



Detecting *Leptospira* in Water: Evaluation of a Proposed Method

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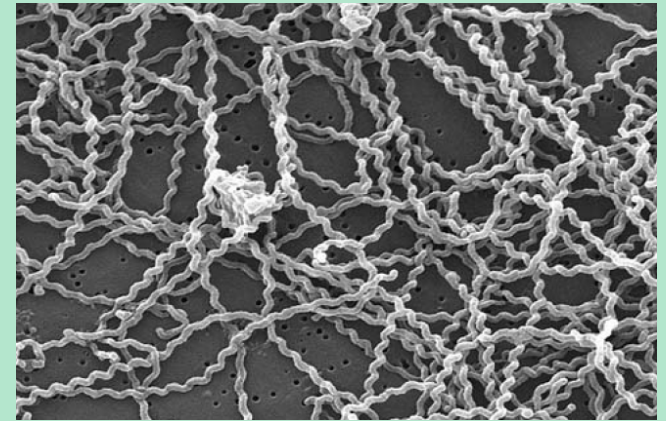
Outline

- Introduction
 - What is Leptospirosis
 - Need for environmental test
 - Goals and objectives
- Study 1: Evaluating a collection tool
 - Filtration: preferred materials, pore sizes, and a quantification technique
- Study 2: Evaluating a detection tool
 - PCR: A sensitive and specific identification tool
- Conclusions



Introduction: What is Leptospirosis?

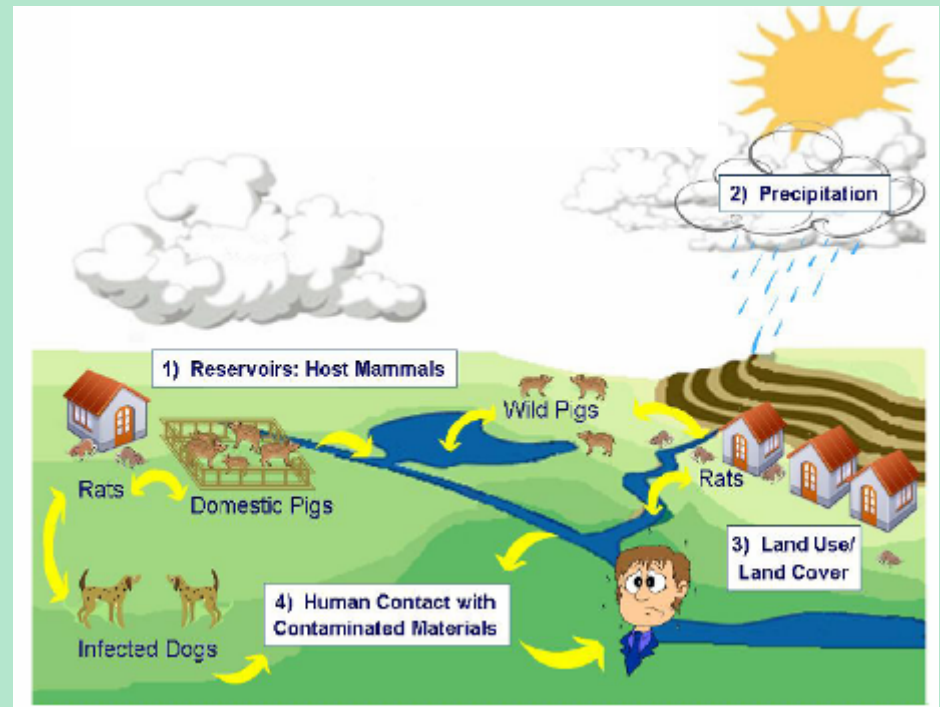
- First recognized by Adolf Weil in 1816
- Mammalian infection by *Leptospira*
- Mechanism of infection
- Symptoms of disease



Introduction: Need for a Test

- Transmission
 - Associated with fresh water
 - Outbreaks fluctuate

- Management
 - Hawaii
 - A. Samoa



- A freshwater test can help!



