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ABSTRACT: *Aeromonas hydrophila* is a gram-negative, rod-shaped, facultative, anaerobic bacterium that is ubiquitous in freshwater and slightly brackish aquatic environments and infects fish, humans, reptiles, and birds. Recent severe outbreaks of disease in commercial channel catfish (*Ictalurus punctatus*) aquaculture ponds have been associated with a highly virulent *A. hydrophila* strain (VAH), which is genetically distinct from less-virulent strains. The epidemiology of this disease has not been determined. Given that *A. hydrophila* infects 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 (*Ardea alba*) were used in this transmission model because these wading birds frequently prey on farmed catfish. Great Egrets that were fed VAH-infected catfish shed VAH in feces demonstrating their potential to spread VAH.

Key words: *Aeromonas hydrophila*, aquaculture, *Ardea alba*, epidemiology, Great Egret, *Ictalurus punctatus*, virulent *Aeromonas hydrophila* (VAH).

INTRODUCTION

In 2009, a virulent strain of *Aeromonas hydrophila* (VAH) caused acute mortalities and chronic disease leading to additional mortalities of catfish (*Ictalurus punctatus*) in western Alabama catfish farms (Pridgeon and Klesius 2011). Between June 2009 and October 2009, VAH caused an estimated loss of more than 1,360 metric tons of market-size catfish (Pridgeon and Klesius 2011). Molecular identification of *A. hydrophila* was achieved by sequencing small portions of the genomes of three isolates cultured from the 2009 outbreak (Pridgeon and Klesius 2011). The 16S-23S ribosomal DNA intergenic spacer region, *cpn60*, *gyrB*, and *rpoD* genes shared 97% to 99% sequence similarities (Pridgeon and Klesius 2011). The three western Alabama isolates had a much lower lethal dose required to kill 50% of the fish (LD_{50}) value in comparison with the 1998 isolate, which suggests greater virulence (Pridgeon and Klesius 2011). Subsequent whole genome analysis of six VAH strains shows that this strain is highly homogeneous with specific molecular markers (Hossain et al. 2013). It is important to understand how VAH is transported between catfish farms. Fish-eating birds, such as Double-crested Cormorants (*Phalacrocorax auritus*), Great Blue Herons (*Ardea herodias*), Great Egrets (*Ardea alba*), and American White Pelicans (*Pelecanus erythrorhynchos*) are frequently found on commercial catfish facilities and may transport bacterial pathogens such as VAH. Observations from outbreaks of disease associated with VAH suggest that VAH primarily kills catfish; other fish have not suffered significant losses.

Some of the clinical signs of VAH septicemia are similar to enteric septicemia of catfish, which includes hemorrhages in the irises, internal and tissue hemorrhages, and ulcers. The VAH spreads throughout the fish and multiplies very quickly. Biochemically, VAH is unusual compared with 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 other *A. hydrophila* isolates, use inositol and are citrate positive.

The epidemiology of VAH infection has not been determined. Anecdotal evidence indicated that west Alabama had a large number of Wood Storks (*Mycteria americana*) scavenging on diseased ponds. The foraging strategy of Wood Storks includes the willingness to prey on both live and sick or dead fish (Coulter et al. 1999); however, because the Wood Stork is an endangered species (USDI-FWS 2006), we used Great Egrets as a model to study the potential for VAH transmission by fish-eating birds. The Great Egret is a common fish-eating bird that frequents catfish ponds and is also attracted to ponds experiencing disease-associated losses (Glahn et al. 1999).

Birds are known to be susceptible to motile aeromonads. Most of the information reported on the relationship between *Aeromonas* and birds is limited to diagnostic submissions of birds that have been killed by *Aeromonas* infection but the history, prevalence, and other factors are not extensively researched (Brittingham et al. 1988). While investigating routine avian diagnostic submissions over a 25-mo period, Shane et al. (1984) found that *A. hydrophila* was isolated from 2% (20 of 1,000 cases). The results suggested that *A. hydrophila* was a facultative opportunistic pathogen of birds. Glunder and Siegmann (1989) concluded the intestines were the primary isolation site of *A. hydrophila*, and the secondary isolation site included the lungs. They found carnivorous, aquatic birds had the highest *A. hydrophila* recovery rate compared with terrestrial avian species (Glunder and Siegmann 1989).

