

Vivian S. Blue. VIBRIO AND AEROMONAS AS AGENTS OF WOUND INFECTIONS FROM ESTUARINE WATERS IN EASTERN NORTH CAROLINA. (Under the direction of Dr. Bernard E. Kane, Jr.) Department of Environmental Health, East Carolina University, March 1987

Vibrio and Aeromonas species occur widely in the environment, particularly in estuarine waters. In recent years, the importance of these species has been recognized in medical and environmental microbiology. Serious and sometimes fatal wound infections have been reported following recreational and occupational exposure to surface waters with Vibrio cholerae and Aeromonas hydrophila as the causative agents. This study is an investigation of the role of Vibrio cholerae and Aeromonas hydrophila as potential pathogens of public health concern.

A survey of the incidence and distribution of freshwater wound infections acquired following occupational and recreational exposure to rivers and estuarine waters was performed in selected areas of eastern North Carolina. Wound swab samples supplied by participating clinics were screened for Vibrio and Aeromonas species in an attempt to document these organisms as agents of infection. Many of these wound cases were the direct result of punctures and bites from shellfish or crustacea. A substantial number of cases were related to activities in seafood manufacturing plants.

Water sampling of selected areas for V. cholerae showed that the organisms are indigenous to estuarine waters. The densities obtained from this study are not a useful measure of public health risk since the virulence of these free-living strains was not assessed. No link can be made showing the free-living strains are causative agents of wound infections.

The data from this survey show that the problem of infection from shellfish and estuarine waters is frequent and abundant, but not all together serious. Many physicians believe that the infections are most prevalent in

seafood processing plants where temperatures may allow the organisms to multiply rapidly and reach an infective level. Levels in recreational waters may be low as shown by the data from this study and therefore pose very little problems as far as infective dose is concerned. An investigation into the infections from seafood processing plants may provide valuable data concerning prevalence and infective levels of Vibrio and Aeromonas.

VIBRIO AND AEROMONAS AS AGENTS
OF WOUND INFECTIONS FROM ESTUARINE WATERS IN
EASTERN NORTH CAROLINA

A thesis
Presented to
the Faculty of the Department of Environmental Health
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In Partial Fulfillment
of the Requirements for the Degree
Master of Science in Environmental Health

by
Vivian S. Blue
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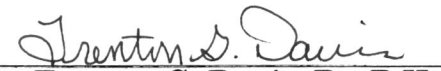
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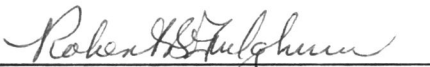
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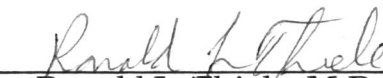

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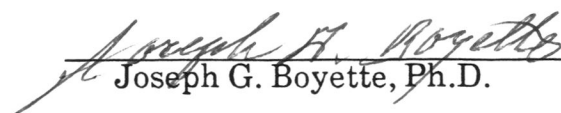
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I. INTRODUCTION

Vibrio and Aeromonas species of bacteria occur widely in the environment, particularly in marine and estuarine waters. Many strains of these species may possess virulent enterotoxins and cytotoxins which have been considered to be potential pathogens for human infection (63). In recent years the importance of these species has been recognized in medical and environmental microbiology (64). Serious, and sometimes fatal wound infections have been reported following recreational and occupational exposure to surface waters (34,38) with Vibrio cholerae and Aeromonas hydrophila as the causative agents. Many of these infections have developed from minor abrasions, lacerations, alligator and crab bites, and aspiration pneumonia due to drowning.

An interest in the environmental epidemiology of these infections was stimulated by two fatal cases that occurred following exposure to the waters of Bay River, North Carolina. One case in 1974 which identified the causative agent as a non-agglutinating Vibrio cholerae, and the other case in 1982 which was caused by Aeromonas hydrophila (23). Clinics in the Bay River area also report several nonfatal cases of infection each week during the summer months.

Although interest in these organisms as the causative agents of infection has been generated, the numbers of Vibrio and Aeromonas organisms isolated from infections and submitted to the Center for Disease Control (CDC) are only a small portion of the numbers of infections that actually occur (63). The numbers submitted to the CDC, however, have increased each year possibly because of an increased awareness rather than to

an increased incidence in the disease (63). Since these infections are not reportable diseases as are some others of public health significance, the importance of these infections and the resultant necessity of invoking preventive measures for control is unknown.

Because of the sometimes fatal wound infections by Vibrio and Aeromonas there is an interest in these agents as a public health problem. Although extensive surveys have been done of estuaries and some marine Vibrios to determine the "presence" of these organisms, the environmental epidemiology and pathogenicity of the fresh water Aeromonas and Vibrios that are responsible for these serious infections has not been established (22). This may be because the organisms are easily mistaken for the Enterobacteriaceae with the "routine" microbiological tests performed by many labs.

The Vibrio and Aeromonas species belong to the family Vibrionaceae. In general, this family is characterized by Bergey's Manual of Determinative Bacteriology (95) as:

Gram negative straight or curved rods with polar or occasionally lateral flagella. Most are oxidase positive and all utilized D-glucose as their carbon and energy source. Most require 2-3% NaCl or seawater base for optimum growth. They are primarily aquatic organisms found in freshwater and seawater. Some species are pathogenic to man as well as frogs and fish.

The family Vibrionaceae has been linked continually with the family Enterobacteriaceae. In fact, the two families have been often referred to as a "superfamily" (95). In 1965, Veron placed into the Vibrionaceae a group of genera that were oxidase positive and motile by polar flagella to differentiate them from the Enterobacteriaceae which are oxidase negative and have peritrichous flagella (95). However, both families have species whose

characteristics are so exceptional that their relationship to the group is questionable. In comparing the genetic and physiological features of the two families, it is obvious that they share numerous similarities suggesting a common evolutionary origin.

This study is primarily concerned with two specific organisms from the family Vibrionaceae: 1) Vibrio cholerae non:01 and 2) Aeromonas hydrophila which are presumed to be possible pathogens to man based on limited medical reports. These organisms have caused outbreaks of gastrointestinal illness and in some instances have been isolated from persons with extraintestinal disease.

A. Vibrio cholerae Non:01

Vibrio cholerae is a gram negative, facultatively anaerobic rod which is motile by polar flagella in liquid media and often by lateral flagella on solid media (95). The growth requirements include 5-15 mM Na⁺ for optimal growth, D-glucose, D-fructose, maltose, and glycerol (95). All Vibrio cholerae non:01 types grow well at 20°C, most at 30°C, a few at 45°C, and none at 50°C.

The non:01 connotation for the species evolved from the attempt to determine taxonomic separation based on serology. The non:01 group V. cholerae was considered to be biochemically similar to the epidemic strains of V. cholerae except that they did not agglutinate in V. cholerae 0-group 1 antiserum (64). These "non-agglutinable vibrios" were previously known as NAG's or NCV's (non-cholera vibrios) (12). They are now most often referred to as non:01 Vibrio cholerae.

The non:01 strains of V. cholerae are widely distributed in the environment. They have been found to exist in many areas of Asia, Europe,

and the United States. They have been isolated from birds, fish, and frogs as well as from freshwater and estuarine environments (95). *Vibrios* are important in the aquatic environment since they constitute a large portion of the aquatic bacterial population (64). In the United States, the non:O1 strains have been isolated from estuaries in Louisiana (21), Texas (29), Florida (29,56), and Mississippi (29). Many strains were also isolated from areas of the Chesapeake Bay (9) where the water had a salinity of 0.4-1.7‰ (20). The incidence of the *vibrio* strains in the Chesapeake Bay waters was correlated with the zooplankton and shellfish (81). The organisms were found to be an important ecological factor to the marine aquatic ecosystem because of their ability as chitin digesters and mineralizers of organic matter (20). The organisms adsorb onto the surfaces of chitinous planktonic copepods and release of their cholera toxin causes Na^+ release from the host, thus creating an environment conducive for optimal growth (99).

In Europe, non:O1 *V. cholerae* strains have been found prevalent in Czechoslovakia (3), Greece (83), Australia (89), rivers in Rumania (82), and drainage ditches in Kent, England (8,69,105). Strains were also found in the estuaries of Germany (67,79,80). In German rivers, the organisms were abundant in water with a pH greater than 7.5 and a salinity of 0.01‰ or greater (12). In the Baltic Sea, it was determined that the organisms were detected in 22% of the seawater samples over a 3 year period (12).

In general, the non:O1 *V. cholerae* strains have been found in sewage, brackish surface waters, estuarine waters, and in association with aquatic animals and plants. Salinity is a definite growth requirement of the organism, but there is considerable variation in what Na^+ level is required for

survival and optimal growth. Miller, Drasai, and Feachem (78) agree that 0.5-3.0‰ is required for maintenance of the aquatic population. Kaper (63) suggests a favorable Na⁺ range of 0.4-1.7‰ while Hood (56) indicated that 1.0-2.5‰ was needed for optimum growth. Singleton (99) agrees closely with Hood suggesting 1.5-2.5‰ but he believes that some organisms will survive with salinity as low as 0.5‰. DePaola (29) found non:01 organisms to survive in U.S. gulf waters in concentrations less than 0.5‰ salinity.

A distinct seasonal cycle has been observed for Vibrio cholerae non:01 from studies performed in Kent, England. Observations done in 1978-1979 at Chetney Marsh indicated that the organisms were not detected until May when the water temperature rose above 9°C (105). The population then reached a maximum of 100/ml in August to September and a gradual decrease in numbers was observed as the water temperature decreased (105). By late October to early November, the population had decreased to undetectable levels. This seasonal incidence of May - October/November has been supported by others such as Muller (80) Lee (69,105), and DePaola (29). As to where and how the organisms survive during the winter remains questionable. However, it has been suggested by Lee that the organisms may survive in estuarine birds or in other estuarine animals eaten by birds such as fish, mollusks, or crustaceans (69). Another possibility is that the organisms might survive in the sediment as suggested by Nalin (81). However, a very low isolation rate from sediment was observed. Although the organism was found to be an important factor to the marine aquatic ecosystem in relation to the zooplankton, it has not been isolated as yet from the plant material during the winter months (105). This suggests that the organism may be more

competitive with the other aquatic bacteria than with sediment and plant surfaces (105).

