1-1-2012

Potential of Great Egrets to be Vectors for the Transmission of s Virulent Strain of Aeromonas Hydrophila between Channel Catfish Culture Pond

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Potential of great egrets to be vectors for the transmission of a virulent strain of *Aeromonas hydrophila* between channel catfish culture pond

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A Thesis
Submitted to the Faculty of
Mississippi State University
in Partial Fulfillment of the Requirements
for the Degree of Master of Science
in Veterinary Medical Sciences
in the College of Veterinary Medicine

Mississippi State, Mississippi

August 2012

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Title of Study: Potential of great egrets to be vectors for the transmission of a virulent strain of *Aeromonas hydrophila* between channel catfish culture pond

Pages in Study: 56

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Aeromonas hydrophila is a Gram-negative, rod shaped, facultative anaerobic bacterium that is ubiquitous to freshwater and slightly brackish aquatic environments and can cause infections in fish, humans, reptiles, and avian species. Recent severe outbreaks of disease in catfish aquaculture have been associated with a highly virulent *Aeromonas hydrophila* strain (VAH) that is genetically distinct from less virulent strains.

Given that *A. hydrophila* is known to infect birds, we hypothesized that fish eating birds may serve as a reservoir for VAH and spread the pathogen by flying to uninfected ponds. Great Egrets were used in this transmission model because these wading birds frequently predate catfish farms. We found that Great Egrets that were fed VAH infected catfish shed VAH demonstrating their potential to spread VAH. Histologically there were changes found in selected tissue samples.

Keywords: *Adrea alba,* aquaculture, *Ictalurus punctatus*, *Aeromonas hydrophila*, Virulent Aeromonas Hydrophila (VAH)

DEDICATION

I would like to dedicate this to the people in my life that have always stood by me through the good times and the bad times. First I would like to thank my parents Mr. & Mrs. Michael Jubirt for always pushing me to follow my dreams and never settle for good enough or second best. Next I would like to thank my sister Regan Jubirt for always being a listening ear. Many days and nights I would call her to discuss my good days with research as well as my bad days, even though she did not understand exactly what I was talking about she would always find a way to make me feel better about the situation. I strive to be like my mentor Dr. Patrice Robinson and I am forever indebted to her for all of her advice, encouraging words, and support throughout this process. She has taught me that no matter what have confidence in yourself, if someone else has done it so can you! Last but not least I would like to thank my grandmother for always encouraging me to never give up and have faith in God that he will see me through to the end. Thank you all from the bottom of my heart!

ACKNOWLEDGEMENTS

I would like to thank my committee members Dr. Larry Hanson, Dr. Fred Cunningham and Dr. Lora Hanson who supported and guided me throughout this process. I would like to thank Dr. Matt Goodwin for supplying us with the VAH specific primers and probes that made this assay possible. Dr. Lester Khoo thank you for supplying the bacterial isolates those were essential in my project. I would like to thank my lab mates Kamalakar Chatla, and Du Nyugen who were always helpful from answering questions to being a listening ear. Lorelei Ford, I owe you a HUGE thank you for always listening and answering my "silly questions", formatting my thesis, and being so kind and patient! I would like to thank Dr. Claudia Hohn for being very supportive throughout this process and always giving me advice. I would also like to thank Allen Shack for being a confidant, math teacher, and supportive friend. I couldn't have analyzed my histological samples without the expertise of Dr. Skip Jack, Thank you. I cannot forget to thank Katie Hanson Dorr, Scott Lemmons, and Paul Fioranelli for braving the freezing cold December mornings to help me take care and feed the birds.

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CHAPTER I

INTRODUCTION

Aeromonas is a genus of facultative anaerobic, rod shaped, Gram-negative bacteria (Sartory, 2002). Aeromonads are indigenous to aquatic habitats and are able to survive with or without a host, ubiquitous, and capable of utilizing available nutrients in their environment (Sartory, 2002). Aeromonads are most frequently found in fresh waters but also occur in brackish waters and the distribution in water is correlated with organic load (Camus, Durborow et al., 1998; Sartory, 2002). Phenotypic and genetic characteristics divide the motile aeromonads into three species, *A. hydrophila, A. caviae,* and *A. sobria* (Krieg, 1984). Motile aeromonads are motile by polar flagella, cytochrome oxidase positive, ferment glucose with or without the production of gas, and are insensitive to the vibriostatic agent R129 (Cipriano, Bullock et al., 1984).

The exact etiology of disease involving aeromonads are not completely understood (Cipriano, Bullock et al., 1984). Motile aeromonads are often referred to as a complex of disease conditions in fishes and the ability to cause disease varies by strain (Cipriano, Bullock et al., 1984; Camus, Durborow et al., 1998). Aeromonads express extracellular toxins and enzymes but the primary toxins produced are hemolysins (Sartory, 2002). A*eromonas hydrophila* and *A. sobria* express the important hemolysin aerolysin and it has been reported that environmental temperatures effect the activity of hemolysins and proteases of aeromonads (Sartory, 2002).

Recent work has demonstrated that bacterial infections caused by motile aeromonad species are exceedingly prevalent and troublesome in fish operations (Camus, Durborow et al., 1998). Aeromonads are highly prevalent in aquatic habitats, this prevalence along with stressful situations has lead to disease in many cultured fish species (Camus, Durborow et al., 1998). Some of the clinical signs seen in infected fish are a distended abdomen, scale profusion disease, tail or fin rot, ocular ulcerations, red rot disease and skin ulcerations (Cipriano, Bullock et al., 1984).

A majority of the information reported on the relationship between *Aeromona*s and birds is limited to diagnostic submissions of birds that have been killed by the disease but the history, prevalence, and other factors are not extensively researched (Brittingham, Temple et al., 1988). Shane *et al*. (1984) investigated the occurrence of *Aeromonas hydrophila* in routine avian diagnostic submissions. The objective of the study was to notate the prevalence of *A. hydrophila* in avian species over a 25 month period (Shane, Harrington et al., 1984). *Aeromonas hydrophila* was isolated from 2% (20 out of 1000 cases) during the test period. From the results, the investigators concluded that *A. hydrophila* behaved as facultative and opportunistic pathogen.

Glunder and Siegmann (1989) researched the relationship between avian species, habitat, food type, and the rate at which *A. hydrophila* could be recovered from post mortem avian submissions. They concluded the intestines were the primary isolation site of *A. hydrophila*, and secondary isolation site included the lungs. In their study they found carnivorous aquatic birds had the highest *A. hydrophila* recovery rate compared to terrestrial avian species (Glunder and Siegmann, 1989).

A virulent strain of *Aeromonas hydrophila* (VAH) emerged as a serious threat to the farm raised catfish industry in 2009 and 2010. This bacterium rapidly kills large

numbers of fish with cumulative mortality often exceeding 50% of the population. West central Alabama was ground zero for the problem but it has spread to East Mississippi, Arkansas and recently to the Mississippi Delta. Historically all three motile Aeromonas species have been routinely found in channel catfish but these infections were generally considered to be secondary to another infection or a debilitating stressor and was not considered to be a significant problem to the farm raised catfish industry. This strain prefers hot climates it is believed to originate specifically in Southeast Asia.

The clinical signs of the VAH septicemia are similar to Enteric Septicemia of Catfish (ESC), which includes hemorrhages in the irises, internal and tissue hemorrhages, and ulcers. The VAH spreads throughout the entire fish and grows very quickly. It has been reported that that Channel Catfish Virus and Viral Hemorrhagic Septicemia are not contributing factors. Biochemically VAH is unusual compared to the more common *Aeromonas* isolates. They have the characteristic ability to ferment many complex sugars, are resistant to vibriostat 0/129 and produce indole, but unlike the others VAH utilizes inositol and is citrate positive.

Several fish pathologists in the southern United States have formed an informal working group to determine factors associated with motile aeromonad septicemia (MAS) outbreaks in the West Alabama region. Motile Aeromonad septicemia is known as the infection caused by motile aeromonads. Among the discussed topics were: 1) what unique characteristics have made the industry in West Alabama most susceptible to MAS? and 2) how is this new disease spreading?. One possibility that was raised is West Alabama has a much higher prevalence of wood storks that scavenge on diseased ponds. Apparently, wood storks are unique among the fish eating bird in that they are more willing to eat dead and decomposing fish. However, wood storks are endangered and a

protected species. Therefore, we proposed to evaluate the potential for fish eating birds to transmit and spread of the strain of *Aeromonas hydrophila* that is responsible for massive catfish deaths in Alabama and East Mississippi using great egrets as the model bird species. In this study we evaluated the ability great egrets to shed viable VAH when fed infected fish and evaluated the potential for VAH to colonize these birds.

CHAPTER II

LITERATURE REVIEW

Taxonomy of *Aeromonas*

Historically the genus *Aeromonas* has been placed within different families (Sartory, 2002). Among gram negative bacteria, the cytochrome-C oxidase positive characteristic of *Aeromonas* distinguishes them from *Enterobacteriaceae*. *Pseudomonas* produces lipase, is not fermentative and does not produce gas from D-glucose, whereas *Aeromonas* has these abilities (Krieg, 1984). Unlike Aeromonas, *Vibrio* has a sheathed polar flagella, a sensitivity to vibriostatic agent O/129, and is commonly found in marine waters (Krieg, 1984). With *Vibrio* sodium is required for growth or stimulates growth but this is not a requirement for Aeromonad growth *(Krieg, 1984)*. The Aeromonads are distinguished from *Pseudomonadaceae* and *Vibrionaceae*; their classification at a specific level is under debate (Krieg, 1984).

Motile Aeromonads are cytochrome oxidase positive, ferment glucose with or without the production of gas, and are insensitive to the vibriostatic agent O/129 (Cipriano, Bullock et al., 1984). There are many factors that contribute to the ever changing classification of this genus (Cipriano, Bullock et al., 1984). The genus *Aeromonas* was transferred from the genera *Bacillus*, *Pseudomonas*, *Proteus*, and *Aerobacter* into its own separate genus (Cipriano, Bullock et al., 1984). Many viewpoints favor a single species for all motile Aeromonads, but traditionally the motile Aeromonads have been divided into three species based on phenotypic and genetic

characteristics: *A. hydrophila, A. caviae, and A. sobria* (Krieg, 1984). However, there are at least 19 different species of *Aeromonas* that have been described, which include *A. hydrophila, A. caviae, A. sobria, A. veronii, and A. schubertii,* and the non-motile, *A. salmonicida.*

Pathogenesis of *Aeromonas* Species

Aeromonads express extracellular toxins and enzymes but their primary produced toxins are hemolysins (Sartory, 2002). *A. hydrophila* and *A. sobria* express the important hemolysin aerolysin (Sartory, 2002). Hemorrhaging and septicemia are common characteristics of infection with motile Aeromonads, and the hemolysins that are responsible could be a major factor in pathogenicity for these bacteria (Santos, Toranzo et al., 1988). However, not all Aeromonads require a hemolysin to be pathogenic (Santos, Toranzo et al., 1988).

Hemolysins are cytolysins that insert themselves into the cell wall and cause the content of the cytoplasm to leak. Two forms of hemolysins (α and β) have been reported for motile Aeromonads (Pollard, Johnson et al., 1990; WHO, 2002). *Beta*-hemolysins cause complete lysis of erythrocytes and a clearing zone on Blood Agar plates. Aeromonads that express *Beta*-hemolysins have been reported to cause diarrheal infections in humans (Santos, Toranzo et al., 1988). *Alpha*-hemolysin, cause cell leakage but no lysis of erythrocytes and are of minor importance in the pathogenesis of *Aeromonas* infections (Pollard, Johnson et al., 1990). Environmental temperatures effect the activity of hemolysins and proteases of aeromonads (WHO, 2002).

Several authors have studied the variation in the extracellular enzymes that motile aeromonads produce. Janda (Janda, 1985) investigated the biochemical and

exoenzymatic properties of *Aeromonas species* by evaluating 127 isolates of *A. hydrophila, A. sobria, and A. caviae (Janda, 1985)*. In the exoenzymatic studies, it was found that *Aeromonas* species produce amylase, DNase, RNase, esterase, lipase, gelatinase, protease, fibrinolysin, and chitinase (Janda, 1985). The enterotoxigenic markers were found predominately with *A. hydrophila* and *A. sobria* species isolates, which suggests that the two species are possibly the major enteric pathogens (Janda, 1985). Aeromonads extracellular proteases have shown to be been lethal to channel catfish (*Ictalurus punctatus*), common carp (*Cyprinus carpio*), and American shad (*Alosa sapidissima*), and several other species (Thune, GRAHAM et al., 1982). Enterotoxins, hemolysins, proteases, hemagglutinins, and endotoxins produced by *Aeromonas* has been the subject of much research (Cipriano, Bullock et al., 1984).

