ul of DNA was obtained from the 0.45µm nitrocellulose filter.**
- **PCR and gel electrophoresis took 4hrs, 40 minutes.**
- ***Leptospira* DNA was successfully detected in all trials.**

DISCUSSION

Filtration

- Results confirm the visual quantification technique works for leptospires in pure suspension.
- Filters made of Nitrocellulose or possessing 0.2µm pore sizes retain the most leptospires.
- Bacterial surfaces stick to negatively charged materials like nitrocellulose, glass, and nylon.

Conclusion: Bacterial recovery is most enhanced by using a nitrocellulose or nylon filter with 0.2µm pores.

*Collaborators at University of Nevada Reno are investigating the behavior of leptospires on filter surfaces and in viscous media using scanning electron microscopy.

DNA Detection

- We have been successful in recovering bacterial DNA from less selective filters, indicating the procedure is sensitive.
- Several factors which impact DNA recovery include: filter material, the extraction kit and protocol, and factors related to the PCR reaction.
- Using a filter with smaller pore sizes will likely enhance DNA recovery and improve the method's sensitivity.

Conclusion: Identifying *Leptospira* in environmental waters may soon be possible. Upcoming research will evaluate the sensitivity of this method with environmental samples. It is hoped the upcoming work will illustrate the strengths and weaknesses of the methodology, and indicate whether the method could be useful for regular environmental monitoring.

FUTURE WORK

1) Evaluate test sensitivity using complex water samples.

- Obtain stream waters and characterize by measuring pH, salinity, total nitrogen, TSS, heavy metals, and amount of other organocompounds.
- Determine the probability of detecting *Leptospira* by using filtration & PCR on stream water samples spiked with serial dilutions of *Leptospira*. Each dilution should be tested several times.

2) Environmental testing: Trial Run

- Results permitting, the test will be implemented in various streams in Hawaii, which are thought to be contaminated with *Leptospira*.

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