Bergey's manual indicates that many Vibrio non:01 strains produce a toxin characteristic of cholera toxin and a few environmental strains produce a toxin resembling E. coli endotoxin (95). Recent genetic evidence has demonstrated that some non:01 strains possess genes encoding cholera toxin (95). West and Lee (105) suggest that some of these non:01 strains may be pathogenic for man. They have been associated with cholera-like disease and have been isolated from superficial lesions and blood cultures (105).

The reservoir of pathogenic non:01 V. cholerae strains is unknown. The organisms survive and multiply in the environment, but what percent of these free living strains are pathogenic for man is unknown (12). There are two theories as to the possible reservoir of these pathogenic strains. The first is that humans are the reservoir. The organisms may exist in a human chronic carrier state where they get into the food and water supplies via infected humans (12). The V. cholerae organisms multiply readily in certain foods and can reach high numbers to cause infection. Foodborne outbreaks of non:01 V. cholerae gastroenteritis have been linked with potatoes in Czechoslovakia (2), eggs (24), and in the U.S. the outbreaks have mostly been due to raw shellfish consumption (28,59,106) especially raw oysters and crabs. Waterborne outbreaks have been associated with this organism. Outbreaks have been traced to India, the Orient, and the Sudan (59). A second theory pertaining to the reservoir of these pathogenic strains is the possibility of the transformation of non:01 strains to 01 strains. It was suggested in the Indian Journal of Medicine in 1964 that a transformation of the somatic antigen may

occur in the environment (12). This theory, however, has not been shown to be true by any investigators to date. Therefore, the source of infection remains unknown for most cases of intestinal and extraintestinal disease due to pathogenic strains of non:01 V. cholerae.

Besides the cholera-like intestinal cases which are the most commonly associated disease from non:01 V. cholerae, the organism has been linked with wound infections. Clark and Steigerwalt (64) demonstrated a lactose positive vibrio capable of extensive tissue damage or death in humans. Other lactose positive vibrio wound infections have been linked to contact with seawater or saltwater shellfish. Cases of wound infections from association with seawater have been found in Sweden (6). Ear infections were noted in Sweden (6) and Belgium (46).

Most of these wound infections displayed a few similar clinical characteristics that began with swelling, erythema, and occasionally pain around the wound site within seven days before the onset of infection (11). Cellulitis then developed rapidly and sometimes spread to surrounding areas. These infections from exposure to seawater were associated with many types of wounds such as lacerations, punctures, abrasions, scratches, crab bites, and chronic ulcers.

B. Aeromonas hydrophila

Aeromonas hydrophila is a gram negative, facultative anaerobe which is motile by polar flagella, but may possess lateral flagella in young cultures (33). It is asporogenous, ferments carbohydrates with the production of acid and gas, produces indophenol oxidase and catalase, and is beta-hemolytic on blood agar (76).

When isolated, the Aeromonads in general must be differentiated from the vibrios, pseudomonads, and Enterobacteriaceae - especially Serratia and E. coli (33). Aeromonas hydrophila can be differentiated from most strains of V. cholerae by its failure to decarboxylate the amino acids lysine and ornithine as well as by producing a positive arginine dihydrolase reaction (33). Most of the Aeromonads can be differentiated from the Pseudomonads by their fermentative rather than oxidative metabolism of certain carbohydrates (33). Most Aeromonas strains grow well on the selective media used to isolate and identify the Enterobacteriaceae such as MacConkey, EMB, and SS agars (33). A combination of characteristics and tests such as the flagellar structure, decarboxylase reactions and indophenol oxidase test will aid in differentiating the Aeromonads from the Enterobacteriaceae (33). However, it is important to emphasize that A. hydrophila still may be easily misidentified because of its morphological and biochemical similarities to these organisms.

Aeromonas hydrophila is found in most freshwater environments throughout the U.S. (49). It was once thought to be a strict freshwater inhabitant and not indigenous to the marine environment (87). This view, however, has been changed by the detection of the organism in marine environments such as off the coast of Puerto Rico in the Atlantic Ocean (50). It was also considered as having a transient existence following sewage or river inputs (87), but the organism's relationship to pollution is not clear in that it has been found in unpolluted waters as well as not being detected in polluted waters. The presence of the organism as a normal inhabitant of the gut was documented by Bras, et al (13) from accounts of the isolation of the

organism from patients with peritonitis, postoperative wound infections, and abscesses. The organism has also been isolated from marine life, soil, food products (14), and was frequently found in chlorinated and unchlorinated drinking water supplies (15,16).

Aeromonas hydrophila grows well in warmer climates and warm water conditions (94). It has been found to grow and survive in a temperature range of 4-45°C and at a pH range of 5.2-9.8 (49,91,63). Hazen et al (49) have suggested an optimum pH range of 5 to 9. Rouf and Rigney (91) determined an optimum temperature for growth of 30°C. Hazen et al (49) also suggested that studies involving sampling throughout the U.S. indicate that temperature was not well correlated with Aeromonas densities. Kaper et al (63) disagrees and concludes that temperature does have a significant effect on densities. A study in the Anacostia River near Washington, D.C., showed that the organism's counts rose in the top and bottom water as well as in the sediment when the water temperature exceeded 20°C and decreased in February when the water temperature dropped to 0.5°C (94). These results were consistent from studies by Rippey and Cabelli (87). Other studies have shown a summer peak in densities in North America and that this peak may be related to the water temperature (64). Another study in four ponds in Rhode Island shows that there is some seasonal correlation with densities of the organism. Densities were maximal from mid-June through October and declined thereafter (87).

Some other variables were found to affect the concentration of the organism. The densities correlated most strongly with total phosphorus, chlorophyll, and Secchi depth indicating an indirect effect of phytoplankton on

A. hydrophila densities (87). Shoreline densities were greater than mean densities especially in the vicinity of rooted aquatic plants (87). A moderate correlation was noted from dissolved phosphorus, nitrogen, and dissolved organic carbon (87). There was little correlation with ammonia, pH, alkalinity, or dissolved oxygen with the density of the organisms (87). It appears that Aeromonas densities were well correlated with indices of eutrophication.

A. hydrophila infections are common among fish and amphibians (38). The organism was once believed to be an opportunistic pathogen in immunologically compromised hosts, but has now acquired status as a primary pathogen in patients without immunological abnormalities (15). Strains from various infections and waters show a variety of extracellular virulence factors such as hemolysin, cytotoxic enzymes, and enterotoxin (15). Aeromonas species isolated from chlorinated drinking water in the U.S. have been shown to possess virulence factors coinciding with enteric disease (17). Kaper et al (15) tested many aquatic isolates for cytotoxicity and found that 70% of the isolates were cytotoxic.

A. hydrophila must also be considered in relation to nonenteric disease. Infections resulting from exposure to waters contaminated with the organism have been reported frequently (33,47). These infections developed from minor abrasions or lacerations obtained while swimming or scuba diving, alligator bites, and even from injuries sustained in a plane crash into the waters of the Florida Everglades (38). In 1974, a 62 year old nurse obtained two puncture lacerations from crab traps while crabbing in shallow brackish water (38). Within 24 hours, cellulitis developed over the entire

injured leg. The patient's temperature became elevated and several flaccid bullae appeared. Eventually necrosis developed and amputation of the extremities was recommended but not performed due to certain surgical risks. Approximately three months later, the patient died. Water samples obtained at the injury site showed fresh cultures of A. hydrophila and cultures from the blood and blister fluid eventually were identified as A. hydrophila also (38).

In 1976, a scalp laceration obtained while diving in a lake in Wisconsin became swollen and inflamed within 24 hours (47). Sampling of the lake showed A. hydrophila to be the major contaminant in the water. Large numbers of the organism were also isolated from the wound itself (47). Another case in 1978 which involved a leg wound due to a diving incident in polluted waters showed the causative agents to be Aeromonas species. This case was perhaps the first recorded instance of a primary infection of soft tissue in a human caused by two species of Aeromonas - A. sobria and A. hydrophila (62). Both isolates exhibited cytotoxicity, but only A. hydrophila demonstrated a cholera enterotoxin-like effect also (62). At the site of the diver's injury, sampling yielded 193 isolates of Aeromonas species of which 172 were classified as biotypes of A. hydrophila (62). Of the 193 isolates, 25-30% were considered to be cytotoxic (62).

Most all of the documented A. hydrophila wound infections follow a characteristic clinical syndrome resembling group A Streptococci infection (47). Within 24 hours, there is an explosive onset of local inflammation accompanied by a moderate fever and leukocytosis (47). Cellulitis is also present.

Although the documentation of A. hydrophila infections in immunologically competent persons is somewhat limited, the increased occurrence or awareness of this type of infection is noteworthy. In a 1 year period, a hospital isolated A. hydrophila from thirteen patients, eight of which had superficial infections (76). In the previous two years the same hospital had only three or four isolates.

C. Objectives

The following study provides an investigation into the possibility of Vibrio cholerae and Aeromonas hydrophila being potential causative agents for public health concern. An interest in the environmental epidemiology of the infections arising from these organisms was expressed by members of the Craven County Medical Society in 1982 (23). The interest was addressed in conjunction with the presentation of a fatal case of Aeromonas that had occurred following exposure to waters of Bay River, N.C. and by a review of the current status of vibrios in estuarine waters (23).

A survey of the incidence and distribution of freshwater wound infections acquired following occupational and recreational exposure to surface waters was performed using three methods - 1) A questionnaire survey of practitioners in the Neuse River area, 2) Direct testing of wound infections from swabs supplied by cooperating clinics in the Neuse River area, 3) A limited survey of the characteristic water quality areas conducive to high Vibrio densities performed through water sampling. The above activities were designed to meet the following objectives:

- 1) Documentation of the incidence and distribution of wound infections acquired following occupational and recreational exposure to surface waters.

- 2) Identification of the causative organisms.
- 3) Assessment of the relationship between the water quality and the incidence of infection.

