ABSTRACT

Title of Document: IDENTIFICATION AND CHARACTERIZATION OF *Aeromonas caviae* ISOLATED FROM CLINICAL SAMPLES COLLECTED DURING THE 2010 HAITIAN CHOLERA OUTBREAK

Philip Wilson Clark, Master of Science, 2017

Directed by: Dr. Rita R Colwell, Distinguished Professor

Marine, Estuarine, Environmental Science

University of Maryland, College Park

Diarrheal disease is a leading cause of child mortality worldwide. Bacteria belonging to the genus *Aeromonas* have been debated as one of the causative agents of gastroenteritis. During the 2010 Haitian cholera outbreak, fecal samples were gathered from 81 patients suffering symptoms of diarrhea, vomiting, and abdominal pain. These isolates were investigated for *Vibrio cholerae* and *Aeromonas* species. Bacteria from these samples were isolated and characterized

using polymerase chain reaction (PCR) and biochemical testing. Identification of *Aeromonas* was performed to species level using multi-locus sequence typing (MLST) analysis. *Aeromonas caviae* was identified in 17 fecal samples (21%). Putative virulence factors in each strain were determined using additional PCR and biochemical analysis. Both *V. cholerae* and *A. caviae* were confirmed in seven of the 17 samples, suggesting potential polymicrobial infection.

# IDENTIFICATION AND CHARACTERIZATION OF *Aeromonas caviae* ISOLATED FROM CLINICAL SAMPLES DURING THE 2010 HAITIAN CHOLERA OUTBREAK

By

Philip Wilson Clark

Thesis submitted to the Faculty of the Graduate School of the
University of Maryland, College Park in partial fulfillment
of the requirements for the degree of
Master of Science
2017

Advisory Committee:
Professor Rita R. Colwell, Chair
Professor Anwar Huq
Professor Wade C. Winkler

**Acknowledgements**

I would like to first thank my advisor, Dr. Rita Colwell. I will never be able to say enough to express my appreciation for all that she has done for me. She is a constant source of support and encouragement and believed in me when I did not. For that and for so much more, I am forever grateful.

Thanks also to Dr. Anwar Huq, who has always challenged me and pushed me to achieve more. He is the frequent source of sound advice and mentorship. Thank you for the opportunity to work with you.

Thank you as well to Dr. Wade Winkler. His infectious excitement for science has been a constant source of inspiration. I hope my level of enthusiasm and creativity in the realm of biology can one day approach his. Thank you again for all you have done.

Thank you to Arlene Chen, Shah Rashed, Dr. Elisa Taviani, Dr. Bradd Haley, Dr. Nur Hasan, Dr. Daniella Ceccarelli, Dr. Richard Stewart, and everyone else in the University of Maryland community who have helped me along this journey.

Thanks to my family. They have been an endless source of love and encouragement when I did not feel I deserved any. My parents and brothers have seen me through thick and thin, and have always lent an ear when it was needed.

To my wife and beautiful daughter, my love for you is endless.

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# Chapter 1 Literature Review

## Agents and Consequences of Diarrheal Disease

Diarrheal diseases remain a major health concern worldwide and account for approximately 17% of all deaths among children five years and younger. This rate is higher than that of AIDS, malaria, and measles combined [1]. Diarrhea is typically the symptom of an infection of the intestinal tract caused by a variety of bacterial, viral, and/or parasitic enteric pathogens. These organisms are spread from person to person through the fecal-oral route, typically by consumption of food or water naturally contaminated or transmission of a fecal pathogen from a carrier of the disease [2, 3].

Bacterial agents include *Vibrio cholerae*, *Shigella* spp. and *Salmonella* spp., as well as enteropathogenic *Escherichia coli* (EPEC) and enterotoxigenic *E. coli* (ETEC) which are globally the most common bacterial pathogenic agents of diarrheal disease [4]. Two of the most frequently reported viral diarrheal pathogens are rotavirus and norovirus [5]. It is estimated that norovirus is responsible for 10% to 38% of global diarrhea-related inpatient and outpatient visits among children under 5 years of age [6]. Less commonly reported diarrheal pathogens include eukaryotic parasitic organisms, e.g., *Giardia intestinalis*, *Cryptosporidium* spp., and *Entamoeba* spp. These are more frequently the agents of diarrheal disease in developed countries. However, globally they are a less frequently determined cause of diarrheal disease [7].

The symptoms of diarrhea include passage of three or more loose to watery stools per day and acute diarrhea can be categorized by its clinical presentation [8-10]. Acute diarrhea can last for a span of several hours to days, presenting with or without blood, the former is referred to as dysentery. The infected person can quickly become dehydrated from loss of body fluid and electrolytes and the dehydration can be worsened by fever and vomiting. Symptoms may vary according to the causative agent of the infection. Dehydration is considered the most severe result of diarrheal disease, as death can quickly follow if fluids and electrolytes are not replenished [11-14].

## Causes and Preventative Measures

By providing access to clean water and implementing simple improvements in waste management, occurrence and mortality rates of diarrheal diseases can be drastically reduced. Unfortunately, many of these diseases occur in developing countries that do not have the physical, economic, or educational resources to easily and rapidly install these capabilities [15-17]. Natural disasters and internal conflicts greatly worsen safe water availability and increase the risk of waterborne diseases, even in industrial and developing countries. In 2005, after Hurricane Katrina made landfall in New Orleans, Louisiana, the Centers for Disease Control (CDC) reported 22 new cases of *Vibrio*-related illness just two weeks after the hurricane. The CDC also strongly recommended any persons working in hurricane-damaged areas to wear extensive protective gear and avoid any exposure to brackish water or seawater, whenever possible [18]. A major flooding event in Bangladesh in 2004 was associated with approximately 17,000

cases of cholera, ETEC, and other diarrheal diseases [19]. Two studies in Indonesia reported exposure to floodwaters was associated with a significant increase in the likelihood of exposure to, and infection by, *Salmonella enterica* serotype Paratyphi A (paratyphoid fever) and *Cryptosporidium parvum* [20].

General conflict and war increase exposure of military personnel and civilians alike to infectious disease agents and, notably, limit access to clean water. American military personnel have reported several instances of diarrheal disease when serving in Iraq, Afghanistan, and Southeast Asia [21-23]. Refugee camps established for civilians fleeing conflicts are essentially incubators for infectious disease, as the refugees often have to tolerate poor, cramped living conditions without regular access to clean water, soap, and proper medical treatment [24-26]. As water becomes a scarce resource in these areas, aid workers become targets of criminal activity as they attempt to deliver food and clean water to civilians living in the war-torn areas. The international charity organization Médecins Sans Frontières (MSF) reported attacks on health facilities and aid workers in Afghanistan, the Central African Republic, South Sudan, the Syrian Arab Republic, and Yemen [27].

Due to these and many related challenges, access to safe drinking water is always an issue that never is completely resolved. Therefore, it is imperative to monitor and characterize waterborne pathogens to achieve a better understanding of the mechanism of infection and epidemiology.

The role of *Aeromonas* spp. in causing diarrheal disease has long been

debated, and the debate is still ongoing. [28]. Nevertheless, the available evidence suggests these bacteria pose a real threat to public health and safety [28, 29].

## Taxonomy of the Genus *Aeromonas*

The study of the Gram-negative, oxidase positive, rod-shaped organisms belonging to the genus *Aeromonas* has been hindered by debate and confusion for more than 100 years. During the late 19$^{th}$ and 20$^{th}$ century, *Aeromonas* species were identified as belonging to at least 15 other genera. Because of this confusion in the classification of *Aeromonas* spp. and debate over identification of *Aeromonas* as its own genus, it is difficult to determine the first instance of the laboratory isolation of any *Aeromonas* species [30].

The earliest likely reference to an *Aeromonas* bacterium was published in 1890, when a then-novel species was isolated from drinking water supplies in the city of Chemnitz, Germany and characterized as *Bacillus punctatus* by Zimmerman [31]. Other early descriptions of potential *Aeromonas* species were published and named separately by Ernst and Sanarelli and correlated with a disease in frogs and subsequently other cold-blooded animals. The genus *Aeromonas* was proposed in 1936 by Kluyver and van Niel, based on fermentative properties unique to Gram negative rods with polar flagella that had been described at the time [32].

During the first few decades following establishment of *Aeromonas* as a genus, aeromonads could be roughly broken into two major groupings based upon their growth characteristics and results of DNA-DNA hybridization experiments.

