1.2.9 Motile *Aeromonas* Septicemia*

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A. Name of the Disease and Etiological Agent

Motile *Aeromonas* septicemia (MAS) is caused by any of three species of the genus *Aeromonas*: *A. hydrophila*, *A. caviae* and *A. veronii* biovar sobria. These species are commonly referred to as motile aeromonads. One strain of *Aeromonas hydrophila* causes particularly devastating MAS in channel catfish and channel x blue hybrid catfish. This strain has distinct characteristics and will be referred to as "virulent *Aeromonas hydrophila*" (VAh).

B. Known Geographical Range and Host Species of the Disease

1. Geographical Range

Motile *Aeromonas* septicemia occurs worldwide, especially in freshwater and brackish water systems. The devastating MAS caused by VAh has a more limited distribution. It was first reported in Alabama in 2009 (Pridgeon and Klesius, 2011) and has since been found in Mississippi and Arkansas.

2. Host Species

Most fish and many aquatic invertebrates are susceptible to disease caused by motile aeromonads. These bacteria also cause disease in amphibians, reptiles, birds and mammals, including humans.

C. Epizootiology

The epizootiology of MAS is complex, only partially understood and likely varies substantially based on the host and water system. The environmental distribution of motile aeromonads has been well studied, with a focus on public health due to concerns of human infections acquired from drinking water, contaminated food and physical contact. These bacterial species are widely distributed in virtually any relevant freshwater or marine environment, including chlorinated tap water (due the ability to form protective biofilms). The density of potentially pathogenic motile aeromonads in natural waters is generally correlated with temperature and organic load. These species can associate with algae, biofilms, and zooplankton and are natural components of the gut flora of many vertebrates and invertebrates. These organisms have the potential to harbor and spread motile aeromonads.

The motile aeromonads appear to be transmitted primarily by horizontal means. Uptake may be by oral or dermal routes with the later occurring after disruption of mucosal defenses. Asymptomatic carriers likely shed the bacterium in feces. Diseased individuals may shed the bacterium from open lesions and are often preyed upon by other fish. Furthermore, motile aeromonads can be saprophytic and likely persist and multiply in decomposing fish.

Most cases of MAS can be correlated to a predisposing factor such as injury, infection with another agent, or an immunosuppressive event such as environmental stress. Motile *Aeromonas* septicemia is often a complicating factor in columnaris disease, saprolegniosis, and several diseases of viral etiology. Furthermore, MAS is sometimes associated with nutritional stress, low dissolved oxygen and handling stress. Typical MAS outbreaks are most common in spring and fall when fluctuating temperatures compromise the immune system.

Many severe outbreaks of MAS caused by VAh have been documented in cultured channel catfish and channel x blue hybrid catfish since 2009, without any identified predisposing primary pathogen or obvious condition that would cause immunosuppression. Severe MAS outbreaks due to VAh in the catfish industry occur primarily in the warm summer months. Losses can progress rapidly, with a large percentage (~20-30%) of the catfish in the pond succumbing to disease within a week from when losses are first observed. These outbreaks frequently occur in larger fish that are at or near market size. In addition, other fish species may succumb to MAS if present in a pond with VAh-associated MAS in the resident catfish population (Figure 1).

D. Disease Signs

Gross pathology of MAS can range from few external or internal signs in peracute cases, to hemorrhagic septicemia in acute cases, to abscesses and large ulcers in chronic cases. Severe MAS outbreaks often display a range of lesions indicating variation in the progression of disease in individuals. External signs seen in fish with typical septicemia include reddened fins, inflammation of the anus, diffuse hemorrhages on the skin, exophthalmia and abdominal swelling (Figure 2). Scaled fish may display protruding scales (lepidorthosis) due to edema of the scale pockets, also fin erosion, focal scale loss and ulcers may be seen (Figure 3). One sign that is commonly seen in catfish with MAS caused by VAh is iridial hemorrhages in the eye (Figure 4). Internal signs include bloody

ascites, diffuse hemorrhages in the intestines, connective tissue, visceral fat and musculature, swollen friable kidney and spleen (Figure 5).