There were many problems in meeting the above objectives, because Vibrio and Aeromonas wound infections are considered as non-reportable to the Center for Disease Control. The number of cases recorded is small and does not likely represent the true incidence and distribution. Since many clinics were not specifically looking for these infections, many cases may have been overlooked. Also, the questionnaires represented data mainly from a physician's recall and limited documentation. The direct testing of wound swabs supplied by interested physicians was an attempt to bridge this gap by hopefully screening specifically for these type infections. However, obstacles were prevalent here also. First, only a very small number of physicians agreed to participate, thus limiting the scope and volume of screening. Second, there was the identification problem of "recognition rather than isolation" of the causative agents. Distinction between V. cholerae and A. hydrophila was difficult due to the morphological and biochemical similarities of these organisms to others. Also, since isolates obtained from environmental surveys may not be pathogens and determination of their pathogenicity is difficult, environmental surveys of organism densities may not be good measures of public health risks.

II. MATERIALS AND METHODS

Since the number of Vibrio and Aeromonas organisms isolated from infections is somewhat limited due to overlooking or misidentifying their existence in routine lab testing, a survey of the incidence and distribution of wound infections with these organisms as possible causative agents was performed. A three part survey was conducted which included the direct testing of wounds swabs from clinics, a response questionnaire to non-participating clinics to obtain further data, and water sampling of selected areas.

A. Wound Swab Testing

Initially, instruction and patient data forms (see Appendix), culture tubes with transport media, and swabs were provided to the cooperating clinics in the Neuse River area. The patient data forms containing information pertinent to the nature of the wound and the most likely site of exposure, along with the wound swab sample were mailed back to the university laboratory. It was recommended that the culture tubes containing the wound swabs in Carey-Blair Transport Medium be mailed immediately upon sampling. A maximum 48 hour transport time was stressed, however if this time limit was not possible, the samples were refrigerated. The North Carolina Division of Health Services Laboratory indicated that refrigeration of the samples in Carey-Blair for up to 72 hours did not seem to affect the organisms (107). The efficiency of Carey-Blair medium has also been verified as being effective in maintaining the viability of V. cholerae for up to four weeks (107). Upon receipt of the data forms and wound samples, each form

samples, each form was checked for the necessary information, and the swab samples were assigned a case number and documented into a log book.

Each wound swab was screened for Vibrio cholerae non:01, Aeromonas hydrophila, and Staphylococcal and Streptococcal organisms.. The testing schemes for the Staphylococcal and Streptococcal organisms were not as extensive as those for Vibrio and Aeromonas since these organisms were only screened as an aid in determining the percentages of the different types of causative agents in the wound infections.

Upon receipt, each swab was streaked on to Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS), Rimler-Shotts Agar (RS), and Nutrient Agar plates and incubated at the optimum temperature for isolation of the organisms. Diagram 1 was utilized for the isolation and identification of Vibrio cholerae non:01.

The colonies growing on the TCBS and Nutrient Agar plates were inspected and gram stained if they were observed to be suspect colonies indicative of V. cholerae non:01. On TCBS, the suspect colonies picked for isolation and identification were of several different colonial types:

1. Dark green, low convex, small, bright.
2. Flat yellow (indicating sucrose fermentation)
3. Pale green, flat.
4. Green, translucent, flat.
5. Green with yellow center, low convex.

On Nutrient agar, Vibrio cholerae non:01 suspect colonies were light iridescent greenish to red and granular in texture. A gram stain was performed on a representative colony from each suspect type and from these all gram negative straight or curved rods were then transferred to MacConkey

agar and Nutrient agar for future testing. On MacConkey agar, observations were made only as to whether the organism grew or not. From the fresh growth on the Nutrient agar, several tests and the inoculation of other mediums were performed. Kligler Iron Agar, (KIA), Triple Sugar Iron Agar (TSI), and Lysine Iron Agar (LIA) slants were inoculated and incubated at 35°C. Readings on these slants were made at 18 and 24 hours after inoculation.

The string test, oxidase test, and catalase test were all performed on the suspect colonies. The string test was done by emulsifying a small loop of growth from the Nutrient agar plate in a drop of aqueous 0.5% sodium deoxycholate solution. A mucous-like string may be seen as the inoculating loop is lifted from the slide indicating a positive reaction. The catalase test was performed by emulsifying a colony with one drop of 30% hydrogen peroxide. Bubbling is indicative of a positive test. For the oxidase test, the General Diagnostics Pathotec Cytochrome Oxidase test strips were used. A representative colony was spread over a small area of the strip. A change in color of the strip to purple indicated an oxidase positive organism. Control organisms were used to assure validity of the test.

As a final test, each suspect colony was inoculated onto a rapid identification test system. The Minitek nonfermenter system was the system of choice because of the wide selection of test disks available. However, if the Minitek system failed to give an acceptable identification, the API test system was used (5). If the API test strip was utilized, two additional tests were performed - motility and OF glucose. To observe for motility, a hanging drop prep was made from a 20 hour Tryptic Soy broth culture. Observed movement

by the organisms was indicative of motility. For the OF glucose test, an OF glucose tube (Flow Laboratories) was inoculated and incubated at 35°C for 24-72 hours and then observed for oxidative or fermentative utilization of glucose. If the organism utilized the glucose through an oxidative pathway, the upper area of the tube above the tube constriction was yellow. A fermentative pathway was observed if yellowing occurred below the tube constriction. In addition to the Minitek and API systems, the Vitek automated identification system was used depending upon its availability.

VIBRIO CHOLERAEE NON:01 ISOLATION AND IDENTIFICATION

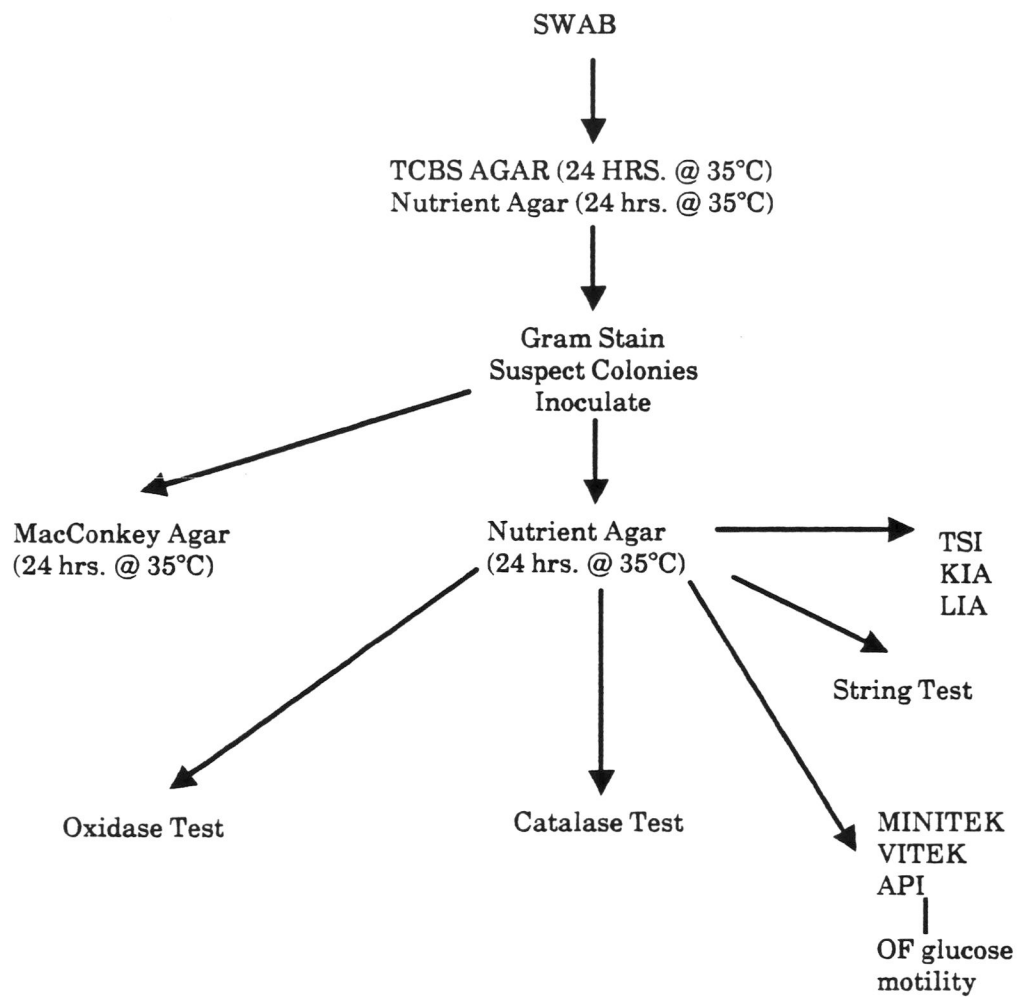


Diagram 1

The test results were compiled and evaluated for each suspect colony type and compared with the presumptive phenotypic identification characteristics of Vibrio cholerae non:01 presented in Table 1.

MacConkey agar	+
TSI	A/A H ₂ S-
LIA	K/K or K/N
KIA	K/A H ₂ S-
String test	+
Catalase	+
Oxidase	+
Lysine decarboxylase	+
Arginine decarboxylase	—
Ornithine decarboxylase	—
Motility	+
OF glucose	Fermentative

Table 1 Biochemical Reactions For Presumptive Identification of Vibrio cholerae non:01 (35,95).

The isolation and identification of Aeromonas hydrophila was performed in a similiar manner to the scheme for V. cholerae as shown in Diagram 2.

The suspect colonies growing on the Rimler-Shotts (RS) agar plates that were chosen for isolation and further testing were:

1. Yellow colonies (indicating maltose fermentation).
2. Yellow with black centers.

Those colonies growing on Nutrient agar that were chosen for testing were spreading and colorless. Occasionally, Aeromonas colonies may appear on TCBS agar. These usually appear as bluish colonies. Retrospective studies concerning the determination of the optimal time for observation and

interpretation of the growing organisms on RS agar have recommended that the plates be read between the 20th-24th hour (52). After the 26th hour, reversal of the fermentation reactions was observed (52). The other biochemical tests and test systems for the presumptive identification of Aeromonas hydrophila were performed on the isolates in the same manner as for Vibrio cholerae non:01. Table 2 below shows the presumptive identification test results for A. hydrophila.

MacConkey agar	+
TSI	K/A H ₂ S- or N/K H ₂ S-
String test	—
Catalase	+
Oxidase	+
Lysine decarboxylase	—
Arginine decarboxylase	+
Ornithine decarboxylase	—
Motility	+
OF glucose	Fermentative

Table 2 Biochemical Reactions For Presumptive Identification of Aeromonas hydrophila (33,93).

