Evaluation of a Method to Detect *Leptospira* in Water: Preliminary Results from a Regional Collaboration

Ilima Hawkins[1], Mark Walker[2], Carl Evensen[1]

[1]University of Hawai'i, Department of Natural Resources and Environmental Management, College of Tropical Agriculture and Human Resources

[2]University of Nevada, Department of Natural Resources and Environmental Sciences

INTRODUCTION

The Disease

Leptospirosis is a growing zoonotic disease of global concern.[1, 2] Approximately 40 to 120 cases/year are reported in the U.S.[3] but is common throughout the tropics.[1,2] Most human infections occur from physical contact with freshwaters contaminated with pathogenic *Leptospira*.[2,4,5]

- Leptospirosis will develop 2 days to 4 weeks after the initial infection[1].
- Biphasic disease with highly variable symptoms.
  - Phase 1: characterized by vague flu-like symptoms that decline on their own.[1,5]
  - Phase 2: 10% of those afflicted develop the second, more serious phase.
    - Symptoms: tissue and organ infection, kidney damage, jaundice, liver failure, and death.[1]
- Treatment involves a course of strong antibiotics, like doxycycline.[5]

A pathogenic Leptospire[1]

The Organism

***L**eptospira* are aerobic, helical, motile bacteria that need fatty acids to grow.[3,5]

- Range from 0.2 to 0.3 microns in diameter; 6 to 30 microns in length.
- Divided into two taxonomical species: pathogenic *Interrogans* and saprophytic *Biflexa*.

Optimal growth conditions:[3,6]

1) Ambient temperatures of 28 to 30ºC
2) A pH of 5.2 to 7.7
3) Moist conditions
4) Minimal exposure to sunlight.

Survival times:[3,6]

1) 3 to 5 days in moist soils.
2) Up to 10 days in fresh water.
3) Up to 4 weeks in sterile tap water (pH 7)
4) Up to 3 days in aerated wastewater
5) 12 to 14 hours in undiluted wastewaters

Animal reservoirs:[4]

Deer, foxes, muskrats, opossums, raccoons, rodents, skunks, cattle, dogs, goats, horses, pigs, and sheep.
*Cats do not carry leptospires.

Pathogenic *Leptospira* require a mammalian host to survive and propagate (in the kidney renal tubules).[2,4]

- Hosts release urine contaminated with *Leptospira.*
- Humans contact infected animals or contaminated materials.[2,4]

The incidence of disease is dependent on four general factors:[7]

- The frequency of *Leptospira* deposition by host populations
- Precipitation patterns
- Regional land use and land cover
- The frequency of human contact with contaminated water (and other materials)

Approaches to Environmental Detection and Project Goals

Detecting leptospires in environmental waters is technically difficult, and no standardized protocol exists.[3]
- Independent studies have had limited success isolating, identifying, and culturing *Leptospira.*[3,8]
- The performance of these procedures have not been evaluated.

Effective management requires identifying and monitoring contaminated waters. This is of special importance for American Samoa, where the disease is new and the full impact is yet be felt.

American Samoa: Effluent Drainage[3]

Kaua'i, Hawai'i: Warning Signs for Hikers [4]

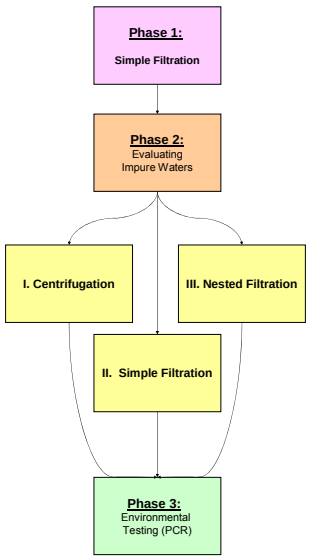
The objective of this work is to evaluate a proposed water testing protocol for *Leptospira Interrogans.* The method involves:

1) Sampling large volumes of stream water
2) Using a concentrating procedure to purify leptospires
3) Applying a molecular test to detect the presence of pathogenic spirochetes in the concentrated sample.

Preliminary results are presented here from a regional research project involving Nevada and Hawai'i, which has broad relevance throughout the American Pacific Islands.

METHODS

Experimental Design



Leptospira Suspensions

- A live culture of *Leptospira icterohaemorrhagiae icterohaemorrhagiae M20* was obtained from the National Veterinarian Services Laboratory, and stored in a dark cabinet at approximately 30ºC.
- The culture is passaged every 5 months in EMJH broth and semisolid media, both at 0.2% wt-w enrichment with 5-flurouracil for sterility.
- After 6 weeks of growth, the quality of a culture is visually affirmed using darkfield microscopy.