The enteropathogenicity of *Aeromonas hydrophila* has been extensively studied. The pathogenicity of *Aeromonas hydrophila* was first investigated by Sanyal *et al*. (1975). Samples of *A. hydrophila* were isolated by the investigators from, potable water, the Ganges River and from fecal samples of adults with diarrhea and non-symptomatic adults and children. The exact cause of the diarrhea was unknown in the compromised patients. They then inoculated rabbit intestinal loops with *Aeromonas* from the collected samples. They found that only certain strains of *Aeromonas hydrophila* caused fluid accumulation in the ligated gut loops. The fluid accumulation in the gut indicated that the majority of strains were enterotoxic regardless of the isolation source.

Annapurna and Sanyal (1977) investigated the enterotoxicity of 50 isolates of *Aeromonas* from domestic animals, chickens, water and children and adults with diarrhea or healthy individuals. Fluid accumulation in the rabbit ileal loops were caused by 47 out of 50 strains of *A. hydrophila* (Annapurna and Sanyal, 1977). Although fluid

accumulation was noted in the rabbit guts, it differed among the strains that were cultured. The authors reevaluated those strains that gave no fluid accumulation and fluid accumulation was eventually noted after two additional passages. The fluid accumulation in the gut from the various sources again showed that the majority of strains were enterotoxic regardless of the isolation site.

A heat-stable and a heat-labile enterotoxin are produced by *Aeromonas hydrophila* (Gemmell, 1984), but the strains that caused diarrhea in normal individuals produced only the heat-labile enterotoxin (Gemmell, 1984). Aeromonads that caused opportunistic infections in immunocompromised patients produced the heat-labile enterotoxin (Gemmell, 1984; Morgan, Johnson et al., 1985).

Some enterotoxins are not cytotoxic and thus may not directly cause cell damage. For example many cytotonics (enterotoxins that morphologically change, but do not kill, the target cell) cause the outpouring of water and chloride ions into the intestinal lumen (Gemmell, 1984). There are two cytotonic enterotoxins expressed by aeromonads. They are classified based on size and the enzymes they activate (Gemmell, 1984). The first toxin has a molecular mass of 70-90KDa and activates adenylate cyclase and the second enterotoxin is 5-10 KDa and activates guanylate cyclase (Gemmell, 1984).

Aeromonas sobria protease (ASP) is another secreted virulence factor. It is serine protease released that cleaves the C5 component of the complement system and releases the C5a (small) and C5b (large) domains (Nitta, Imamura et al., 2008). The protease releases impaired C5b domains which are unable to form the membrane attack complex. In conjunction with a nonfunctional membrane attack complex the protease releases large concentrations of C5a domain, which disables the neutrophils and interrupts further pathway signaling.

The cell envelope of Aeromonads plays a pivotal role in their toxicity. Gram-negative bacteria have a cytoplasmic membrane, peptidoglycan layer, and outer membrane (Wiese, Reiners et al., 1996). The outer membrane contains lipopolysaccharides (LPS See Fig1.). The LPS has either an oligo- or polysaccharide section attached to the A lipid component. The lipid A component is what attaches the LPS to the outer membrane and porins (pores which are an additional essential membrane protein) allow hydrophilic substrates to pass through them. The function of LPS layer in Gram-negative bacteria has been hypothesized to help maintain structure and aid in protection from innate defenses.

Another important virulence factor associated with the cell envelope of *A. hydrophila* is the Type Three Secretion System (T3SS). *Aeromonas hydrophila* uses the T3SS to inject effector proteins into host cells (See Figure 2) (Vilches, Jimenez et al., 2009). The following bacterial functions have been shown to have controlling effects on the T3SS: Lateral and Polar flagella, O-antigen LPS, Quorum-sensing system, Pyruvate dehydrogenase complex, and PhoPQ two-component system. The authors concluded that precise timing and coordination of all the aforementioned bacterial functions are essential to *A. hydrophila* infectious capabilities.

Aeromonads also have an S-layer that may be a virulence factor. The S-layer is a structural envelope component that is found in the outermost cell membrane of many bacterial species (Ilk, Kosma et al., 1999; Sara and Sleytr, 2000). S-layers are composed of several subunits that come together to form oblique, square, or hexagonal lattice configurations (Ilk, Kosma et al., 1999). Research has shown that S-layers are also in a two-dimensional crystalline array, which combines to cover the entire cell (Sara and Sleytr, 2000; Messner, Steiner et al., 2008). S-layers have been identified in Gram-

positive and Gram-negative bacteria and most are 5nm to 25nm thick (Sara and Sleytr, 2000). S-layer glycoproteins from the *Bacillaceae* family have O-linked glycans that are comprised of N- or 0-glycosidic linkages (Sleytr and Sara, 1997; Messner, Steiner et al., 2008). The S-layers have various lattice types and are made of glycoprotein subunits that are roughly spaced 2.5nm to 3.5 nm apart (Sara and Sleytr, 2000). The arrangement of the lattice type structures resemble the O-antigen component of the lipopolysaccharide section in Gram-negative bacteria (Sleytr and Sara, 1997). The S-layers provide support to the outer membrane and contributes to the virulence capabilities of pathogenic bacteria (Sleytr and Sara, 1997; Sara and Sleytr, 2000).

Natural Sources of *Aeromonas* Species

Water Sources

Aeromonas spp. are commonly found in sludge, sewage, and surface water (Glunder and Siegmann, 1989; Egwari and Aboaba, 2002). Selective differential media can be used to culture *Aeromonas hydrophila* for the quantitative evaluations from environmental sources. These media include starch ampicillin (SA) , ampicillin dextrose (AD), peptone- beef extract-glycogen (PGB), ampicillin-trehalose (mA), lactose bile salts (m-Endo), brilliant green bile salts-starch (BGBSS) (Handfield, Simard et al., 1996). The strains of *Aeromonas hydrophila* appeared differently on each media type. *Aeromonas hydrophila* on AD appeared yellow with dark center and on BGBSS purple with dark center. *Aeromonas hydrophila* grown on mA appeared yellow, on PBG green with dark center, and on SA yellowish (Handfield, Simard et al., 1996).

The presence of *Aeromonas hydrophila* in water samples can be confirmed using the API 20E system and biochemical tests. The following biochemical tests confirmed

Aeromonas hydrophila: production of gas from glucose, *L*- arabinose, and salicin fermentation, growth at 42 ° C, esculin hydrolysis, and utilization of *L*- arganine and *L*- ornithine *(Handfield, Simard et al., 1996)*.

Motile Aeromonads are among the most abundant bacteria found in fresh and brackish waters (Camus, Durborow et al., 1998). *Aeromonas hydrophila* is more prevalent in cleaner waters as *Aeromonas caviae* is reportedly more frequent in fecal polluted waters (Pianetti, Falcioni et al., 2005). *Aeromonas spp.* can survive extended periods of time with limited nutrients by entering a starvation survival state and are highly resilient pathogens (Pianetti, Falcioni et al., 2005). These pathogens can also be isolated from biofilms on surfaces of pipes and bottles (Pianetti, Falcioni et al., 2005).

Fish Sources

Bacterial infections caused by motile aeromonad species are prevalent and troublesome in fish operations (Camus, Durborow et al., 1998). Motile aeromonads are widely distributed in nature and are recognized worldwide for their abilities to cause septicemia in poikilothermic and homeothermic species (Cipriano, Bullock et al., 1984; Santos, Toranzo et al., 1988). The prevalence of aeromonads along with stressful situations that occur in aquaculture has lead to disease in many cultured fish species (Camus, Durborow et al., 1998). The infections caused by motile Aeromonads have been referred to many names such as motile Aeromonad septicemia (MAS), motile Aeromonad infections (MAI), and red pest (Camus, Durborow et al., 1998). The most common disease caused by motile Aeromonads is hemorrhagic septicemia. Motile Aeromonad hemorrhagic septicemia outbreaks have been reported in populations of

Common Carp, Channel Catfish, Striped Bass, and Largemouth Bass (Cipriano, Bullock et al., 1984).

Aeromonas spp. can affect fish acutely, chronically and covertly (Cipriano, Bullock et al., 1984). Environmental stress is a common factor that determines how severe the disease will present in fish populations (Cipriano, Bullock et al., 1984). The infection can occur in any species, age, or sex of fish, but it is seen most frequently in younger fish (Camus, Durborow et al., 1998). Bacterial virulence, condition of the host, and the degree of genetic resistance are also other factors that affect how the disease is expressed (Cipriano, Bullock et al., 1984). Some of the clinical signs seen in infected fish are a distended abdomen, scale profusion, tail or fin rot, ocular ulcerations, and skin ulcerations (Cipriano, Bullock et al., 1984). The chronic outbreaks cause low level mortality over an extended period and are more often associated with external ulcers. Acute outbreaks often have a very high mortality, a short onset and more frequently display hemorrhagic septicemia (Camus, Durborow et al., 1998). If an *Aeromonas* outbreak should occur in a fish operation every attempt should be made to identify and to correct any predisposing factors in the operations (Camus, Durborow et al., 1998). Other means of disease prevention are to ensure that seines and harvest equipment have been properly sanitized. These prevention techniques can often correct the disease problem and reduce future outbreaks.

Reptile Sources

In addition to fish, *Aeromonas spp.* cause disease in avian species, mammals including humans, reptiles, and amphibians (Pasquale, Baloda et al., 1994). Pasquale *et al*. reported on an outbreak of *A. hydrophila* in illegally imported turtles at a pet shop in

Naples, Italy. Of the 100 turtles, 95 died from an acute infection in 10 days. The turtles exhibited clinical signs of lethargy, refusal to eat, and depression and gross pathology was performed on 21 turtles. Samples were taken from the kidney, liver, lungs, and the heart. *Beta*-hemolytic *A. hydrophila* was cultured from the affected turtle, which is recognized as a human pathogen. This could pose a public health concern since turtles are a growing house hold pet and could possibly be a vector for disease from turtles to humans, special precautions should be taken.

Infectious stomatitis caused by *Aeromonas* has been demonstrated in multiple species of snakes (Page, 1966; Goldstein, Agyare et al., 1981). The Ecuadorian king snake (*L. doliata*) Tree boa (*Boa endyris cooki*); Mexican boa (*Constricter Mexicana*); and Water snake (*N. rhombifera*) species have reportedly been pathogenic hosts for *A. hydrophila* (Page, 1966).

Ulcerative inflammations caused by aeromonads in the skin of frogs and toads are common at temperatures of 20°C and below (Page, 1966). As an opportunistic pathogen whenever a stressor is involved A. hydrophila becomes pathogenic. In frogs *A. hydrophila* is naturally found in the gastrointestinal track but commonly colonizes the skin and visceral organs (Hill, Newman et al., 2010). Hill *et al*., reported on the first concurrent infection with *A. hydrophila, Mycobacterium spp*., and *Batrachytrium dendrobatidis* in African Clawed frog. Some of the clinical signs observed in *A. hydrophila* infection include ulcerations, lethargy, and anorexia (Hill, Newman et al., 2010). The authors believed infrequent water changes, stress, and *Mycobacterium spp*. infections lead to the A. *hydrophila* infection.

In another case report red leg disease caused by *A. hydrophila* killed 13 Chinese brown frogs (*Rana chensinensis*). The frogs were hyperemic and had hemorrhages in the

gastrointestinal tract and legs and these characteristics are commonly found in frogs that have red-leg disease(GE, ZHANG et al., 2012). The authors identified two strains of *A. hydrophila.* Strain A had a higher virulence and was isolated from a larger number of isolates compared to strain B (GE, ZHANG et al., 2012).

Human Sources

The most commonly discussed mesophilic motile Aeromonads are the causative pathogens of gastroenteritis in children and adults (Sartory, 2002). *Aeromonas spp.* are linked with two types of gastroenteritis. The cholera-like gastroenteritis usually requires medical attention (Subashkumar, Thayumanavan et al., 2006). The more severe dysenteric gastroenteritis causes loose stools filled with blood and mucus (Rahman and Willoughby, 1980). *Aeromonas* infections also cause Cellulitis and Myonecrosis, especially in immunocompromised patients (Rahman and Willoughby, 1980; Chou, Tsai et al., 2004). A wide range of virulence has been documented with motile Aeromonad isolates from humans (Santos, Toranzo et al., 1988). *Aeromonas hydrophila* is wildly known as the causative agent for diarrhea in immunocompromised children and adults (Subashkumar, Thayumanavan et al., 2006). Sample strains of *A. hydrophila* taken from man and water sources were enterotoxic regardless of isolation site (Annapurna and Sanyal, 1977). Proteases, slime, secreted hemolysins, and enterotoxins have been found to be expressed by several clinical and environmental isolates collected by investigators (Davis, Chretien et al., 1978).