AEROMONAS HYDROPHILA ISOLATION AND IDENTIFICATION

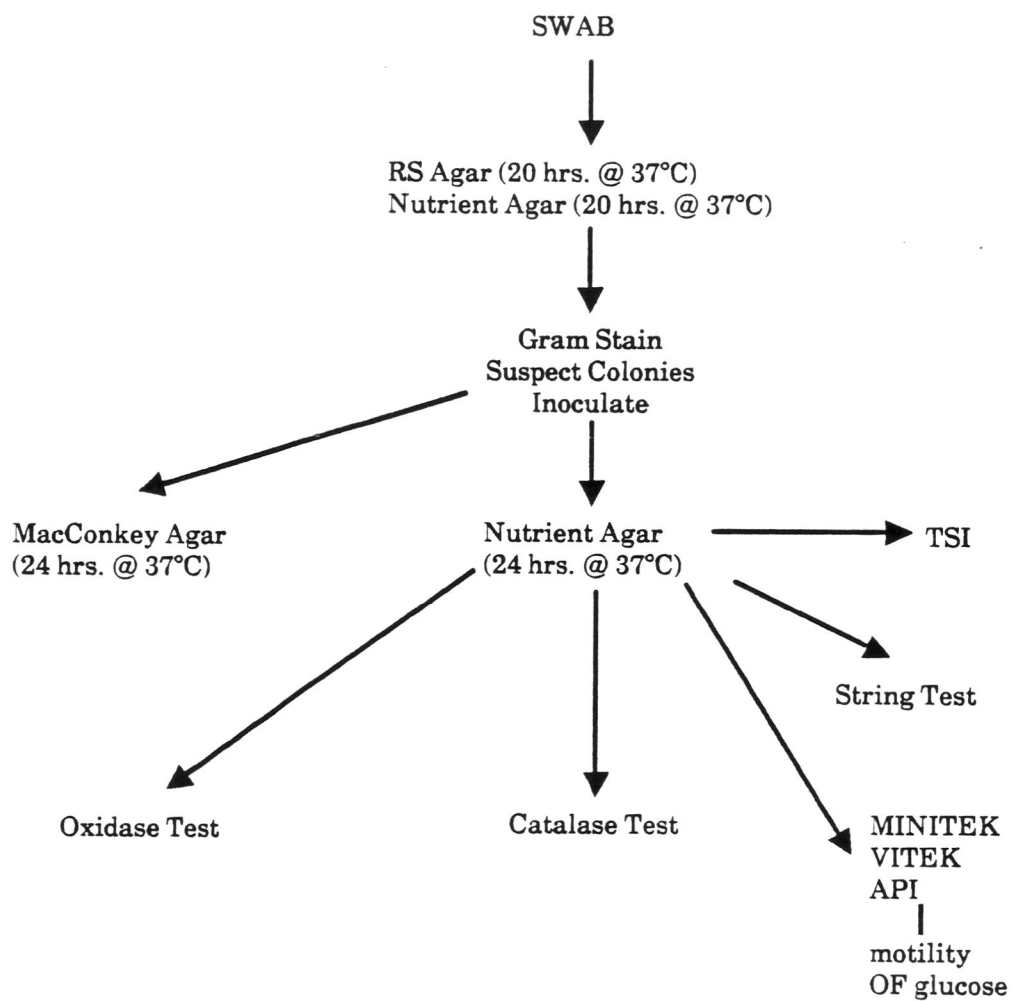


Diagram 2

Limited screening tests were also performed for the presumptive identification of Staphylococcal and Streptococcal organisms as shown in Diagrams 3 and 4.

All colonies growing on Streptosel Agar were gram stained. Those cells which appeared to be oval or spherical and occurred in pairs or chains were streaked on to blood agar and checked for their hemolytic action. Most wound infections may be caused by Group B Streptococci which exhibit Beta hemolysis which appears as a clear, colorless zone around the streptococcus colonies. All beta hemolytic colonies were then exposed to Bacitracin (0.04 units/disk) to differentiate presumptively between Group A and Group B streptococci. Both Group A and B are beta hemolytic, but only Group A is positive showing a zone of inhibition (clearing) around the disk placed on a confluent lawn of culture. Group B is resistant and no zones of inhibition are usually seen.

Pathogenic, coagulase-positive cocci form shiny, convex, black colonies on Tellurite Glycine Agar (TG). All colonies growing on the TG agar were gram stained and observed for gram positive cocci in clusters. Those colonies meeting this description were subjected to the coagulase test. This test was performed using rabbit plasma with ethylenediaminetetraacetate (EDTA). The tube method was employed where a 0.5 ml amount of plasma was inoculated with one suspect colony. The tube was gently mixed and placed in a 37°C water bath. The tube was examined periodically up to four hours. Any degree of clotting is regarded as positive for enzyme producing strains. All gram positive cocci were tested on the STAPH-IDENT test system for species type confirmation.

STREPTOCOCCI IDENTIFICATION (PRESUMPTIVE)

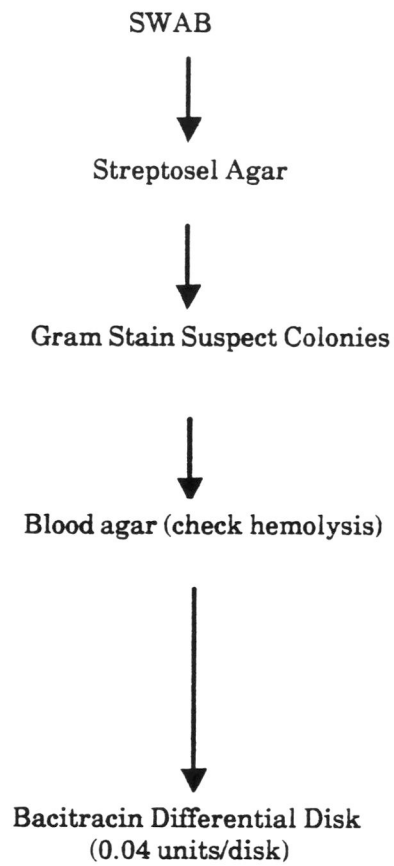


Diagram 3

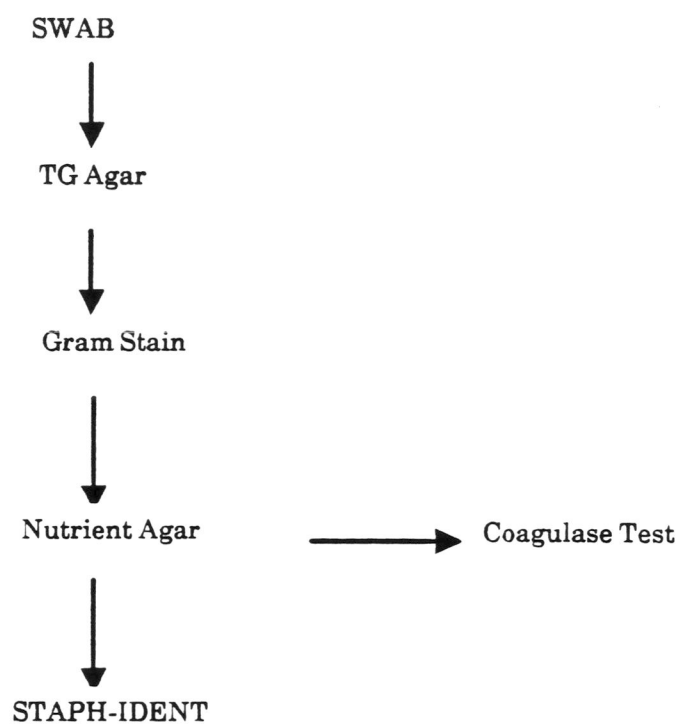
STAPHYLOCOCCI IDENTIFICATION (PRESUMPTIVE)

Diagram 4

B. Response Questionnaire

Water-related wound infection surveys were sent to sixty physicians in Coastal North Carolina areas to supplement and gather additional data concerning Vibrio and Aeromonas infections. It was hoped that these data would help with the documentation of the incidence and distribution of water-related occupational and recreational wound infections. The surveys were mailed only to those physicians whose specialties might involve the treatment of water-related wound infections in their practice. These specialties included pediatrics, general practice, emergency medicine, pathology, and public health. A listing of physicians and their specialties by county was obtained from the ECU School of Medicine for use in this environmental survey. The following counties were surveyed: Craven, Pamlico, Jones, Beaufort, Hyde, Martin, Washington, Tyrrell, Onslow, Pasquotank, Camden, Currituck, Carteret and Dare.

Each survey was accompanied by a cover letter explaining the purpose for the survey (see Appendix for cover letter and survey questionnaire). Nine questions were addressed on the survey. The questions requested information concerning the number of water-related wounds treated each year by the physician, what percent of these were not characteristic of Staphylococcus, and the clinical features of the non-staphylococcal infections. Additional questions were then asked concerning the treatment of known Vibrio and Aeromonas infections such as the numbers of cases and the severity of the cases. Finally, the physicians were asked to recall if there were any particular type of wounds that were common to the non-staphylococcal infections and

whether there were any geographical locations that proved to have a high incidence of these type of infections.

C. Water Survey

In order to assess the relationship between the water quality and the incidence of water-related wound infections, an environmental investigation was performed by sampling various areas believed to be conducive to high Vibrio densities. The sites were chosen based on physical and chemical characteristics favorable to the organisms survival as well as the frequency of recreational use. These sites were selected because several of the reported severe or fatal V. cholera non:01 infections had occurred in similar areas (23).

Aeromonas densities were not assayed in this survey. Although information on Aeromonas densities may seem useful, extensive surveys and attempts to correlate Aeromonas with water quality and red sore disease in fish have been made. The results have not been conclusive. There is general agreement that Aeromonas is an allochthonous organism in estuarine waters and may have a role in red sore disease (87). It was not felt that this limited survey would contribute sufficiently to the role of this organism in wound infections to justify the additional analytical procedure needed to quantify Aeromonas densities in these few samples.

