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Experience with Mortalities of Cultured Catfish *Ictalurus punctatus* (Rafinesque 1818) and *I. punctatus* X *I. furcatus* (Valenciennes 1840) caused by Highly Virulent Strains of *Aeromonas hydrophila*

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ABSTRACT

Epizootic outbreaks of motile aeromonad septicaemia (MAS) due to *Aeromonas hydrophila* infections in channel catfish *Ictalurus punctatus* (Rafinesque 1818) and hybrid catfish *I. punctatus x I. furcatus* (Valenciennes 1840) spread through West Alabama and East Mississippi, United States of America in 2009 and have been seasonally recurrent, with losses in the millions of pounds. While *A. hydrophila* is typically considered an opportunistic pathogen, no other primary aetiologic agent has been found. Mortalities are as high as 60 % in ponds and primarily affect larger fish, causing diseases characteristic of MAS infections that include severe skin ulceration and haemorrhage, generalized petechiation, ascites, marked splenomegaly with necrosis, and gastric haemorrhage. A multistate research group (Alabama, Arkansas, Louisiana and Mississippi) examined *A. hydrophila* isolates, which were found to be highly clonal by phylogenetic analyses of gene sequences (*atpD, dnaJ, dnaX, gyrA, gyrB, recA, rpoD*). A representative strain of the hypervirulent *A. hydrophila* pathotype (vAh), ML09-119, was found to be more virulent in channel catfish than historical *A. hydrophila* strains associated with traditional, opportunistic infection (tAh). Bar coded sequencing of numerous vAh and tAh isolates identified unique genomic regions associated with the epizootic isolates. A diagnostic polymerase chain reaction (PCR) targeting a unique vAh-associated genetic locus has been developed to differentiate vAh strains from tAh strains.

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Some of these genes were associated with *myo*-inositol metabolism, which corresponds with the ability of epizootic strains to utilize *myo*-inositol. Genes from lysogenic bacteriophage, O-antigen biosynthesis genes and transposases were also uniquely present in the epizootic strains. Collectively, these data support the conclusion that lateral gene transfer has contributed to the pathogenicity of epizootic *A. hydrophila* strains. Further research will need to be conducted to determine the specific contribution of the unique genetic loci to *A. hydrophila* virulence.

Keywords: catfish, motile *Aeromonas* septicaemia, United States of America, virulent *Aeromonas hydrophila*

Introduction

The role of horizontal genetic transfer, as it relates to enhanced bacterial virulence in aquaculture, is the focus of this contribution, which highlights the effect that one plasmid can have on an industry as well as trade worldwide (Lee et al. 2015). Mobile genetic elements, consisting of plasmids, insertion sequences, transposons, phages and integrons are ubiquitous in bacteria and figure prominently in horizontal gene transfer between bacteria. By enabling the acquisition of genes to promote survival in various environments, these mobile genetic elements have profound implications for human and animal health, where the ability of bacteria to acquire antimicrobial resistance and additional virulence factors leads to enhanced pathogenicity, disease and mortality. Such a situation has occurred internationally with *Vibrio parahaemolyticus* in farmed shrimp, as well as in *Aeromonas hydrophila* in farmed catfish in the United States of America and in farmed carp in the People's Republic of China (Lee et al. 2015; Rasmussen-Ivey et al. 2016b). The following is a brief review of our experience and understanding of this disease in the farmed catfish industry.

Currently, there are 47 recognized species of *Aeromonas* (Euzéby 1997). The members of this genus are ubiquitous in the environment, particularly in aquatic habitats, and thrive over a wide range of temperatures, pH and turbidities (Janda and Abbott 2010). Within *Aeromonas*, motile aeromonads are a group of loosely associated species (*A. hydrophila*, *A. sobria*, *A. veronii*, *A. caviae*, among others) that have similar biochemistry, genetics and serology (Cipriano and Austin 2011). This mesophilic group is typified by *A. hydrophila*, which has an optimal temperature range of 35–37 ^{o}C and is well known to cause disease in multiple species of fish. *Aeromonas hydrophila* was the first motile aeromonad species named; in addition to causing fish disease, this species can be associated with opportunistic infections in many vertebrates, including humans (Janda and Abbott 1998). Disease caused by *A. hydrophila* and other motile aeromonads is often referred to as motile *Aeromonas* septicaemia (MAS), which is characterized by widespread systemic infection (Grizzle and Kiryu 1993). The primary species responsible for MAS are *A. hydrophila*, *A. sobria* and *A. caviae* (Plumb 1999).