Occurrence of *Aeromonas* in Avian Species

A majority of the information reported on the relationship between *Aeromonas* and birds is limited to diagnostic submissions of birds that have been killed by the disease

but the history, prevalence, and other factors are not extensively researched (Brittingham, Temple et al., 1988).

There have been several case reports on the occurrence and pathogenic effects of *Aeromonas* in birds. These disease cases include a 10 year old male ostrich (*Struthio camelus*) demonstrating necrotizing enteritis and septicemia (Franca, Walker et al., 2009), an African Grey Parrot (Akkoc, Kocabiyik et al., 2008), with hemorrhaging in the skull and ascites, a captured Ground-Hornbill (*Bucorvus abyssinicus*) with petechial hemorrhages in the lungs, liver, kidney, epicardium, endocardium, and in the serosa (Ocholi and Kalejaiye, 1990), canaries (*Serinus canaries*) with enteritis, a toucan (*Ramphastos toco*) with nephritis, and two cockatiel (*Nymphicus hollandicus* (Panigrahy, Mathewson et al., 1981).

The occurrence of *Aeromonas hydrophila* in routine avian diagnostic submissions to notate the prevalence of *Aeromonas hydrophila* in avian species over a 25 month period was investigated by Shane *et al*. (1984). Twenty isolates of *A. hydrophila* were obtained from 15 bird species, and 70% of the case occurred during the winter months they concluded that the climatic change stressors predisposed the birds to *Aeromonas* infections. *A. hydrophila* was isolated from 2% of the submissions during the test period. From the results, the investigators concluded that *A. hydrophila* behaved as a facultative and opportunistic pathogen.

Shane *et al*., conducted an additional survey to investigate the prevalence and pathgenicity of *A. hydrophila* in live, companion, and exotic avian species (Shane and Gifford, 1985). The bacterial samples from the various species were isolated from the liver, lung and brain (Shane and Gifford, 1985). *Aeromonas hydrophila* was recovered in only 8% of the 141 specimens sampled. The results revealed that chicks and poults were

highly susceptible to *Aeromonas* via the subcutaneous and yolk sac routes and a mortality rate of 80-100% occurred within 48 hrs of exposure. The ducklings sampled were unaffected and did not respond to the *A. Hydrophila.* The present study revealed that the prevalence of *A. hydrophila* is found widely in multiple avian species and can be isolated from multiple organs.

Although the normal intestinal flora of wild birds has been investigated, it is not well documented. Glunder and Siegmann researched the relationship between wild birds habitat, food type, and the rate at which *A. hydrophila* could be recovered from post mortem avian submissions and documented the habitat, nutrition and climate areas (Glunder and Siegmann, 1989). Most samples were obtained from birds that were dead when submitted a few that were killed for necropsy. The intestines were the primary isolation site of A. *hydrophila*, but isolates were also found in the lungs. *Aeromonas hydrophila* isolated from the intestines was found less frequently in terrestrial avian species (3.4%) versus aquatic birds (18.5%). *Aeromonas hydrophila* was isolated more frequently in carnivores (12%) versus omnivores (8.4%) and herbivores (7%).

In summary, A. *hydrophila* has several virulence factors that contribute to its disease causing ability. Virulence factors such as hemolysins, cytotoxins, and S-layer cause opportunistic infections in species ranging from fish to humans. Predisposing factors such as stress or injury and optimal environmental conditions greatly facilitate *Aeromonas* associated disease outbreaks. Our research investigated the interaction of a specific strain of *A. hydrophila* in the intestinal flora of Great Egrets (*Ardea albus*). The objective of our study was to evaluate the potential of Great Egrets to be vectors for the transmission of VAH between channel catfish culture ponds.

CHAPTER III

DEVELOPMENT OF A VIRULENT *AEROMONAS HYDROPHILA* TRANSMISSION MODEL USING GREAT EGRETS

Abstract

Aeromonas hydrophila (AH) is a gram negative, motile, rod shaped bacterium. Aeromonads are ubiquitously found in multiple aquatic environments ranging from lakes to slightly brackish waters. A highly virulent *Aeromonas hydrophila* strain (VAH) has been responsible for severe disease outbreaks in catfish operations in West Alabama, Arkansas, East Mississippi, and Mississippi Delta.

We developed a system to differentiate and quantify VAH from other environmental motile aeromonads along with other bacterial species. After molecular identification we selectively differentiated only viable aeromonads using a two step process, culture extractions then quantification. This assay development allowed us to reliably evaluate only potentially disease causing bacteria. The purpose of this study was to examine the potential of viable disease causing VAH and its potential transmission to uninfected catfish ponds.

Keywords: *A*quaculture, *Ictalurus punctatus*, *Aeromonas hydrophila*, Virulent Aeromonas Hydrophila (VAH).

Background

Aeromonas spp. are gram negative, oxidase positive, facultative anaerobes. Biochemically Aeromonads are divided into two subgroups, non-motile psychotrophs (*A. salmonicida*) and motile mesophils (motile aeromonads). Motile aeromonads are found ubiquitously in many aquatic environments, which include lakes, streams, and slightly brackish waters and commonly cause disease in cultured fish species. *Aeromonas hydrophila* is the causative agent for diseases such as Motile Aeromonad Septicemia, Red Sore disease, and Ulcerative Infections in fish (Janda and Abbott, 2010). Some of the clinical signs seen in infected fish are a distended abdomen, scale protrusion, fin rot, ocular and skin ulcerations (Cipriano, Bullock et al., 1984). The opportunistic nature of motile aeromonads coupled with environmental stressors commonly leads to disease outbreaks.

In 2009 a specific strain of *Aeromonas hydrophila*, virulent *Aeromonas hydrophila* (VAH), was responsible for acute to chronic mortalities in West Alabama catfish operations (Bebak, Hemstreet et al., 2010). Between June and October of 2009 VAH caused an estimated loss of more than 3 million pounds of market sized catfish (Pridgeon and Klesius, 2011).

The goal of this project was to develop a reliable method to differentiate and quantify VAH in samples that have high numbers of motile aeromonads and a mixture of others species of bacteria. Such an assay would be of use to quantify VAH in feces, mud, surfaces and eutrophic water and allow us to evaluate potential vectors and reservoirs for this important pathogen. *Aeromonas hydrophila* is naturally found in pond water on aquaculture facilities, the gut flora of fish and birds therefore we needed a method to identify only live bacteria and to differentiate VAH from environmental AH. To

differentially identify only viable bacteria we chose to evaluate isolates using a two step process, first culture then quantification and identification. In this study we evaluated culture and molecular identification methods that were conducive to quantitative analysis. We found that DNA hybridization probes could be used to identify VAH but the non-radio isotopic based assay was not suited for colony hybridizations. Then we developed a system based on culturing the bacteria on selective media, molecular identification, and using VAH specific qPCR primers and probes. The qPCR primers and probes used in our assay were developed by Dr. Mark Liles, Dr. Andrew Goodwin, and Dr. Matt Griffin comparing the genomic sequences of VAH to non-pathogenic AH. AH specific qPCR primers and probes were used to differentiate other bacterial species from mixed fecal population (Wang, Wang et al., 2009).

Materials and Methods

Bacterial Isolates and Preparation of Genomic DNA

Aeromonas isolates used in this study were from fish disease diagnostic cases from various laboratories (Table 1).

Each isolate was grown overnight on Criterion Ampicillin Dextrin (AD) Agar a selective isolation media for *Aeromonas* species (Hardy Diagnostics, Santa Maria, California). Single colonies were isolated and grown overnight in 5 ml of Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Sparks, Maryland). Samples from overnight broth cultures were archived by mixing 900 µl of cultures with 450 µl of 50% sterile glycerol (IBI5762 Shelton Scientific-IBI, Peosta, Iowa) and freezing at -80°C.

The Genomic DNA was isolated from overnight *Aeromonas hydrophila* cultures (500 µl) using the Puregene genomic DNA isolation system (Qiagen, Valencia, CA) following the manufacturer's protocol for DNA purification from gram-negative bacteria. DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE).

VAH PCR

Aeromonas hydrophila isolate AL09 #2 (pre-confirmed VAH strain) was selected to determine if Primer sets 2369, 2968, and 2395 (Table 2) were reliable at differentiating VAH samples from non-VAH samples. Isolate AL09 #2 was selected and amplified using the above-mentioned PCR primer sets. Dr. Mark Liles at Auburn University and Drs. Brian Scheffler and Geoffrey Waldbieser at the Catfish Genetics Laboratory (USDA-ARS) sequenced the genomes of 6 VAH strains and 6 non-virulent A. *hydrophila* strains and compared the sequences to the ATCC type strain (which is not related to catfish VAH). They found several regions that were specific for VAH. They selected 26 PCR primer sets and found that the three aforementioned primers gave reliable VAH specific product. The reactions were preformed in a final volume of 25ul containing 50-100 ng of template (1 µl), .25 µl of each primer, 2 µl dNTP (TAK 4030, Takara Bio, Madison, Wisconsin), 2.5 µl 10mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$ 10X Buffer A. 1.25 units Fisher BioReagents *Taq* DNA Polymerase (0.25 µl). The reaction volume was adjusted to 25 µl with UltraPure water. The extracted DNA sample was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE). Amplification of the target sequence was carried out using suggested thermocycling conditions: 95°C for 30 seconds, followed by 30 cycles of 95°C for 30 seconds, 66°C for

15 seconds, and 72°C for 30 seconds, with the concluding cycle of 72°C for 5 minutes. This reaction was performed using an MJ Research thermal cycler (PTC-200, Applied Biosystems). After PCR amplification, 5ul of each PCR product was visualized using a 1% agarose gel, 1% ethidium bromide stain (FB-BP1302-10, Fisher Scientific, Houston, Texas) and detected using UV transillumination (ChemiImager 5500, Alpha Innotech Corporation, San Leandro, California). The three primer sets as expected yielded a 346-350 base pair (bp) product which was confirmed using 100 bp DNA ladder (Invitrogen, 1397847, Grand Island, NY).

Slot-blot and Colony DNA Hybridization Assays

Probes were generated for use in DNA Dot-Blot Hybridization using PCR products described above and isolate AL09 #2 as template. Each PCR product was visualized by electrophoresis on 1% agarose. Then PCR products from the three primer sets were purified using Montage PCR kit per the manufactures instructions (Millipore, UFC7PC250, Bedford, MA).

Slot-blot analyses were carried out using one of the three probes in each assay. Target DNA (1ug/well, of each bacterial isolate) was applied to Hybond™-N+ positively charged nylon membrane (Amersham Biosciences, RPN303B, UK Limited) using the Convertible® Filtration Manifold System (Life Technologies, GIBCOBRL) according to manufacturer's protocols. A total of 100 ng of DNA Probe was labeled using Amersham ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare, RPN300, Piscataway, NJ) according to the manufacturer's protocol. After blotting each membrane was UV cross-linked using application program C2 on GS Gene Linker UV chamber (BioRAD, 165-5031), and then hybridization and stringency washes were carried out

using a hybridization oven (Unitherm, Natick, MA). Detection was performed exposing the Amersham Hybond™-N+ film (Amersham Biosciences, RPN303Bmm, UK Limited) film for 30 seconds and developing using an X-ray film processor.

Probe 2968 was used in colony hybridization assays. This assay was used to detect VAH isolates vs. non-VAH isolates grown overnight on AD selective media. VAH isolate AL09 #2 (positive control) and TN95-04 (negative control) were grown overnight in 5 ml of BHI broth, serially diluted, plated on AD selective media and grown overnight. Plates with 50-100 colonies were processed following Protocol D and Protocol A of Amersham ECL Direct Nucleic Acid Labeling and Detection System (GE Healthcare, RPN300, Piscataway, NJ).