The psychrophilic strains, containing isolates currently residing within the species *Aeromonas salmonidica*, were nonmotile and typically associated with fish diseases. The mesophilic group was more similar to *Aeromonas hydrophila* and was associated with a variety of human infections [29].

The first laboratory isolation of *Aeromonas caviae* was achieved in 1936 by Liu, who isolated it from septic guinea pigs and initially named the isolate *Pseudomonas caviae* [33]. This isolate and subsequent isolates were later regrouped into a subspecies of *Aeromonas punctata* and finally labelled as its own species by Popoff and Véron in 1976 [34].

The latest edition of *Bergey's Manual of Systemic Bacteriology* recognizes a total of 14 species of *Aeromonas*, identified primarily based on DNA-DNA hybridization and 16S sequence analysis [35]. Recently, further multi-locus sequence typing (MLST), using multiple housekeeping genes, and results of advances in genome sequencing, this categorization has been expanded. Thus, as of 2017, there are currently a total of 36 species and 12 subspecies recognized in the list of prokaryotic names with standing in nomenclature (http://www.bacterio.net) and available from the Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures.

## *Aeromonas* as a Human Pathogen

Because they possess to a broad range of extracellular enzymes, the aeromonads have long been associated with disease in both cold blooded animals and humans, causing multiple types of infections. The most recognized

mesophilic species commonly associated with some form of human infection are *A. hydrophila, A. sobria,* and *A. caviae* [29].

Actual virulence and disease caused by *Aeromonas* species depends on several variable factors and the disease process in *Aeromonas* is not completely understood in all areas of infection. However, *Aeromonas* species have been shown to possess genes enabling motility, adhesion, multiplication within a host, as well as potentially virulent enzymes secreted by Type II and Type III secretion systems, all of which can be considered requirements for a microorganism to be pathogenic [36].

Clinical infections can be separated into two broad categories: intestinal and non-intestinal infections. Non-intestinal infections, commonly a result of exposure to polluted freshwater, include tissue infections and septicemia, and have been well documented in the literature [37-40]. Exposure to polluted water is especially likely to result in an infection in those who suffer severe physical trauma or who are immunocompromised at the time of exposure. Wound infections, sometimes accompanied by septicemia, often result in cellulitis, while cases of necrotizing fasciitis requiring limb amputation have also been reported [41]. Cases of septicemia without initial wound infection or in an immunocompromised patient have also been reported and the initial source(s) of infection have been considered unclear [29].

### *Aeromonas* as a Cause of Gastroenteritis

The potential for aeromonads to colonize and cause gastroenteritis in

humans has previously been the subject of extensive debate, based on the perceived lack of true clonal outbreaks and apparent non-fulfillment of Koch's postulates since several strains were isolated and a low number of acute illnesses resulted from the single human challenge study that has been done [28]. Multiple studies have shown that *Aeromonas* strains causing diarrhea in patients were a identical to those found in local drinking water and other food sources considered the potential source of infection. A study by Khajanchi et al. (42) indicated conservation of genetically identical virulence factors and indistinguishable pulsed-field gel electrophoresis (PFGE) patterns between clinically and environmentally isolated strains of *Aeromonas* species. Results of the study presented here also show multiple patients from widely geographically distant regions of Haiti were shedding strains of *Aeromonas* that are presumed genetically identical as shown by multi-locus sequence typing (MLST). These results suggest that clonal outbreaks are indeed possible and may have already occurred.

Koch's postulates were anecdotally fulfilled by a gastrointestinal infection of *A. trota* when a laboratory worker accidentally ingested some of a liquid culture, as shown by Carnahan, et al. [42]. The results of the laboratory accident may be difficult to replicate in animal systems, due to evidence showing very stringent requirements for colonization and infection after ingestion of an *Aeromonas* species [28]. Researchers must consider the type of strain, if more than one strain is present, the immune status of the patient, and the fact that multiple strains and species have been shown to play a role in contributing to the

virulence of *Aeromonas* [43].

It is also possible that *Aeromonas* spp. play a role in polymicrobial infections, as they have been isolated from clinical fecal samples in conjunction with other pathogens. Historically, *Aeromonas* spp. have been co-isolated from diarrhea samples along with both bacterial and viral pathogens, such as *Vibrio cholerae, Vibrio parahaemolyticus, E. coli,* and Norwalk-like viruses [44-46]. Few reports have investigated this interaction or potential for co-infection, so future studies investigating the capability of *Aeromonas* spp. to function as an enteric pathogen should take this possibility into account.

Animal models have also helped reinforce the hypothesis that *Aeromonas* spp. have the ability to colonize the human gut. Dos Santos et al. demonstrated the ability of five clinically isolated strains of *A. caviae* to successfully adhere to the colonic mucosa of the rabbit intestine, establishing a biofilm on its surface [47].

The findings from all of these reports place the above-mentioned challenge study into question. Multiple strains were examined, but considering the multitude of factors required for infection and even shedding of virulent *Aeromonas* species, further studies using volunteer challenges are needed. Real world outbreaks have suggested that very specific cell concentrations or host conditions are required for infection to occur, which calls into question the ability for laboratory controlled challenge experiments to accurately mirror real world conditions. A model generated by Teunis and Figueras estimated the median dose

for a 1% risk of illness to be just 0.9 ingested colony-forming units (CFU), calculated from exposure events [28].

## Intestinal Infections Caused by *Aeromonas* spp.

*Aeromonas*-associated diarrhea has been well-documented as a worldwide health issue and reported in both industrialized and developing nations. The patient population can vary, with *Aeromonas* gastroenteritis diagnosed in all age groups, although a greater risk of infection has been reported in children under the age of five, as well as in those considered immunocompromised [48]. A review of the literature by Holmberg and Farmer reported finding *Aeromonas* spp. were found in the stools of 0.04% to 21% of those diagnosed with gastrointestinal disease versus 0% to 10% of healthy controls [49]. These frequencies are relatively similar to isolation incidences of *Salmonella, Shigella*, and *Campylobacter* spp. in ill versus healthy persons, all of which are well known and characterized causes of enteric disease in humans [50].

Gastroenteritis caused by *Aeromonas* spp. can have an incubation period of 1 to 2 days [49]. Stools produced during *Aeromonas* gastroenteritis have been described as dysenteric and watery, sometimes accompanied by abdominal pain and vomiting. The most commonly reported symptoms associated with an *Aeromonas* infection are an inflammation of the large and small intestine, watery diarrhea, and abdominal pain. It is estimated that these symptoms account for approximately 75% to 89% of all cases of *Aeromonas* gastroenteritis [29]. One study noted a high frequency (60%) of vomiting in younger children suffering from *Aeromonas* gastroenteritis [49]. Another study in Hong Kong noted that a

person with *Aeromonas* gastroenteritis had an average of 8.6 loose or watery stools per day, with symptoms lasting for three days and accompanied by mild to moderate dehydration [51]. Much less common is *Aeromonas* gastroenteritis associated with mucus and blood in the stools. Previous studies have reported these symptoms present in 3% to 22% of cases [51-53].

On a few rare, documented occasions, *Aeromonas* gastroenteritis has presented with cholera-like symptoms, with multiple rice watery stools and vomiting, resulting in severe dehydration. Kumar and Kumar described such a case in a 2-year-old girl in India, who was hospitalized and able to recover within two days [54]. Champsaur et al. described another similar case of cholera-like symptoms with *A. caviae* being recorded as the sole isolated pathogen. In this case, a 67-year-old woman lost 13 liters of "rice-water" stools and recovered after seven days of hospitalization [55]. Other similar cases have been documented in Cuba and Libya [56, 57].

## Virulence Factors

Numerous strains of *Aeromonas* species that have been characterized or sequenced carry features that are assumed to be necessary for causing gastroenteritis in humans. However, the precise mechanisms of *Aeromonas* infections are not completely understood, so it is unclear which virulence factors are absolutely required for infection. Some studies have tried to correlate specific virulence factors with increased severity in symptoms, but no single factor or set of virulence genes has been consistently identified as a required indicator for the ability to cause or worsen gastroenteritis [29].

Polysaccharides produced on the exterior of the cell, such as capsules, S-layers, and extracellular polysaccharides have all been well characterized and documented, and are important in attachment and colonization of a host organism [47]. Biofilms formed by the excretion of polysaccharides aid in establishing an anchored community within the host and the bacteria can then avoid attacks by a local immune system [58]. Lipoplysaccharaides in the cell envelope have also been well characterized in some *Aeromonas* spp. and are often correlated with high immunostimulatory activity that can present a wide range of clinical manifestations in both septicemia and tissue infections [59-61].