The following eight water sampling sites were chosen:

<u>Site</u>	<u>Location</u>	<u>Sampling Time</u>
1	Wilson Creek	10:00 am
2	Brice Creek	10:30 am
3	Trent River(between the Railroad and Hwy 17 Bridge)	10:40 am

<u>Site</u>	<u>Location</u>	<u>Sampling Time</u>
4	Flanners Beach (shore)	11:20 am
5	Flanners Beach (50 cm depth)	12:00
6	Goose Creek	12:50 pm
7	Upper Broad Creek (near power cables)	1:10 pm
8	Bridgeton	2:50 pm

The samples were taken on June 5, 1985 between 10:30 and 2:50 pm. Each water sample was collected in a one gallon polypropylene jug that had been sanitized with 200 ppm chlorine and scalding hot water. Before sampling, each jug was rinsed with the water from each sampling site. Water samples were taken at a depth of approximately 25 cm below the surface and were held in insulated containers to maintain the sample near the collection temperature for transport to the laboratory.

Before sampling, salinity, pH, dissolved oxygen, turbidity, and the temperature were taken at each site. A DRT 150 HF model turbidometer (HF Instruments, Bolton, Ontario) was used to obtain the dissolved oxygen and temperature (celsius), the salinity was determined by using a YSI model 33 salinity - conductance - temperature meter (Yellow Springs Instruments, Ohio), and the pH was obtained using a Fisher Accumet 420 pH meter (Fisher Scientific, Pennsylvania).

The water samples were analyzed for total and fecal coliforms as well as for Vibrio cholerae. The fecal coliform counts on the water samples were determined by using the five dilution multiple-tube fermentation procedures

described in Standard Methods using A-1 medium (4) and a 10^{+1} to 10^{-2} dilution series. These dilutions were selected in accordance with previous experiences cultivated from determining the character of the waters in the sampling areas.

The analysis of the Vibrio cholerae densities in the water samples was performed by filtering water from each site through a 0.45 micron Gelman GN-6 filter until filtration stopped. The number of milliliters filtered was then recorded for use in future calculations. Following filtration, each membrane filter was homogenized with alkaline peptone water in a high speed blender. The volume of alkaline peptone water added for homogenation was adjusted so that each milliliter of the homogenated mixture was equivalent to approximately 12 ml of filtered seawater. Thus 8.3 ml of the homogenate represented 100 ml of seawater. From this determination, a multiple-tube test was performed using inocula such that the resulting dilution series was equivalent to 10^{+3} to 10^0 ml. One tube only was used for the 10^{+3} dilution which was equivalent to the remaining homogenate in the jar after all the other 15 dilution tubes were inoculated. The 10^{+2} to 10^0 dilution tubes were inoculated with decimal fractions of the calculated value 8.3 mls homogenate which was equivalent to 100 ml of seawater. Therefore, the 10^{+2} tubes were inoculated with 8.3 ml of homogenate, the 10^{+1} tubes with 0.8 ml, and the 10^0 tubes with .08 ml. All of the tubes were incubated for 24 hours at $35^{\circ}\text{C} \pm 1^{\circ}\text{C}$. Vibrio cholerae densities were determined using the most probable number (MPN) method. The MPN's were determined by using Thomas' formula from Standard Methods (4).

Each tube showing turbidity (which indicates growth) was saved and TCBS and TGA agar plates were streaked from each turbid broth. Both plates were incubated at 35°C for 24 hours and representative colonies from each plate were isolated and streaked on T₁N₁ agar slants. Representative suspect Vibrio cholerae colonies were similar to the colonial morphological types picked from TCBS in the wound swab analysis procedures previously described. The T₁N₁ inoculated slants were incubated for 24 hours at 35°C.

A tube labeling system was designed to maintain and organize the handling and processing of the samples. The labeling scheme was as follows:

- 1st digit 1,2,3,4,5,6,7, or 8 to correspond to sample site
- 2nd digit 0,1,2, or 3 to correspond to dilution
- 3rd digit B or E or 1,2,3,4,5,6 to correspond to colonial morphology
- 4th digit 1,2,3,4,5-10 an arbitrary number assigned to each colony isolate to distinguish between them

The colonial morphology digits were determined from the following scheme:

Suspect colonies picked from the TGA plates were typed as:

- B = Flat translucent with halo
- E = Flat translucent with halo and opaque center

Suspect colonies from TCBS were designated as:

- 1 = Dark green, bright, semi-flat
- 2 = Flat yellow
- 3 = Pale green, flat
- 4 = Green, translucent, flat
- 5 = Green, semi-flat, with yellow center

It was observed during the selection of the suspect colonies that the halos on the gelatin (TGA) plates were more apparent when the plates were cold, so each plate was refrigerated for a few minutes before transferring colonies. Also, the yellow on TCBS was not always apparent unless the plates were warm, so each plate was left in the incubator at 35°C for about one half hour before transferring colonies to T₁N₁.

After 24 hours incubation at 35°C, the T₁N₁ inoculated slants were used to inoculate tubes containing tryptone broth with 0, 3, and 7% NaCl. The T₁N₁ slants and the inoculated tubes were then incubated 24 hours at 35°C. After 24 hours incubation, the suspect colonies from each T₁N₁ slant that showed growth at 0 and 3% but not 7% NaCl were retained for further testing. All salt tubes and other T₁N₁ slants not meeting this criteria were discarded.

The T₁N₁ slants containing cultures which grew at 0% and 3% NaCl were then gram stained and tested for oxidase, catalase, string test, and inoculated on to TSI and LIA agar slants if they were observed to be gram negative curved or straight rods. Several Minitex disks were also inoculated to obtain the carbohydrate utilization requirements of the organisms. The Minitex disks used were lysine, ornithine, arginine, mannitol, inositol, dextrose (aerobic), and dextrose (anaerobic). The morphological and biochemical test results were then analyzed and determinations were made as to whether or not the isolates met the criteria for consideration as a presumptive Vibrio cholerae.

Those isolates meeting the following test requirements were considered to be presumptive Vibrio cholerae:

Growth at 0 and 3% but not 7% NaCl

TSI A/A * H₂ S(-) gas (-)

LIA K/K or K/N** H₂ S(-) gas (-)

String test	+
Oxidase	+
Catalase	+
Lysine	+
Ornithine	+
Arginine	—
Mannitol	+
Inositol	—
Dextrose (Aerobic)	+
Dextrose (Anaerobic)	+

* acid slant/acid butt

** alkaline slant/alkaline or neutral butt

III. RESULTS AND DISCUSSION

A. Wound Swab Samples

Initially thirty-five post cards were sent to physicians in the Neuse River area requesting their participation in this survey. Seven physicians from New Bern, Bayboro, and Aurora replied that they would participate .

Each physician indicated that he might supply anywhere from 0.5-5 samples per week and others 3 to 4 per season. A total of eight case samples were actually received during time period of August 1984 to October 1985.

The following is a case by case analysis of each wound sample received:

CASE #1

Patient:	White male 31 years Unemployed
Activity:	Sorting crabs
Cause of injury:	Crab fin
Location of injury:	Bay River (Paradise Shores)
Date of injury:	July 31, 1984
Type of wound:	Puncture
Description of infection:	Cellulitis
Identification of microorganisms:	<u>Proteus vulgaris</u> <u>Enterobacter cloacae</u> (low selectivity 1/367) <u>Proteus species</u>

CASE #2

Patient: White male 27 years Commercial fisherman
 Activity: Picking up crab
 Cause of injury: Blue crab bite
 Location of injury: Pamlico County (Upper Broad Creek)
 Date of injury: September 8, 1984
 Type of wound: Puncture
 Description of infection: Cellulitis
 Identification of microorganisms: Staphylococcus aureus
Micrococcus species

CASE #3

Patient: White male 90 years Retired
 Activity: Dressing crabs
 Cause of injury: Crab bite
 Location of injury: Unknown
 Date of injury: August 22, 1984
 Type of wound: Bite
 Description of infection: Erythema, purple discoloration,
 vesicles, swelling, septicemia, shock
 Identification of microorganisms: Staphylococcus aureus
Proteus species

CASE #4

Patient:	Black male 47 years Seafood plant employee
Activity:	Cut from putting up paneling in seafood plant
Cause of injury:	Knife cut
Location of injury:	Eastman Creek
Date of injury:	October 23, 1984
Type of wound:	Laceration
Description of infection:	Cellulitis
Identification of microorganisms:	<u>Staphylococcus</u> species Unidentified gram negative rod

CASE #5

Patient:	Black male	73 years	Retired
Activity:	Fishing		
Cause of injury:	Fish fin		
Location of injury:	Oriental Harbor (Neuse River) 50 yds of Kerr Scott Bridge		
Date of injury:	October 5, 1984		
Type of wound:	Puncture		
Description of infection:	Local redness		
Identification of microorganisms:	<u>Staphylococcus</u> species Pseudomonas species		

CASE #6

Patient: White male 22 years Crab Processor
Manager
Activity: Cleanup
Cause of injury: Crab debris
Location of injury: Bay River
Date of injury: September 16, 1984
Type of wound: Puncture
Description of infection: 1.5 cm redness and swelling
Identification of microorganisms: Staphylococcus species

CASE #7

Patient: White male 59 years Farmer
Activity: Fishing
Cause of injury: Shrimp fin
Location of injury: Unknown
Date of injury: August 8, 1985
Type of wound: Unknown
Description of infection: Peculiar violaceous cellulitis, pustules
Identification of microorganisms: Staphylococcus species
Enterobacter species

CASE #8

Patient:	White female 56 years	Seafood Processor
Activity:	Weighing crabmeat	
Cause of injury:	Unknown	
Location of injury:	Seafood Manufacturing Plant, Oriental, NC	
Date of injury:	September 25, 1985	
Type of wound:	Unknown	
Description of infection:	Unknown	
Identification of microorganisms:	<u>Staphylococcus aureus</u> <u>Enterobacter cloaceae</u>	

Six of the eight sample cases were mailed within the same day of sampling and the remaining two samples were mailed and received within five days of sampling. All procedures for identification of the causative agents in the wound samples were started within one day of receipt of the sample from the physicians.

Media and reagents for the testing procedures were made up fresh prior to use in each case and the incubation times were strictly adhered to. Quality control organisms were used for each new batch of media to check the performance of the typical reactions of each media type. The testing procedures varied from sample to sample and it appeared that the API-20E quick test system was the most successful in identifying the organisms. The Minitek system gave limited assistance in providing some biochemical reactions results for consideration. The Vitek automated system was available for use on only one occasion, but proved to be beneficial in that case.