Natural infection in catfish has four forms: septicaemia, focal visceral lesions, cutaneous, and asymptomatic (Meyer 1975). Fish with MAS typically have marked external hyperaemia and haemorrhage, swollen abdomens and exophthalmia/ panophthalmitis that may progress to ocular rupture (Plumb 1999). Internally, there is generalized hyperaemia and petechiation, severe multifocal necrosis in the liver and kidney, renomegaly, splenomegaly, variable splenic necrosis, bloody ascites and flaccid intestines containing haemorrhage and yellow mucus (Plumb 1999; Cipriano et al. 2001).

Disease attributable to *A. hydrophila* has been reported in many fish species including channel catfish *Ictalurus punctatus* (Rafinesque 1818) (Miller and Chapman 1976; Grizzle and Kiryu 1993; Baumgartner et al. 2016), minnows and baitfish, common carp *Cyprinus carpio* (Linnaeus 1758), gizzard shad *Dorosoma cepedianum* (Lesueur 1818) (Rock and Nelson 1965), grass carp *Ctenopharyngodon idella* (Valenciennes 1844) (Deng et al. 2009), striped bass *Morone saxatilis* (Walbaum 1792), largemouth bass *Micropterus salmoides* (Lacépède 1802) (Miller and Chapman 1976) and tilapia (Abu-Elala et al. 2015). *Aeromonas hydrophila* is found abundantly in almost all freshwater environments including domestic tap water, sediment and sewage, as well as being part of the normal flora on the skin and in the intestines of fish (Hazen et al. 1978; MacMillan and Santucci 1990; Brandi et al. 1996). Therefore, the mere presence of *A. hydrophila* by itself is not indicative of poor environmental quality or impending disease outbreaks in fish populations.

Disease potential of the opportunistic, traditional *A. hydrophila* strains (tAh) is based on complex interactions between multiple biotic (host and bacterium) and abiotic (climate, water chemistry, etc.) factors (Janda 1991; Cipriano et al. 2001). *Aeromonas hydrophila* is considered to be an opportunistic or secondary pathogen, where pre-existing diseases, weakened immune systems, injury, crowding or poor water quality (e.g. low oxygen, high ammonia or extreme temperatures) provide the bacterium an opportunity for tissue infection (Miller and Chapman 1976; Walters and Plumb 1980; Plumb 1999). In culture ponds, where environmental stressors abound, it is common to find more than one pathogen in a single moribund fish. For example, catfish with motile aeromonad infections are often co-infected with *Flavobacterium columnare* or *Edwardsiella ictaluri*, giving credence to the idea that motile aeromonads are opportunistic (Rock and Nelson 1965; Hawke and Thune 1992). In catfish pond disease outbreaks, it is uncommon to find MAS without the presence of another significant bacterial, fungal or parasitic pathogen (W. Baumgartner, personal observation). Despite the historical assignation as an opportunistic pathogen, *A. hydrophila* can indeed act as a primary pathogen. Within the hypervirulent *A. hydrophila* pathotype (vAh), strain J-1 (categorized as sequence type 251 [ST251]) was first reported in 1989 in association with epizootic outbreaks in Chinese carp (Chen and Lu 1991). Since then, vAh strains (NJ-35, ZC1) have been recognized as the causative agents of severe MAS outbreaks (MASv) in farmed grass carp in the People's Republic China, resulting in losses exceeding US$74 million annually (Deng et al. 2009; Rasmussen-Ivey et al. 2016b).

Aeromonas hydrophila reference isolates (vAh and tAh) are equipped with an arsenal of virulence factors, which cumulatively contribute to disease; they include secretion systems (type II, and some have a complete type VI), biofilm formation, flagella, haemolysins, O-antigens, S-layers, collagenase, elastase, lipase, metalloprotease and serine protease, among others (Rasmussen-Ivey et al. 2016a). Furthermore, the genus *Aeromonas* has a complex array of mobile genetic elements with the ability to transfer resistance and virulence genes between strains and different species (Piotrowska and Popowska 2015).