Introduction: Goals and Objectives

Project goal: To propose and evaluate a two-part test which detects pathogenic *Leptospira* in freshwater

- Challenge 1: Collection
- Objective: Evaluate how efficiently *Leptospira* can be filtered

- Challenge 2: Identification
- Objective: Determine the sensitivity of a PCR-based test for *Leptospira* under real-world conditions



Study 1: Evaluating a Collection Tool

→ Filters commonly used to collect waterborne bacteria

→ Little is known about filtering *Leptospira*

→ *Leptospira* morphology is “unconventional”

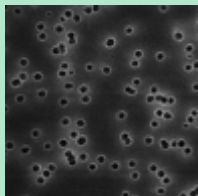
- **Objectives:**

- Evaluate how filter materials and pore sizes collect *Leptospira*

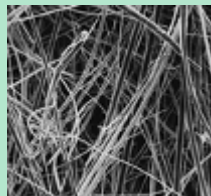
- Determine whether filtration is an effective collection tool for *Leptospira*

Study 1: Evaluation of a Collection Tool

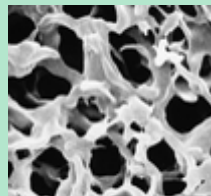
- Many types of filters:
 - Hydrophobic vs hydrophilic
 - Pore size
 - Manufacturing process
- Examined 5 materials, 4 pore diameters



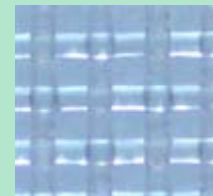
Isopore



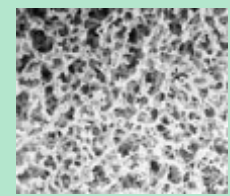
Glass
Fiber



Durapore



Nylon
Mesh



Nitro-
cellulose



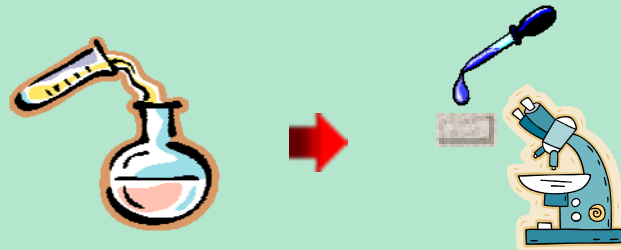
Filter Characteristics

Filter (catalog number)	Type	Name	Material	Pore Diameter
GE* Nitrocellulose-Mixed Esters of Cellulose Membrane (E02WP04700)	Hydrophobic Water sampling	<i>0.2μm NC</i>	Nitrocellulose	0.20 μ m
Millipore Isopore Membrane filter (HTTP04700)	Hydrophilic	<i>0.4μm Isopore</i>	Polycarbonate	0.40 μ m
Fisher (09-719-555)	Hydrophobic Water sampling	<i>0.45 μm NC</i>	Nitrocellulose	0.45 μ m
Millipore (AP1504700)	Hydrophilic Prefilter for coarse debris removal	<i>0.8μm Glass</i>	Glass fiber	0.8 μ m
Small Parts Inc (CMN-0040)	Hydrophobic nylon mesh sheet	<i>40.0μm Nylon</i>	Nylon mesh	37 μ m
Millipore Durapore (GVWP04700)	Hydrophilic Liquid purification	<i>0.22μm D.P.</i>	Polyvinylidene fluoride	0.20 μ m
Millipore Durapore (HVLP04700)	Hydrophilic Liquid purification	<i>0.40μm D.P.</i>	Polyvinylidene fluoride	0.40 μ m