Quantitative PCR

Primers and probes for *A. hydrophila* specific qPCR were designed by (Wang, Wang et al., 2009). This qPCR assay was used to determine the amount of *A. hydrophila* cultured on selective media spread-plates in all Great Egret fecal samples during the trial period. VAH specific primers and probes designed by Dr. Matthew Griffin were used to quantitatively determine the amount of VAH present in all Great Egret fecal samples during the study. Genomic DNA of A. *hydrophila* isolate AL09 #2 and ATCC *F. columnare* were used as positive and negative controls, respectively, for *A. hydrophila* specific qPCR. *A. hydrophila* VAH isolate AL09 #2 and, non-VAH isolate AL97-91 DNA were used as positive and negative controls for the VAH qPCR respectively. In order to quantify VAH in each sample a standard curve was created using VAH isolate AL-09 #2. Ten fold serial dilutions were used in all qPCR that contained DNA copy number from 1.00E+07 to 1.00E+09.

The qPCR reactions were preformed in a final volume of 25ul containing 30 ng of template (10 µl), .5 µl of each primer, 2.5 uM dNTP (TAK 4030, Takara Bio, Madison, Wisconsin) (.5ul), 10mM Tris-HCl, 50mM KCl, 1.5 mM $MgCl_2$ 10X Buffer A (2.5ul), 1 unit Takara Hot Start Version *Taq* DNA Polymerase (.2 µl). The reaction volume was adjusted to 25 µl with UltraPure water (10.3 µl). The amplification mixtures were subjected to an initial incubation of 1 cycle of 2 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C, and a final cycle of 65°C for 30 seconds. This reaction was performed using a Stratagene Research thermal cycler (Mx3005P, Stratagene, La Jolla, CA).

Heat Inactivated VAH

VAH isolate AL09#2 and non-VAH isolate AL97-91 were grown overnight in 5 ml of Brain Heart Infusion (BHI) broth. 300ul of overnight broth was brought to and Optical Density of .4 and the VAH isolate was heat killed in dry heat incubator (BioRad, 01087) for 30 minutes at 75°C. Heat killed VAH was diluted in non-VAH bacteria in four ten-fold serial dilutions and cultured from Ampicillin Dextrin selective plates. After overnight incubation at 37° C the growth was harvested from each plated dilution. The genomic DNA was isolated from each plated dilution using the Puregene genomic DNA isolation system (158388, Qiagen, Valencia, CA) following the protocol for "DNA Purification from Gram-Negative Bacteria Using the Gentra Puregene Yeast/Bact. Kit". Next 30 ng from each cultured plate was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, DE) and later used in qPCR analysis.

Fecal Samples

Approximately 1 g feces were scraped from the concrete floor, placed in a sterile plastic transport bag (Nasco, Whirl-Pak, Fort Atkinson, WI) and transported to the lab within 1 hour. In the laboratory, approximately 0.1g of feces was added to a 1.5 ml microfuge tube, weighed, then diluted 1:10 in PBS. Then the sample was vortexed and particulate matter removed by centrifuging at 3 x1000 for 30 seconds. One hundred microliters of this suspension was spread on to AD agar plates. A series of 5 ten–fold dilutions were made and 100 microliters of each dilution was spread onto AD agar plates. Plates were then incubated at 37°C for 24 hours, yellow convex translucent colonies were tested for cytochrome oxidase C (Becton Dickinson, Sparks, MD) counted and colony forming units of presumptive Aeromonas per gram of feces was calculated. A characteristic colony from each counted plate was identified to species using API 20E strip test per manufacturer's directions (BioMerieux, REF 20160, USA). Genomic DNA was isolated from overnight growth of the first dilution-AD agar plates. Genomic DNA of *A. hydrophila* isolate AL09 #2 and ATCC *F. columnare* were used as positive and negative controls, respectively, for *A. hydrophila* specific qPCR.

Table 1 Archived *A. hydrophila* isolates listed by name and PCR product result

Sample Origin	Sample I.D.	API 20 E ID Result[1]	PCR Result for VAH
Avian Fecal Sample	1064	7047127	P
Avian Fecal Sample	1065	7047127	P
Avian Fecal Sample	1066	7047126	P
Avian Fecal Sample	1067	7047324	P
Avian Fecal Sample	1068	6247124	N
Avian Fecal Sample	1069	7247144	N
Avian Fecal Sample	1070	7247104	N
Avian Fecal Sample	1071	7247145	N
Avian Fecal Sample	1072	7247124	N
Avian Fecal Sample	1073	6007126	P
AL97-91	1074	7047126	P
AL09-150 B	1075*	7047126	P
TN97-08	1077	7047106	N
MN98-04	1078	7007126	N
ML09-119-1	1079*	7047126	P
TN95-04	1080	-	N
AL10-63 Kidney	1081	7047125	N
AL10-121 Kidney	1082	7047127	N
AL09-123	1083	4002124	N
AL09 #2	1084	7047126	P
AL09 #1	1085	7047126	P
AL09 #4	1086	3047326	P
GA97-22	1087	7047127	P
AL09-74	1088*	7047127	P
AL09-79	1089*	7047126	P
AR 2010	1090*	1067326	P
AL06-06	1091	7047117	N

N=Negative

P=Positive

*= Known VAH sample

Note: Each API code number represents the sum of positive results for three tests: from left to right 2-nitrophenyl-betagalactosidase (4), Arginine (2),and Lysine (1); Ornithine (4), Citrate (2), and hydrogen sulfide production(1); Urea (4), Tryptophan (2), and Indole (1); Sodium pyruvate (4), Gelatinase (2), and Glucose (1); Mannitol (3), Inostitol (2), and Sorbitol (1); Rhamnose (4), Sucrose (2), and Melibose (1); Amygladin (4), Arabinose (2), and Oxidase (1)

Table 2 Archived *A. hydrophila* isolates listed by name and intensity of hybridization result for each probe

Sample I.D.	Probe Result for VAH	Probe Result for VAH	Probe Result for VAH
1064	3	3	3
1065	3	3	3
1066	1	1	1
1067	1	1	1
1068	3	3	3
1069	3	3	3
1070	3	3	3
1071	3	3	3
1072	3	3	3
1073	3	3	3
1074	1	1	1
1075	1	1	1
1077	3	3	3
1078	3	3	3
1079	1	1	1
1080	3	3	3
1081	3	3	2
1082	3	3	3
1083	3	3	3
1084	1	1	2
1085	1	1	1
1086	1	1	1
1087	2	2	2
1088	1	1	1
1089	1	1	1
1090	1	1	1
1091	3	3	3
	Probe 2369	Probe 2395	Probe 2968
1=Strongest band			
2=Mild band			
3=No band			

Results

Culture and evaluation of case isolates

All diagnostic isolates grew well on Ampicillin Dextrin (AD) agar producing large yellowish colonies. After incubation API 20 E molecular identification was taken for isolates 1064-1091 (Table 3).

VAH Primers

In the beginning of our assay development we used primer sets 2369, 2968, and 2395 on bacterial isolates from VAH outbreaks and non-VAH fish disease cases. The aforementioned primer sets were designed to reliably differentiated VAH samples from non-VAH samples. The PCR on isolates 1075, 1079, 1088, 1089, and 1090 gave the expected 354 bp product (Figure 1). The three primer sets were used on known VAH sample 1084 and each lane yielded a ~354 bp product on 1% agarose gel.

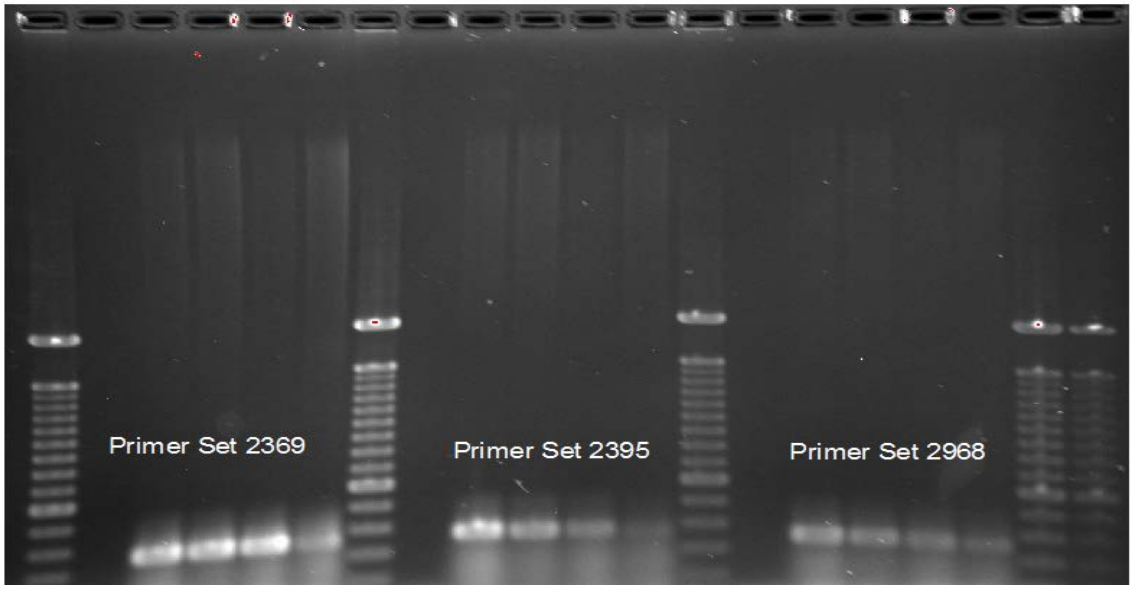

Figure 1 PCR results confirming base pair product generated by PCR primer sets 2369, 2968 and 2395.

DNA template VAH Sample AL-09# 2, Lane 1 negative control and lanes 2-5 are Unknown Sample AL-09#2.

Dot Blot DNA Analysis and Hybridization

Our goal was to develop a technique that would allow the differentiation of VAH in multiple bacterial isolates simultaneously. By using a slot/dot blot analysis we could analyze well over 48 DNA samples at one time. The slot blot analysis was carried out

using PCR products of primer sets 2395, 2395, and 2968 and probes to identify which primer sets would give the specificity and strongest detection signal. After bacterial analysis and quantification a probes were generated for use in the DNA Dot-Blot Hybridization using VAH positive bacterial isolate 1084. We found that all three primer sets gave an equally strong detection signals and could differentiate VAH among our *Aeromonas hydrophila* samples (Figure 3).

However, dot blot analysis requires individual colony picking, DNA extraction and dot application and is thus a labor-intensive method to use for quantitating VAH in mixed samples. Therefore we evaluated the use of our DNA probes for colony hybridization using the horseradish peroxidase-based, Amersham ECL Direct Nucleic Acid Labeling and Detection System. We found that this detection system was sensitive to endogenous peroxidases expressed by *A. hydrophila* and attempts to inactivate this enzyme using hydrogen peroxide destroyed the DNA on the blot.