Since 2009, pond outbreaks caused by an emergent clade of vAh have resulted in the loss of more than 20 million pounds of market-size farmed catfish (*Ictalurus punctatus* and *I. punctatus* x *I. furcatus*) in Alabama and in Mississippi. Outbreaks of MASv began in West Alabama in April 2009 and continued through September. That year, MASv was documented on at least 48 farms with an estimated loss of 3 184 000 lbs of catfish. In the spring and summer of 2010, the disease re-emerged and spread to at least 60 farms (including the 48 affected in 2009), with estimated losses of 2 400 000 lbs of catfish. Data from subsequent years are similar, with losses of over 2 million lbs of catfish per year (W. Hemstreet, Alabama Fish Farming Center, personal communication). Contaminated fish, water and seining/hauling equipment are likely sources for spreading this disease.

This disease has continued to spread through West Alabama farms and is now the most commonly diagnosed pathogen at the Alabama Fish Farming Center (diagnosed in 35 % of case submissions) (Hemstreet 2015). In East Mississippi, the same trend has occurred; MASv was the most commonly diagnosed disease from 2012 to 2015, and it was diagnosed in 35 % of case submissions in 2015. From 2013 to 2015, MASv cases have also occurred on catfish farms in the Mississippi delta. In 2015, at least five West Mississippi catfish operations and two Arkansas operations reported having MASv outbreaks. In affected farms, MASv was characterized by an acute onset of anorexia followed by high mortality rates of up to 50 to 60 %, predominantly in the large market-size fish. Affected fish had clinical signs typically seen in MAS caused by other aeromonad strains and species, with dermal haemorrhage and ulcers, and multi-organ necrosis.

Because *A. hydrophila* was initially considered a secondary pathogen, efforts focused on the identification of an underlying primary pathogen or condition. Through cooperative efforts (Auburn University; Mississippi State University, College of Veterinary Medicine in east Mississippi (MSU-CVM) and Thad Cochran National Warmwater Aquaculture Center in the Mississippi delta, University of Arkansas-Pine Bluff, Louisiana State University, and the United States Department of Agriculture Agricultural Research Service, (USDA ARS)), underlying primary bacterial disease agents (including known pathogens such as *E. ictaluri*, anaerobic bacteria or obligate intracellular bacteria), viruses, parasites, environmental conditions, genetic factors and other known causes of disease were ruled out.

At the same time, research was conducted to determine if the *A. hydrophila* isolates represented an emerging primary pathogen of catfish. The MASv isolates from channel catfish ponds had been previously typed as *A. hydrophila* based on biochemistry (API 20E assay) and 16S rRNA gene sequencing (100 % identity to known *A. hydrophila* strains). Interestingly, phenotypic characterizations by API 20E assay indicated that vAh strains had a unique biochemical profile within *A. hydrophila*, in that they ferment inositol. Based on phylogenetic analysis of the DNA gyrase B-subunit gene (*gyrB*) (Yanez et al. 2003), virulent strains of *A. hydrophila* were grouped together as a single clade that could be distinguished from all other *A. hydrophila*.

Aeromonas hydrophila Genomics

The 4.7 Mb genome of *A. hydrophila* type strain ATCC 7966 reveals considerable metabolic versatility, reflecting its ability to cause disease in multiple hosts and persist in aquatic environments (Seshadri et al. 2006). It appears able to use a large number of substrates for growth and is capable of inactivating a large number of toxic compounds. A large number of potential virulence genes were detected, several of which encoded secreted proteins. A functional repeat in toxin (Rtx) with an actin cross-linking domain was also found (Suarez et al. 2012). Pan-genome analysis of *Aeromonas* species revealed higher pathogenic potential and antimicrobial resistance in *A. hydrophila* compared to *A. veronii* and *A. caviae* (Ghatak et al. 2016). Recently the genomes of many *A. hydrophila* isolates, including vAh strains, have been completed (Tekedar et al. 2013, 2015; Pridgeon et al. 2014a,b; Pang et al. 2015; Rasmussen-Ivey et al. 2016b; Yang et al. 2016), which include AL06-06 from goldfish, virulent Chinese carp strain JBN2301, vAh strain AL09-71 and catfish pond isolates pc104A and ML90-119^{T} (type strain for initial studies).