Proteases are a well-studied class of enzymes that catalyze the cleavage of peptide bonds and contribute to invasion of the pathogen within the host by causing direct damage to host tissue and the initial immune defense [62]. Laishram and Pennathur were able to purify an alkaline thermostable metalloprotease from a strain of *A. caviae* [63]. Cascón et al. were able to demonstrate a specific protease (*ahyB*) essential for pathogenicity of *A. hydrophila* in fish and required to maintain proteolytic activity. The sequence of this protein shares 61% and 53% identity with metalloproteases of organisms capable of causing disease, namely *Helicobater pylori* and *V. cholerae*, respectively, suggesting similar capability as a pathogenicity factor [64].

Another extracellular class of virulence factor produced by *Aeromonas* spp. includes the pore-forming toxins, aerolysin and hemolysin. These enzymes cause osmotic lysis and destruction of erythrocytes in the human immune system [62]. A study by Wong, et al. [65] demonstrated that the aerolysin and hemolysin

profile of *Aeromonas* is significantly correlated with cell culture cytotoxicity, suggesting these enzymes, or specific combinations of these enzymes, play an important role in human pathogenesis. These genes also show similarity to the aerolysin and hemolysin in *V. cholerae*, where they also contribute to enhanced virulence.

Both cytotoxic and cytotonic extracellular toxins have been characterized in *Aeromonas* spp. [36, 66]. The cytotoxic enterotoxin encoded by the gene *act* is an aerolysin-related pore-forming toxin capable of provoking degeneration of crypts and villi of the small intestine, and is typically found in isolates recovered from the stool of patients suffering diarrhea [67, 68]. It is considered responsible for hemolytic, cytotoxic, and enterotoxic activities of *A. hydrophila*, and considered by some to be one of the main virulence factors.

Cytotonic enterotoxins do not result in the degeneration of the epithelium, but are more closely similar in action to the cyclic adenosine monophosphate (cAMP) and prostaglandin-mediated mechanism of cholera toxin in intestinal epithelial cells. These toxins increase the production of cAMP and prostaglandins resulting in the forced export of massive amounts of nutrients from epithelial cells, making them readily available to enteric pathogens and causing diarrhea in an infected patient. These are divided into heat-labile (*alt*) and heat-stable (*ast*) sub-categories [69, 70].

In general, *Aeromonas* spp. secrete a wide range of extracellular enzymes, such as lipases, amylases, chitinase, nucleases, and gelatinases. It is unknown

what exact roles these play in pathogenicity, but they demonstrate the potential to readily adapt to environmental changes and contribute to a large metabolic diversity which could aid in survival during host infection [36]. A few lipases have been previously described and have been linked to a significant increase in both hemolytic and cytotoxic activity and overall pathogenicity in both fish and mice [71, 72].

Type III and VI secretion systems (T3SS and T6SS) have been documented as playing a critical role in the virulence of Gram-negative pathogens, acting as transport mechanisms for toxins and other protein products across the outer membrane into host cytoplasm. The T3SS contains an injectisome, consisting of about 20 proteins that assemble into a needle-like structure anchored to the envelope, capable of translocating proteins into the cytosol of the host cells. Functioning T3SS have been described in both *A. salmonicida* and *A. hydrophila* [73-76]. A T6SS works similarly, with a phage-tail-spike-like injectisome, again capable of translocating toxins into a host cell. Only a few characterized strains of *A. hydrophila* contain functional T6SS and have been linked to some involvement in virulence [77, 78].

Finally, the ability of locomotion, using flagella, is a key factor in colonization and invasion of the host mucosa. Within the gut, motility and chemotaxis are required for the pathogen to reach the target tissue. The lateral and polar flagella of *Aeromonas* spp. are also capable of acting as adhesions, aiding in attachment to host cells. Lateral flagella additionally have a required role in biofilm formation, another key factor in pathogenicity [79-81].

Because of the rather unclear status of *Aeromonas* as an enteric pathogen and the still yet to be defined mechanism of infection, it is important to monitor clinical strains for all of the above virulence factors, with the expectation that a defining pattern will emerge that will allow for a better understanding of aeromonads as pathogens.

## *Aeromonas* in the Environment

Aeromonads are essentially ubiquitous in the microbial biosphere and have been isolated from diverse ecological niches, frequently found as part of the flora of vertebrates and invertebrates, as well as in soil and both fresh and salt water. *Aeromonas* species have been previously isolated from a broad range of organisms, spanning several different phyla [29]. Several *Aeromonas* species containing genes encoding virulence factors have been isolated from the surface and intestinal tract of both tilapia fish (*Tilapia nilotica*) and catfish (*Clarias betrachus*), the feces of the primate long-tailed macaque (*Macaca fascicularis*), houseflies (*Musca domestica*), and the hepatopancreas of a giant freshwater prawn (*Macrobrachium rosenbergii*) [82-84].

A study by Hazen et al. [85] identified viable *A. hydrophila* in 135 of 147 (91.8%) natural aquatic habitats sampled in the United States and Puerto Rico. *Aeromonas* spp. have been repeatedly isolated from rivers, lakes, ponds, estuaries, drinking water, groundwater, wastewater, and sewage, with concentrations varying from lows of <1 CFU/ml to highs of $10^8$ CFU/ml [29]. The abundance of *Aeromonas* reservoirs provide ample opportunities for exposure and increased potential for causing disease in humans through ingestion or access to open

wounds [86-88].

Notably, the clinically relevant species, *A. hydrophila, A. caviae*, and *A. veronii bv.* sobria, considered responsible for a large majority of human infections are those *Aeromonas* spp. most frequently isolated from environmental water sources and food [29]. Since they belong to the mesophilic group of *Aeromonas* spp., rates of their isolation and reported infections of the gastrointestinal tract are affected by seasonality and local climate. Most studies have found that they are more commonly isolated during the warmer months of the year or from consistently warmer regions of the globe [89].

# Chapter 2 *Aeromonas* spp. Isolated from Patients During the Cholera Outbreak in Haiti in 2010

## Introduction

Members of the genus *Aeromonas*, Gram-negative, rod-shaped, facultative aerobes, are gaining recognition as an emerging enteric opportunistic or primary pathogen [28]. They are considered ubiquitous in the environment, having repeatedly been isolated from both fresh and salt water, soil, and from both vertebrate and invertebrate animals. Due to their ubiquity in the environment, the potential for human exposure is nearly constant. Human infections caused by *Aeromonas* species include soft tissue and wound infections, bacteremia, and gastroenteritis [29]. The current edition of *Bergey's Manual of Determinative Biology* recognizes 14 species of *Aeromonas*, but only three of them, *A. caviae*, *A. hydrophila*, and *A. veronii* biovar *sobria* have been associated with gastrointestinal disease [35].

Though the specific role of *Aeromonas* in gastroenteritis is still being debated, many putative virulence factors that appear to contribute to gastroenteritis have been identified. Several functioning cytotoxin, enterotoxins, hemolysins, and secretory systems have been well characterized in several strains of all clinically relevant *Aeromonas* spp. [36]. Some environmental and clinical strains of *A. caviae* that have been isolated were found to carry genes encoding a Shiga toxin similar to that coded by *stx1*, the Shiga toxin gene found in *Escherichia coli* O157:H7, and other strains of Shiga toxin-producing *E. coli* (STEC) [90, 91]. *A. caviae* has also demonstrated the ability to adhere to and

colonize the colonic mucosa of rabbit intestine [47]. However, attempts to fulfill Koch's postulates with other animal models during a human volunteer study challenged the hypothesis that *Aeromonas* spp. are a direct causative agent of gastroenteritis [92]. It is possible that numerous factors must be considered when determining the enteropathogenicity of *Aeromonas* species, such as the immune status of the patient, the specific species and strains involved, the discrepancy in expression of virulence factors in different environmental conditions, and the possible involvement and collaboration of other pathogens present in the intestinal tract [29]. *Aeromonas* spp. have been isolated along with other enteric pathogens such as Norwalk-like viruses, *Vibrio cholerae,* and enterotoxogenic *E. coli* (ETEC) [44-46]. Yet *Aeromonas* spp. remain important, if still ill-defined, players in gastrointestinal infection. Therefore, it is necessary to identify and characterize putative virulence factors in each clinical isolate of *Aeromonas* spp. shed from persons suffering symptoms of gastrointestinal infection.