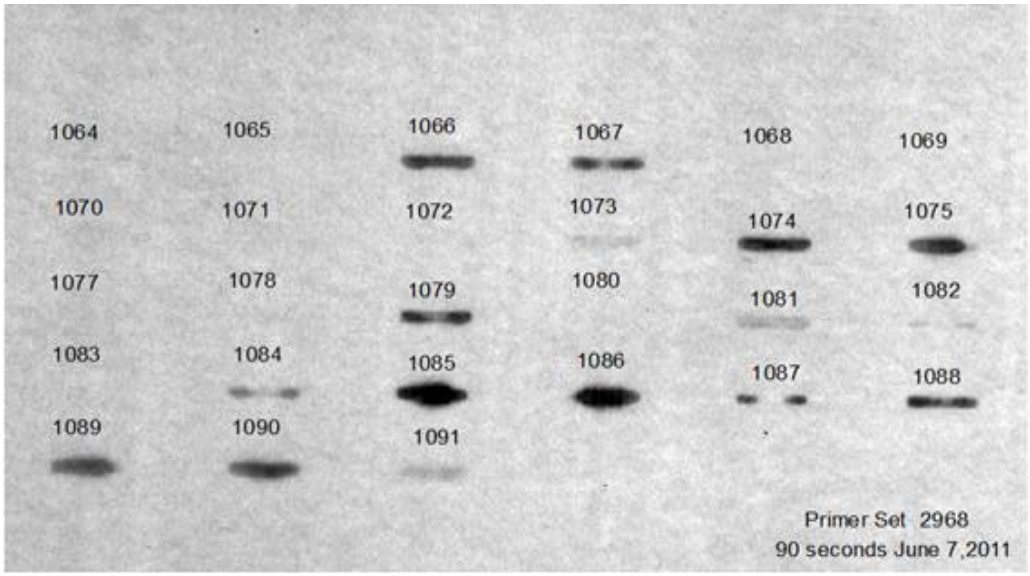

Figure 2 DNA Slot-blot of field isolates using probe 2968

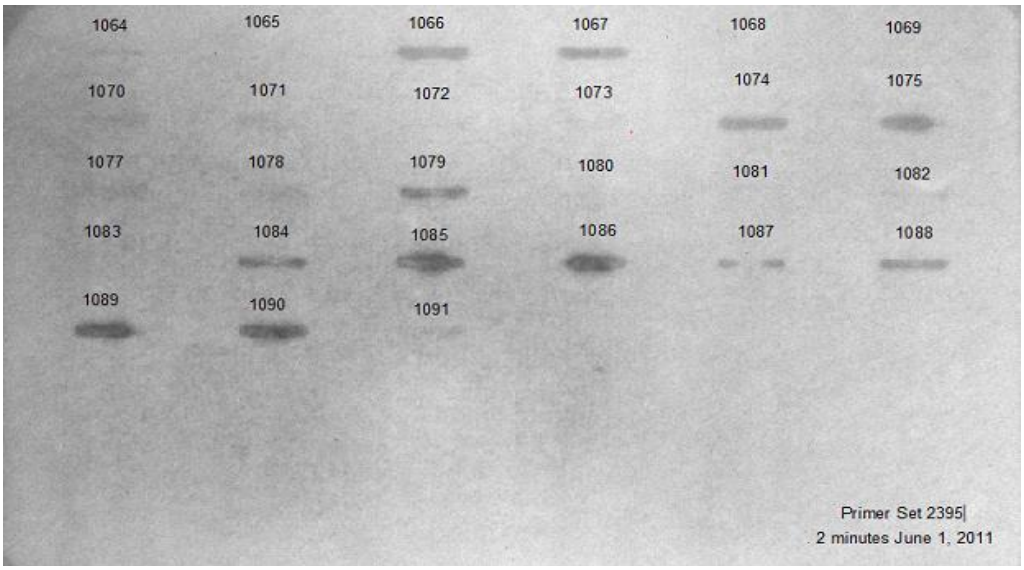

Figure 3 DNA Slot-blot of field isolates using probe 2395

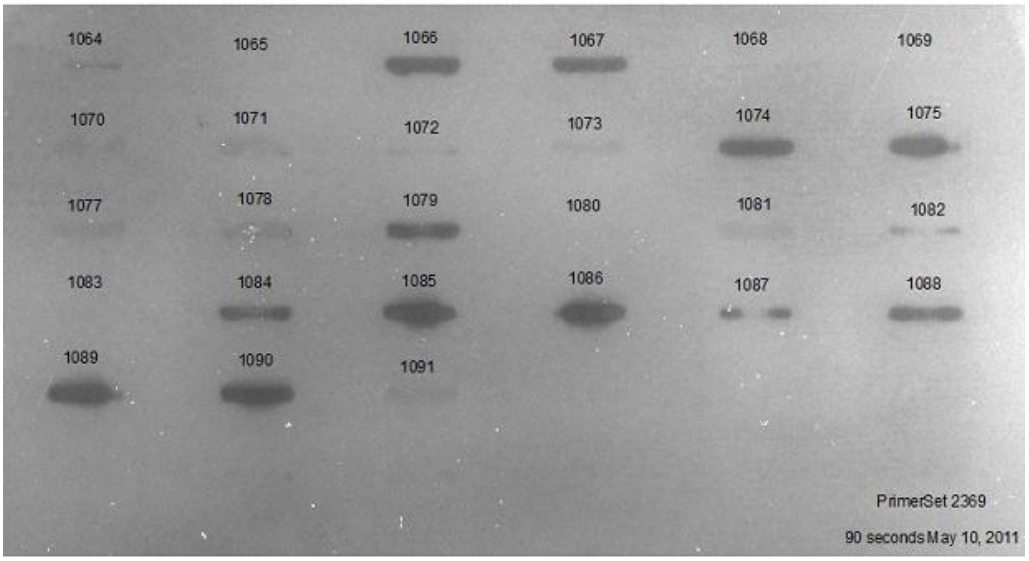

Figure 4 DNA Slot-blot of field isolates using probe 2369

Virulence Identification Using qPCR Primers and Probes

A. hydrophila and VAH specific qPCR

An alternative method to colony counts for quantifying bacterial colonies from plates is to quantify the growth using qPCR. Two different qPCR assays were needed to specifically identify and quantify *A. hydrophila* and VAH strains from other species of bacteria on a spread plate. The *A. hydrophila* qPCR assay used in our study were designed by (Wang, Wang et al., 2009) and proved to be highly effective at differentiating *A. hydrophila* from other species of bacteria in great egret fecal samples. VAH qPCR assay was developed by Dr. Mark Liles, Dr. Andrew Goodwin and Dr. Matthew Griffin comparing the genomic sequences of VAH to non-pathogenic AH, this created our VAH specific qPCR primers & probes. The VAH qPCR assay was used to quantitatively determine the amount of VAH present in all great egret fecal samples during the study.

For Days 0-7 each bacterial isolate type was confirmed to be VAH or non-VAH isolate. These results were supported by our initial microbiological tests that identified each isolate as AH. Quantitative PCR analysis identified which isolates shed by test birds were VAH.

Table 3 Primers and probes used in PCR and qPCR

NAME	SEQUENCE	PRODUCT LENGTH
PCR Primers		
Contig_2968F	TCTTAAAGCGACAGCATACCGCTCA	338 bp
Contig_2968R	TGGGCGGCTGCATTGGTCATG	
Contg_2369F	TGGCGTCCGACCAAAACGCC	346 bp
Contig_2369R	CCCGTGCGGATAGGAACTGGC	
Contig_2395F	TGAACGAATGTGAATTCGCTTGCCA	350 bp
Contig_2395R	ACTTTGGCCAAGGAGATAACCCCA	
qPCR Primers and Probes		
Hot Aero Left-20	CTATTACTGCCCCCTCGTTC	
Hot Aero Right-19	ATTGAGCGGTATGCTGTCG	
Hot Aero Probe-26	FAM-TCAAGCGTTCATAAAGTGCCGAGTCA-BHQ	
AHA-F	GCCGTCGAAACCAACGTAGA	
AHA-R	CAACACCTGGTCCGGTATCG	
AHA-Probe	FAM-CAGCAGAAACTTGCCACTCGGTCTG-BHQ	

Heat Inactivated VAH

Our goal was to find a good method to quantify viable VAH in environmental and fecal samples so that mechanisms of transmission and spread can be evaluated. By growing the bacteria on plates before doing qPCR we assumed that any contribution to the PCR by dead bacteria would be trivial. To test this we evaluated heat killed VAH diluted in viable non-VAH A. hydrophila isolates by culturing and qPCR. We found that the VAH specific qPCR did not detect non –viable VAH. The primers and probes only detect potentially disease-causing VAH.

Sensitivity of Assay

In each of the qPCR assays a standard curve was used to quantitatively identify the amount of target DNA that was in each unknown fecal sample. A standard curve was created using ten-fold serial dilutions with DNA. An additional standard curve using

colony forming units was created using ten-fold serial dilutions of dilute VAH into non-VAH bacteria. The CFU standard curve was created using 4.5 x 10^5 CFU of non-VAH.

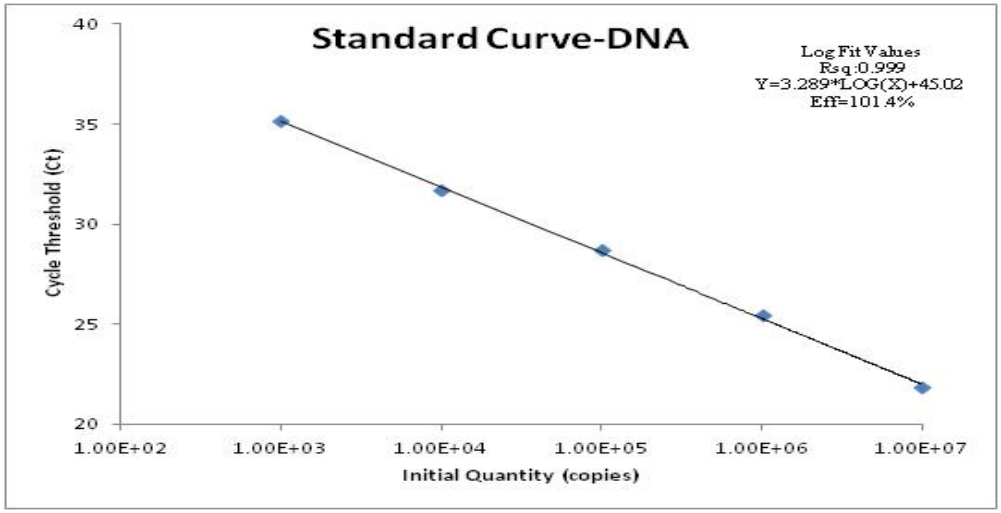

Figure 5 Standard Curve for use in qPCR created with DNA template AL09#2

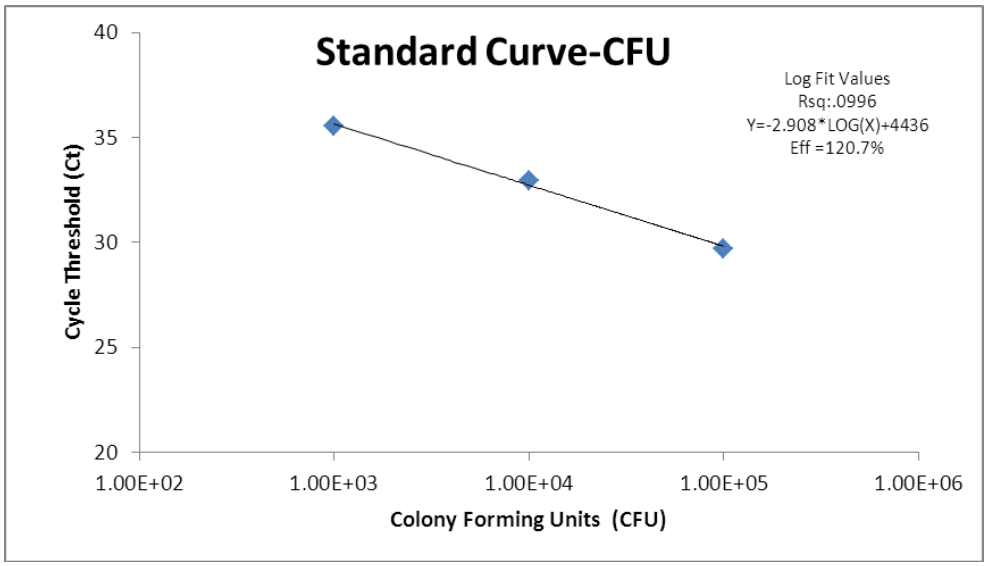

Figure 6 Correlation of qPCR threshold cycle to colony froming units of VAH in mixed cultures.

VAH isolate AL09#2 was mixed in serial dilutions in a stock of non-VAH isolate AL09-123 and placed on AD plates to produce a combined total of 6.3 x 10^7 colonies. DNA was from the bacteria harvested from the AD plate and used as template for the qPCR.

Application to diagnostic samples

After DNA isolation, extraction and multiple microbiological tests on isolates, the bacterial growth of the whole plate was DNA extracted and evaluated by qPCR analysis. Each isolate that was identified as *A. hydrophila* in microbiological test was found to be *A. hydrophila* using the qPCR primers and probes.

Discussion and conclusions

This study evaluated the use of spread plate culture in combination with molecular identification for evaluating the presence of VAH in environmental samples and feces. . We evaluated the effectiveness of our system by using dilutions of viable VAH or killed VAH in a non-VAH strain of *A. hydrophila*.

Several different selective culture medias have been used by others. Handfield *et al*., reported the most reliable isolation occurred with Ampicillin Dextrin selective media but not all isolates could be cultured on it (Handfield, Simard et al., 1996). We tested all of our *A. hydrophila* cultures on AD agar plates and found that all grew well at 37°C. Therefore we used this medium for our selective two step process.