Locations provided for the sites of injury by the physicians proved to be rather vague in most instances. Only one location could be specifically pinpointed. The other areas were too general to assume a reasonable site location. Since the patients were the only source for this knowledge, and there might have been some time lapse before the visit to the physician, it was assumed that this information may have been forgotten or the patient was just unaware of the time and location of the injury.

Five of the eight wound cases were directly caused by contact with shellfish, specifically crabs. Most were due to bites or punctures from fins. The remaining wounds were due to activities in seafood processing plants.

No specific correlations in the clinical descriptions of the wounds were noteworthy with the exception that four cases showed cellulitis. Redness and swelling was apparent in a few cases and a purple discoloration was noted in two cases.

In identifying the causative agents of the wound infections, five different microorganisms were isolated. The most commonly isolated organisms was Staphylococcus species in seven of the eight cases. More specifically, Staphylococcus aureus was apparent in three of the seven instances. Enterobacter species was isolated in three of the eight cases with Enterobacter cloacae being identified in two out of the three instances. The third most occurring organism was Proteus species in two of the eight cases. Proteus vulgaris was specifically identified in one case. Micrococcus species and Pseudomonas species were also isolated from the wounds. An unidentified gram negative rod was also isolated from Case #4 but no specific

identification profile could be established by biochemical or quick test procedures to make a reasonable assumption to the identity of the organism.

None of the cases showed Aeromonas hydrophila or Vibrio cholerae to be causative agents. In realizing the morphological and biochemical similarities of these two organisms with those in the family Enterobacteriaceae, there is a great possibility that there was a chance of misidentifying the organisms using the recommended general laboratory methods. However, all of the isolates did exhibit a negative indophenol oxidase test which immediately eliminates the Vibrio and Aeromonas species which are positive for this reaction.

It is known that many laboratories are unfamiliar with the Vibrio species and do not identify them. The CDC reports that the Vibrio species have been incorrectly called Pseudomonas, A. hydrophila, Pleisiomonas shigelloides, and Serratia marcescens (11). There is also some confusion with infections caused by Erysipelothrix insidiosa which is often obtained from contact with shellfish (11). Chromobacterium violaceum also provides difficulty to the proper identification of the Vibrio and Aeromonas species (12).

B. Water Survey

The following chart summarizes the physical, chemical, and bacteriological characteristics indicative of each sampling site:

<u>Site</u>	<u>Location</u>	<u>Salinity</u> %	<u>pH</u>	<u>Dissolved</u> <u>Oxygen</u> (mg/l)	<u>Turbidity</u> (NTU)	<u>Temp.</u> °C	<u>Vibrio</u> <u>cholerae</u> MPN/100ml	<u>Fecal</u> <u>Coliform</u> MPN/100ml
1	Wilson Creek	0.05	7.8	7.8	4	28	1.13	4
2	Brice Creek	0.60	7.2	5.9	2.4	28	0.13	800
3	Trent	0.86	7.5	5.5	4.8	28	0.13	13
4	Flanners Beach	1.30	7.8	6.5	5.3	28	<0.06	<2
5	Flanners Beach (50 cm)	1.35	-	-	-	-	0.06	<2
6	Goose Creek	1.38	8.0	7.2	3.6	30	0.13	50
7	Upper Broad Creek	1.25	8.1	6.6	4.6	29	0.06	4
8	Bridgeton	0.48	8.1	6.6	9.0	29	<0.06	11

Presumptive Vibrio cholerae were isolated from six of the eight sampling sites. The Wilson Creek site had the highest density of vibrios, followed by Brice Creek, Trent, and Goose Creek. Flanners Beach was very low and Flanners and Bridgeton did not reveal any presumptive indication of V. cholerae organisms.

With one exception the physical and chemical values for each site were not exceptional. The Wilson Creek site had the highest Vibrio density and the lowest salinity, lower than the range that is normally expected for survival of Vibrios. Had this site also showed a high Fecal Coliform density it might have indicated a source of domestic wastes. The Vibrio densities are similar to those reported for the Chesapeake Bay (63).

Brice Creek showed a high fecal coliform density which may indicate sewage out flow, land runoff, or possibly septic tank drainage. In comparing the Vibrio and E.coli densities for the areas, it is apparent that the presence of

V. cholerae does not correlate well with fecal coliform levels. This is probably explained by the observations of Hood and Ness who suggest that V. cholerae survives better in estuarine waters than does E. coli (55). In effect, this would create a potential problem in terms of the public health significance and shellfish safety. If the validity of fecal coliforms as a good indicator of water quality is examined here, one might assume that since the indicator organism does not survive as long as the pathogen, other means of determining high levels of V. cholerae in water and shellfish must be found (55).

The water survey indicated the presence of V. cholerae organisms in these areas, but this is not greatly significant in that it has already been established that V. cholerae is indigenous to estuarine waters worldwide (3,20,21,29,83,89). Since the pathogenicity of these free-living strains has not been assessed because of the difficulty and lack of a competent test, no conclusions may be derived pertaining to a health risk to the public. Testing of this sort would be especially difficult since the environmental isolates might lose their virulence under laboratory conditions. Pathogenicity of these organisms is further complicated since the reservoir of pathogenic non:O1 V. cholerae strains is unknown (12). Since the isolates obtained from this environmental study may not be pathogens, the V. cholerae densities obtained here are probably not useful in measuring a public health risk.

This study was unable to accurately assess any correlation between water quality and the incidence of water-related wound infections based on the data obtained. It was hoped that by determining high V. cholerae densities at the locations where there was a high incidence of wound infections caused by V. cholerae organisms, a significant relationship between

the two could be formulated. This attempt was hampered by many problems. First, there was very minimal participation from physicians in supplying wound swabs. Some swab samples were not documented accurately as to the specific location of the injury. This was not helpful in determining the water sampling sites. No Vibrio organisms were determined to be the causative agent in any of the wound swabs tested. Since no specific sites were identified, it was decided that an overall assessment of Vibrio densities in several general public use areas might provide more useful information.

In looking at the biochemical profile of the isolates presumptively identified from the water survey, it is interesting to note that many of them could easily be identified as V. cholerae or V. parahaemolyticus. There is very little difference in characteristics between the two. V. parahaemolyticus can survive however in a salinity concentration higher than V. cholerae. It has been shown to survive in 8% NaCl and some strains have been viable in 10% NaCl (93). Even though the portion of the tests where the isolates were inoculated into different % NaCl tubes should differentiate the two species, it is still possible for the organisms to change their environmental characteristics under laboratory conditions. Considering the salinity of the sampling sites from this survey, it might be reasonable to assume that many of these isolates may very well be V. parahaemolyticus.

Observations of specific test results revealed that the lysine test may have failed to perform accurately giving negative results for all of the isolates. The control organisms used to show that the medium performed correctly clarified the test reactions in that they gave valid results for the medium lots.

There is also a possibility that these isolates could possibly be *Vibrios* other than *V. cholerae* or *V. parahaemolyticus*, such as *V. alginolyticus* or *V. vulnificus*. These organisms will not usually grow in 0% NaCl and like the higher salinity levels such as 10% NaCl. To obtain the proper dihydrolase and decarboxylase reactions to differentiate these species, the medium must have a final concentration of 0.5-1% NaCl (12). In the tests performed for this study, the mediums used were not adjusted for NaCl concentration since *V. cholerae* was the organism being screened for and it will grow in the absence of NaCl.

C. Response Questionnaire

Sixty water-related wound infection surveys were sent to selected physicians in Coastal North Carolina. Of the sixty sent out, twenty three surveys were received showing a 38% response from the physicians. From the twenty three surveys returned, it was difficult to determine which physicians responded and where their practice was located. This problem was mainly due to not having a required identification signature on the survey forms. Most of the physicians merely filled in the data requested and did not identify themselves, however, five physicians did acknowledge who they were. The approximate location of where some the unidentified forms came from was ascertained from the return postal stamp on the envelopes or from hospital seals.

From the twenty three surveys received, eleven stated that they regularly saw patients with water-acquired wound infections in their practice. The number of wound infections treated ranged from 8-100 + cases per

physician. The other twelve physicians responded that they rarely treated water-acquired wounds and provided no valuable supplemental data.

Although the questionnaire revealed a variety of information, several physicians were in agreement that crab bites and punctures from crab and fish fins were an abundant source of Aeromonas and Vibrio infections. A few physicians even stressed the high incidence of these infections in seafood processing plants.

It appeared from the questionnaire that the physicians who felt that there was a definite high incidence of these infections in their area were the only ones to respond with relevant information.

IV. CONCLUSION

Assessing the possibility of Vibrio cholerae and Aeromonas hydrophila as causative agents for public health concern is a difficult task. More informative data than that gathered from this study is needed in the areas of ecology, epidemiology, pathogenicity, and identification methodology of the organisms to determine the importance of taking preventive action for control.

Both organisms are widely distributed geographically and they are apparently indigenous in estuarine waters. The reservoir of the pathogenic strains is unknown. The source of infections is questionable also except that most patients have a history of exposure to seawater or shellfish. The two major theories as to the source of the pathogenic strains (12), have not been challenged to the point of providing a reasonable conclusion to the reservoir dilemma. Most tests for Vibrio and Aeromonas reveal the presence of the organisms but not their pathogenicity. This provides a difficult situation in that the free-living strains cannot be linked confidently as causative agents of wound infection. If an infection incurs from a cut or wound at a specific location, and a water analysis reveals high levels of Vibrio or Aeromonas, there are no conclusive procedures to assess a public health risk. Colwell and Kaper have stated that if studies were performed in relation to Vibrios and their relationship to the zooplankton and aquatic invertebrates, the epidemiology and pathogenicity of the organisms might be better understood (20). Perhaps comprehensive study into the Vibrio/aquatic ecosystem association would provide a better understanding of the pathogenic mechanisms of these organisms.