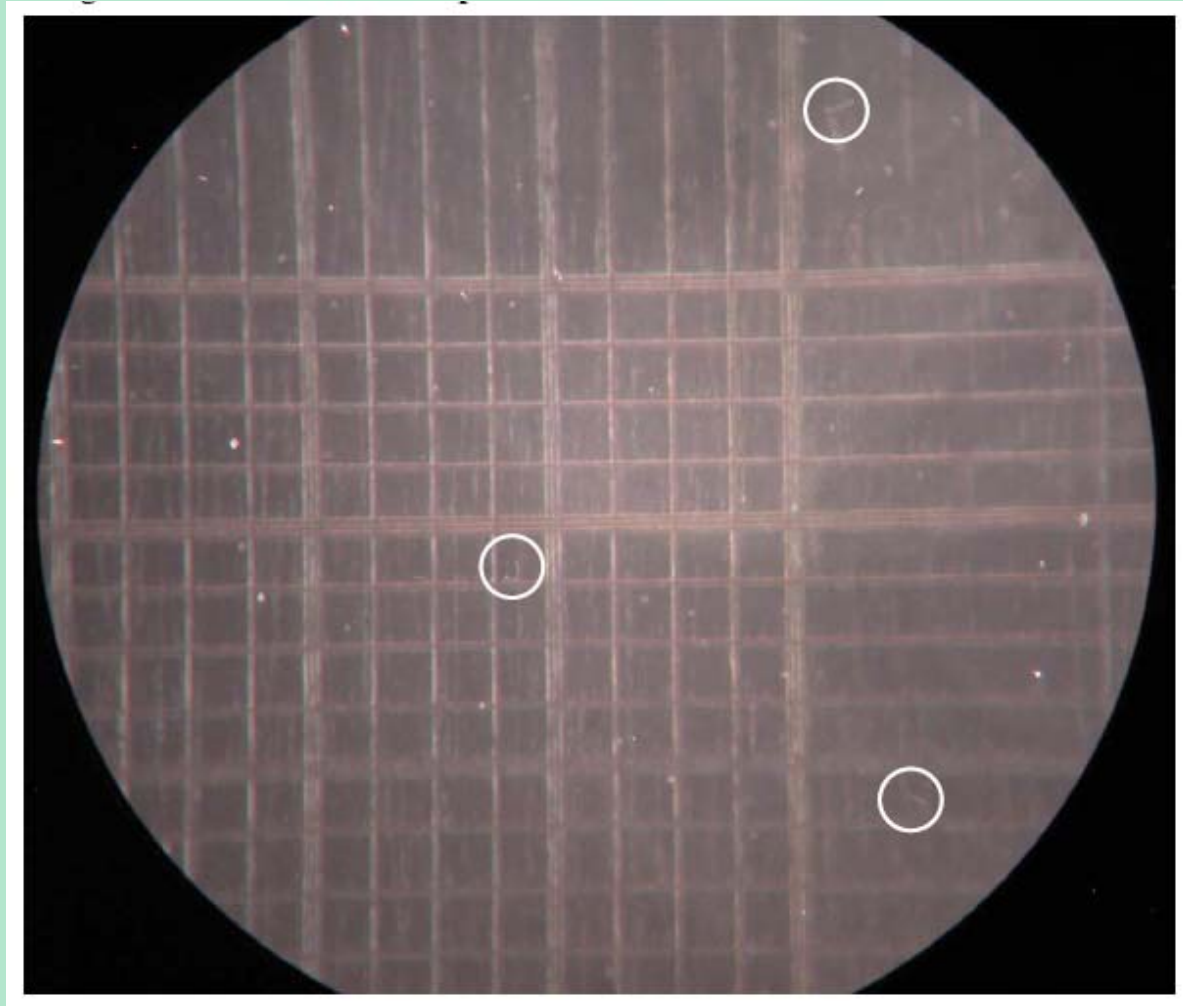
Study 1: Methods

1. Generating & quantifying a *Leptospira* suspension

- A. NVSL → *Leptospira interrogans copenhageni icterohaemorrhagiae M-20*
- B. Cultured in liquid & semisolid EMJH
- C. Diluted 500 μ L liquid stock into 30mL 0.01 M PBS
- D. Quantify 10 times with a Petroff Hauser Chamber



Petroff Hauser Chamber



Study 1: Methods

→ Selected 10 replicates because:

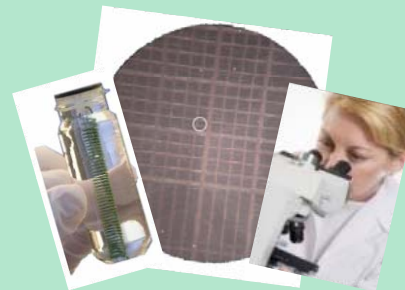
$$N \geq [z^2\theta^2] \div d^2$$

* The observed cell concentration deviated by 0 - 4.75 cells/mL

2. Filtered and quantified flow through

A. Vacuum filter three 10mL samples

B. Quantify each of 3 filtrates 10 times



Study 1: Methods

- Calculating percent transmittance

$$\frac{[\text{Avg Flow Through}]}{[\text{Avg Starting Concentration}]} \times 100 = \% \text{ Transmittance}$$

