Assessing the Influence of Land-Based Discharges (Streams, Storm Drain, and Groundwater) on the Concentrations and Ratio of Four Human Pathogenic Marine Vibrio spp. in Four Categories of Coastal Water Environments of Hawaii

Introduction

The majority of the available information concerning *Vibrio* species (spp.) is based on studies conducted in temperate regions. Information on the prevalence and ecology of these species in tropical areas, such as Hawaii, is limited. Four pathogenic *Vibrio* spp. (*V. alginolyticus, V. cholerae, V. parahaemolyticus, V. vulnificus*) have been documented to cause human infections in Hawaii. *V. vulnificus* infections have recently resulted in two deaths related to exposure to Hawaii's coastal waters. One death occurred on the island of Hawaii and the second death occurred on Oahu (Wilson 2006). Over the past 20 years, four isolated cases of *V. cholerae* infections were confirmed on Oahu in individuals that did not travel outside the state (Leidemann 2005). The source of these infections could not be determined, and contaminated seafood was considered to be the most likely source. In addition, infections of *V. parahaemolyticus* associated with undercooked crabs (Barker 1974) and *V. alginolyticus* infections in surfers with cuts have also occurred in Hawaii (Blake et al. 1980).
Prior to the present study there have been no studies conducted to determine the prevalence of these pathogens in the coastal waters of Hawaii. Thus, the major goal of this study was to obtain data concerning the prevalence of pathogenic *Vibrio* spp. in Hawaii's coastal waters and to determine their potential public health significance to individuals who use these waters for recreational purposes.

Problems and Research Objectives

Current Problems Concerning *Vibrio* spp. Enumeration in Coastal Waters

Several problems have been identified in assessing issues related to *Vibrio* spp.:

1. None of the existing methods to enumerate *Vibrio* spp. from water samples have been approved by the United States Environmental Protection Agency. Thus, there is a need to collect additional data and to identify an appropriate enumeration method.
2. No systematic studies exist to determine concentrations of pathogenic *Vibrio* spp. in various types of Hawaiian coastal waters used by humans.
3. Concern of human exposure to *Vibio* spp. has been raised—testing for exposure to *Vibrio* spp. in recreational coastal waters cannot proceed until environmental data are collected and measurement methods are approved. Thus, data gathered from this study will provide the first step in determining the potential risk from *Vibrio* spp. at various Hawaiian coastal water sites.

The principal objectives of this proposal are to:

1. To determine the prevalence of the four human pathogenic *Vibrio* spp. (*V. alginolyticus, V. cholerae, V. parahaemolyticus, V. vulnificus*) in coastal waters approved for recreational use which are characterized by negligible to varying degrees of land-based runoff.
2. To determine the prevalence of the four human pathogenic *Vibrio* spp. in confined coastal waters, which are not approved for recreational use and are characterized by high degrees of contamination from land-based runoff.

3. To determine if conditions of low salinity and warm water temperature (>30°C), which characterize some coastal water sites on the island of Hawaii, are selective for the growth of the four human pathogenic *Vibrio* spp.
4. To determine the prevalence of the four human pathogenic *Vibrio* spp. in sediments of coastal waters as a possible source of these pathogens into the water column.
5. To determine whether raw sewage and various treatments of sewage (primary, secondary and disinfected) that are discharged into Hawaiian coastal waters, are possible sources for the four human pathogenic *Vibrio* spp.

Methodology

Sample Categories

Sampling sites on the islands of Oahu and Hawaii were divided into four water-quality categories based on their use and the degree of impact by land-based runoff.

1. Primary Coastal Beaches Approved for Swimming (Oahu): these sites consist of the more popular beaches on Oahu and are characterized by no obvious impact by land-based runoff. Thus, waters at these sites are generally characterized as having high salinity and low turbidity.
2. Secondary Coastal Beaches Approved for Swimming (Oahu): these sites consist of less popular beaches on Oahu and are characterized by variable impact by nearby land-based runoff. Thus, waters at these sites are generally characterized as having lower salinity and higher turbidity than waters at primary coastal beaches.
3. Coastal Harbors, Ponds, and Canals Not Approved for Swimming (Oahu): these sites are confined coastal bodies that have poor circulation with the open ocean. They are highly susceptible to land-based runoff and, as a result, have variable salinities with high turbidity. Because these sites are contaminated with land-based runoff, they are not approved for primary contact recreational use. However, human activity and exposure to these waters are acknowledged to occur. The prime example of this type of site is the Ala Wai Canal where a documented case of accidental human contact with water at this site, resulted in infection and death by *V. vulnificus*.
4. Approved Swimming Sites (Island of Hawaii): these study sites were selected based on past evidence of *V. vulnificus* wound infections. While beaches on Oahu are largely represented by sandy swimming beaches, the beaches on the island of Hawaii are characterized by a rocky coastline because the island is geologically younger than Oahu. Coastal water sites used for recreation on the island of Hawaii are often large rock-enclosed coastal ponds. Many of these sites are impacted by land-based runoff, generally susceptible to freshwater input and are characterized by low and variable salinity.