E. Disease Diagnostic Procedures

1. Presumptive diagnosis

The presumptive diagnosis of MAS is usually based on clinical signs and the isolation and identification of *A. hydrophila*, *A. veronii* biovar sobria, or *A. caviae* using traditional biochemical tests or with the aid of commercial biochemical assays such as API 20E, API 20NE (BioMérieux, Durham, NC), Sensititre (TREK Diagnostic Systems, Inc., Cleveland, OH) or BBL Crystal Enteric/Nonfermenter (E/NF; BD Diagnostics, Sparks, MD). However, biochemical characteristics are variable and often result in the misidentification at the species level (Solar *et al*. 2003, Lamy *et al.* 2010). These tests may also misidentify at the genus level, with *Vibrio* being the most common misidentification. It is suggested that resistance to the vibriostatic agent O/129 be used as a supplemental test when using these systems (*Vibrio* and *Plesiomonas* will have a zone of inhibition around a 150 µg O/129 disk).

a. Routine culture

The motile aeromonads grow rapidly on any common nutrient rich agar plates such as TSA or BHIA at 20 to 37°C. Aseptically sampling the kidney tissue of fish with acute MAS will usually produce relatively pure cultures with moderate to high numbers of colonies. Chronic MAS or external lesions may produce mixed colony types. Because motile aeromonads are often secondary pathogens and grow quickly, they can overgrow other, possibly more clinically important, bacterial species. Also, MAS outbreaks should be evaluated for underlying viral infections. Isolating motile aeromonads from open lesions, external surfaces and GI cultures are facilitated using semi selective media such as Rimler Shotts agar (Shotts and Rimler 1973) or Ampicillin dextrin agar plates (Hardy Diagnostics, Santa Maria, CA). On standard culture media the colonies are cream colored, round, slightly raised and often alpha hemolytic on blood agar. These bacteria are Gram negative short rods (~0.5 x 1µm), that are highly motile, oxidase positive, catalase positive, produce indole, ferment and oxidize glucose usually with the production of gas and are resistant to the vibriostatic agent O/129 and novobiocin. Carbohydrate utilization is variable among the species and strains of motile aeromonads. However, the VAh strains are relatively unique in they can utilize inositol (with a preferred form being *myo*-inositol) and citrate as sole carbon sources whereas most motile aeromonads do not.

b. Selective growth of Virulent *Aeromonas hydrophila* (Vah)

The following protocol allows identification of virulent *A. hydrophila* (VAh) strains that utilize *myo*-inositol as a sole carbon source, a phenotype expressed in VAh strains due to the presence of a unique genetic pathway for *myo*-inositol catabolism (Hossain et al., 2013). There are two variants of this assay, the plate or broth formats. Either may be used to discriminate VAh strains from more typical *A. hydrophila* strains that lack the capacity to use *myo*-inositol. The plate assay may be useful for fish tissues, providing a preliminary identification of primary disease isolates as VAh strains, to be followed by more quantitative assays (M9I broth or VAh-specific PCR assay).

To prepare the M9 minimal basal medium, dissolve 11.28 g of 5× M9 minimal salts (Difco, BD) in 968 ml of purified water. For the M9I petri plate assay, include 15 g agarose per L (the use of agar as a solidifying agent permits very weak growth of non-VAh strains). Autoclave the M9 minimal medium at 121°C for 15 min. Allow the medium to cool to 55°C and add 30 ml of filter sterilized solution of 10% *myo*-inositol and 2 ml of sterile 1.0 M

$MgSO_4$ solution. After mixing the M9I agarose, pour approximately 30ml of the medium into a 15 cm diameter petri plate. Tissues from a MAS affected channel catfish may be streaked directly onto a M9I plate to isolate *myo*-inositol utilizing VAh strains. Growth will be observed by 24 hours and a significant lawn with isolated colonies will be observed by 48-72 hours post-inoculation.

For the more quantitative M9I broth assay, grow the bacterial culture overnight at 30°C in tryptic soy broth (TSB) medium (or other appropriate medium). Pellet the bacteria by centrifugation at 10,000 × g for 5 min. Resuspend bacterial cells in M9I broth and repeat the centrifugation step to wash the cells. Next, resuspend the cells in M9I broth to an OD_{600} = 0.5. Dilute the bacterial cell suspension 1:100 in M9I broth. Then add 100 µl of the 1:100 diluted bacterial suspension into 1.9 ml of M9I broth. It is recommended to use three replicates for each isolate. After recording the initial OD_{600}, incubate the culture with shaking at 30°C and record the OD_{600} at 24, 48 , and 72 hours. VAh strains will show significant growth by 48 hours in M9I broth and achieve maximum OD_{600} by 72 hours post-inoculation. To date, a 100% correlation has been observed between the capacity of VAh strains to utilize *myo*-inositol and the presence of a unique genetic locus targeted by the quantitative PCR (qPCR) assay (Griffin et al., 2013).