Further research needs to be conducted concerning the identification procedures used for identifying V. cholerae and A. hydrophila in the laboratory. Since laboratories do not routinely screen for these organisms, they are not usually familiar with the organisms diagnostic traits. It might prove beneficial if the laboratories would include screening for the Vibrio and Aeromonas species in their testing of wound infections in patients who have been exposed to seawater. Identification of the organisms is not an easy task. Difficulty arises in making conclusive identification within the Vibrionaceae family on the species level as well as with other families such as the family Enterobacteriaceae. Several of the Vibrio species are differentiated from each other based on growth in NaCl and decarboxylase reactions. These tests however are not 100% conclusive in some cases since a few strains may have been isolated that do not conform to the majority. V. cholerae and Aeromonas hydrophila seem to be the causative agents of choice in most wound infections presumed to be of Vibrionaceae origin, but there are others in the family that cause similar wound clinical features. V. parahaemolyticus, V. vulnificus, and V. alginolyticus have been isolated and identified as agents for wound infection. Less is known about these three strains than V. cholerae and A. hydrophila and it would appear that these latter two are referred to most often based on the limited testing performed to identify the causative agent. Perhaps if a more detailed and comprehensive identification methodology were applied, these three lesser known species might become more commonplace as originators of wound infection. To enhance the identification process, it would be helpful if the laboratories could be validated in their testing procedures of the Vibrio and Aeromonas species. Possibly a positive

control organism such as V. vulnificus could be used to upgrade the test methodology and at the same time check the accuracy and consistency of the isolation and identification techniques of the laboratories.

The data gathered from this survey did not provide any significant information relating to the epidemiological aspects of V. cholerae and A. hydrophila acquired wound infections. The epidemiological characteristics were distorted because of the limited numbers of cases and the incompleteness of data from each case. It would appear from the data already available that V. cholerae and A. hydrophila provide a minor public health problem to apparently healthy people. The problem of infection from seawater and shellfish is frequent and abundant, but not all together serious. Serious or life threatening complications seem to be a rare occasion in healthy individuals. The problem of concern may lie with the immunocompromised patients. Serious and sometimes death producing situations arose in patients who were noted as having some known underlying disease especially of systemic severity. Many of the underlying diseases included from these cases were diabetes, ulcers, alcoholism, leukemia, and other hepatic disease (12).

Based on data and conversations with physicians, it is likely that many Vibrio and Aeromonas wound infections arise from contact with shellfish especially in seafood processing plants. They seem to believe that the infections are more prevalent from this source more so than from recreational water activities. Cuts from handling and cleaning shellfish may become infected more readily from touching raw seafood that has been held at temperatures which allow the organisms to multiply rapidly and reach an infective level. Levels in recreational waters may be low as shown by the data

from this study and therefore the organisms pose very little problems as far as infective dose is concerned. An investigation into the infections from seafood processing plants may provide some valuable data concerning prevalence and infective levels of Vibrio and Aeromonas as agents of infection.

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APPENDIX A

LABORATORY MEDIA AND REAGENTS

I. Microbiological Media

A. Thiosulfate Citrate Bile Salts Sucrose Agar (TCBS)

Commercially available from BBL

B. Rimler - Shotts Medium (102)

L-Lysine HCl	5.0 g
L-Ornithine HCl	6.5 g
Maltose	3.5 g
Sodium thiosulfate	6.8 g
L-Cysteine HCl	0.3 g
Bromothymol blue	0.03 g
Ferric ammonium citrate	0.8 g
Sodium deoxycholate	1.0 g
Novobiocin	0.005 g
Yeast extract	3.0 g
NaCl	5.0 g
Agar	13.5 g
Distilled water	1000 ml

Adjust pH to 7.0. Heat to a boil for 1 minute and dispense into petri dishes.

C. Nutrient Agar (BBL)

D. Kligler Iron Agar (BBL)

E. Triple Sugar Iron Agar (BBL)

F. Lysine Iron Agar (BBL)

G. MacConkey Agar (BBL)

H. Streptosel Agar (BBL)

I. Tellurite Glycine Agar (BBL)

J. Blood Agar Base (Infusion Agar) (BBL)

K. Taurocholate Gelatin Agar (TGA) (102)

Bacteriological gelatin	30 grams
Bacto-Tryptone (Difco)	10 g

Yeast extract	1 g
Sodium taurocholate	5 g
NaCl	10 g
Agar	15 g
Distilled water	1000 g

Heat to a boil for 1 minute. Adjust the pH to 8.5. Autoclave for 15 minutes at 121° C.

L. Carey-Blair Transport Medium (BBL)

M. T₁N₁ Agar (102)

Bacto-Tryptone (Difco) 10 g

NaCl 10 g

Agar 20 g

Adjust to pH 6.9. Autoclave 15 minutes at 121° C.

N. 1% Tryptone Broth

With 0% NaCl: 5 g tryptone per 500ml distilled water

With 3% NaCl: 5 g tryptone and 15g NaCl per 500ml distilled water

With 7% NaCl: 5 g tryptone and 3.5 g NaCl per 500 ml distilled water

Heat until dissolved. Dispense in tubes and autoclave for 15 minutes at 121°C.

O. Lauryl Tryptose Broth (BBL)

P. Brilliant Green Lactose Broth (BBL)

Q. EC Broth (BBL)

R. Alkaline Peptone Water (102)

Bacto-Peptone (Difco) 10 grams

NaCl 10 g

Distilled water 1000 ml

Heat until dissolved and adjust pH to 8.5. Dispense into tubes and autoclave for 10 minutes at 121° C.

II. Reagents

- A. Oxidase Test (General Diagnostics)
Strips impregnated with N,N-dimethyl-p-phenylenediamine and α -naphthol
- B. String Test (35)
Sodium deoxycholate, 0.5% solution in distilled water
- C. Catalase Test (35)
Hydrogen peroxide, 30% solution in distilled water.
- D. Coagulase Test (BBL)
Coagulase Plasma, Rabbit with EDTA.
- E. Bacitracin Differential Disk (BBL)
Taxis A Disc 0.04 units of Bacitracin.

III. Suppliers

- A. Analytab Products (API 20E and STAPH-IDENT)
200 Express Street
Plainview, New York 11803
- B. Flow Laboratories (Uni-OF-Tube)
25 Lumber Road
Roslyn, New York 11576
- C. BBL Microbiology Systems (Minitex)
P.O. Box 243
Cockeysville, Maryland 21030
- D. General Diagnostics (Pathotec)
Morris Plains, New Jersey 07950
- E. McDonnell Douglas Health Systems (Vitek)
595 Anglum Drive
Hazelwood, Missouri 63042

APPENDIX B

IDENTIFICATION TEST RESULTS FOR WOUND SWAB SAMPLES

CASE #1

Colonial morphology on TCBS agar - Green with black center (Isolate 1)

Colonial morphology on Nutrient agar - Greenish-beige, shiny, opaque (Isolate 2)

Beige, shiny, transparent edges (Isolate 3)

No growth on Streptosel, TG, and RS agars.

	<u>Isolate 1</u>	<u>Isolate 2</u>	<u>Isolate 3</u>
MacConkey agar	Positive	Positive	Positive
Gram stain	Tiny gram negative rods	Gram negative coccobacilli	Tiny, gram negative rods
KIA	k/black H ₂ S+ gas +	k/black H ₂ S+ gas +	k/black H ₂ S+ gas +
Oxidase	Negative	Negative	Negative
Catalase	Positive	Positive	Positive
String Test	Negative	Negative	Negative

Rapid Identification System

Isolate 1	API 20E System <u>Proteus vulgaris</u>	PROFILE NUMBER	0076001
Isolate 2	API 20E <u>Enterobacter cloacae</u>	PROFILE NUMBER	1305533
Isolate 3	API 20 E <u>Proteus vulgaris</u>	PROFILE NUMBER	0076001

CASE #2

Colonial morphology on Nutrient agar - Flat, opaque, off-white, smooth (Isolate 1)
 Flat, opaque, golden yellow (Isolate 2)

Colonial morphology on TG agar - Shiny, black, opaque (Isolate 3)

No growth on TCBS, RS, and Streptosel agars.

	<u>Isolate 1</u>	<u>Isolate 2</u>	<u>Isolate 3</u>
MacConkey agar	Negative	Negative	Negative
Gram stain	Gram positive cocci	Gram positive cocci	Gram positive cocci
Coagulase	Negative	Positive	Positive

Rapid Identification System

Isolate 1	API STAPH-IDENT Presumptive <u>Micrococci</u>	PROFILE NUMBER	0000
Isolate 2	API 20E <u>Staphylococcus aureus</u>	PROFILE NUMBER	1570
Isolate 3	API 20 E <u>Staphylococcus aureus</u>	PROFILE NUMBER	5700

CASE #3

- Colonial morphology on Nutrient agar - Beige, shiny, swarming, semi-transparent (Isolate 1)
- Colonial morphology on TG agar - Gray/black, shiny, raised (Isolate 2)

No growth on TCBS, RS, and Streptosel agars.

	<u>Isolate 1</u>	<u>Isolate 2</u>
MacConkey agar	Positive	Negative
Gram Stain	Short, gram negative rods	Gram positive cocci
KIA	A/A H ₂ S-gas -	-----
Oxidase	Negative	-----
Catalase	Positive	Positive
Coagulase	-----	Positive
String test	Negative	-----

Rapid Identification System

Isolate 1	API 20E <u>Proteus vulgaris</u>	PROFILE NUMBER	0076030
Isolate 2	API STAPH-IDENT <u>Staphylococcus aureus</u>	PROFILE NUMBER	7700

CASE #4

Colonial morphology on TCBS - Convex, opaque, yellow, shiny
(Isolate 1)

Colonial morphology on Nutrient agar - Convex, opaque, beige, shiny
(Isolate 2)

No growth on TG, RS, and Streptococcal agars.

	<u>Isolate 1</u>	<u>Isolate 2</u>
MacConkey agar	Positive	Negative
Gram Stain	Small, gram negative rods	Gram positive cocci
KIA	K/A H ₂ S- gas -	-----
Oxidase	Negative	-----
Catalase	Positive	Positive
String test	Negative	-----
Coagulase	-----	Negative

Rapid Identification System

Minitex and API systems did not identify Isolate 1.

Isolate 2	API STAPH-IDENT <u>Staphylococcus warneri</u>	PROFILE NUMBER	6600
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CASE #5

Colonial morphology on TCBS - Small, blue/gray, opaque (Isolate 1)

Colonial morphology on Nutrient agar - Convex, opaque, white (Isolate 2)

No growth on TG, RS, and Streptosel agars.