The distinctions between coastal waters on the islands of Hawaii and Oahu are related to the geological ages of lava that formed the two islands, and the corresponding weathering effects on the lava rock formation at coastal water sites. In this regard, Oahu is 2–3 million years old whereas the island of Hawaii is less than 1 million years old (and still forming). Age and weathering controls the hydrogeology of each island, and over time, sedimentary deposits form

a relatively impermeable rock called caprock. This caprock is prevalent on Oahu while not on the island of Hawaii. As a result, when it rains on Oahu, the caprock prevents groundwater from flowing out into the coastal waters and forces it to percolate underground. On the island of Hawaii however, the absence of caprock causes the groundwater to be readily discharged into coastal waters and ponds. This leads to ponds with low salinity waters which is a condition known to be conducive for the growth of bacteria such as pathogenic *Vibrio* spp. Some of these low salinity coastal ponds are also characterized by high water temperature generated by the discharge of lava-heated groundwater into coastal areas. Some of these thermal ponds have previously been identified as sources for the transmission of *V. vulnificus* to humans. A case of a person swimming in a thermal pond, becoming infected and dying from *V. vulnificus* has been documented.

Sample Collection

At all primary and secondary recreational beaches, water samples were collected (in sterile, one-liter plastic sampling bottles) in waist deep water. Harbor, canal, and pond sites were sampled from shore using a sampling bucket and were then similarly collected in sterile one-liter plastic bottles. Sediment samples were taken in knee deep water using a strainer containing a nylon mesh. Three samples were taken from each site, placed into sterile Whirl-Pak bags, and transported to the laboratory for analysis. All Oahu samples were analyzed within three hours of collection.

Water samples from the island of Hawaii were similarly collected into sterile one-liter plastic bottles. Due to transport times to the Oahu laboratory, these samples were processed within eight hours of collection.

Using a sampling pole, sewage samples from the Hawaii Kai, Sand Island, and Wahiawa Wastewater Treatment Plants on Oahu were collected in sterile half-liter plastic bottles and transported to the laboratory. Samples were analyzed within three hours of collection.

All samples were transported for analysis to the Oahu laboratory in a cooler without ice.

Chemical Parameters of Water Samples (Turbidity and Salinity)

Water samples were measured for turbidity and salinity. Turbidity was measured using a Hach Turbidometer and recorded as Nephelometric Turbidity Units. Salinity was measured using a refractometer and recorded as parts per thousand (ppt). Both turbidity and salinity measurements were used to determine whether sites were impacted by freshwater/groundwater discharge.

Bacteriological Analysis of Samples

Water and Sewage Samples

Membrane filtration was used for the enumeration of both total marine bacteria and vibrio bacteria. A peptone buffer (PB) solution containing 0.1% peptone and 3% NaCl (Azanza et al. 1996) was used to dilute samples prior to filtration. Water samples were diluted as needed with sterile PB and 25 ml portions were filtered through a 0.45 μm Gelman filter and placed onto either marine agar (MA), thiosulfate citrate bile salts sucrose (TCBS) agar, or CHROMagar Vibrio (CV) agar. All media was prepared in accordance with manufacturer specifications. Inoculated agar plates were incubated for twenty-four hours at 35°C. After the incubation period, colonies on each plate were counted. Approximately 20% of turquoise colonies, 20% of mauve colonies, and 10% of colorless colonies were picked off CV plates using sterile toothpicks and streaked for isolation on fresh CV plates. Isolated colonies were then streaked onto tryptic soy agar (TSA) + 0.5% NaCl plates for biochemical testing.

Sediment Samples

Bacteria were eluted from sediment samples using a procedure modified from that used by the United States Geological Survey (Myers et al. 2003). Sediment samples were mixed using a sterile wooden spatula and 100 g sub-samples were placed into sterile half-liter plastic bottles. A total of 200 ml of PB was added to each bottle, followed by vigorous hand shaking for five minutes to elute the bacteria from the sediment to the water or supernatant phase. The bottle was then left undisturbed for thirty seconds to allow the sediments to settle. The supernatant was then transferred into a separate sterile bottle. This sediment elution process was done twice and the two supernatants were combined. The combined supernatant samples were processed in a manner similar to that described earlier for the water samples and isolates were picked off the CV plates as described above. A small aliquot (10 g) of each sediment was placed onto a drying dish and dried in a 105°C oven for twenty-four hours to determine the dry weight of the sediment.