We evaluated the potential for fish-eating birds to transmit and spread a virulent strain of *A. hydrophila* using the Great Egret as the model bird species. We evaluated the ability of Great Egrets to shed viable VAH when fed infected fish and the potential for VAH to colonize those birds.

MATERIALS AND METHODS

Study population

All work was registered and conducted under the supervision of the US Department of Agriculture, Wildlife Services–National Wildlife Research Center (NWRC), Institutional Animal Care and Use Committee, using approved protocols to ensure humane handling and use. Ten Great Egrets were captured at commercial catfish fingerling ponds, under Federal Collection Permit MB019065-0, in the Mississippi Delta using soft catch leg hold traps or a rocket net as described by King et al. (1998) and transported to the NWRC Mississippi Field Station avian test facility. Egrets were tested before starting the trial to confirm they were negative by fecal cultures for VAH. Each egret was weighed and marked with a unique leg band. Birds were individually housed in 3.3×3.3×2 m (L×W×H) cages containing shallow plastic feeding tanks filled with fresh water changed daily. Birds were fed live channel catfish ad libitum throughout the 10-d quarantine and 7-d study periods. Body weight was obtained by placing birds in a preweighed burlap sack and weighing them on a digital scale; cloacal temperatures were obtained by digital thermometer and recorded for the prestudy and poststudy period.

Six test birds were fed VAH-injected channel catfish, and four control birds were fed noninjected catfish for 3 consecutive days. Injected catfish were produced by anesthetizing them with tricaine methanesulfonate (MS222, Western Chemical, Ferndale, Washington, USA) in 100 mg/L of water, followed by intraperitoneal injections with 0.5 mL of an overnight bacterial culture in brain-heart infusion broth containing approximately 1×10^8 colony-forming units (CFUs) of *A. hydrophila* isolate AL09 2, a confirmed VAH. Daily feed logs were used to calculate the number and grams of fish consumed. The total amount of VAH ingested by each egret was calculated because each fish was injected with the same amount of VAH. The amount of VAH each egret ingested depended on the number of infected catfish fingerlings consumed and ranged from 9.0×10^8 CFUs to 2.55×10^9 CFUs. After 3 d of feeding VAH-injected catfish to treatment birds (days 0–2), all egrets were fed noninjected fish for the remainder of the trial (days 3–7). Feces of egrets were collected daily and included samples collected

during quarantine (days −10 to −1), the morning before initial feeding of VAH-infected catfish (day 0), during the VAH challenge (days 0–2), and for 5 d thereafter (days 3–7).

Identification of virulent *Aeromonas hydrophila*

Approximately 1 g of feces was scraped from the concrete floor of each egret pen, placed in a sterile plastic bag (Nasco, Whirl-Pak, Fort Atkinson, Wisconsin, USA), and transported to the laboratory within 1 h. In the laboratory, approximately 0.1 g of feces was added to a 1.5-mL microfuge tube, weighed, and diluted 1:10 in phosphate-buffered saline. The sample was vortexed, and particulate matter removed by centrifuging at 3,000 × G for 30 s. A 100-µL suspension was spread onto ampicillin dextrin (AD) agar plates (Hardy Diagnostics, Santa, California, USA). A series of five 10-fold dilutions were made, and 100 µL of each dilution was spread onto AD agar plates. Plates were then incubated at 37 C for 24 h, yellow convex translucent colonies were tested for cytochrome oxidase C (Becton Dickinson, Sparks, Maryland, USA), counted, and the CFUs of presumptive *Aeromonas* per gram of feces was calculated. Microbiologic tests were performed on each characteristic colony type, and a characteristic colony from each counted plate was identified to species using an API 20E strip test per manufacturer's directions (REF 20160, BioMérieux, Durham, North Carolina, USA). Genomic DNA was isolated from overnight growth of the first dilution-AD agar plates using the Puregene genomic DNA isolation system (158388, Qiagen, Valencia, California, USA) following the protocol for "DNA Purification from Gram-Negative Bacteria Using the Gentra Puregene Yeast/Bact. Kit." DNA was quantified using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Rockland, Delaware, USA). We performed VAH- and *A. hydrophila*–specific quantitative PCR (qPCR) on 0.1 µg of genomic DNA from the *A. hydrophila* isolate AL09 2a; non-VAH isolate AL97-91 and ATCC *F. columnare* were used as positive and negative controls.

