EXECUTIVE SUMMARY

IMPORTANCE OF THE STUDY

Aeromonas hydrophila is an emerging human pathogen that causes both gastrointestinal and non-intestinal diseases in children and adults. These bacteria are isolated from freshwater, salt water, and a variety of foods and produce an impressive array of virulence factors. The organism is becoming increasingly resistant to chlorination in water and to multiple antibiotics. As a result, the Environmental Protection Agency placed this organism on the "Contaminant Candidate List", and monitoring of US water supplies for the presence of *Aeromonas* species began in 2002.

In a 16-month study of the presence of *A. hydrophila* in Indiana drinking water, 7.7% of the biofilm samples were positive for this organism, indicating its potential for regrowth and ability to contaminate distribution systems. Based on PCR analysis, 43, 70, and 30% of the *Aeromonas* isolates obtained by the US Environmental Protection Agency (EPA) (a total of 205 examined) from drinking water from 16 utilities in 4 states harbored *alt, act*, and *ast* genes (these genes encode heat-labile [Alt] cytotonic enterotoxin, cytotoxic enterotoxin [Act], and heat-stable cytotonic enterotoxin [Ast]), respectively. All of these enterotoxins contribute to gastroenteritis in humans and animals (1). Recent studies indicated after disinfection with 1 mg/l of chlorine, 10% of the pipes had aeromonads and that *A. hydrophila* in biofilms could survive up to 0.6 mg/l of monochloramine, which could remove *E. coli* biofilms. The presence of various virulence factors in *Aeromonas* spp. and their prevalence in drinking water reinforces the need to examine the health risk of this water-borne pathogen to better define the quality guidelines for drinking water (20). As a result, this organism is included in the "Contaminant Candidate List" by the EPA. Further, the increasing resistance of *Aeromonas* spp. to chlorination in water and to multiple antibiotics poses added health risk factors.

A recent study conducted in Lebanon recovered *Aeromonas* spp. from both the chlorinated water network as well as from the untreated underground water source (50). Enteropathogenic *Aeromonas* spp. were also found to be commonly present in untreated drinking water obtained from wells in Libya (51). The spp. of *Aeromonas* recovered from such water supplies included *A. hydrophila* and *A. caviae* as well as *A. veronii* and *A. jandaei*. Many studies have demonstrated the ability of *Aeromonas* to survive and grow in drinking water supplies, despite water treatment strategies such as rapid/slow sand filtration, hyperchlorination/direct filtration and the use of granular activated carbon (10, 52, 53). Studies have shown that water temperature and free chlorine are the main factors which affect the growth of *Aeromonas* in water distribution systems (54). Sisti *et al*.'s study (54) observed a rapid decline in viability of *A. hydrophila* at low temperature (5°C), whereas at 20°C (the temperature resembling water in distribution systems during the summer), *A. hydrophila* displayed a greater resistance to chlorine (from 0.2-0.25 mg/l concentration).

The presence of this pathogen in fresh vegetables has been noted (55, 56). The incidence of enteropathogenic *Aeromonas* spp. in minimally processed, ready-to-eat vegetables, could therefore present a potential food hazard. When decontamination procedures were evaluated to control the *Aeromonas* contamination of minimally processed vegetables, it was noted that strains of *A. caviae* and *A. hydrophila* were resistant to chlorination in water, from 0.1-0.5 mg/l free chlorine (57). A study conducted by Chamorey *et al.* (58) indicated a minimum concentration of 0.95 ppm of chlorine needed for efficient reduction of *A. hydrophila*. Further, findings by Sisti *et al*. (54) demonstrated that normal concentrations of chlorine used in tap water (0.2-0.25 ppm) did not reduce *Aeromonas* spp. at 20°C; however, a slight decline in bacterial numbers was noted at a cooler temperature of 5°C. Uyttendaele *et al*. (57) observed that decontamination with a lactic acid

solution, and not chlorine, showed the most potential to reduce *Aeromonas* spp. and to guarantee prolonged shelf-lives of fresh-cut vegetables.

Other studies have shown that reconstituted pesticides may present a suitable environment for the survival and growth of pathogenic *Salmonella*, *Shigella*, *E. coli* O157:H7, and *Listeria monocytogenes* (59, 60). A recent investigation in Australia indicated that dam water used to reconstitute the fungicide Kumulus saw a predominance of *Aeromonas* spp. after incubation (61). The preharvest application of pesticide solutions onto vegetable produce may be an important additional source of contamination by enteropathogens such as *Aeromonas*.

Despite the association of virulence factors with aeromonads isolated from drinking water, there is increasing evidence that strains isolated from the environment generally belong to different groups than clinically associated strains.

RESEARCH OBJECTIVES

The purpose of our study under this AwwaRF contract was to identify virulence traits that could differentiate pathogenic from non-pathogenic strains of *Aeromonas* in water (surface and ground) supplies. Using rigorous statistical analysis methods, we could discriminate stool *Aeromonas* isolates from those from water, as the former produced significantly higher cytotoxic activity and lactones (indicator of quorum sensing).

APPROACH AND GOALS ACCOMPLISHED

During funding from the AwwaRF (2003-2006), we analyzed 242 isolates of *Aeromonas* that were obtained from water and clinical specimens. These isolates were subjected to biochemical identification and DNA fingerprinting and analyzed for the presence of various virulence factors by genetic methods and biological/functional assays. The purpose of this study was to identify critical virulence factors in *Aeromonas* isolates that contribute to disease state in the host. We also performed biochemical characterization and DNA fingerprinting on *Aeromonas* isolates of water and clinical origin so that we can trace specific *Aeromonas* strains that could lead to human infections by consuming contaminated water.