Presumptive Identification Using Biochemical Testing

Presumptive human pathogenic *Vibrio* spp., which formed turquoise, mauve, and colorless colonies on CV plates were picked and streaked for purification on TSA + 0.5% NaCl agar. A twenty-four hour culture of the isolates grown on this medium was used to speciate the colonies based on results of two additional biochemical tests (sucrose fermentation, growth at various levels of salt) following guidelines published by the U.S. Food and Drug Administration Bacterial Analytical Manual and reported by DePaola and Kaysner (2004). To test for sucrose fermentation, isolates from TSA + 0.5% NaCl agar were streaked onto TCBS agar. The formation of yellow colonies after a twenty-four hour incubation period indicated sucrose fermentation. Isolates were also tested for salt tolerance by observing for growth in nutrient broth containing various concentrations of NaCl (0%, 6%, 8%,10%). Salt tubes were inoculated by homogenizing a single colony from a TSA + 0.5% NaCl plate into a tube of 2%

saline broth and then using 0.1 ml of the homogenate to inoculate each of the (0%, 6%, 8%, and 10%) salt tubes. After incubation at 35°C for seven days the presence (or absence) of growth was documented (Choopun et al. 2002).

Genetic Confirmation of Presumptive Isolates Using Species-Specific PCR Primers

More recently, a new genetic test called PCR has been used to speciate most bacteria. For genetic testing, the DNA of presumptive isolates was extracted according to the method described by Lee et al. (2004). Briefly, isolates were grown overnight at 35°C in 1 ml of tryptose soy broth containing 1.5% NaCl. The culture was then boiled at 100°C on a heat block for five minutes and centrifuged for five minutes at 10,000 RPM in a microcentrifuge (Lee et al. 2004). The supernatant was then used directly for PCR reactions. Species-specific PCR primers used in this study to confirm the identity of *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* are listed in Table 1. These primers target specific regions of the DNA of each species The specific products generated by the use of these primers have corresponding molecular weight, which were visualized as bands on 1.5% agarose gels.

Table 1. Gene targets and references of PCR primers used for confirmation of *V. cholerae, V. parahaemolyticus,* and *V. vulnificus* isolates.

Species	Target Gene	Reference
V. cholerae	16S-23S rDNA ISR	Chun et al., 1999
V. parahaemolyticus	pR72H	Lee et al., 1995
V. vulnificus	16S rDNA	Kim and Jeong, 2001

Principal Findings and Significance

1. What is the prevalence of the four human pathogenic *Vibrio* spp. (*V. alginolyticus, V. cholerae, V. parahaemolyticus, V. vulnificus*) at primary coastal beaches on Oahu where the greatest exposure to swimmers occur, and where the water is characterized as having consistently high salinity (35–36 ppt) and negligible impact from land-based runoff?
   - The results showed clear prevalence (100%) of *V. alginolyticus*, the least virulent human pathogenic *Vibrio* sp., and the absence of the three more virulent *Vibrio* spp. (*V. cholerae*, *V. parahaemolyticus, V. vulnificus).*
   - The first significance of these findings is that since most people swim at primary recreational beach sites on Oahu, the expected exposure and risk of infection by virulent *Vibrio* spp. (*V. cholerae, V. parahaemolyticus, V. vulnificus*) is minimal.
   - The second significance of these findings is that good quality coastal waters with high salinity prevent growth and prevalence of highly virulent *Vibrio* spp.

2. What is the prevalence of the four human pathogenic *Vibrio* spp. at secondary recreational beaches on Oahu where less exposure (as compared with primary beaches) to swimmers occur, and where the water is characterized by variable degrees of land-based runoff resulting in variable salinities (6.0–32 ppt)?

- The results showed the primary prevalence of *V. alginolyticus* (100%), and the secondary prevalence of *V. vulnificus* (40%) and *V. parahaemolyticus* (20%), and the absence of *V. cholerae* at these sites.
- The first significance is that, as with primary swimming beaches, at these secondary sites the least pathogenic *Vibrio* sp., *V. alginolyticus*, is the most predominant.
- The second significance is that at these same sites, where water salinity is lowered due to land-runoff, the more virulent *Vibrio* spp. (*V. parahaemolyticus, V. vulnificus*) were also prevalent. This indicates, in agreement with previous studies, that the prevalence of these two species are highly influenced by water salinity.
- The third significance is that because relatively few people swim at secondary swimming beaches on Oahu, the expected exposure and risk of infection to most swimmers to the more virulent *Vibrio* spp. (*V. parahaemolyticus, V. vulnificus*) is minimal.