Phase 1: Simple Filtration

1) 250µL of NVSL *L.I.I.M20* stock was mixed into10mL of 0.01M PBS solution at 0.2% wt-wt 5-flurouracil.
2) The concentration of leptospires was visually assessed using a Petroff Hauser counting chamber and darkfield microscopy.
   - The suspension was vortexed for 8 seconds
   - A 9 µL aliquot was placed on the counting chamber
   - The specimen was viewed under darkfield microscopy at 400x.
   - The total number of leptospires within the counting grid was determined
   - 10 repetitions of counts were performed.
3) The suspension was vacuum filtered (10 to 25 kPa)
4) Microscopic assessment was used to estimate the filtrate concentration as described above.
5) An average concentration was calculated for the starting suspension and filtrate.
6) Triplicate repetitions were performed for each filter.

*The following filters were evaluated: 0.2 µm nitrocellulose, 0.22 µm Durapore (polyvinylidene fluoride), 0.4 µm nitrocellulose, 0.45 µm Durapore, 0.8 µm glass fiber, 40 µm nylon mesh.

Phase 2: Evaluate Impure Waters

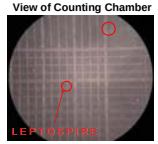
- Beginning now

RESULTS

Results are reported as the percent of the starting suspension that passed through the filter

(Filtrate Concentration ÷ Starting Concentration) x 100

View of Counting Chamber



- Results from a 1-way ANOVA indicate filter performances differed significantly at a 0.1 probability level.
- 50% of the variability of filter performances is due to filter-specific characteristics like pore size and membrane material (R2 ≈ 50%).
- Figure 2 illustrates: 1) Smaller pore diameters passed lower proportions.
  2) Filter materials may not greatly affect performance.

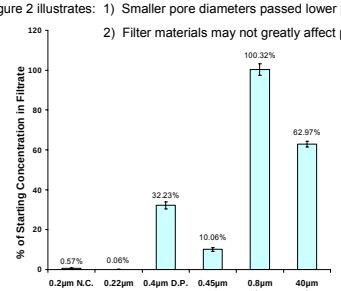


Figure 2: *Values represent average percent yields of three trials per filter. 95% confidence interval bars are also shown. Abbreviations include: N.C. for nitrocellulose, D.P. for Durapore by Whatman.*

- The 0.2 µm Nitrocellulose passed more leptospires than 0.22 µm Durapore.
- 0.4 µm Durapore passed more than the 0.45 µm Nitrocellulose.
- The 0.8 µm glass fiber filter yielded approximately 100% of the starting concentration in the filtrate. Further examination using a blank control demonstrated a high number of visual artifacts, making it inappropriate for microscopic work.
- The 40 µm nylon mesh demonstrated the highest filtrate recovery.

DISCUSSION

Filters most commonly used for microbial filtrations (0.2µm ; 0.45µm N.C.were challenged with *Leptospira icterohaemorrhagiae icterohaemorrhagiae M20*

The results of the ANOVA and 95% CIs indicate three important findings:

1) Affirm visual quantification using the Petroff Hauser chamber works for leptospires in pure solution. Future studies of *Leptospira* may benefit from using this method.
2) Pore sizes do affect filter performances with *Leptospira* suspensions. R^2 may have been low due to a pattern of similar performance levels with similar pore sizes.
3) Although literature and previous work has demonstrated selectivity is influenced by filter materials, no pattern was observed between materials of similar sizes.

Recovery from 0.2µm - 0.45µm membranes was low and ranged between 0.06% - 32.32%. Preliminary conclusions are:

- *Leptospira* are difficult to filter.
- They are long and spiral, and may tangle or damage easily on rough filter surfaces.
- Nitrocellulose, glass, and nylon are negatively charged, and are likely to hold onto bacterial surfaces.

Our collaborators at University of Nevada Reno are investigating the behavior of leptospires on filter surfaces using scanning electron microscopy. If the linear leptospires tangle on membrane surfaces, they may be easily resuspended when the surface matrix is smooth and orderly.

FUTURE WORK

Future work to develop an optimal filtration and detection procedure will now move into Phase 2 of the experimental design. Three common purification techniques will be tested against *Leptospira* suspensions containing particulate matter, and spirochete detection will be performed using Polymerase Chain Reaction technology. Work will proceed as follows:

1. **Optimize a PCR protocol by testing starting suspensions, used filters, and filtrate for *Leptospira***
2. **Simulate real-world conditions by adding soil particles and organic matter to suspensions. Bacterial isolation will be attempted using centrifugation, single membrane filtration, and nested (multiple) filters of decreasing gradients.**
3. **The most efficient method will be used to test stream water from Manoa Valley, Hawai'i, which is known to be contaminated with *Leptospira.***

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