Analysis of genome sequences from ML09-119 and other vAh strains, including those isolated from Asian carp, revealed that the vAh strains are highly similar whereas the tAh genomes were highly variable and lacked many of the gene sequences present in the vAh strains (Hossain et al. 2014; Rasmussen-Ivey et al. 2016b). This result was supported from a concatenated phylogeny based on seven evolutionarily conserved gene sequences (Hossain et al. 2014) and from a core genome phylogenetic analysis of *Aeromonas* spp. (Rasmussen-Ivey et al. 2016b), which demonstrated the highly clonal nature of these recently emerged *A. hydrophila* isolates. Interestingly, many vAh isolates obtained recently from MASv outbreaks in Mississippi are more closely affiliated with Asian carp isolates compared to vAh strains isolated from MASv outbreaks in Alabama (Rasmussen-Ivey et al. 2016b). It is possible that vAh emerged from a common *A. hydrophila* ancestor carried by grass carp or other carp species, which have been used in aquaculture ponds in the United States of America for weed control since the 1960s (Hossain et al. 2014).

Analysis of the vAh-associated unique genetic regions revealed genes that are present in vAh and absent from tAh strains, including genes located within predicted genomic islands, suggesting their acquisition through lateral gene transfer (Fig. 1 and 2) These vAh-associated genes are predicted to be involved in many functions, including *myo*-inositol catabolism, prophage structure and regulation, transposases and other genes with low percent similarity to known *A. hydrophila* gene sequences.

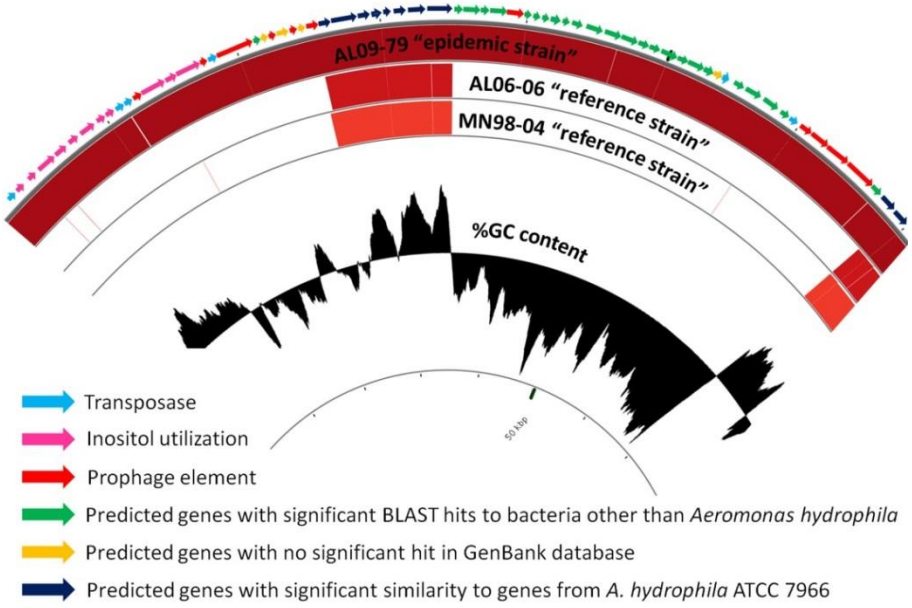

Fig. 1. Comparative genomic analysis of a contig from vAh strain ML09-119 against vAh strain AL09-79 and two tAh strains (inner rings) along with percent GC content. Graph represents BLASTn comparison using CGView (Grant and Stothard 2008).

Fig. 2. Comparative whole genome predicted gene-based analysis of all confirmed vAh (n=26) and tAh isolates (n=15).[1]

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

One of the important genotypic differences in the vAh isolates concerns the organization of the type six secretion system (T6SS) operon. Chinese carp isolates (J-1, NJ-35, ZC1) contain a majority of the proteins necessary for a functional T6SS, while Alabama and Mississippi vAh isolates form a subclade where the majority of core proteins (9 of 13) are lacking (Rasmussen-Ivey et al. 2016b). Immersion challenge data in catfish (see following) found that Asian strain ZC1 was less virulent than American catfish isolates, a finding that seems counter-intuitive given the assumption that T6SS should engender greater pathogenicity. However, the functionality of the T6SS in catfish vAh isolates is currently unknown; the reduced T6SS may somehow allow enhanced evasion of the fish immune system (Rasmussen-Ivey et al. 2016b) (Fig. 3).