2. Confirmatory diagnosis

Genomic DNA based assays are the most commonly used methods to confirm a motile aeromonad identification.

a. DNA extraction and controls.

DNA is extracted from affected fish tissues (lesion, posterior kidney) or bacterial cells using a commercially available kit sucha as DNeasy Blood and Tissue kit (Qiagen, Valencia, CA, USA) following the manufacturer's protocol. The DNA can be quantified and evaluate for purity spectrophotometrically. Alternatively, a suspension of approximately 2 µl of an individual bacterial colony can be suspended in 100 µl of sterile PBS heated to 95°C for 5 min and used as PCR templates. All PCR assays should use a representative negative control (a non-*Aeromonas* or non-VAh bacterial isolate, on non-infected tissue) prepared using the same reagents. Likewise a positive control should be included in each analysis to rule out assay failure.

b. Molecular analysis to confirm motile *Aeromonas* species

Confirmation and more precise identification to the species or strain level is accomplished using sequence analysis of the 16S ribosomal RNA gene or housekeeping genes such as *rpoD* (which encodes sigma factor 70 of RNA polymerase) or the *gyrB* genes (which encodes the β-subunit of DNA gyrase) (Yanez *et al.* 2003, Soler *et al*. 2004). A more rapid confirmation that the organism is an aeromonad can be achieved using genus specific PCR targeting specific sequences of the glycerophospholipid-cholesterol acetyltransferase gene (GCAT) (Chacon *et al.* 2002, Soler *et al.* 2002) or the 16S ribosomal RNA gene (Kong *et al.* 1999) (Table 1).

c. Conventional PCR for detection of VAh strain

The VAh strain can be differentiated from other *Aeromonas* spp. by amplifying a 167-bp region of a predicted open reading frame unique to VAh strains (Griffin et al., 2013; Tekedar et al., 2013). Reactions consist of 13 µl of PCR super mix, 20 pmol of each

primer, 5 µl of template (genomic DNA or cell suspension) and nuclease-free water to bring the total volume to 25 µl, using thermal cycling conditions listed in Table 1.

d. Quantitative PCR for detection of VAh strain

Quantitative detection of the VAh strain can be achieved using the above mentioned primers and an internal oligonucleotide hydrolysis probe (2968P; 5'-FAM-TCAAGCGTT/ZEN/CATAAAGTGCCGAGTCA-BHQ-3') (ZEN probes available from Integrated DNA Technologies, standard hydrolysis probes are also effective). Reaction conditions are contingent on the requirements of the DNA polymerase used in the reaction. The VAh-specific qPCR assay was validated using two different commercially available qPCR master mix formulations: (1) the IQ supermix™ (BioRad, Hercules, CA) was used when genomic DNA isolated from broth culture or infected fish tissues was the target or (2) the Taqman® Environmental Master mix 2.0 (Life Technologies, Grand Island, NY, USA) was used when genomic DNA was isolated from substrates with potential inhibitory components. Reactions consist of 7 µl of PCR super mix, 20 pmol of each primer, 2 pmol of probe, 5 µl of DNA template and nuclease-free water to a total volume of 15 µl. Amplifications are performed on a real-time PCR platform programmed for a 2-step amplification cycle based on the specific recommendations for each super mix. The reactions with the IQ super mix™ use a 3 min initial denaturation at 95°C followed by 40 cycles of 95°C for 10 sec and 65°C for 30 sec. The reactions that use the Taqman® Environmental Master Mix require an initial denaturation step of 95°C for 15 min followed by 40 cycles of 95°C for 15 sec and 65°C for 1 min.

F. Procedures for Detecting Subclinical Infections

Because motile aeromonads are ubiquitous, there has been little emphasis on optimizing and validating methods for the detecting subclinical infections. Low levels of motile aeromonads can be cultured from the posterior kidney of apparently healthy fish using standard media. Also, these bacteria are also common in the gut of fish. Selective media such as Rimler Shots medium or Ampicillin Dextrose medium facilitates culture from external surfaces and the gut. The use of VAh-specific PCR and/or VAh-selective medium that uses *myo*-inositol as a sole carbon source will aid in the surveys or inspections of populations for the VAh strain. PCR can also be used on gut samples or environmental samples if commercially available products appropriate to the sample substrate (feces, sediment, pond water, etc.) to remove potential inhibitory agents.