On January 12, 2010, a 7.0 magnitude earthquake struck Haiti, t 25 kilometers southwest of the Haitian capital of Port-au-Prince, with the epicenter located underneath the city of Léogâne [93]. Three more aftershocks were recorded, two registering at magnitude 5.9 and a third at 5.5. Much of the country was already dealing with inadequate infrastructure, including lack of access to clean drinking water and only substandard medical facilities being available to a large portion of the population. Additionally, due to a lack of adequate reinforcement structure of many buildings, much of the capital and surrounding area lay in ruins. It is estimated that three million people were affected by the

earthquake, making up nearly one third of the entire population of the country [94]. With extensive damage done to already poor sewage and drinking water facilities, the potential for a waterborne outbreak of any kind was very high [95].

The first cases of Haitian patients exhibiting symptoms of vomiting and watery diarrhea to be reported were on October 19$^{th}$ and 20$^{th}$ of 2010 in Bocozel and Deschapelles, towns located in the Artibonite Department, both located along the Artibonite River. Specimens collected from these cases were sent to the Laboratorie National de Santé Publique (LNSP) in Port-au-Prince for diagnosis. On January 21, 2010, eight of these samples tested positive for *Vibrio cholerae* O1 and, therefore, were the first confirmed cases of cholera in Haiti since 1960. However, a later study showed *V. cholerae* to be present in Haiti as early as 2008 [96] . *V. cholerae* was isolated from acute diarrhea victims throughout the country within a month and cholera was confirmed in all ten departments of Haiti [95, 97].

This study was designed to determine if *Aeromonas* spp. had any role in the outbreak by examining clinical fecal samples collected from 81 Haitian patients suffering symptoms of gastroenteritis within the first three weeks of the 2010 Haitian cholera outbreak. Biochemical analysis, followed by polymerase chain reaction (PCR) amplification of an *Aeromonas*-specific 16S sequence was used for initial identification to genus. Multi-locus sequence typing (MLST) was employed for species identification of all strains. Isolates of *A. caviae* were characterized using biochemical analysis, including virulence traits, antimicrobial susceptibility, and presence of 16 genes encoding putative virulence factors.

## Methods

### Sample Collection and Bacterial Isolation

During a three-week period, early in the 2010 Haitian cholera outbreak, a fecal sample was collected from each of 81 individuals experiencing symptoms of gastroenteritis or typical "cholera-like" symptoms, normally rice-water diarrhea, vomiting, and abdominal pain. Samples were collected at the Nuestros Pequeños Hermanos (NPH) Haiti Saint Damien Pediatric Hospital in Tabarre, Haiti, at rural mobile health clinics near the city of Saint-Marc, or at the individual's residence. In total, the samples included at least one individual from each of 18 towns across eight Arrondissements of Haiti (Fig. 1).

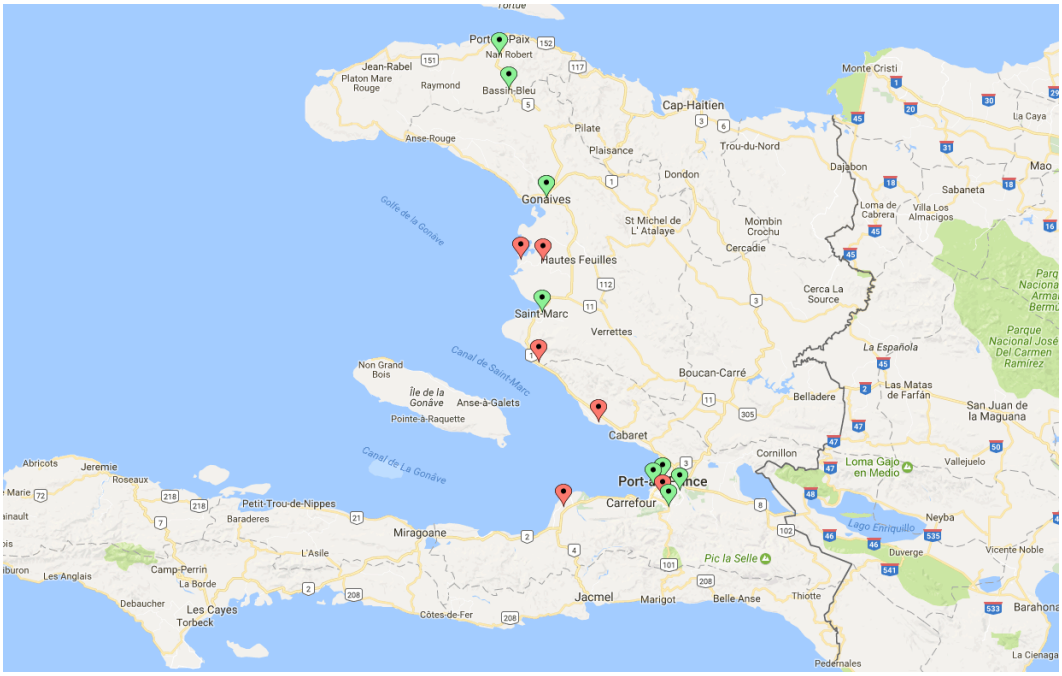


**Figure 1**. Source and distribution of clinical sampling locations. All samples were collected in November 2010, during the first weeks of the 2010 Haitian cholera outbreak. Green markers indicate sites where *A. caviae* was isolated from at least one clinical sample.

The 81 fecal samples were streaked onto thiosulfate-citrate-bile-salts sucrose (TCBS) agar and grown overnight at ambient room temperature. All colonies on TCBS agar were transferred and cultured in Luria-Burtani (LB) agar stabs in 1.5 mL microcentrifuge tubes, and sealed with parafilm for transport. After a three-day transport at ambient conditions, the LB agar was removed and cut in half. Half was used to streak directly onto TCBS agar, and the other half was incubated in 50 ml of alkaline phosphate water (APW) for eight hours at 37˚C. After incubation for eight hours, the top of the broth was skimmed and streaked onto TCBS for overnight incubation at 37˚C. Colonies displaying unique morphologies were picked, in duplicate, if identical colonies had formed. Stock cultures were maintained at -80°C in LB broth containing glycerol at 25% (v/v).

For bacterial DNA extraction, each isolate was grown in 5 mL LB broth at 37°C. After overnight growth 1 ml of broth culture was centrifuged for 10 minutes at 8000xg. Bacterial DNA was extracted using the Qiagen DNA Mini Kit according to the manufacturer's instructions. DNA was stored in nuclease free water at -20°C.

**PCR Reactions**

Genomic DNA extracted from yellow, sucrose fermenting colonies on TCBS was subjected to PCR, employing gene probes for *V. cholerae* transmembrane regulatory protein (*toxR*) and outer membrane protein (*ompW*) [98, 99]. Isolates positive for these genes were identified as *V. cholerae* and further categorized as O1, O139, or non-O1/O139 using the Hoshino multiplex assay. A primer previously developed for the 16S region in *Aeromonas* spp. was

used for identification of isolates as *Aeromonas*. The 16S rRNA gene of all non-*V. cholerae* was sequenced and genus level identification performed using the online search BLAST tool.

Species identification and genomic diversity of the putitative *Aeromonas* isolates were determined using multi-locus sequence typing (MLST) of six housekeeping genes: DNA gyrase subunits A and B (*gyrA*, *gyrB*); RNA polymerase sigma factor 70 (*rpoD*); DNA polymerase subunits gamma and tau (*dnaX*); a DNA chaperone (*dnaJ*); and ATP synthase F1 beta subunit (*atpD*). Primers were used as previously described (Table 1) [100]. The resulting sequences were concatenated and aligned with sequences from 25 other *Aeromonas* species. Phylogenetic analyses were conducted using the MEGA7 program [101].

Virulence capabilities of confirmed strains of *Aeromonas caviae* were presumptively characterized by presence of genes encoding known virulence factors. Primers used in this PCR analysis targeted genes for various proteases, hemolysins, and exotoxins as previously described. Genes encoding effector proteins of type III secretion systems (T3SS) and type VI secretion systems (T6SS) were targeted (Table 1).