There are several methods to identify target DNA from mixed samples. In our assay development we wanted a rapid method that was also highly selective and would work well with traditional spread plate method of bacterial enumeration, so we evaluated the potential the colony hybridization method. First we used primers that selectively amplify DNA sequences that are unique to VAH. We then used these products as probes in DNA slot blot analyses to determine if the probes were specific for VAH. Our slot blot analysis demonstrated that probes derived from PCR products of all three primer sets gave an equally strong detection signal and could differentiate VAH among our *A. hydrophila* samples. We evaluated the use of our DNA probes for colony

hybridization using a horseradish peroxidase-based nucleic acid labeling and detection system. We found that this detection system was sensitive to endogenous peroxidases expressed by *A. hydrophila* and gave false positive hybridization signals on our blots. Several attempts were made to inactivate the enzyme, but all procedures that eliminated false positives damaged the DNA to give false negatives. As an alternative to colony blots we investigated the use of qPCR on DNA extracted from whole plate cultures and found very good correlations between VAH concentrations and input colony counts. Using the two step process there was no residual signal as a result of dead bacteria. Also, we found that the qPCR assays we less subjective and less labor intensive (per sample) than hybridization assays.

We investigated the utility of two qPCR interpretation test using VAH and AH specific primers and probes. Each isolate underwent API 20E microbiological test, whole plate extractions, DNA extraction, and finally qPCR analysis. Our daily fecal samples from each great egret were tested to confirm if they shed *A. hydrophila* in their feces. Next we wanted to confirm if they shed VAH as well as the amount of viable bacteria that was able to successfully pass through the intestinal tract. Using our VAH specific primers and probes allowed us to identify which birds were vectors for VAH, the duration of shedding, and the quantity (CFU) they shed.

Several measures were taken to ensure the quantified bacterial isolates were viable. We investigated the sensitivity of qPCR using whole plate extracts and colony counts. Each GREG fecal sample was quantified using a standard curve created with target DNA. In the standard curves using target DNA and curve using colony forming units (CFU), we observed similar trends. The CFU standard curve used a theoretical ratio of ten-fold dilutions of VAH that was diluted into non-VAH bacteria. The CFU standard

curve was created using 4.5×10^5 CFU of non-VAH and 6.33×10^4 CFU of VAH bacteria. The ratio of CFU decreased each ten-fold dilution in a step wise manner as we expected per our trend line After further analysis heat killed VAH that was diluted into non-VAH bacteria was not detected by our qPCR assay, only viable potentially disease causing bacteria were detected. It was important to confirm that our assay only detected viable VAH as the purpose of this study was to examine the potential of viable disease causing VAH and it transmission to uninfected catfish ponds.

CHAPTER IV

POTENTIAL OF GREAT EGRETS TO BE VECTORS FOR THE TRANSMISSION OF MOTILE AEROMONAD SEPTICEMIA BETWEEN CHANNEL CATFISH CULTURE POND

Abstract

Recent severe outbreaks of disease in catfish aquaculture have been associated with a highly virulent *Aeromonas hydrophila* strain (VAH) that is genetically distinct from less virulent strains. *Aeromonas hydrophila* (AH) which is a Gram-negative, rod shaped, facultative anaerobic bacterium is typically opportunistic in nature. Unlike AH, VAH is considered to be a highly virulent primary pathogen. Aeromonads can cause infections in fish, humans, reptiles, and avian species.

Great Egrets were used in this transmission model because these wading birds frequently predate catfish farms. Through a two-step identification assay we concluded that Great Egrets that were fed VAH infected catfish shed VAH during trial period. We concluded that fish eating birds may serve as a reservoir for VAH and can potentially spread the pathogen by flying to uninfected ponds.

Keywords: *Adrea alba,* aquaculture, *Ictalurus punctatus*, *Aeromonas hydrophila*, Virulent Aeromonas Hydrophila (VAH).

Background

The leading aquaculture industry in the United States is commercial catfish production. According to the National Agricultural Statistics Service (NASS), Alabama, Mississippi, and Arkansas are the top producing states of channel catfish (*Ictalurus punctatus*). United States catfish sales totaled 403 million dollars in 2010 which increased 8 percent from the previous year (NASS). Due to environmental factors and disease the commercial catfish industry suffers enormous losses every year.

Members of the bacterial genus *Aeromonas* are important pathogens in aquaculture and are separated into two major groupings, the motile mesophiles and non-motile psychrophiles (Janda and Abbott, 2010). Motile Aeromonads are cytochrome oxidase positive, ferment glucose with or without the production of gas, and are insensitive to the vibriostatic agent O/129 (Cipriano, Bullock et al., 1984). Motile Aeromonads are commonly found to cause disease in warm water cultured fish despite being a part of their normal intestinal micro flora (Cipriano, Bullock et al., 1984). The opportunistic nature of *Aeromonas* coupled with environmental stressors such as high water temperatures, low dissolved oxygen and high nitrite levels has lead to severe outbreaks. (Camus, Durborow et al., 1998). In addition to outbreak associated with environmental stressors seasonal outbreaks typically emerge in the early spring and fall (Cipriano, Bullock et al., 1984). These bacterial outbreaks caused by motile aeromonad species are exceedingly prevalent and troublesome in fish operations (Camus, Durborow et al., 1998).

Aeromonas hydrophila is the causative agent for diseases such as Motile Aeromonad Septicemia, Red Sore disease, and Ulcerative Infections in fish (Janda and Abbott, 2010). Some of the clinical signs seen in infected fish are a distended abdomen,

scale protrusion, fin rot, ocular and skin ulcerations (Cipriano, Bullock et al., 1984). The virulence of the bacterium, condition of the host, and the degree of genetic resistance are other factors that affect the degree of disease expression (Cipriano, Bullock et al., 1984). *Aeromonas hydrophila* has also been found to cause disease in reptiles, birds and gastrointestinal problems in humans.

In 2009, a specific virulent strain of *Aeromonas hydrophila* (VAH), was responsible for acute to chronic mortalities in West Alabama catfish operations (Bebak, Hemstreet et al., 2010). Between June and October of 2009 VAH caused an estimated loss of more than 3 million pounds of market sized catfish (Pridgeon and Klesius, 2011). Molecular identification on small portions of the genomes of three isolates of *Aeromonas hydrophila* cultured from the 2009 outbreak were sequenced and analyzed by Pridgeon *et al*. The 16S-23S rDNA intergenic spacer region, cpn60, gyrB, and rpoD genes shared 97 to 99% sequence similarities (Pridgeon and Klesius, 2011). The three West Alabama isolates had a much lower LD50 value in comparison to a 1998 isolate, which suggest a higher virulence (Pridgeon and Klesius, 2011). It is important to understand how VAH is transported to various catfish operations in Alabama and neighboring states. Many species of fish eating birds such as double-crested cormorants (*Phalacrocorax auritus*), great blue herons (*Ardea Herodias*), great egrets (*Ardea alba*) and the American white pelican (*Pelecanus erythrorhynchos)* are frequently found on commercial catfish operations. These fish-eating birds are responsible for substantial losses to the industry and may serve as vectors of bacterial pathogens such as VAH.

In the present study, we evaluated the potential of great egrets (GREG) to be vectors for the transmission of VAH between channel catfish culture ponds. The transmission potential was determined by feeding the birds VAH injected catfish and

culturing their feces daily for VAH. Then at termination, the birds were necropsied and evaluated for VAH infection by culture histopathology and serology.

Materials and Methods

Study population

Eleven Great Egrets were captured at commercial catfish fingerling ponds in the Mississippi Delta using soft catch leg hold traps as previously described (King D.T. , 1998) and transported to the National Wildlife Research Center Mississippi Field Station avian test facility. We tested the Egrets prior to starting the trial to confirm they were negative by fecal cultures for VAH. All great egrets were pre-bled, weighed and marked with a unique leg band. Great egrets were individually housed in 3.3 m x 3.3 m x 2 m (L x W x H) cages containing shallow plastic tanks filled with water. The water was changed daily and the great egrets were fed an *ad libitum* diet of live catfish throughout the study. Daily health inspections were conducted and weights were taken pre and post study period.

Six birds (test birds) were fed VAH infected fish and four birds (control birds) were feed non-infected VAH fish for three consecutive days. VAH infected fish were produced by anesthetizing them with Tricane Methane Sulfonate (MS222) at a rate of 100 mg/L of water and intraperitoneal injections with 0.5 ml of bacterial culture containing ~$1x10^8$ colony forming units (CFU) of VAH *Aeromonas hydrophila*. Daily feed logs were kept to calculate the number of fish grams and number of fish eaten. This allowed us to calculate the amount of VAH ingested as each fish was injected with the same amount of VAH. Throughout the trial each treated great egret ingested different amounts of VAH infected catfish fingerlings ranging from 9.0×10^8 CFU to 2.55×10^9

CFU. The fish (injected or non-injected for control birds) were placed in the shallow plastic feeding tanks filled with water. After Day 3 all birds were feed non-injected fish for the duration of the trial. At initiation of the challenge, during the VAH feeding and five days post feeding (Day 3-7), feces were sampled from each bird daily.

Identification of virulent Aeromonas hydrophila

Fecal samples were serially diluted and cultured on Ampicillin Dextrin (AD) agar to determine colony counts as described in Chapter III. Each colony count was evaluated for cytochrome oxidase and biochemical profile using API 20E strips. The bacteria from the least diluted plates were harvested and DNA extracted for VAH specific Aeromonas specific PCR as described in Chapter III.

Immunological assay

Protein preparation assay

In our immunological assay we used the Micro BCA™ Protein Assay kit (Thermo Scientific, 23235) to process serum samples for the Duck (*Anas Platyrhynchos*), Goat (*Capra hircus*), Chicken (*Gallus gallus*), Great Egret (*Ardea alba*), and Double Crested Cormorant (*Phalacrocorax auritus*). Per the manufacturer's directions we prepared the diluted albumin standards, Micro BCA working reagents, and produced a linear working range for the samples.

Western blot assay

After Micro BCA™ Protein Assay preparation the serum samples were directly used for SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis to determine if the Horseradish Peroxidase (HRP) labeled Goat anti-bird IGg would

detect immunoglobulin (Ig) in the serum samples from our species of interest (Alpha Diagnostics, San Antonio, Texas. Dilutions of GREG, chicken, goat (negative control), double crested cormorant and duck serum samples in Laemmli buffer (Bio Rad,, Hercules, CA) were loaded on a 8% polyacrlyamide gradient gel and separated at a constant voltage of 100V for 1.5 hours using a Bio-Rad apparatus (170-3970, Hercules, CA). Proteins were transferred electrophoretically to a methanol saturated polyvinylidene fluoride (PVDF) membrane (Amersham Biosciences, UK Limited) by blotting at constant 100V for 1.5 hours using Bio-Rad apparatus. The membrane was blocked overnight in a 4°C cold room with constant agitation in blocking buffer and washed four times for 5 minutes in Tris buffered saline-Tween 20 and one time for 5 minutes in TBS. The membrane was incubated for 1 hour with agitation in Primary antibody solution containing horseradish peroxidase conjugated Goat anti-bird IgG (Alpha Diagnostic Intl, 90520). After four more washes with TBS-Tween 20 and TBS, 1-Step™ TMB-Blotting (Thermo Scientific) was added to visualize our desired product. The immunostaining was stopped after 5-10 minutes with double distilled water. A positive result on the immunoblot was a 150 kDA product because the approximate size of IgG is 150 kDA.

Bacterial agglutination

Bacterial agglutination titration was done using Pre and Post serum samples from GREG using a modification of the method described by (Roberson, 1990). Bacterial isolates AL09 #2 and *E. ictaluri* were grown overnight in 10 ml of Brain Heart Infusion (BHI) broth (Becton, Dickinson and Company, Sparks, Maryland). The bacterial suspension was adjusted to approximately 10^9 organisms per ml by a spectrophotometer.

The absorbance reading was set to 600nm at an O.D. of .75. The bacterial suspension was spun down, resuspended in saline and 0.3 % formalin was added. The prepared bacterial suspension was kept refrigerated for later use. Two fold dilutions were preformed with saline and serum and bacterial suspension was later added to each well on 96-well U-bottom microdilution plate. The first well started with a 1:10 and ended with a observable titer response with 1:160 dilution.

Great Egret Necropsy

At the conclusion of the trial each GREG was necropsied and histological and bacterial samples were taken. The samples were taken from: mouth, nasal, upper intestine, lower intestine, kidney, eye, esophagus, and lungs. Bacteria swabs from the samples were cultured on Ampicillin Dextrin media. All histological samples were fixed in 10% formalin buffer and embedded in paraffin. All histological samples were also H & E stained.

Statistical Analysis

The influence of VAH dose consumed versus bacterial load shed was analyzed using analysis of variance (ANOVA) using SPSS computer software. No significant correlation between dose fed and amount of bacteria shed was found.