- Averaged 3 trial values for each filter

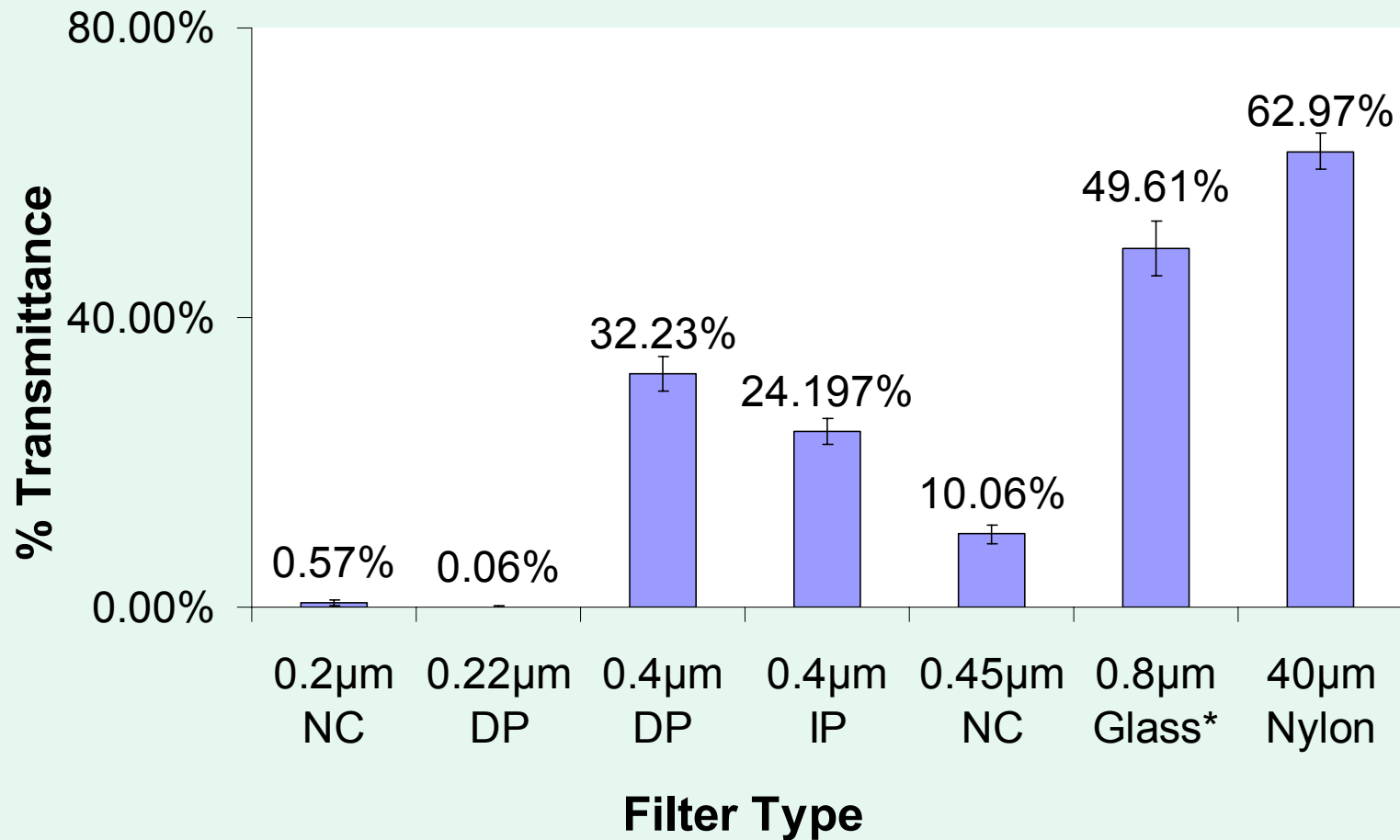
- Corrected glass fiber results

- Transmittance over 100%
- Filtered sterile DI H₂O and found visual artifacts
- Generated corrective factor