G. Procedures for Determining Prior Exposure to Etiological Agent

Most freshwater fish are repeatedly exposed to motile aeromonads. The detection of low level VAh in some fish in a population using the methods described above would indicate that the fish had been exposed. There is considerable antigenic diversity among the species and strains of motile aeromonads but the VAh strain isolates are relatively homogenous and fish do develop antibodies in experimental exposures (Pridgeon and Klesius 2011), suggesting the potential to use serology to screen for previous exposure to VAh.

H. Procedures for Transportation and Storage of Samples

See Section 1, 1.1.1 General Procedures for Bacteriology.

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Table 1. Primers and conditions for *Aeromonas* specific PCR assays.

Specificity/ Target sequence	Primers (5'-3')	Denaturation	Annealing	Extension	Cycles/ Product length
Genus GCAT[1]	f:CTCCTGGAATCCCAAGTATCAG r:GGCAGGTTGAACAGCAGTATCT	94°C, 1 min	56°C, 1 min	72°C, 1 min	35/ 237 bp
Genus rDNA[2]	f:GGAAACTTCTTGGCGAAAAC r:GGTTCTTTTCGCCTTTCCCT	94°C, 1 min	60°C, 1 min	72°C, 1 min	35/ 550 bp
VAh strain/ Unique ORF[3]	f:CTATTACTGCCCCCTCGTTC r:ATTGAGCGGTATGCTGTCG	95°C, 15 sec	60°C, 1 min		40/ 167 bp

[1]Glycerophospholipid-cholesterol acetyltransferase gene PCR (Soler et al. 2002). The first cycle includes an initial incubation at 95°C for 3 min and the last cycle ends with an incubation at 72°C for 3 min.

[2]Targets a conserved portion of the intergenic spacer and the 23S ribosomal RNA gene (Kong *et al.* 1999). The first cycle includes an initial incubation at 94°C for 2 min and the last cycle ends with an incubation at 72°C for 3 min.

[3]Targets a region of a predicted open reading frame unique to VAh strains (Griffin et al., 2013; Tekedar et al., 2013). The first cycle includes an initial incubation at 95°C for 5 min.

Figure 1. Channel catfish (top) and a Fathead minnow (bottom) from a commercial catfish pond experiencing a MAS epizootic caused by VAh. Pictures by William Hemstreet.

Figure 2. An MAS affected channel catfish with diffuse external hemorrhages. Picture by William Hemstreet.