Results

Virulent *Aeromonas hydrophila* confirmation

We developed a system based on culturing bacteria on selective media, molecular identification, and using AH and VAH specific qPCR primers and probes (see chapter 3). Throughout the duration of the trial (Days 0-7) each bacterial isolate underwent a two-

step identification system. First microbiological tests were performed on each characteristic colony type. Table 4 shows an overview of API results for VAH stock that was injected into all catfish fingerlings and bacterial isolates cultured from GREG feces. The last column shows the exceptions that we found with some of our API test results. For example with the Mannitol oxidation test 1 GREG tested negative and the other 5 tested positive.

Table 5 identifies each bacterial isolate that was cultured from control birds and test birds for the quarantine period and throughout the duration of the trial. Before the start of the trial we found the study population did not shed *A. hydrophila*, indicating that the great egrets used in our study were VAH free. After the treated GREG ingested VAH infected catfish fingerlings they shed AH.

The second step in our assay was to evaluate if the AH strain was in fact VAH by harvesting and extracting DNA from highest concentration spread plate. Each isolate we found to be AH in GREG samples was later identified as AH or VAH. Table 7 shows each day the great egrets shed VAH. All GREG stopped shedding by Day 4. Figure 7 shows the amount of VAH that was ingested and shed by each test great egret.

Table 4 Overview of BioMerieux API results for VAH stock and treated birds

API Test[1]	VAH Stock	Treated Birds	Exceptions to Bird Isolates
ONPG	+	+	
ADH	+	+	
LDC	+	+	
ODC	-	-	(+1/6)
Citrate	-	-	(+3/6)
H2S	-	-	(+2/6)
Urea	-	-	(+1/6)
TDA	+	+	(-3/6)
Indole	+	+	
VP	+	+	
Gelatinase	+	+	
Glucose	-	-	(-2/6)
MAN	+	+	(-1/6)
Inositol	-	-	
Sorbitol	-	-	
RHA	-	-	
SAC	+	+	(-1/6)
MEL	-	-	(+1/6)
AMY	-	-	(+2/6)
ARA	-	-	(-3/6)
Oxidase	+	+	

1. API test name: Beta-galactosidase (ONPG), arginine dihydrolase (ADH), Lysine (LDC), Ornithine (ODC), hydrogen sulfide production (H2S), Tryptophan (TDA), Indole, Sodium Pyruvate (VP), Mannitol (MAN), Rhamnose (RHA), Melibiose (MEL), Amygladin (AMY), Arabinose (ARA), Cytochrome oxidase C (Oxidase)

Table 5 BioMerieux API 20 E daily fecal test results

	Bird ID	Quarantine	Day 1	Day 2	Day 3
Control		*P. shigelloides*	*	*	*
		P. shigelloides	*	*	*
		E. coli	*P. shigelloides*	*	*
		P. shigelloides	*	*	*
Treated		*P. fluorescens*	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
		P. shigelloides	*	*A. hydrophila*	*A. hydrophila*
		P. shigelloides	*	*	*A. hydrophila*
		P. shigelloides	*A. hydrophila*	*	*A. hydrophila*
		E. coli	*A. hydrophila*	*A. hydrophila*	*A. hydrophila*
		E. terrigena	*	*	*
		Day 4	**Day 5**	**Day 6**	**Day 7**
Control		*	*	*P. shigelloides*	*V. fluvialis*
		V. fluvialis	*	*P. shigelloides*	*
		*	*P. shigelloides*	*	*
		V. fluvialis	*V. fluvialis*	*V. fluvialis*	*V. fluvialis*
Treated		*	*	*	*
		A. hydrophila	*	*	*
		*	*	*	*
		*	*P. shigelloides*	*	*
		A. hydrophila	*	*	*
		A. hydrophila	*	*	*

Table 6 Quantitative Real-Time PCR of detected *A. hydrophila* and *Virulent Aeromonas hydrophila* detected in fecal samples during trial period

	Day 1	Day 2	Day 3	Day 4	Day 5	Day 6	Day 7
Treated							
GREG 33	◊ *	◊ *	◊	◊			
GREG 25	◊ *		◊				
GREG 32				◊			
GREG 27			◊				
GREG 28	◊ *	◊	◊				
GREG 30		◊	◊	◊			
Control							
GREG 35							
GREG 31							
GREG 37							
GREG 36							
VAH = ◊ AH= *							

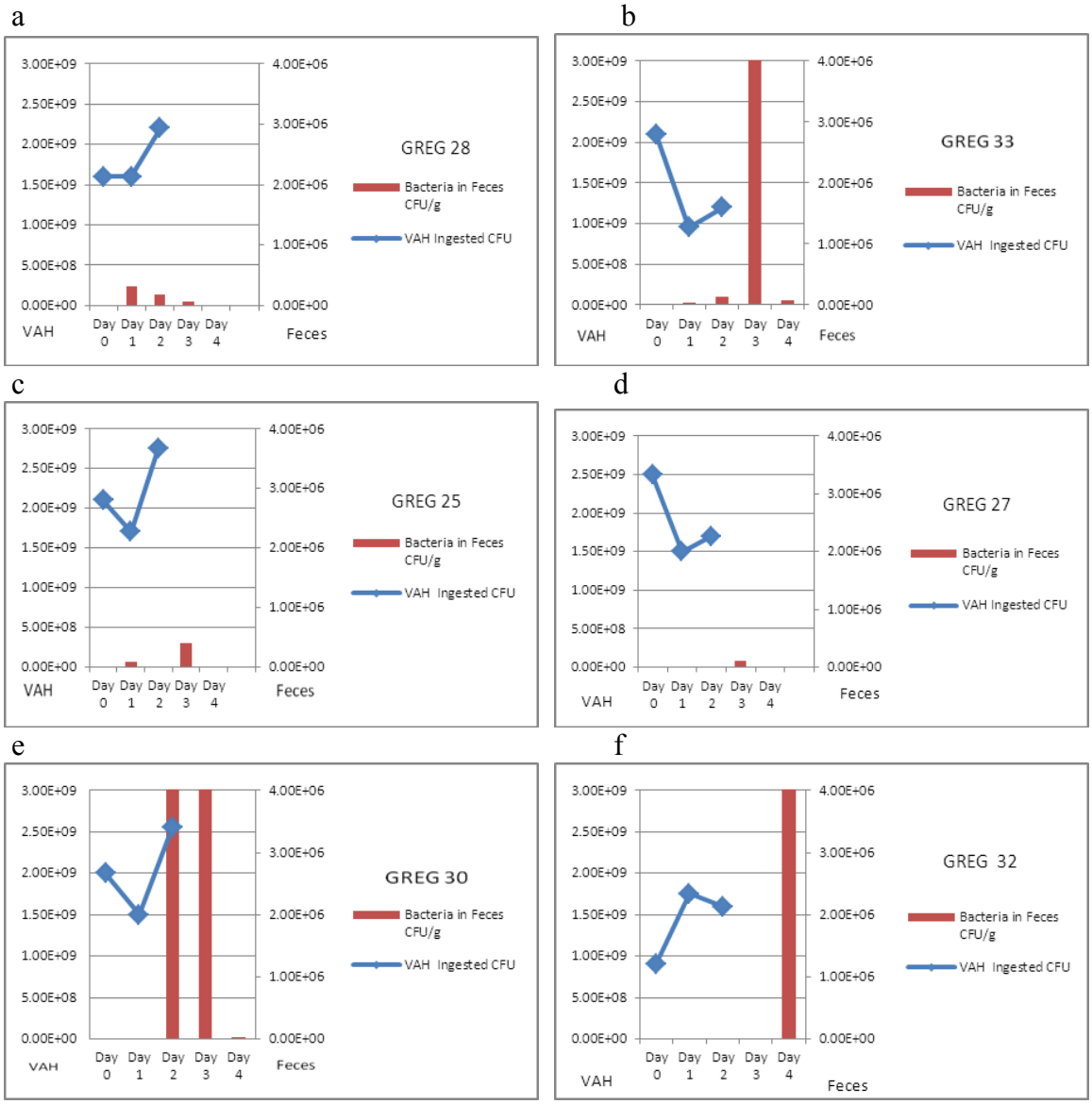

Figure 7 Quantity of VAH ingested (right axis) and quantity of viable VAH (left axis) shed by each great egret

Serum antibody levels

We used western blot analysis to determine if commercial anti-bird Ig was specific for GREG antibodies and those of other important birds that frequent catfish aquaculture ponds. We found that the commercial Ig did detect chicken Ig well in

contrast it did not detect GREG, American white pelican, double crested cormorant or Duck Ig in Western blots (Figure 8).

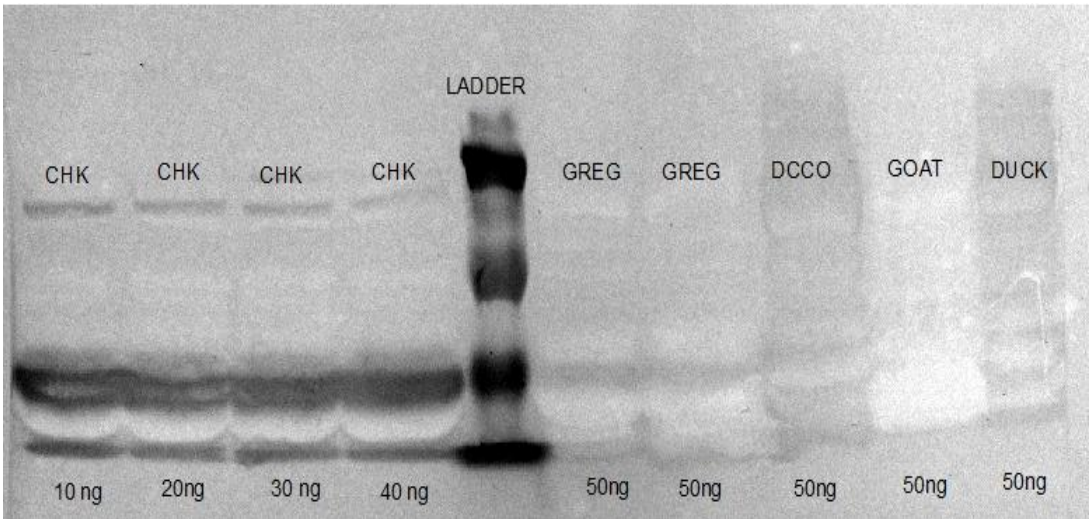

Figure 8 Western Blot Analysis of serum samples from chickens (CHK), great egrets(GREG), ducks, double crested cormorants (DCCO) and goat, as a negative control using goat anti-bird IG (Alpha Diagnostic Intl, 90520) as a primary antibody

Therefore we evaluated the VAH specific antibodies in the GREG serum samples used by evaluating, bacterial agglutination titers using pre- and post-challenge blood samples. There was minimal titer response to our negative control bacteria *E. Ictaluri.* This was used to determine if the birds had previous immunity to VAH. Our titration agglutination showed there was a minimal titer response in the control and test birds (Table 7).

Table 7 Bacterial agglutination analysis of pre and post serum samples from each great egret using virulent *Aeromonas hydrophila* (VAH) and *Edwardsiella ictaluri* as a negative control to evalutae pre and post titer responses

GREG ID and treatment	VAH pre	VAH post	difference	E. I. pre	E. I. post	difference
VAH fed*						
32	0	0	0	3	3	0
27	2	4	2	3	5	2
28	5	4	-1	3	3	0
30	3	4	1	2	3	1
mean	2.5	3.0	0.5	2.75	3.5	0.75
Control						
35	2	2	0	4	5	1
31	3	0	-3	2	2	0
37	5	3	-2	0	3	3
36	4	3	-1	3	3	0
mean	3.5	2.0	-1.5	2.25	3.25	1.0

* Treated GREG 33 was not sampled and the post sample of Treated GREG 25 was unusable and their data were not included in this table.

Great Egret Necropsy

After the bacterial swabs were cultured isolates were processed and analyzed using qPCR assay; VAH was isolated from two great egrets in our study population. VAH was detected in the nasal cavity of GREG 32, and the nasal and mouth cavities from GREG 30.

Statistical Analysis

Although all fish that were fed VAH and shed VAH there was not a significant correlation between the amount of VAH consumed the day before and the concentration of VAH in the feces if we focused on the VAH fed GREG on days 1-4 (R= .201 and sig= .346).