Great Egret necropsy

At the conclusion of the trial (day 7), birds were necropsied, and histologic samples and bacterial swabs were collected. The samples were obtained from oral and nasal mucosa, upper and lower intestine, kidney, eye, esophagus, and lungs. Bacterial samples were cultured on AD agar plates. *Aeromonas*-like colonies were evaluated for cytochrome oxidase C activity, and the biochemical profiles of oxidase-positive, gram-negative colonies were determined using API 20E strips. The DNA was extracted from cultures for molecular confirmation. All histologic samples were fixed in 10%-buffered formalin, embedded in paraffin, sectioned, and stained with H&E.

Molecular confirmation of VAH

After the bacterial swabs were cultured, isolates were processed and analyzed using qPCR assay. We used VAH-specific qPCR (Griffin et al. 2013) and *A. hydrophila*–specific qPCR (Wang et al. 2009) on the DNA extracts from the growth of the 10^{-1} dilution plate of fecal samples and from necropsy samples. The qPCR reactions were performed in a final volume of 25 µL containing 30 ng of template (10 µL), 20 pmol of each primer, 10 pmol of FAM/BHQ double-labeled probe (MWG), 2.5 µM dNTP (TAK 4030, Takara Bio, Madison, Wisconsin, USA) (0.5 µL), 10 mM Tris-HCl, 50 mM KCl, 1.5 mM $MgCl_2$ 10× Buffer A (2.5 µL), and 1 unit Takara Hot Start Version *Taq* DNA polymerase. The reaction volume was adjusted to 25 µL with ultrapure water (10.3 µL). The amplification mixtures were subjected to an initial incubation of one cycle of 2 min at 95 C, followed by 40 cycles of 15 s at 95 C, and a final cycle of 65 C for 30 s with a Stratagene research thermal cycler (Mx3005P, Stratagene, La Jolla, California, USA).

Statistics

The influence of VAH dose consumed versus subsequent bacterial load shed was evaluated in the treated birds using analysis of variance (ANOVA) with SPSS computer software (IBM Corporation, Armonk, New York, USA). Fish consumption data were grouped for each egret by three periods, respectively: quarantine period (day −10 through day −1), during treatment period (days 0–2), and posttreatment (days 3–7). We tested for treatment effects and treatment by time (period) interactions on average fish consumption and average fed fish using mixed-effects ANOVA, with pen numbers as random factor to account for repeated measures of response variables. We also compared mean body temperature (C) of egrets between treatments in the three periods using mixed ANOVA. We compared means between treatments for each period using least-squares means and the Tukey-Kramer adjustment for multiple comparisons if the interaction was significant. All analyses were conducted using SAS (SAS, Cary, North Carolina, USA) version 9.22 at an α level of 0.05.

RESULTS

Fish consumption and physiologic changes

The egrets were readily captured and consumed the VAH-injected fish, but there were no differences in consumption before ($P=0.657$) or after ($P=0.997$) the treatment period between treatment and control birds. However, during the treatment period, egrets of the control group consumed fewer fish than did egrets of treatment group ($P\leq0.001$).

Mean fish consumptions (means±SD) of controls were 84.100±34.44, 188.08±24.35, and 226.43±11.78 g for before treatment, during treatment, and after treatment periods, respectively. Mean fish consumptions of treatments were 105.03±35.51, 262.33±52.03, and 206.29±37.62 g for before, during, and after treatment periods, respectively. Body temperature did not differ between treatment and control birds during quarantine, during the infection phase, or after VAH challenge ($P=0.927$). Mean body temperatures (±SD) of control egrets were 40.389±0.289, 41.370±0.362, and 40.315±0.778 C for days 11, 17, and 21, respectively. Mean body temperatures of treatment egrets were 40.286±0.415, 41.659±0.291, and 40.214±0.456 C for period days 11, 17, and 21, respectively.

Necropsy and histopathology revealed no notable lesions in any of the birds.