3. What is the prevalence of the four human pathogenic *Vibrio* spp. in confined coastal waters such as harbors, canals, and ponds, which are not approved for swimming?
   - Coastal water at these sites are consistently contaminated by land-based runoff resulting in salinity ranging from 3 to 35 ppt. Moreover, as these coastal waters are not effectively flushed out by open ocean waters, they are considered unsuitable for swimming. However, primary human contact, including swimming, occasionally occurs at these sites.
   - The results showed the primary prevalence of *V. alginolyticus* (100%), and the secondary prevalence of *V. parahaemolyticus* (40–71%) and *V. vulnificus* (0–57%), and the absence of *V. cholerae*.
   - The significance of these findings is that, as these sites are not designated for swimming, people should not be having direct contact with these waters. In the absence of primary contact exposure, the risk for transmission of *Vibrio* spp. to humans should be negligible. However, if people were to swim at these sites, they would be exposed to three of the four known human pathogenic *Vibrio* spp. (*V. alginolyticus, V. parahaemolyticus, V. vulnificus*) that were tested for in this study.

4. What is the prevalence of the four human pathogenic *Vibrio* spp. at coastal water sites on the island of Hawaii, which are used for swimming?
   - Coastal waters of the island of Hawaii are very diverse and differ from the coastal waters on Oahu. Most coastal water sites on the island of Hawaii are characterized as being contaminated by land-based, sub-surface water discharges and having salinity ranging from 8 to 33 ppt.
   - The results showed primary prevalence (89–100%) of *V. alginolyticus* and the secondary prevalence of *V. parahaemolyticus (*22–50%) and *V. vulnificus* (22–100%) at these sites. 100% prevalence of *V. vulnificus* also occurred in the thermal ponds that were tested, along with the absence of *V. cholerae* from any of the coastal sites.
   - The first significance of these findings is that coastal water sites on the island of Hawaii are designated for swimming but the number of people who use these sites are substantially less than those who use coastal waters on Oahu. However, people who swim in coastal waters on the island of Hawaii appear to be at greater risk of becoming infected

with *V. parahaemolyticus* and *V. vulnificus* than swimmers on Oahu because swimming sites used by the public on the island of Hawaii are mainly comprised of low salinity waters.

- The second significance of these findings is that when the salinity of coastal waters was lowered by land-based runoff and water temperature rose due to sub-surface lava activity, the prevalence of the more virulent *Vibrio* spp. (*V. parahaemolyticus, V. vulnificus*) also increased.

5. What is the prevalence of the four human pathogenic *Vibrio* spp. found in the coastal water sediments on the island of Oahu?
   - The results showed that the prevalence of pathogenic *Vibrio* spp. in coastal sediments followed a trend similar to the prevalence of such pathogens in coastal beach-water samples. *V. alginolyticus* was prevalent in both primary and secondary beach sediment while *V. parahaemolyticus* and *V. vulnificus* was only prevalent in secondary beach sediment. *V. cholerae* was not recovered from sediment samples.
   - The first significance of these results is that sediments can be seen as a source of human pathogenic *Vibrio* spp. into the water column, and may possibly be the most stable source of the three human pathogenic *Vibrio* spp.
   - The secondary significance is that when swimmers cut themselves from coastal water sediments, they may be exposed to high concentrations of pathogenic *Vibrio* spp.

6. What is the prevalence of the four human pathogenic *Vibrio* spp. in human raw sewage, primary treated, secondary treated and disinfected sewage?
   - The results showed that *V. parahaemolyticus* and *V. vulnificus* were sporadically present in raw sewage and in effluent having received primary treatment at all three tested wastewater treatment plants. *V. cholerae*, on the other hand, was consistently recovered from raw sewage and from effluent having received primary treatment at all three tested wastewater treatment plants.
   - The first significance of these results is that some humans are infected with and are shedding the three most virulent *Vibrio* spp. (*V. cholerae, V. parahaemolyticus, V. vulnificus*) into the sewage system.
   - The second significance is that human sewage is the only known environmental source of *V. cholerae* in Hawaii. The low salinity of wastewater favors growth of *V. cholerae*. Accidental release of raw sewage can introduce these pathogens into coastal waters that may lead to public health consequences for users of such waters.

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