	<u>Isolate 1</u>	<u>Isolate 2</u>
MacConkey agar	Positive	Negative
Gram Stain	Medium, gram negative rods	Gram positive cocci
KIA	K/K H ₂ S-gas -	-----
Oxidase	Positive	-----
Catalase	Positive	Positive
Coagulase	-----	Negative
String test	Negative	-----

Rapid Identification System

Isolate 1	API 20E Fluorescent <u>Pseudomonas</u> group	PROFILE 2210004 NUMBER
Isolate 2	API STAPH-IDENT <u>Staphylococcus epidermidis</u>	PROFILE 3000 NUMBER

CASE #6

Colonial morphology on Nutrient agar - Flat, opaque, cream, shiny (Isolate 1)

No growth on TCBS, RS, TG, and Streptosel agars.

Isolate 1

MacConkey agar	Negative
Gram Stain	Gram positive cocci
Coagulase	Negative

Rapid Identification System

Isolate 2	API STAPH-IDENT <u>Staphylococcus saprophyticus</u>	PROFILE 2601 NUMBER
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CASE #7

Colonial morphology on Nutrient agar - Cream, spreading, opaque, grainy (Isolate 1)
Smooth, opaque, shiny, white (Isolate 2)

No growth on RS, TG, TCBS, and Streptosel agars.

	<u>Isolate 1</u>	<u>Isolate 2</u>
MacConkey agar	Positive	Negative
Gram Stain	Short, gram negative rods	Gram positive cocci
KIA	K/black H ₂ S-gas -	-----
Oxidase	Negative	-----
Catalase	Positive	Positive
String test	Negative	-----
Coagulase	-----	Negative

Rapid Identification System

Isolate 1	API 20E <u>Enterobacter aerogenes</u>	PROFILE 5304773 55 NUMBER
Isolate 2	API STAPH-IDENT <u>Staphylococcus saprophyticus</u>	PROFILE 2201 NUMBER

CASE #8

- Colonial morphology on TCBS agar - Yellow, flat, green transparent edges (Isolate 1)
- Colonial morphology on Nutrient agar - Off-white, opaque, flat, shiny (Isolate 2)
Cream, shiny, opaque, flat (Isolate 3)

No growth on RS, TG, and Streptosel agars.

	<u>Isolate 1</u>	<u>Isolate 2</u>	<u>Isolate 3</u>
MacConkey agar	Positive	Positive	Negative
Gram stain	Small, gram negative rods	Small, gram negative rods	Gram positive cocci
KIA	k/black H ₂ S+ gas +	k/black H ₂ S+ gas +	-----
Oxidase	Negative	Negative	-----
Catalase	Positive	Positive	Positive
String Test	Negative	Negative	-----

Rapid Identification System

Isolate 1	API 20E <u>Enterobacter cloacae</u>	PROFILE NUMBER	3305563 55
Isolate 2	API 20E <u>Enterobacter cloacae</u>	PROFILE NUMBER	3305563 55
Isolate 3	API STAPH-IDENT <u>Staphylococcus aureus</u>	PROFILE NUMBER	7740

APPENDIX C

WATER SURVEY TEST DATA AND SAMPLE CALCULATIONS

Fecal Coliforms MPN

<u>SITE</u>	DILUTION			
	10+1	10+0	10-1	10-2
1	2	0	0	0
2	5	5	3	0
3	4	0	0	0
4	0	0	0	0
5	0	0	0	0
6	5	2	0	0
7	2	0	0	0
8	3	1	0	0

Vibrio cholerae MPN

<u>SITE</u>	DILUTION				<u>#POSITIVE ISOLATES</u>
	10+3	10+2	10+1	10+0	
1	1/1	2/5	2/5	3/5	8
2	0	1/5	0	1/5	2
3	0	1/5	0	1/5	2
4	0	0	0	0	0
5	0	0	1/5	0	1
6	0	0	1/5	1/5	2
7	0	0	0	1/5	1
8	0	0	0	0	0

Sample Calculations

Thomas' formula for estimating most probable numbers (MPN)

$$\text{MPN/100 ml} = \frac{\text{no of positive tubes} \times 100}{\sqrt{\begin{matrix} \text{(ml sample in} \\ \text{negative tubes)} \end{matrix} \times \begin{matrix} \text{(ml sample in} \\ \text{positive tubes)} \end{matrix}}}$$

$$\text{Site 1} \quad \frac{8 \times 100}{\sqrt{(332) (1555)}} = 1.13/100 \text{ ml}$$

$$\text{Site 2} \quad \frac{2 \times 100}{\sqrt{(1454) (1555)}} = 0.13/100 \text{ ml}$$

IDENTIFICATION PROFILES FOR PRESUMPTIVE V. CHOLERA ISOLATES

	TCBS	Gelatin	19% TRYPTONE + NaCl	TSI				LIA				STRING	OXIDASE	CATALASE	GRAM	LYSINE	ORNITHINE	ARGININE	MANNITOL	INOSITOL	DEXTROSE A	DEXTROSE AN
				02	3%	7%	S	B	S	B	S	STRING	OXIDASE	CATALASE	GRAM	LYSINE	ORNITHINE	ARGININE	MANNITOL	INOSITOL	DEXTROSE A	DEXTROSE AN
				02	3%	7%	S	B	S	B	S	STRING	OXIDASE	CATALASE	GRAM	LYSINE	ORNITHINE	ARGININE	MANNITOL	INOSITOL	DEXTROSE A	DEXTROSE AN
	V.C.	YCFT SM	TPHSM F	✓	+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	V.P.	GCFT SM	TPHSM F	✓	-	+	+	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
SITE 1	1361				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	1282				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	1264				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	1185				+	+	-	A	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	1121				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	1122				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	1083				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	1031				+	+	-	A	A	-	-	K	N	-	-	+	+	-	+	-	+	+
SITE 2	2211				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	2083				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
SITE 3	3282				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	3015				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
SITE 5	5182				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
SITE 6	6083				+	+	-	A	A	-	-	K	N	-	-	+	+	-	+	-	+	+
	6181				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+
SITE 7	7015				+	+	-	K	A	-	-	K	N	-	-	+	+	-	+	-	+	+

TSI : A=yellow
K=purple, magenta

GELATIN : H=hazy edges
Tp=transperent
Sm= < 3mm
L= > 3mm
F=flat

LIA : A=yellow, gray
N=purple
K=deep purple
R=red (Proteus)

TCBS: Y=yellow
G=green
C=dark center
F=flat
T=translucent edges
Sm= < 3mm
L= > 3mm

APPENDIX D

FORMS, LETTERS, AND QUESTIONNAIRES

A. WOUND SWAB TESTING

INSTRUCTIONS

Samples are to be taken only from infections that were acquired during water-related occupational or recreational activities (boating, fishing, swimming, etc.). If it is known or presumed to be a staph or strep infection, we prefer not to receive a specimen. Our primary interest is infections by Aeromonas, Vibrios or other organism of aquatic habit. We recognize that it is not often possible to distinguish the probable organism by clinical presentation.

Procedures:

1. Use the sterile swab to obtain a specimen.
2. Insert the swab into the tube so that the swab is immersed in the medium.
3. Break the stick so that when the tube is capped the cap will force the swab nearly to bottom of the tube.
4. Wrap the tube with the completed information form.
5. Mail as soon as possible. If mail will be delayed, refrigerate until mail time.

EAST CAROLINA UNIVERSITY
DEPARTMENT OF ENVIRONMENTAL HEALTH

Environmental Survey of Water-Related Wounds

We are trying to determine if there are any common conditions of water quality, circumstance, or geographic location that are related to the incidence of water related wound infections. We would appreciate it if you would voluntarily supply the following information. Your identity and any personal data will be confidential.

To be completed by patient or clinic:

- I. Patient data: Sex ____ Age ____ Race ____ Occupation _____
- II. Cause of injury: (For example: broken glass, fish fin, rope burn, etc.):

- III. Activity when injured (For example: water skiing, wading, cleaning fish, etc.):

- IV. Where did the injury occur: (Please be as specific and clear as possible, naming the body of water, cove, beach, shore or other information so that we may be able to identify and possibly sample the water in the same location.

- V. Date and time of injury: _____
- VI. Other helpful comments: _____

The above information is voluntarily supplied. I understand no individual personal identification will be revealed and that the purpose of this survey is environmental assessment, not clinical or therapeutic.

Thank you for your cooperation.

Information from Clinic or Physician:

Patient # _____ Type of wound _____

Any underlying or contributing factors such as diabetes, liver disease, etc.?

Description of infection (cellulitis, etc.): _____

Culture collected by _____ (name) Date Collected _____

Date mailed: _____

B. REPONSE QUESTIONNAIRE SURVEY

Dept. of Environmental Health
School of Allied Health
ECU
Greenville, NC 27834

Dear Sir:

A limited number of physicians in the area have been actively participating in an environmental survey of water related wound infections. Since these infections are not reportable to the CDC, the nature of the problem is undefined. To supplement and gather data from additional sources for this project, we are attempting to survey your area to perceive the extent of this problem.

We would appreciate your help by voluntarily supplying information on the enclosed questionnaire about any wound infections that you may have treated. All information or comments that you may have will be greatly appreciated!

Sincerely,

Vivian Blue

Water-Related Wound Infection Survey

1. I rarely see patients with water-acquired wounds due to the nature of my practice.

True _____ False _____
2. I estimate that I treat _____ wound injuries per year due to water-related activities such as waterskiing, wading, fishing, cleaning fish, etc.
3. Approximately what percent of the above injuries were complicated by infections?
4. Approximately what percent of these infections would be described as not being characteristic of Staphylococcus?
5. Is there a common distinctive clinical feature of the non-staphylococcal infections?
 _____ Yes _____ No. If yes, please give a brief description (rapid cellulitis, etc.)
6. Have you treated any known Vibrio or Aeromonas wound infections? If yes, estimate the number you have treated in the past year _____; past three years _____.
7. Do you recall any severe illness from these infections _____ Yes _____ No.
 Number _____.
8. Based on your recall and your own practice, do you believe there are any geographic locations in your area which are proven to have a higher incidence of wound infections? If yes, comment.
9. Based on your recall and experience, are there any particular types of wounds that are more likely to result in non-staphylococcal infections?
10. Comments:

Thank you for your cooperation!