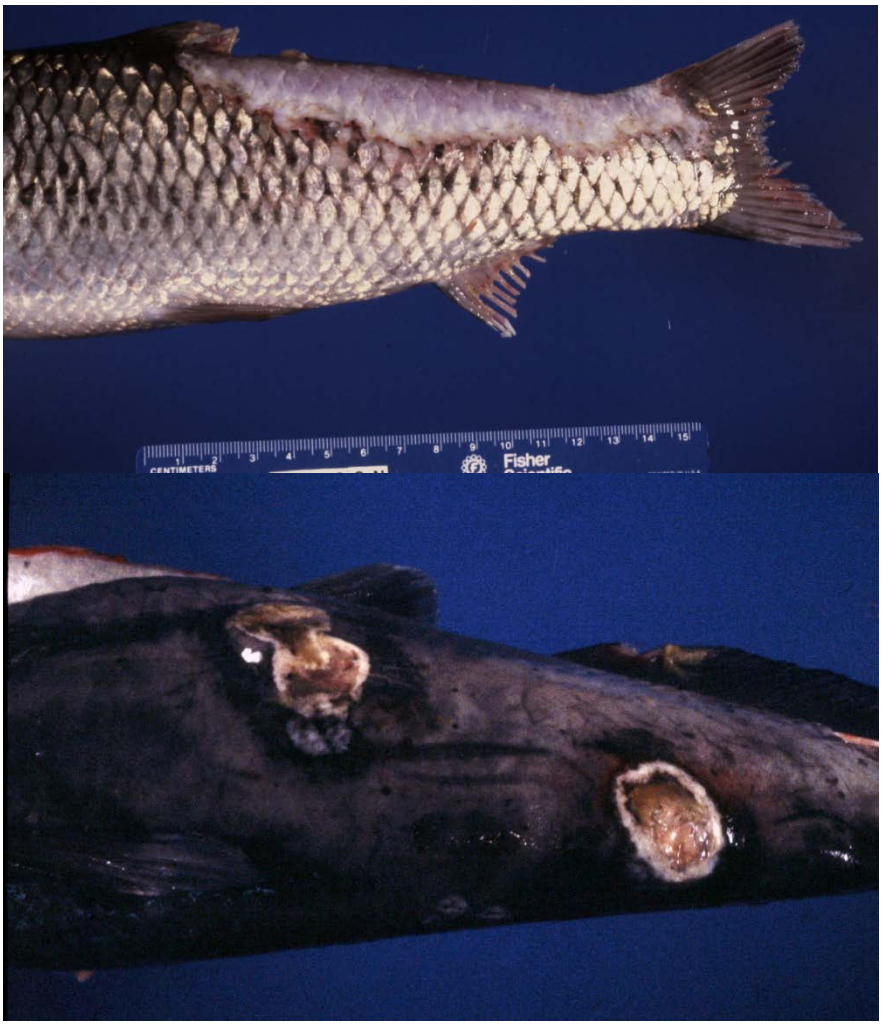

Figure 3. Scale loss and fin erosion in a grass carp with MAS (top) and dermal ulcers on a channel catfish with MAS (bottom). Pictures by William Hemstreet.

Figure 4. Hemorrhages in the eye of a channel catfish with MAS caused by VAh (left) and more extensive eye damage (right). Pictures by William Hemstreet.

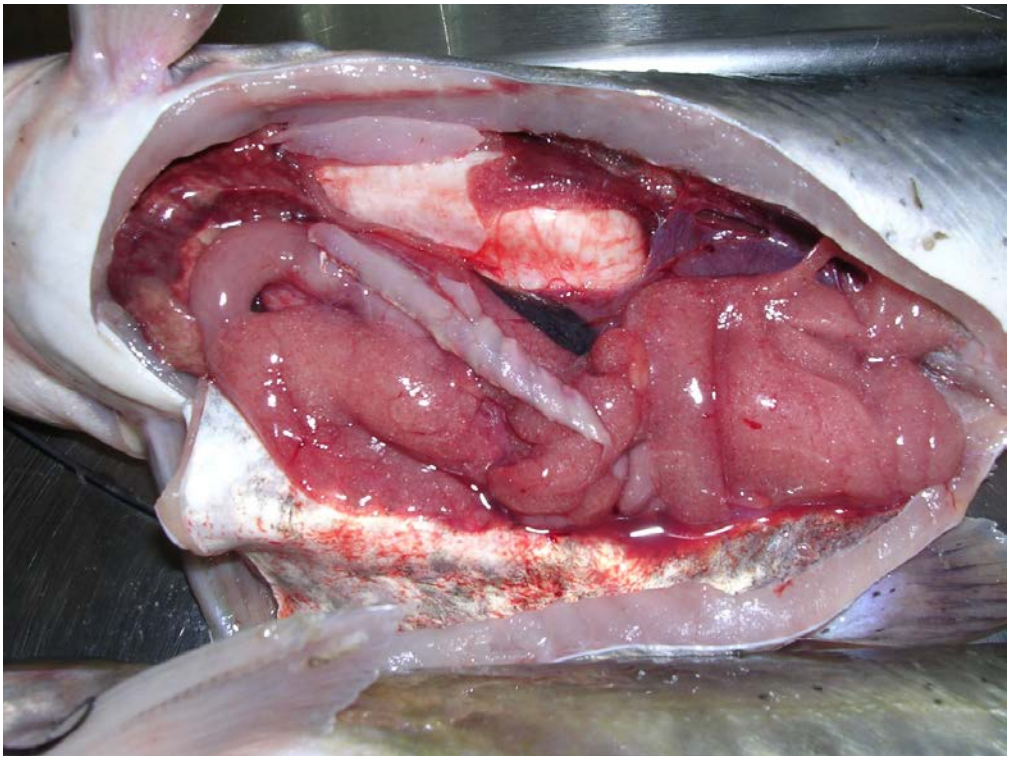

Figure 5. Hemorrhagic visceral organs and adipose tissue and bloody ascites in a channel catfish with MAS caused by VAh. These lesions are common in fish with acute MAS. Pictures by William Hemstreet.