Virulent *Aeromonas hydrophila* confirmation of shedding

Bacterial cultures of all Great Egret feces collected during the quarantine period were negative for *A. hydrophila*. The most common bacterium isolated was *Plesiomonas shigelloides*. The DNA extracted from the least-diluted culture plate from each bird was negative for VAH using qPCR as well. During the trial, we evaluated the presence of, and quantified, viable VAH in feces. The mean amount of VAH ingested and excreted varied by day (Fig. 1). All treated birds shed VAH at some point during the trial, whereas none of the control birds shed VAH. Four of the six Great Egrets shed VAH on multiple days. All egrets stopped shedding by day 4. When comparing the amount of VAH ingested (total CFU) and the subsequent concentration of VAH shed in the feces by individual egret (CFU/g), there was considerable variation. We applied a linear mixed model with naturally logged excretion amount as the response variable (y) and naturally logged ingested amount as the predictor (x) using an individual identification number as a random variable. The regression coefficient of the logged ingested amount (x) was −2.592 ($t=-1.027$, df=5, $P=0.352$). This was consistent with the result of the Spearman correlation of naturally logged excretion and naturally logged ingested amount ($r=-0.409$, $P=0.187$).

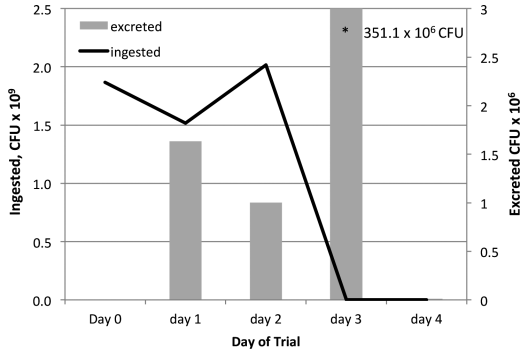

FIGURE 1. Average daily amount of virulent *Aeromonas hydrophila* ingested (total colony-forming units [CFU]) and excreted (CFU/g wet feces) by treated Great Egrets (*Ardea alba*).

We isolated VAH from swabs collected during necropsy from two Great Egrets in our study population. The swabs were collected on day 7, which was 5 d after the final VAH challenge of the treatment birds. We detected VAH in the nasal cavity of one egret, and the nasal and mouth cavities from another.

DISCUSSION

Multiple studies have shown that, on a molecular level, VAH is similar to other strains of *A. hydrophila* with some

variations. Virulent *A. hydrophila* can perform inositol and arabinose oxidation, and it is estimated to be 200 times more virulent to channel catfish than other strains of *A. hydrophila* is (Pridgeon and Klesius 2011). This finding was confirmed with mortality experiments showing west Alabama isolates had a much lower LD_{50} value compared with a nonvirulent *A. hydrophila* isolate (Pridgeon and Klesius 2011). We suspect 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 using contaminated equipment. We also suspect that the natural transmission routes for VAH involve scavengers and predators. In this study, we explored VAH transmission by a predator. Many species of piscivorous waterbirds are found on commercial catfish facilities and are responsible for substantial economic losses to the industry from predation. *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, each bacterial isolate underwent a multistep identification system. After API 20E identification, qPCR analysis was performed on the highest-concentration spread plate per day from each Great Egret. All treatment birds that were fed VAH-infected catfish (days 0–2) shed VAH in their feces, but there was no consistent trend between the birds and the number of days they shed VAH. Four of the six egrets shed VAH on multiple days. All egrets continued to shed VAH after they were no longer being fed VAH-infected fish. However, there were substantial variations among birds and 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 is that we were able to culture VAH from the nasal and mouth cavity of two of the three highest shedders. We have found that during the initial period after capture, birds consume increasing amounts of fish and then intake levels off for a period before decreasing. This pattern was observed in both groups of birds.

Because internal body temperature was not elevated during treatment, and no lesions were observed at necropsy in treated birds, it does not appear that the VAH was pathogenic to the Great Egrets. However, VAH was detected in the nasal cavity, and the nasal and mouth cavities from two egrets. Both were from the treated group, and no VAH was detected in control birds. This demonstrates some potential of VAH to colonize the Great Egret. Although *A. hydrophila* can proliferate at the body temperature of birds, VAH may have preferentially colonized these areas because the outer extremities are cooler than internal body temperatures. We hypothesize that the greater consumption of fish by treated birds during the treatment period is compensatory consumption and have observed this in other studies that we have conducted.

Great Egrets show strong potential as an agent for the transmission of VAH among catfish ponds. This study contributes to our understanding of how VAH may be spread. We were able to elucidate the transmission potential of VAH through our assay. We conclude that fish-eating birds may serve as a reservoir (colonized nasal area) for VAH and can spread the pathogen by flying to uninfected ponds. Reduction of predatory scavenging on commercial catfish operations may also help reduce losses to the industry caused by VAH.

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