**Table 1**. Primers used in this study targeting housekeeping genes for MLST analysis (*gyrB, rpoD, gyrA, dnaJ, dnaX, atpD*), and for putative virulence factors (remaining primers).

| Locus | PCR Primers | | | |
|---|---|---|---|---|
| | Product Name | Primer Name | Sequence | Ref. |
| *gyrB* | DNA gyrase subunit B | gyrB-F | TCCGGCGGTCTGCACGGCGT | [100] |
| | | gyrB-R | TTGTCCGGGTTGTACTCGTC | |
| *rpoD* | RNA polymerase, sigma 70 factor | rpoD-F | ACGACTGACCCGGTACGCATGTA | [100] |
| | | rpoD-R | ATAGAAATAACCAGACGTAAGTT | |
| *gyrA* | DNA gyrase subunit A | gyrA-F | ATGAGCGATCTGGCCAGAGA | [100] |
| | | gyrA-R | CGCGCCTTGTTCACCTGATA | |
| *dnaJ* | Chaperone HSP40, co-chaperone with DnaK | dnaJ-F | CGAGATCAAGAAGGCGTACAAG | [100] |
| | | dnaJ-R | CACCACCTTGCACATCAGATC | |
| *dnaX* | DNA polmyeraseIII/DNA elongation factor III, tau and gamma subunits | dnaX-F | CCATACGTTTGAACAAGTGG | [100] |
| | | dnaX-R | AGGGCATCGCGCATGCTGCC | |
| *atpD* | F1 sector of membrane-bound ATP synthase, beta subunit | atpD-F | CGGCATCAAGGTTATCGACCTG | [100] |
| | | atpD-R | ACCAGCGGATCCAGCTGACG | |
| *ela* | Elastase | ela-F | CGGGTGCACTACAGCCGCAA | This study |
| | | ela-R | CGGGCCGGGTCCTCGAAGTA | |
| *AChly* | *Aeromonas caviae* hemolysin | AChly-F | CTGGCTGATCCGCGGCAACA | This study |
| | | AChly-R | GTACCAGGCGTTGCCACCCC | |
| *pla* | Lipase | lip-F | CA(C/T)CTGGT(T/G)CCGCTCAAG | [102] |
| | | lip-R | GT(A/G)CCGAACCAGTCGGAGAA | |
| *ascF-G* | T3SS structural proteins | ascFG-fwd | ATGAGGTCATCTGCTCGCGC | [103] |
| | | ascFG-rev | GGAGCACAACCATGGCTGAT | |
| *ascV* | T3SS structural proteins | ascV-F | ATGGACGGCGCCATGAAGTT | [104] |
| | | ascV-R | TATTCGCCTTCACCCATCCC | |
| *aexU* | Effector toxin protein | AexU-F | TGGTGAACCGGCGCAAAGTG | [103] |
| | | AexU-R | ATATGAGCCAGCGCAGCCAG | |
| *laf* | Lateral flagella | lafA-F | GGTCTGCGCATCCAACTC | [105] |
| | | lafA-R | GCTCCAGACGGTTGATG | |
| *flaA* | Polar flagella | flaA-F | CAACCGCAACGCCAACGACG | This study |
| | | flaA-R | CGCGCGGCACTGACGTTTTC | |
| *alt* | Heat-liable cytotonic toxin | alt-F | AAAGCGTCTGACAGCGAAGT | [106] |
| | | alt-R | AGCGCATAGGCGTTCTCTT | |
| *ast* | Heat-stable cytotonic toxin | ast-F | ATCGTCAGCGACAGCTTCTT | [106] |
| | | ast-R | CTCATCCCTTGGCTTGTTGT | |
| *act* | Cytotoxic toxin | act-F | AGAAGGTGACCACCAAGAACA | [107] |
| | | act-R | AACTGACATCGGCCTTGAACTC | |

**Biochemical Analysis**

Two layers of a milk agar plate were prepared individually by separately autoclaving 2% (w/v) non-fat dry milk (Nestle) solution and 1.5% agar solution. When preparing plates, the solutions were cooled and mixed prior to pouring to approximately half the volume of the dish. After the agar had solidifyied, an overlay of Marine Agar 2216 (Difco) was added to form an upper nutrient layer. 10 μL of overnight broth culture of each strain in LB broth was spotted onto the surface of the medium and incubated overnight at 37˚C. Visible areas of clearing in the lower milk layer surrounding each cluster of growth was considered an indication of positive proteolytic activity [101].

A modified Congo red agar assay was used to detect biofilm production, as previously described by Hassan et al. The Congo Red Agar (CRA) medium was prepared with brain heart infusion broth (Oxoid) 37 g/L, sucrose 50 g/L, and 10 g/L agar. The Congo red indicator was separately prepared in an 8 g/L solution and mixed with the other components of the medium after sterilization. Plates were inoculated with each isolate and incubated overnight at 37˚C. Black colonies with a dry crystalline consistency indicated biofilm production [108].

Hemolytic activity was determined by growth on Tryptic Soy Agar with 5% sheep blood (Hardy Diagnostics). Areas of agar around the colonies that were transparent and yellow were recorded as beta-hemolysis. Green discoloration of the agar was recorded as alpha-hemolysis.

**Antimicrobial susceptibility**

The disc diffusion method was used to investigate antibiotic susceptibility of all strains to a range of antibiotic classes, most which have been previously cited as an effective treatment against *Aeromonas* infections [109-112]. Single colonies of overnight cultures on LB agar were picked, suspended in 3.0 mL sterile saline, and diluted to obtain turbidity of 0.5 McFarland turbidity standard. Sterile swabs were used to inoculate Mueller-Hinton agar and antibiotic discs were applied to the surface of the agar. After incubating overnight at 37˚C, the diameter of the zone of inhibition was measured and compared to the manufacturer's standards.

## Results

From the 81 fecal samples, a total of 161 unique colony morphologies were identified on TCBS, an average of two unique morphologies per stool sample. Each morphology type was picked in duplicate, yielding 310 total isolates. MLST analysis identified *Aeromonas* spp. in 17 (21%) of the fecal samples, and *V. cholerae* O1 and *V. cholerae* non-O1/O139 were identified in 39 (48%) and 17 (21%) of the samples. Both *V. cholerae* and *Aeromonas* spp. were isolated from the same fecal sample in seven cases, while no other pathogen was identified in the remaining ten. Ten of the 310 cultures could not be recovered after glycerol storage. The remaining isolates identified by 16S rRNA sequencing were found to belong to the genera *Enterococcus*, *Proteus*, and *Citrobacter* (Table 2).

**Table 2.** List of samples, strain ID, patient corresponding isolation sites, and species (NG – no growth, unable to recover after transport)

| Patient | Isolate | Sample | Species | Patient Town | Arrondissement | Department |
|---|---|---|---|---|---|---|
| **1** | A | 1 | *V. cholerae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **2** | A | 1 | *V. cholerae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| | C | 1 | *V. cholerae* | | | |
| **3** | A | 1 | *A. caviae* | Saint-Marc | Saint-Marc | Artibonite |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **4** | A | 1 | *E. faecalis* | Gonaïves | Gonaïves | Artibonite |
| **5** | A | 1 | *P. mirabilis* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *NG* | | | |
| **6** | A | 1 | *V. cholerae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *A. caviae* | | | |
| | B | 2 | *A. caviae* | | | |
| **7** | A | 1 | *V. cholerae* | Grande Saline | Dessalines | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| **10** | A | 1 | *P. mirabilis* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| **11** | A | 1 | *P. mirabilis* | Gonaïves | Gonaïves | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| **12** | A | 1 | *P. mirabilis* | Drouin | Saint-Marc | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *E. cloacae* | | | |
| **13** | A | 1 | *P. mirabilis* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *E. faecalis* | | | |
| | B | 2 | *E. faecalis* | | | |
| **14** | A | 1 | *A. caviae* | Chansolmes | Port-de-Paix | Nord-Ouest |
| | A | 2 | *A. caviae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | C | 1 | *E. gilvus* | | | |
| | D | 1 | *P. mirabilis* | | | |
| | E | 1 | *E. faecium* | | | |
| **15** | A | 1 | *P. mirabilis* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| **16** | A | 1 | *P. mirabilis* | Grande Saline | Dessalines | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| **17** | A | 1 | *V. cholerae* | Gonaïves | Gonaïves | Artibonite |

| | | | | | | |
|---|---|---|---|---|---|---|
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *A. caviae* | | | |
| | B | 2 | *A. caviae* | | | |
| | C | 1 | *V. cholerae* | | | |
| **18** | A | 1 | *P. mirabilis* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **19** | A | 1 | *V. cholerae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *A. caviae* | | | |
| | B | 2 | *A. caviae* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 3 | *P. mirabilis* | | | |
| **20** | A | 1 | *V. cholerae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **21** | A | 1 | *V. cholerae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| **22** | A | 1 | *V. cholerae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *A. caviae* | | | |
| | B | 2 | *A. caviae* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **23** | A | 1 | *V. cholerae* | Bassin Bleu | Port-de-Paix | Nord-Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *A. caviae* | | | |
| | B | 2 | *A. caviae* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **25** | A | 1 | *A. caviae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *A. caviae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **26** | A | 1 | *A. caviae* | Saint-Marc | Saint-Marc | Artibonite |
| | A | 2 | *A. caviae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **27** | A | 1 | *P. mirabilis* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *P. mirabilis* | | | |
| **28** | A | 1 | *V. cholerae* | En Plein | Gonaïves | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *A. caviae* | | | |
| | B | 2 | *A. caviae* | | | |
| | C | 1 | *P. mirabilis* | | | |