Study 1: Results



Transmittance of Leptospira





Study 1: Results

Transformation of % Transmittance Data

	0.2 μm	0.4 μm	ArcSin of 0.2 μm	ArcSin of 0.4 μm
Hydrophobic	0.57%	10.98%	0.64	1.27
Hydrophilic	0.44%	9.58%	0.13	1.39

2-Way ANOVA on % Transmittance

Source	DF	SS	MS	F	P
Material	1	0.11	0.11	7.51	0.025
Pore Size	1	2.67	2.67	187.8	0.000
Interaction	1	0.30	0.30	21.42	0.002*
Error	8	0.11	0.01		
Total	11	3.19			

Study 1: Discussion

- Optimal filter:
 - Small pores
 - Hydrophobic materials
- Potential challenge:
 - Clogging
 - Options:
 - Process a smaller volume
 - Use larger pore size
 - Use nested filtration
 - Combination of solutions
- Recommend:
 - 0.2 μ m Nitrocellulose
 - 0.22 μ m Durapore
 - Corrective factor



Study 2: Analysis of a Detection Method

- Need a robust, sensitive detection technique
- Previous efforts
 - Culturing
 - Fluorescently labeled antibodies
 - Genetic analysis with the polymerase chain reaction
- PCR
 - Developed in 1986 by Kary Mullis
 - Very specific
 - Increasingly used in a variety of biological fields



Study 2: Analysis of a Detection Method

PCR is sensitive:

1. Obtain good quality DNA
2. Annealing temperature

Some studies successful with PCR

No standardized method exists

*Implication of negative results uncertain

Objective: Determine the sensitivity of a PCR test for *Leptospira* under real-world conditions.

1. Identify a relationship between the number of leptospires & the frequency of obtaining a positive result
2. Identify water characteristics that significantly impact test reliability

Study 2: Methods

Overview:

Evaluating PCR under Real-World Conditions

- 1) Collected water from three streams
- 2) Generated serial dilutions, filtered
- 3) Extracted total DNA, checked quantity and quality
- 4) Concentrated DNA; washed with ethanol
- 5) Tested samples for leptospirens using PCR



Study 2: Methods

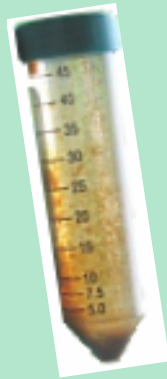
1. Water Samples

- Manoa, Waihe'e, and Waikele streams
- O'ahu water quality study by NAWQA



Study 2: Methods

- Characterizations:
 - Manual TSS and TDS



- Agricultural Diagnostic Service Center:

pH, EC, total N, boron, calcium, copper, iron, potassium, magnesium, arsenic, manganese, molybdenum, sodium, phosphorus, zinc, cadmium, chromium, nickel, lead, selenium, vanadium.

Study 2: Methods

2. Filtering serial dilutions of *Leptospira*

- Quantify *Leptospira* suspension
- 50mL aliquots of stream water
- Serial dilutions: **10^0 , 10^2 , 10^3 , 10^4 , 10^5** cells/50mL water
- 5 replicates, 2 negative controls
- Vacuum filtered: 0.2 μ m nitrocellulostic filters
- Filters stored or processed



Study 2: Methods

3. DNA Extractions

- MoBio's UltraClean Water DNA Isolation Kit
 - *LabNet flat-top vortex adapter
- Final volume = 3mL
- NanoDrop 1000 spec & v3.3.0 software
- Poor $A_{260 / 280}$ & $A_{260 / 230}$ ratios





Average Quantity and Purity of Recovered DNA				
Stream	Dilution	Total DNA (ng/uL)	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

Study 2: Methods

4. Concentration and Washing

- Manoa samples: MoBio protocol 1st
- All samples: Bioline's co-precipitant pink protocol
 - Used half the recommended volume (3 μ L)
 - * 2 EtOH washes
- Resuspended in 20 μ l sterile DI H₂O
- DNA analysis not possible

