Development and Application of an Enteric Pathogens Microarray

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Study Overview

- Pilot study to develop a diagnostic microarray to detect the presence or absence of multiple infectious agents associated with gastrointestinal illness simultaneously from clinical specimens.
- HIV+ patients from the East Bay AIDS Center with acute, chronic and no diarrhea will be recruited to provide a stool specimen and brief questionnaire on gastrointestinal symptoms and potential risk factors.
- Sampling design is a case-control study with two comparison arms.
- Stool specimens will be analyzed using standard microbiological methods and by the novel microarray.
- Pilot study will focus on designing a microarray with up to 300 targets representing 30-50 bacterial and protozoan organisms. We hope to seek future funding to develop a larger array that will include viruses.

What is a microarray?

- A microarray is a glass slide onto which ss DNA fragments are adhered at fixed points (“spots” or “probes”).
- A single slide can hold tens of thousands of probes, each probe is related to a single gene.
- Microarrays exploit the preferential binding of complementary nucleic acid sequences.
- Samples of fluorescently labeled mRNA or cDNA are washed over the array as in a nucleic acid hybridization test, except on a substantially larger scale.
- Target gene sequences from the sample hybridize to their complementary sequence in the “spots”.
- To quantify hybridization, the array is scanned, excited by a laser and the fluorescence intensity of each spot measured.

The motivation...

- Though recent developments in molecular analysis techniques have increased the sensitivity with which enteric pathogens can be detected, they remain inefficient as each organism has to be tested for individually.
- Microarrays are uniquely suited to mass screening and offer the potential to maximize efficiency as various PCR products or oligonucleotides each representing an individual hybridization test, can be spotted on a single microarray and assayed simultaneously.
- This can potentially be a rapid and highly sensitive method that ultimately may be less costly than convention tests and alter the way in which epidemiologic studies can evaluate stool specimens for the presence of pathogens.

Principle Objectives

- To develop a pilot "enteric pathogens microarray" to detect various bacterial and protozoan organisms.
- To validate the microarray by:
  - determining the sensitivity, specificity, predictive values (+/-) and ROC characteristics of the microarray using pathogen specific nucleic acids from known positive samples.
  - directly comparing the microarray results with results from standard microbiological tests and evaluating agreement of the two techniques using the same clinical specimens.
- To evaluate the association of specific infectious organisms with acute, chronic and no diarrhea in HIV+ individuals using standard clinical microbiology and the microarray.
- To determine if infectious agents previously unrecognized as potential pathogens are associated with symptoms of gastrointestinal illness.

Microarray Development: Probe Selection

- Need to develop a set of probe sequences (oligonucleotides) to place on the array which are unique markers for the particular pathogen of interest (often these represent virulence genes or segments with high heterogeneity)

- Two probe selection pathways:
  - Directed placement of primer or oligonucleotide sequences from the literature and current PCR tests. Candidate array probes from this pool will be evaluated using bioinformatics tools for specificity, uniqueness and potential for cross-reactivity with other targets on the array.

  - Systematic search of known gene databanks for all genes belonging to key bacteria and protozoa involved in GI illness. This will allow us to include organisms in the array for which we do not currently have target sequences. Organisms will be grouped by functional clusters and unique target sequences identified. *(This is a bioinformatics scheme currently beyond my understanding!)*

Microarray Development: Array Fabrication, Sample Preparation and Analysis

- CNR Genomics Facility will serve as the site for microarray printing, scanning and analysis. They will also provide consulting, training and other support services.
- Bacterial and protozoan total RNA will be isolated from stool specimens
- Generate labeled cDNA probes from the RNA (dUTP/ dCTP coupled to Cy3, Cy5 dyes)
- Labeled target cDNA will be applied to the array, incubated and washed using standard protocols.
- Array will be scanned and the amount of dye bound to each microarray feature identified and quantitated.

Application of the EPM: Case-Control Study on Causes of Diarrhea

- The pilot microarray will be used to characterize fecal carraige rates of infectious organisms in HIV+ individuals with acute, chronic and no diarheal symptoms.
- Isolation rates and the types of organisms seen will be compared between these three groups.
- Principle objective is to evaluate the association of specific organisms with each of the three groups using results from both standard microbiology techniques and the microarray.

The Three Groups Being Compared

- Patients with **“acute” diarrhea**- have GI symptoms (vomiting, diarrhea, cramps, vomiting, bloody diarrhea and/or fever) that are new, different or more sever than the normal pattern of diarrhea experienced by the patient. Suspected by clinician to be of infectious etiology. This group represents our “cases”.

- Patients with **“chronic diarrhea”-** have diarrheal symptoms (2 or more loose stools per day for two weeks or greater) that are not different from their normal pattern of diarrhea. Diarrhea may be due to medication and may or may not represent enteric infection.