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|---|---|---|---|---|---|---|
| | C | 2 | *P. mirabilis* | | | |
| **29** | A | 1 | *P. mirabilis* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *P. mirabilis* | | | |
| **30** | A | 1 | *P. mirabilis* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **31** | A | 1 | *P. mirabilis* | Delmas | Port-au-Prince | Ouest |
| | A | 2 | *Enterococus* | | | |
| | B | 1 | *NG* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **32** | A | 1 | *V. cholerae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **33** | A | 1 | *NG* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| | C | 1 | *NG* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **34** | A | 1 | *P. mirabilis* | Petion Ville | Port-au-Prince | Ouest |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | C | 1 | *C. braakii* | | | |
| **35** | A | 1 | *P. mirabilis* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *E. faecalis* | | | |
| | B | 2 | *E. faecalis* | | | |
| **36** | A | 1 | *V. cholerae* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **37** | A | 1 | *V. cholerae* | En Plein | Gonaïves | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **38** | A | 1 | *V. cholerae* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **39** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **40** | A | 1 | *V. cholerae* | Arcahaie | Arcahaie | Ouest |
| | A | 2 | *V. cholerae* | | | |

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|---|---|---|---|---|---|---|
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **41** | A | 1 | *V. cholerae* | | | |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *V. cholerae* | | | |
| | B | 2 | *V. cholerae* | | | |
| **42** | A | 1 | *V. cholerae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| **43** | A | 1 | *V. cholerae* | Delmas | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *V. cholerae* | | | |
| | B | 2 | *V. cholerae* | | | |
| **44** | A | 1 | *V. cholerae* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *E. faecalis* | | | |
| | B | 2 | *E. faecalis* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **45** | A | 1 | *V. cholerae* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *E. faecalis* | | | |
| | B | 2 | *E. faecalis* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **46** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *V. cholerae* | | | |
| | B | 2 | *V. cholerae* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **47** | A | 1 | *V. cholerae* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **48** | A | 1 | *V. cholerae* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *V. cholerae* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **49** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *Enterococcus* | | | |
| | B | 2 | *Enterococcus* | | | |
| **50** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |

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|---|---|---|---|---|---|---|
| | B | 2 | *P. mirabilis* | | | |
| **51** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **52** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **53** | A | 1 | *A. caviae* | Cabaret | Arcahaie | Ouest |
| | A | 2 | *A. caviae* | | | |
| | B | 1 | *NG* | | | |
| | B | 2 | *P. mirabilis* | | | |
| | C | 1 | *E. faecium* | | | |
| | D | 1 | *E. faecalis* | | | |
| | E | 1 | *P. mirabilis* | | | |
| **54** | A | 1 | *A. caviae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *A. caviae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **55** | A | 1 | *V. cholerae* | Delmas | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *Enterococcus* | | | |
| | B | 2 | *V. cholerae* | | | |
| | C | 1 | *Enterococcus* | | | |
| | C | 2 | *V. cholerae* | | | |
| **56** | A | 1 | *V. cholerae* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **57** | A | 1 | *V. cholerae* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **58** | A | 1 | *A. caviae* | Petionville | Port-au-Prince | Ouest |
| | A | 2 | *A. caviae* | | | |
| | B | 1 | *NG* | | | |
| | B | 2 | *NG* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **59** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *V. cholerae* | | | |
| | B | 2 | *P. mirabilis* | | | |
| | C | 1 | *P. mirabilis* | | | |
| | C | 2 | *P. mirabilis* | | | |
| **60** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |

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|---|---|---|---|---|---|---|
| | B | 1 | *Enterococcus* | | | |
| | B | 2 | *Enterococcus* | | | |
| **61** | A | 1 | *V. cholerae* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **62** | A | 1 | *V. cholerae* | Delmas | Port-au-Prince | Ouest |
| | A | 2 | *P. mirabilis* | | | |
| | B | 1 | *V. cholerae* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **63** | A | 1 | *V. cholerae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **64** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| | C | 1 | *Enterococcus* | | | |
| | C | 2 | *Enterococcus* | | | |
| **65** | A | 1 | *V. cholerae* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *A. caviae* | | | |
| | B | 2 | *A. caviae* | | | |
| **67** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| **68** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **69** | A | 1 | *V. cholerae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **70** | A | 1 | *V. cholerae* | Montrouis | | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **71** | A | 1 | *V. cholerae* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **72** | A | 1 | *V. cholerae* | Arcahaie | Arcahaie | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **73** | A | 1 | *A. caviae* | Léogâne | Léogâne | Ouest |
| | A | 2 | *A. caviae* | | | |

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|---|---|---|---|---|---|---|
| **74** | A | 1 | *A. caviae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *A. caviae* | | | |
| **75** | A | 1 | *A. caviae* | Tabarre | Port-au-Prince | Ouest |
| | A | 2 | *A. caviae* | | | |
| **76** | A | 1 | *NG* | Port-au-Prince | Port-au-Prince | Ouest |
| | A | 2 | *NG* | | | |
| | B | 1 | *NG* | | | |
| | B | 2 | *NG* | | | |
| **77** | A | 1 | *V. cholerae* | Plaine | Gonaïves | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **78** | A | 1 | *V. cholerae* | Croix-des-Bouquets | Croix-des-Bouquets | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **79** | A | 1 | *V. cholerae* | Arcahaie | Arcahaie | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **80** | A | 1 | *V. cholerae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |
| **81** | A | 1 | *V. cholerae* | Cite Soleil | Port-au-Prince | Ouest |
| | A | 2 | *V. cholerae* | | | |
| | B | 1 | *P. mirabilis* | | | |
| | B | 2 | *P. mirabilis* | | | |

Alignment of sequences of the concatenated *rpoD-gyrB-atpD-dnaJ-dnaX-gyrA* genes of the *Aeromonas* isolates showed highest nucleotide sequence similarity to *Aeromonas caviae* (Fig. 2a, 2b, 3). Neighbor-joining phylogenetic trees indicated the isolates to be largely non-clonal, clustering closely with strains *A. cavaie* CECT838 (ATCC 15468), originally isolated from Guinea pigs, and *A. caviae* MDC51, and *A. caviae* MDC52, both originally isolated from snails [100]. MLST analysis revealed three distinct evolutionary groups and a fourth outlier, isolate 6B-1. None of these clustered groups were isolated from stool samples collected only from a specific geographical location within Haiti.