Study 2: Methods

5. PCR for Detection

- Primers: gyrase subunit B sequence
Only identifies pathogenic *Leptospira*
- Expected product size: 502 bp
- Used Promega's GoTaq Flexi PCR kit: 50 μ L rxns:
31.75 μ L dH₂O, 4 μ L MgCl²⁺, 10 μ L GoTaq buffer, 1 μ L primer,
1 μ L dNTPs, 1 μ L DNA, 0.25 μ L Taq.
- Amplification:

		94°C – 5min
	/	94°C – 1min
35 cycles		51.7°C – 1min
	\	72°C – 1.5min
		72°C – 10min



Study 2: Methods

6. Gel Electrophoresis

- 10uL product run on 1.5% agarose gel, 94V, 45 min.
- Gel soaked in 5 μ g/mL et.br. solution, 20 min.
- Kodak DC 290 camera and Kodak 1D 3.5 software
- Logistic regression performed on positive results

Study 2: Results

- Stream Water Quality
 - All streams: TDS > TSS
 - Manoa: highest total N, P
 - Waihe'e: highest metals, lowest TSS
 - Waikele : highest EC, Na, Mg, Fe, Ca, Ma
- *Many values exceeded DOH standards
 - All streams, all seasons: total N, Se, & Ni
 - All streams, dry season: total P
 - Waihe'e: Pb