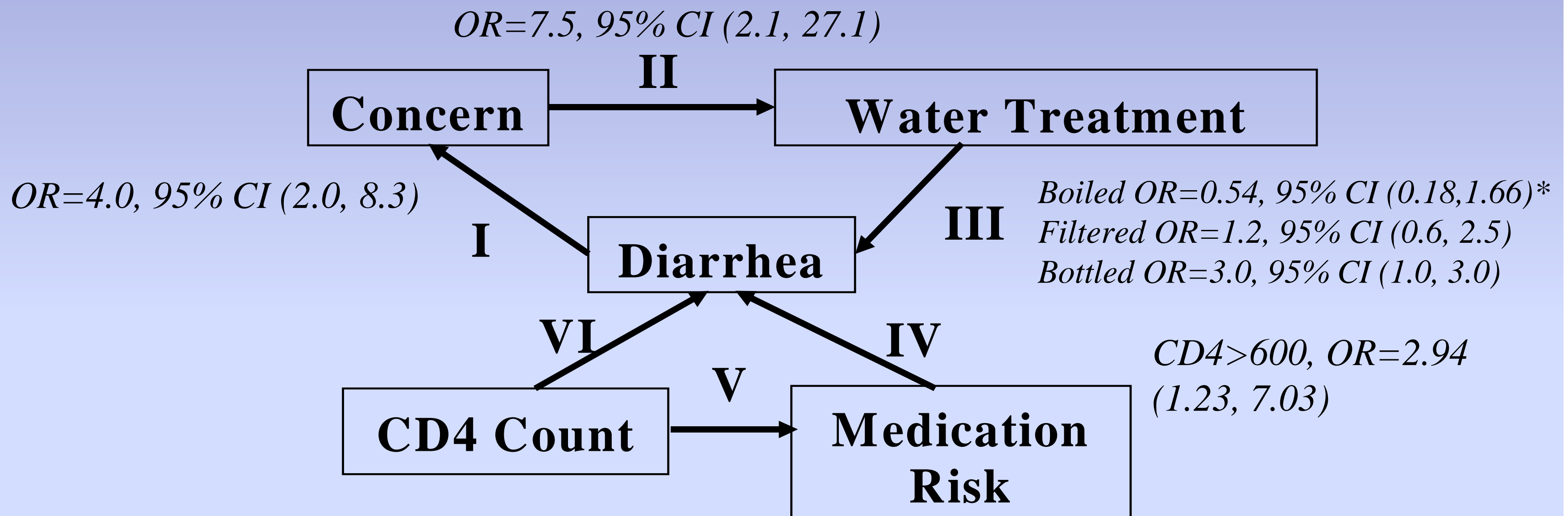
- Patients with **“no diarrhea”-** may have asymptomatic infection with recognized enteric pathogens or other organisms whose pathogenicity is not known or well defined.

Enrollment and Study Procedures

- 150 participants will be recruited from the East Bay AIDS Center during regular clinic visits and asked to provide a stool sample and complete a brief questionnaire on GI symptoms and risk factors.
- Enrollment in the two comparison groups will be matched on time to the cases (would have like to match on CD4 count and sex, but cannot due to cost constraints).
- HIV viral load, CD4 count and HIV medications will be abstracted from medical records for statistical modeling.
- Use clustering techniques to investigate the grouping of participants (acute, chronic, no diarrhea) with respect to specimen results. We will identify those organisms that best distinguish the three groups.

Model of Causal Linkages from HIVWET Cross-Sectional Survey

Eisenberg JNS, Wade TJ, Charles S, Vu M, Hubbard A, Wright CC, Levy D, Jensen P, Colford JM Jr. Risk factors in HIV-associated diarrheal disease: The role of drinking water, medication and immune status. Epidemiology and Infection, 2002, 128(1): 73-82



♦ These linkages represent the *a priori* hypothesis that (I) increased diarrhea causes an increased concern for drinking water quality, which in turn (II) leads to increased boiled or bottle water use, which in turn (III) decreases the prevalence of diarrhea.

♦ Linkages IV-VI illustrate the impact of medication on diarrhea and the interaction between medication related diarrhea and CD4 count.

**Not significant due to insufficient sample size. A sample size of 520 would be needed to achieve 80% power.*

Potential Future Microarray Studies

- More comprehensive enteric pathogens microarray with viruses (collaboration with CDHS VRDL) applied to HIV+ individuals recruited through SF Community Consortium Clinics in SF and East Bay for similar study.

- Use to evaluate incidence and prevalence of enteric pathogens among the elderly enrolled in waterborne disease trial (Sonoma WET) and other waterborne disease studies.

- Develop a microarray for characterizing gene expression from kidney tissue in children with Wilm's Tumor and evaluating associations with race, disease progression and other variables.