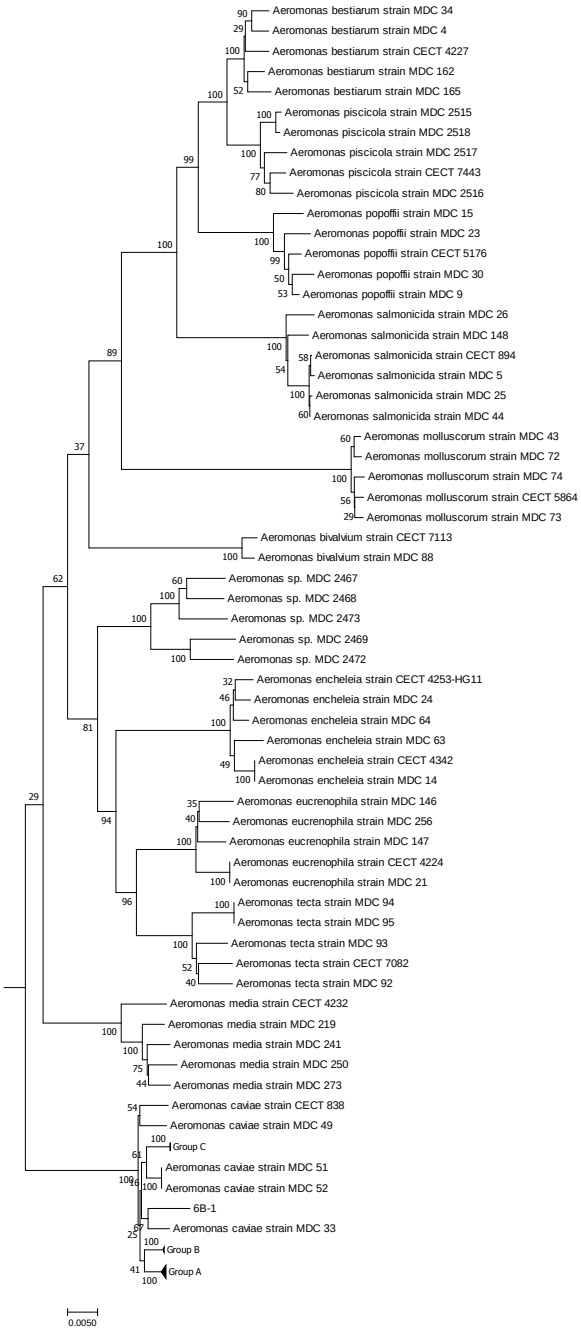


**Figure 2a** Phylogenetic tree of *Aeromonas* spp. based on five housekeeping genes. Evolutionary history inferred using the Neighbor-Joining method [113]. Percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to each branch [114]. Evolutionary distances were computed using the Maximum Composite Likelihood method [115]. The analysis involved 125 nucleotide sequences at a total of 3666 positions. Analysis conducted using MEGA7 [101].