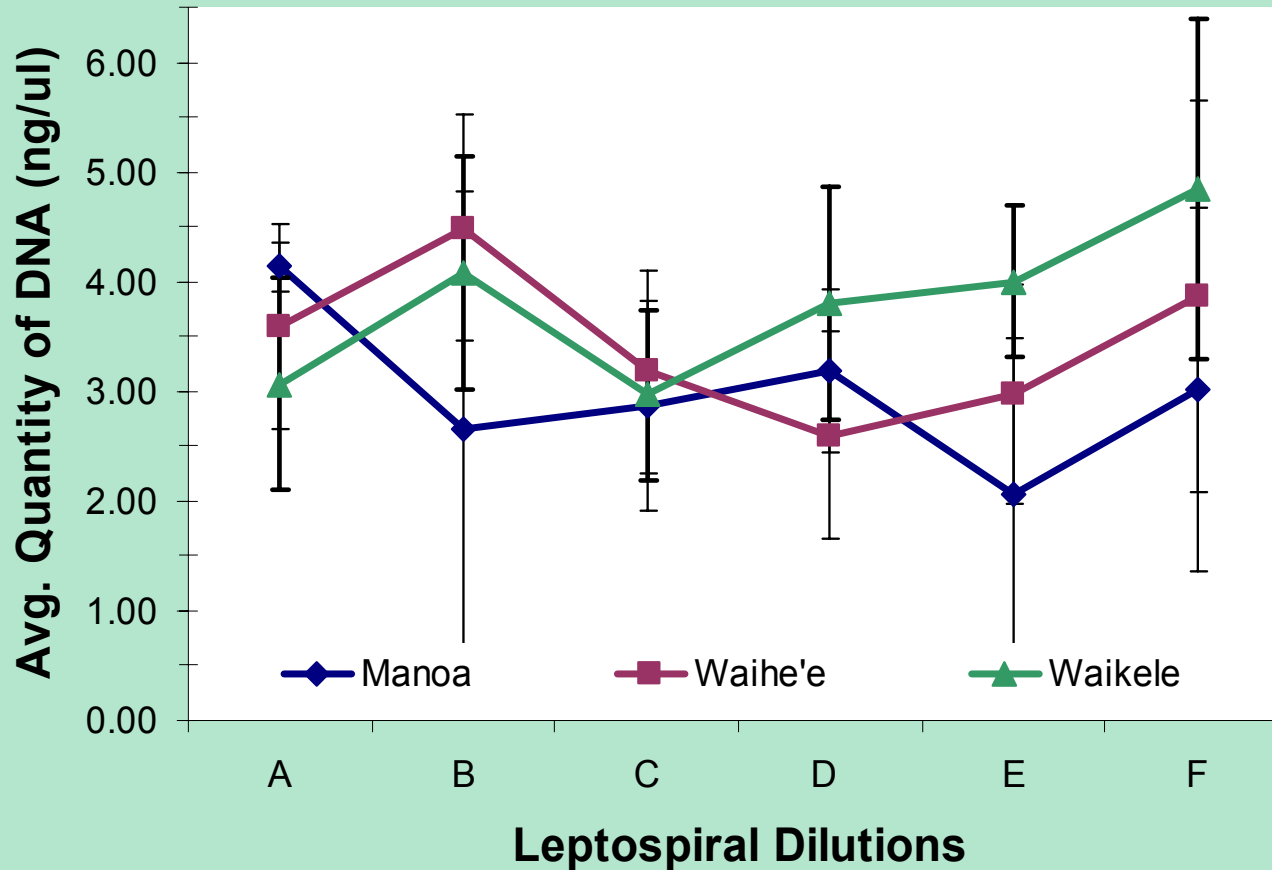


Water Quality Measurements

Characterizations	Manoa	Waihe'e	Waikele
TSS (mg/L)	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 (ug/L)	18	137	30
*B (ug/L)	50	23	100
*Ca (ug/L)	11120	9770	18740
*Cd (ug/L)	1	12	0
*Co (ug/L)	4	22	10
*Cr (ug/L)	14	53	30
*Cu (ug/L)	40	23	0
*Fe (ug/L)	20	0.00	70
*K (ug/L)	2080	1640	2920
*Mg (ug/L)	10560	6370	18450
*Mn (ug/L)	0.00	0.00	10
*Pb (ug/L)	20	147	10
*Na (ug/L)	18120	13580	78490
*Ni (ug/L)	9	33	20
*Zn (ug/L)	20	2	20
*N (ug/L)	2270	410	1510
*Se (ug/L)	27	196	30
*P (ug/L)	160	110	110

Study 2: Results

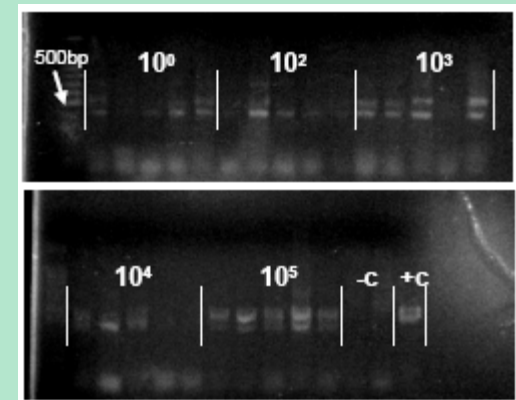
Average Amount of Total Microbial DNA Collected



Study 2: Results

- Manoa Stream Set

- At least 3 out of 5 samples of each dilution were amplified
- Contamination not likely:
- This method is able to detect pathogenic leptospiral DNA at low levels
- Binary logistic regression:



Frequency of (+) Results

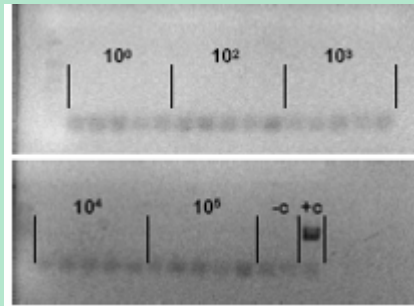
	10 ⁰	10 ²	10 ³	10 ⁴	10 ⁵	-C
Positive Results	3	3	4	3	5	0
Frequency	0.6	0.6	0.8	0.6	1.0	0

DF = 1; P = 0.066

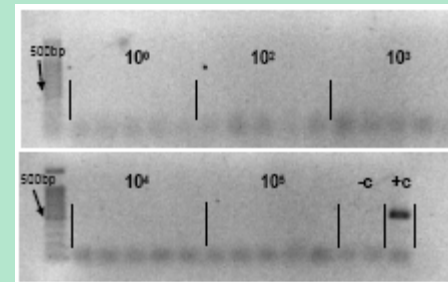
Study 2: Results

- Waihe'e and Waikele Stream Sets

Waihe'e

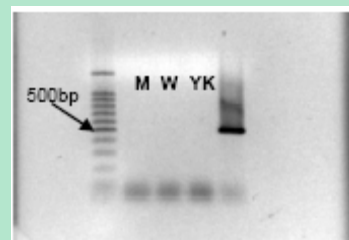


Waikele



- One 10^5 sample from each stream set purified
→ QIAQUICK PCR Purification Kit

10^5 Purified Samples



Study 2: Discussion

Results indicate:

- Microbial DNA was recovered consistently
- Method has potential
- Not reliable enough for routine testing yet
- If experiment worked, correlated:
 - (+) results with # of leptospire
 - (+) results environmental properties of water



Conclusions: Project Summary

Goal: Evaluate an environmental test for pathogenic *Leptospira* in water

Filtration is an efficient means of collection

– Capture rate can be significant

- *Leptospira* can pass through 0.2 μ m pores
- Maximum retention observed at 99.04%

– Clogging not of significant concern

- Amount of suspended solids vary
- Many different goals for environmental testing
 - Routine monitoring vs studies of high-flow events
- If clogging is problematic, methodology may be switched:
Centrifugation and MoBio's UltraClean Soil DNA Isolation Kit



Conclusions: Project Summary

PCR has potential but requires more work

- DNA is consistently recovered
- Co-precipitants are not recommended
- Alternative concentration/purification options:
 - Use a spin column + PCR Purification kit
 - MoBio's PowerClean DNA Cleanup Kit
 - MoBio's UltraClean PCR Purification Kit
 - QIAGEN'S QIAQUICK PCR Purification Kit
- Evaluate PCR again with additional replicates



Conclusions: Project Summary

Provides the ability to:

- Identify and monitor contamination
- Serovar identification
- Perform routine testing
- Development of public protection strategies
- Improve land management strategies

An environmental test would benefit:

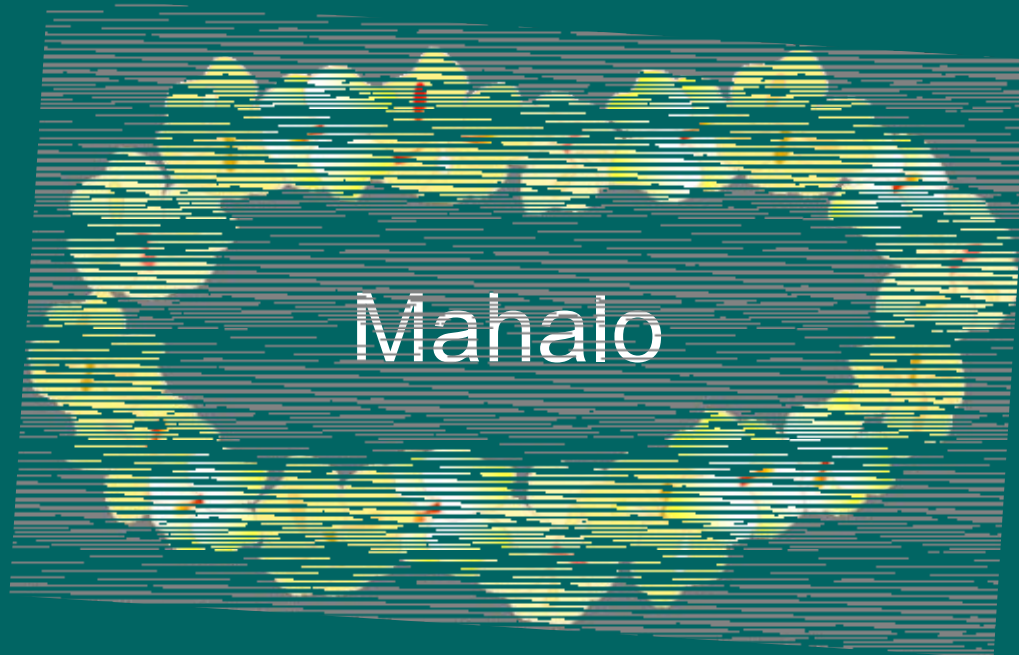
Government agencies

Public confidence

Academia

Public health

This research should continue!



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Dedicated to:

Georgette J. Buchner & Thelma Neilsen

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