Discussion and conclusions

Although VAH is newly recognized variant of a common pathogen, much work is being done to elucidate its virulence factors, mechanisms and transmission potential. Our study used VAH isolates from 2009 West Alabama Outbreak. This specific strain of AH was responsible for massive mortalities between June and October of 2009 in multiple catfish operations (Bebak, Hemstreet et al., 2010; Pridgeon and Klesius, 2011). Millions of pounds of market sized catfish were reported to be lost during this VAH outbreak (Pridgeon and Klesius, 2011). Although there is not a specific identification key for VAH, multiple studies have found that molecularly VAH is similar to AH with variations. VAH can perform inositol and arabinose oxidation and it is estimated to be 200 times more virulent to channel catfish than AH (Pridgeon and Klesius, 2011). This finding was confirmed with mortality experiments that found West Alabama isolates had a much lower LD50 value in comparison to non-VAH isolate (Pridgeon and Klesius, 2011). We suspect that there are two main modes of pond to pond transmission for VAH, human and natural. The human transmission route involves moving infected fish, handling contaminated water, and contaminated equipment. Also we suspect that natural transmission routes for VAH involve scavengers and predators. In this study we explored a predatory transmission vector for VAH. Many species of wading birds are found on commercial catfish operations and are responsible for substantial economic losses to the industry. *Aeromonas hydrophila* is known to infect birds and we hypothesized that fish eating birds may serve as a reservoir for VAH and spread the pathogen by flying to uninfected ponds.

Throughout the duration of the trial (Days 0-7) each bacterial isolate underwent a multi-step identification system. After API 20E identification, qPCR analysis was

performed on the highest concentration spread plate per day from each GREG. All birds were fed VAH infected catfish for Day 0- Day 2 and we found each treated bird shed VAH. There was no consistent trend between the birds and the number of days they shed VAH. Four of the six great egrets, GREG 33, 25, 28 and 30, shed VAH multiple days. Interestingly, all GREG continued to shed VAH after they were no longer being fed VAH infected fish (Figure 7). After analyzing their bacterial load ingested and feces we saw a large difference in the amount of VAH that each GREG shed. Each treated great egret ingested different amounts of VAH infected catfish fingerlings ranging from 9.0×10^8 CFU to 2.55×10^9 CFU. This same pattern was observed with quantity of VAH shed. Our statistical analysis did not show a significant correlation with the amount of bacteria fed and the amount detected in the feces. However, our results may be influenced by substantial variations between birds, and variation in bacteria survival in feces. A more controlled analysis would be needed to determine the mechanisms that influence the concentrations of bacteria shed. One important observation that we made in this study is that two of the three highest shedders we were able to culture VAH from the nasal and mouth cavity. VAH had colonized these two locations in the great egrets. Also, we found through bacterial agglutination titer test our test study population did not have any significant titer responses to ingesting VAH infected catfish fingerlings. The control birds did not have any notable titer changes.

Each GREG was necropsied and histological and bacterial samples were taken. The samples were taken from: mouth, nasal, upper intestine, lower intestine, kidney, eye, esophagus, and lungs. No lesions were observed on any of our histological samples. After the bacterial swabs were cultured and isolates processed and analyzed using qPCR assay, VAH was isolated from two GREG in our study population. VAH was detected in

the nasal cavity of GREG 32, and the nasal and mouth cavities from GREG 30. Both great egrets were treated birds and no VAH was detected in control birds. It is likely that VAH was able to colonize these areas because the outer extremities are much cooler compared to high internal temperatures found on other locations.

In summary GREG show strong potential as a vector for the transmission of VAH to catfish ponds. To our knowledge this was the first study that investigated potential natural vectors VAH vectors and helps us understand how it may be spread. We were able to shed light on the transmission potential of VAH through our assay. We concluded that fish eating birds may actually serve as a reservoir (colonized nasal area) for VAH and can potentially spread the pathogen by flying to uninfected ponds. Hopefully this study can lead to ways to control predatory scavenging on commercial catfish operation that can help reduce losses to the industry caused by VAH outbreaks.

REFERENCES

Akkoc, A., L. A. Kocabiyik, et al. (2008). "Burkholderia cepacia and Aeromonas hydrophila Septicemia in an African Grey Parrot." **32**(3): 233-236.

Annapurna, E. and S. C. Sanyal (1977). "Enterotoxicity of Aeromonas Hydrophila." J Med Microbiol **10**(3): 317-324.

Bebak, J., W. Hemstreet, et al. (2010). "Aeromonas hydrophila: Observations of the Alabama industry in 2010."

Brittingham, M. C., S. A. Temple, et al. (1988). "A survey of the prevalence of selected bacteria in wild birds." J Wildl Dis **24**(2): 299-307.

Camus, A. C., W. G. Durborow, et al. (1998). "Aeromonas Bacterial Infections-Motile Aeromonad Septicemia." Southern Regional Aquaculture Center **478**.

Chou, S. Y., C. C. Tsai, et al. (2004). "Aeromonas hydrophila orbital cellulitis in a patient with myelodysplastic syndrome." J Chin Med Assoc **67**(1): 51-53.

Cipriano, R. C., G. L. Bullock, et al. (1984). "Aeromonas Hydrophilia and Motile Aeromonad Septicemias of Fish." Fish Disease Leaflet (1984)(68).

Davis, W. A., 2nd, J. H. Chretien, et al. (1978). "Snake-to-human transmission of Aeromonas (Pl) shigelloides resulting in gastroenteritis." South Med J **71**(4): 474-476.

Egwari, L. and O. O. Aboaba (2002). "Environmental impact on the bacteriological quality of domestic water supplies in Lagos, Nigeria." Rev Saude Publica **36**(4): 513-520.

Franca, M., R. L. Walker, et al. (2009). "Aeromonas species associated with necrotizing enteritis and septicemia in an adult male ostrich (Struthio camelus)." Avian Dis **53**(2): 310-316.

GE, M.-x., Y.-y. ZHANG, et al. (2012). "Examination of red-leg disease and its pathogen, Rana temporaria chensinensis." African Journal of Microbiology Research **6**(4).

Gemmell, C. G. (1984). "Comparative study of the nature and biological activities of bacterial enterotoxins." J Med Microbiol **17**(3): 217-235.

Glunder, G. and O. Siegmann (1989). "Occurrence of Aeromonas hydrophila in wild birds." Avian Pathol **18**(4): 685-695.

Goldstein, E. J., E. O. Agyare, et al. (1981). "Aerobic bacterial oral flora of garter snakes: development of normal flora and pathogenic potential for snakes and humans." J Clin Microbiol **13**(5): 954-956.

Handfield, M., P. Simard, et al. (1996). "Differential media for quantitative recovery of waterborne Aeromonas hydrophila." Appl Environ Microbiol **62**(9): 3544-3547.

Hill, W. A., S. J. Newman, et al. (2010). "Diagnosis of Aeromonas hydrophila, Mycobacterium species, and Batrachochytrium dendrobatidis in an African clawed frog (Xenopus laevis)." J Am Assoc Lab Anim Sci **49**(2): 215-220.

Ilk, N., P. Kosma, et al. (1999). "Structural and functional analyses of the secondary cell wall polymer of Bacillus sphaericus CCM 2177 that serves as an S-layer-specific anchor." J Bacteriol **181**(24): 7643-7646.

Janda, J. M. (1985). "Biochemical and exoenzymatic properties of Aeromonas species." Diagn Microbiol Infect Dis **3**(3): 223-232.

Janda, J. M. and S. L. Abbott (2010). "The genus Aeromonas: taxonomy, pathogenicity, and infection." Clin Microbiol Rev **23**(1): 35-73.

King D.T. , J. D. P. e. a. (1998). "Two Capture Techniques for American White Pelican and Great Blue Herons." Colonial Waterbirds **21**(2): 3.

Krieg, N. R., Ed. (1984). Bergey's manual of systematic bacteriology. Baltimore :, Williams & Wilkins.

Messner, P., K. Steiner, et al. (2008). "S-layer nanoglycobiology of bacteria." Carbohydr Res **343**(12): 1934-1951.

Morgan, D. R., P. C. Johnson, et al. (1985). "Lack of correlation between known virulence properties of Aeromonas hydrophila and enteropathogenicity for humans." Infect Immun **50**(1): 62-65.

Nitta, H., T. Imamura, et al. (2008). "Production of C5a by ASP, a serine protease released from Aeromonas sobria." J Immunol **181**(5): 3602-3608.

Ocholi, R. A. and J. O. Kalejaiye (1990). "Aeromonas hydrophila as cause of hemorrhagic septicemia in a ground-hornbill (Bucorvus abyssinicus)." Avian Dis **34**(2): 495-496.

Page, L. A. (1966). "Diseases and Infections of Snakes: A Review." J Wildl Dis **2**(4): 111-126.

Panigrahy, B., J. J. Mathewson, et al. (1981). "Unusual disease conditions in pet and aviary birds." J Am Vet Med Assoc **178**(4): 394-395.

Pasquale, V., S. B. Baloda, et al. (1994). "An Outbreak of Aeromonas hydrophila Infection in Turtles (Pseudemis scripta)." Appl Environ Microbiol **60**(5): 1678-1680.

Pianetti, A., T. Falcioni, et al. (2005). "Determination of the viability of Aeromonas hydrophila in different types of water by flow cytometry, and comparison with classical methods." Appl Environ Microbiol **71**(12): 7948-7954.

Pollard, D. R., W. M. Johnson, et al. (1990). "Detection of the aerolysin gene in Aeromonas hydrophila by the polymerase chain reaction." J Clin Microbiol **28**(11): 2477-2481.

Pridgeon, J. W. and P. H. Klesius (2011). "Molecular identification and virulence of three Aeromonas hydrophila isolates cultured from infected channel catfish during a disease outbreak in west Alabama (USA) in 2009." Dis Aquat Organ **94**(3): 249-253.

Pridgeon, J. W. and P. H. Klesius (2011). "Virulence of Aeromonas hydrophila to channel catfish Ictaluras punctatus fingerlings in the presence and absence of bacterial extracellular products." Dis Aquat Organ **95**(3): 209-215.

Rahman, A. F. and J. M. Willoughby (1980). "Dysentery-like syndrome associated with Aeromonas hydrophila." Br Med J **281**(6246): 976.

Roberson, B. (1990). Bacterial Agglutination. Techniques in Fish Immunology. Haven, SOS Publications. **1**.

Santos, Y., A. E. Toranzo, et al. (1988). "Virulence properties and enterotoxin production of Aeromonas strains isolated from fish." Infect Immun **56**(12): 3285-3293.

Sara, M. and U. B. Sleytr (2000). "S-Layer proteins." J Bacteriol **182**(4): 859-868.

Sartory, D. P. (2002). Aeromonas. Guidelines for drinking-water quality, Addendum Microbiological agents in drinking water. Geneva, World Health Organization**:** 1-17.

Shane, S. M. and D. H. Gifford (1985). "Prevalence and pathogenicity of Aeromonas hydrophila." Avian Dis **29**(3): 681-689.

Shane, S. M., K. S. Harrington, et al. (1984). "The occurrence of Aeromonas hydrophila in avian diagnostic submissions." Avian Dis **28**(3): 804-807.

Sleytr, U. B. and M. Sara (1997). "Bacterial and archaeal S-layer proteins: structure-function relationships and their biotechnological applications." Trends Biotechnol **15**(1): 20-26.

Subashkumar, R., T. Thayumanavan, et al. (2006). "Occurrence of Aeromonas hydrophila in acute gasteroenteritis among children." Indian J Med Res **123**(1): 61-66.

Thune, R. L., T. E. GRAHAM, et al. (1982). "Extracellular Products and Endotoxin from Aeromonas hydrophila: Effects on Age-0 Channel Catfish." Transactions of the American Fisheries Society **111**(3): 404-408.

Vilches, S., N. Jimenez, et al. (2009). "Aeromonas hydrophila AH-3 type III secretion system expression and regulatory network." Appl Environ Microbiol **75**(19): 6382-6392.

Wang, H. B., D. C. Wang, et al. (2009). "[Development of TaqMan real-time PCR in detection of Aeromonas hydrophila]." Zhonghua Yu Fang Yi Xue Za Zhi **43**(7): 611-614.

WHO (2002) "Guidelines for drinking water quality, Addendum: Microbiological agents in drinking water, 2nd Edition, Aeromonas."

Wiese, A., J. O. Reiners, et al. (1996). "Planar asymmetric lipid bilayers of glycosphingolipid or lipopolysaccharide on one side and phospholipids on the other: membrane potential, porin function, and complement activation." Biophys J **70**(1): 321-329.