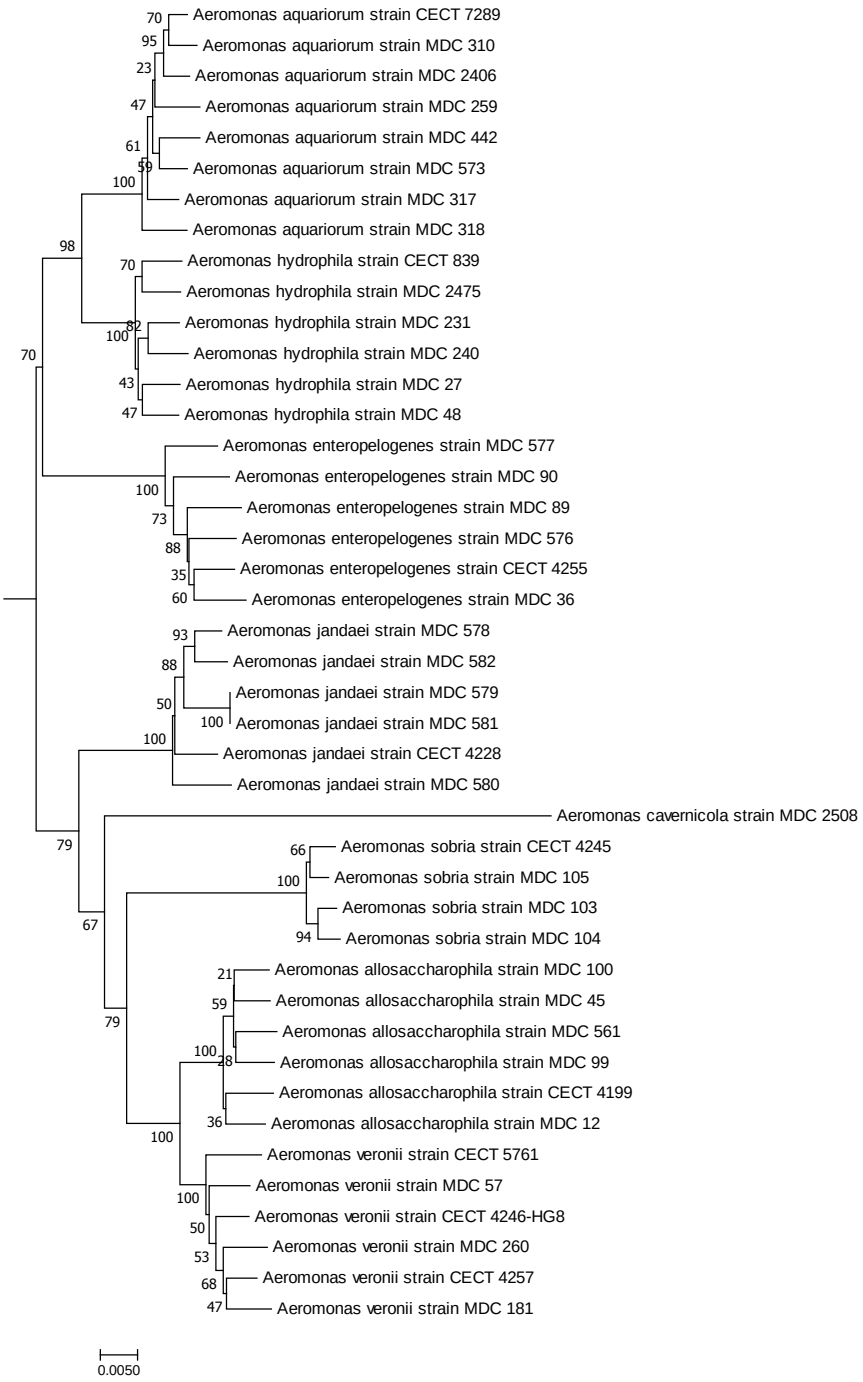


**Figure 2b** Additional phylogenetic tree of *Aeromonas* species based on five housekeeping genes.

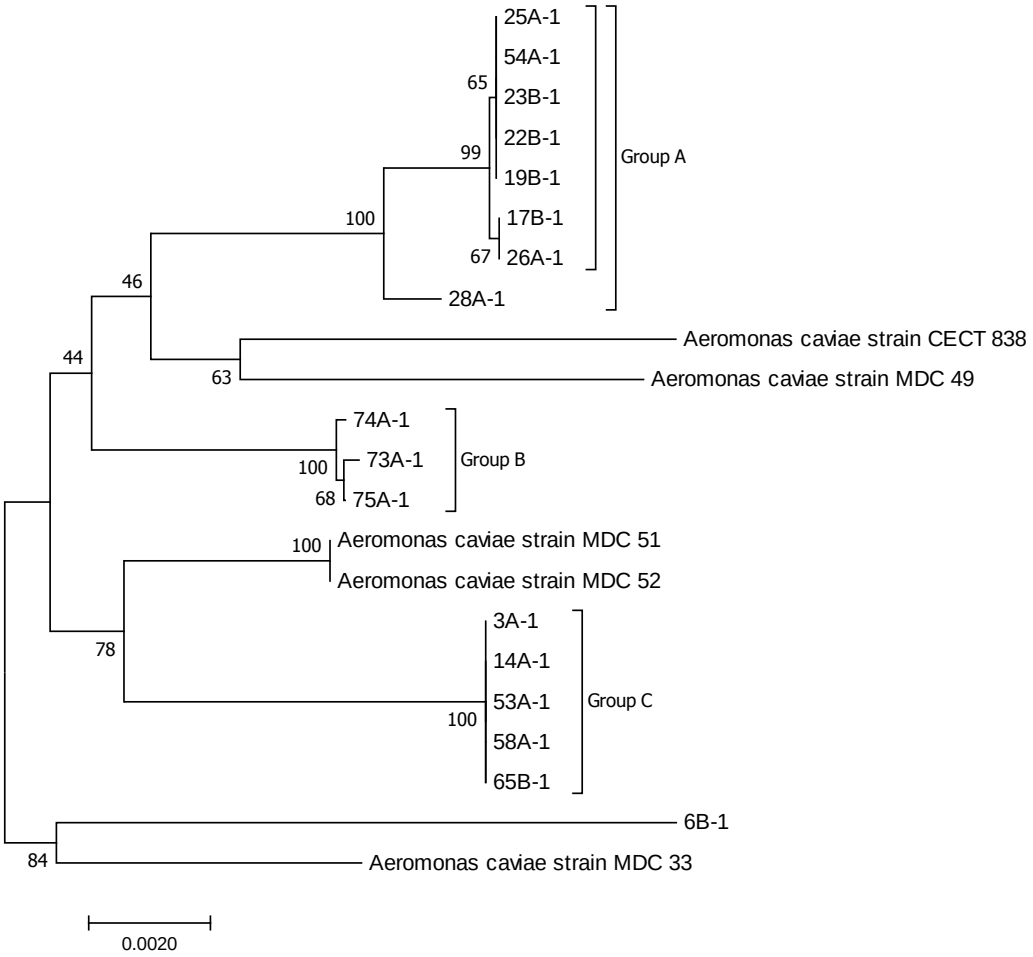


**Figure 3** Phylogenetic tree based on MLST analysis of*A. caviae* and the Haitian strains isolated in this study.

Three strains from a single phylogenetic outgroup of the MLST analysis demonstrated a unique colony morphology when grown on LB agar, that was suggestive of abundant biofilm production. After incubation for 24 hours, no hemolytic activity was observed on blood agar. However, after incubation for 48 hours at room temperature, the centers of growth of strains became yellow and transparent, indicative of beta-hemolysis. All strains produced clearing zones on skim milk plates, indicative of protease activity, and were confirmed to produce biofilm based on results of the Congo Red Agar assay.

Five putative virulence-related genes were detected in all 17 isolates: an elastase extracellular metalloprotease (*ahyB*); lipase (*pla*); flagella A (*flaA*); serine protease (*asp*); and T3SS effector protein (*aexU*). Four strains (24%) carried a gene for a hemolysin previously identified in other strains of *A. caviae* (AChlyA) and 13 (76%) possessed known genes coding for lateral flagella (*lafA*). The other virulence determinants were not detected.

Interestingly, 14 strains (82%) showed either resistance or intermediate resistance to ampicillin in the disc diffusion assay. All were resistant or showed intermediate resistance to trimethoprim/sulfamethoxazole, erythromycin, and tetracycline. Eight strains (47%) were resistant or, showed intermediate resistance to streptomycin, and another set of eight strains were resistant to kanamycin. All isolates were sensitive to ciprofloxacin, chloramphenicol, aztreonam, and imipenem.

**Table 3** Antimicrobial resistance pattern for each strain based on disc diffusion assay. Each is indicated as susceptible (S), intermediate (I), or resistant (R) to each antibiotic.

| Strain | Resistance (R), Intermediate Resistance (I), or Susceptibility (S) of Strains | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Ampicillin | Ciprofloxacin | Chloramphenicol | Trimethoprim/ Sulfamethoxazole | Streptomycin | Tetracycline | Aztreonam | Kanamycin | Imipenem |
| 3A-1 | R | S | S | R | I | R | S | S | S |
| 6B-1 | R | S | S | R | R | R | S | S | S |
| 14A-1 | R | S | S | R | R | R | S | S | S |
| 17B-1 | S | S | S | R | S | R | S | R | S |
| 19B-1 | R | S | S | R | S | R | S | R | S |
| 22B-1 | S | S | S | R | S | R | S | R | S |
| 23B-1 | R | S | S | R | S | R | S | R | S |
| 25A-1 | R | S | S | R | S | R | S | R | S |
| 26A-1 | R | S | S | R | S | R | S | R | S |
| 52B-2 | S | S | S | R | I | R | S | R | S |
| 54A-1 | R | S | S | R | I | R | S | S | S |
| 58A-1 | I | S | S | R | I | R | S | R | S |
| 65B-1 | R | S | S | R | I | R | S | S | S |
| 73A-1 | R | S | S | R | I | R | S | S | S |
| 74A-1 | R | S | S | R | S | R | S | S | S |
| 75A-1 | R | S | S | R | S | R | S | S | S |

**Table 4** Profiles of putative virulence genes present in each isolate as determined by PCR analysis.

| Strain | Presence or Absence of Putative Virulence Genes | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Elastase (*ela*) | *A. caviae* hemolysin (*AChlyA*) | Lipase (*pla*) | TTSS Structural Proteins (*ascF-G* \| *ascV*) | | Effector toxin (*aexU*) | Lateral flagella (*laf*) | Polar flagella (*flaA*) | Heat-stable cytotonic toxin (*ast*) | Heat-liable cytotonic toxin (*alt*) | Aerolysin (*asp*) | Cytotoxic enterotoxin (*act*) |
| 3A-1 | + | + | + | - | - | + | + | + | - | - | + | - |
| 6B-1 | + | + | + | - | - | + | - | + | - | - | + | - |
| 14A-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 17B-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 19B-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 22B-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 23B-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 25A-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 26A-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 52B-2 | + | - | + | - | - | + | + | + | - | - | + | - |
| 54A-1 | + | + | + | - | - | + | + | + | - | - | + | - |
| 58A-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 65B-1 | + | - | + | - | - | + | + | + | - | - | + | - |
| 73A-1 | + | + | + | - | - | + | + | + | - | - | + | - |
| 74A-1 | + | - | + | - | - | + | - | + | - | - | + | - |
| 75A-1 | + | - | + | - | - | + | - | + | - | - | + | - |

## Discussion

In this study, 21% of the fecal samples collected in Haiti in November, 2010, were positive for *A. caviae*, similar to previously established isolation rates from patients suffering gastroenteritis [49]. The exact role of *A. caviae* during the early weeks of the 2010 Haitian cholera outbreak remains unclear since the conclusion was rapidly made that all cases presenting to the hospitals were cholera victims. All *A. caviae* strains exhibited virulence potential when tested using skim milk agar, blood agar and were found to carry genes encoding potential virulence factors. However, because the exact mechanism of pathogenicity of *Aeromonas*-associated gastroenteritis is not yet fully known, and no single virulence factor or set of virulence factors have been directly linked with illness caused by *Aeromonas* spp., it is difficult to define these factors as the cause of the symptoms of enteric disease of each individual from whom the samples had been collected.

Unfortunately, detailed records or repeat samples from each patient could not be obtained due to the turbulent social, political, and physical environment of the first few weeks of the devastating disease outbreak. Sample collection often was done at patient homes by a physician working alone, with priority clearly and appropriately placed on rapid treatment, rather than sample collection, filling out questionnaires, or consideration of further data collection long-term.

Many studies have hypothesized that *Aeromonas* spp. may interact synergistically with other pathogens to cause diarrhea [44-46]. Severity, duration, and symptoms of each individual providing a sample would have provided insight

into this possibility, particularly for those cases where *V. cholerae* was the only pathogen isolated, compared to cases where both *V. cholerae* and *A. caviae* were isolated.

However, studies monitoring incidence of *Aeromonas* spp. in clinical fecal samples are important in deciding whether to accept or reject the hypothesis that *Aeromonas* spp. cause gastroenteritis in humans. A few previous studies established clonal outbreaks of *Aeromonas* infection, with a single genetically similar *Aeromonas* spp. isolated from both clinical fecal samples and the environment. Here, we report three groups of genetically identical clinical isolates collected essentially simultaneously from several distant geographic regions of Haiti. For example, isolate 23B-1 from Bassin-Bleu, is located in the same MLST group as 54A-1, from a sample collected in Cité Soleil, 142 kilometers away. These data suggest one or more clonal strains of *A. caviae* were endemic in Haiti during late 2010.

Symptoms of *Aeromonas*-related gastroenteritis that have been reported in earlier studies have varied widely, ranging from moderate acute diarrhea to "cholera-like" symptoms [29, 55, 57]. Thus, clinical diagnoses, based on symptoms alone, as is often done in rapid treatment during major outbreaks, may not be reliable in identifying the etiological agent. Particularly in resource-limited areas such as Haiti, diagnosis is performed based on symptoms alone, out of necessity, to provide rapid treatment and the infrastructure for laboratory identification of the clinical isolates often is not available in that developing country. Both *Aeromonas* spp. and *Vibrio cholerae* are ubiquitous in the

environment, have similar routes of person to person transmission, and often have been reported to cause identical symptoms [54-57]. It may be difficult to fully understand the exact role *A. caviae* played in the initial onset of the Haitian cholera epidemic, but these results clearly indicate the importance of monitoring for *Aeromonas* spp. in major outbreaks of enteric disease. It is concluded from the results of this study that there is a clear need to investigate further the role played by *Aeromonas* spp. in disease.

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