We specifically measured N-(butanoyl)-L-homoserine lactones (BHLs) using *Chromobacterium violaceum* CV026 as the reporter strain to quantitate quorum sensing in this pathogen. Our data also indicated that we should cautiously interpret genetic data (based on dot blot hybridization), as many gene sequences in various *Aeromonas* isolates could be significantly diverged, resulting in our inability to detect them under high stringency conditions. A typical example was the absence of cytotoxic enterotoxin gene (*act*) in several *Aeromonas* isolates. However, when biological assays were performed, we could neutralize the cytotoxic activity associated with Act on host cells using specific polyclonal antibodies to Act. Currently, all of the cytotoxic factors produced by *Aeromonas* isolates are not known and our laboratory is actively engaged in identifying such toxic factors. Based on our microarray analysis data, it was apparent that both Act and the type 3 secretion system (T3SS) were crucial in inducing animal lethality by producing various inflammatory mediators. By employing both the *in vitro* tissue culture model (e.g., murine macrophages and human colonic epithelial cells) and an *in vivo* mouse model of infection, we noted a significantly reduced induction of genes that were involved in immune-related responses when the host was infected with the *act/aopB* mutant compared to when infection occurred with the wild-type *A. hydrophila*. The *aopB* gene is an important component of the T3SS. We verified microarray analysis data by performing real-time RT-PCR, as well as ELISA and immunohistochemistry.

CONCLUSIONS

Taken together, these data indicated a role of both the bacterial virulence factors and host responses in *Aeromonas*-associated infections. Our data also indicated the importance of both the correct identification of *Aeromonas* species and DNA fingerprinting in correlating human infections that are acquired through contaminated water. There is a small subset of *Aeromonas* isolates (e.g. belonging to a specific hybridization group and species) as well as with a unique pulsotype that lead to disease in the host. Therefore, a correlation between a particular group of *Aeromonas* with a unique pulsotype and set of virulence factors they produce will be crucial in differentiating pathogenic and non-pathogenic aeromonads.

By cluster analysis, we identified 3 interesting sets of *Aeromonas* isolates that had similar pulsotype based on pulse field gel electrophoresis (PFGE) and virulence gene patterns.

If a stool isolate and water isolate are truly linked (i.e., an *Aeromonas* isolate in water causes an infection resulting in the isolate being shed in stool), we should see that the two isolates have both similar PFGE and similar virulence patterns. As shown in **Table XXXIX**, we had three sets of isolates from stool and water that were indistinguishable by PFGE.

The virulence patterns for these seven isolates are reported in **Table XXXIX**. NM-14 and NM-35, as well as NM-22 and NM-35, had identical patterns of the distribution of virulence genes and similar biological activities. Because these isolates had indistinguishable PFGE patterns and similar virulence patterns, these data suggest that isolates NM-14 and NM-35 as well as NM-22 and NM-33 were truly identical. If they were epidemiologically linked, this would suggest NM-14 is a waterborne infection of NM-35 and likewise, NM-22 is a waterborne infection of NM-33. Likewise, isolates in set 3 had indistinguishable PFGE patterns and virulence patterns and thus could represent water to human transmission.

One exciting prospect of this cluster analysis approach is that if NM-14/NM-35 and NM-22/NM-33 are, in fact, the same strain, their virulence signature may represent the group of waterborne *Aeromonas* strains capable of gastrointestinal infections in humans. Interestingly, based on our biochemical identifications, NM-22 (stool) and NM-33 (water) isolates were identified as belonging to *A. caviae*/*media* Group. Isolate NM-14 also belonged to the same group, while NM-35 was identified as atypical/*Aeromonas* species. We plan to reexamine NM-35 isolate for its identification profiling. Likewise, isolates AA-14, AA-15, and AA-16 were identified as belonging to *A. caviae/media* Group. These data are very exciting and shed light on which particular *Aeromonas* water isolates could lead to disease in humans.

RECOMMENDATIONS

It is becoming increasingly clear that only a small subset of *Aeromonas* strains belonging to specific hybridization groups cause gastroenteritis in humans (11). Based on a set of genotypic and phenotypic traits possessed by *Aeromonas* species, the primary objective of our statistical analysis was to discriminate *Aeromonas* strains that infect and cause acute gastroenteritis in humans from those non-virulent strains common in drinking water supplies that are benign to humans. We analyzed 165 *Aeromonas* isolates from water utilities within the USA and human patient populations with diarrhea (*n=52*) that were either receiving water from these utilities or were reported positive for *Aeromonas* culture. In addition, we examined 25 reference strains, which included water, diarrheal and other clinical isolates. All of these cultures were examined for their biochemical characterization, fingerprinting by pulse field gel electrophoresis (PFGE), and the presence of a set of 11 virulence factors. We identified three independent sets in which pulsotypes and virulence signatures from waterborne *Aeromonas* isolates were indistinguishable from those

in human stool samples. Are these isolates the most virulent and have they the potential to cause disease in humans?

Our studies indicated that many of the genetic virulence traits are more prevalent and the phenotypic virulence traits are expressed at a higher level for the stool isolates than the water isolates and this pattern holds for all three species of *Aeromonas* (e.g. *A. hydrophila*, *A. caviae*, and *A. veronii*).

FUTURE RESEARCH

Based on our research and additional studies, we believe that now predictive models can be developed to discriminate between pathogenic and non-pathogenic aeromonads. Funding from the AwwaRF provided us the crucial information needed to establish parameters that should be considered in developing such models.