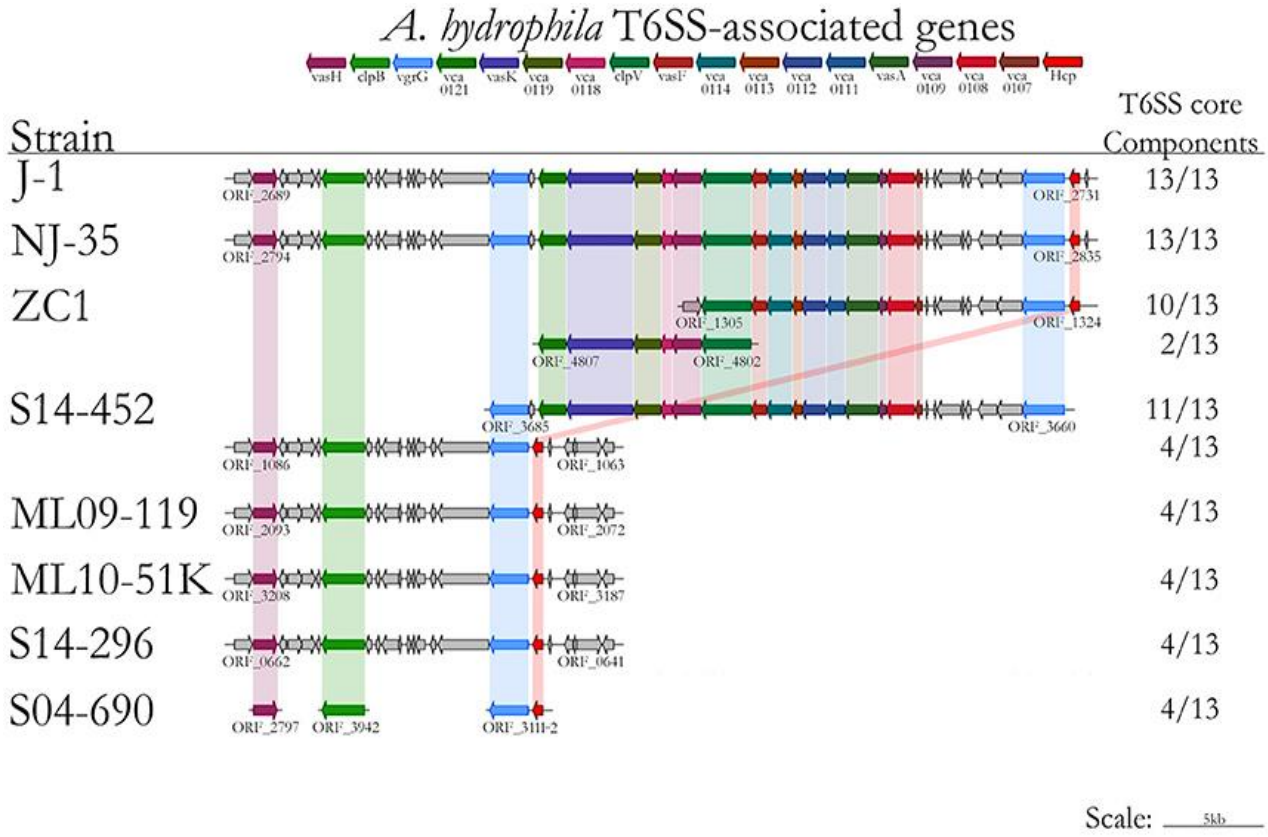

Fig. 3. Type VI secretion system gene prediction using T346 Secretion System Hunter, with results including strains included in the immersion catfish challenge (ML09-119, MNL10-51K, S04-690, S14-296, S14-452 and ZC1) and representatives from Chinese strains (J-1, NJ-35 and ZC1).[1]

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

Myo-Inositol Catabolism

Phenotypic experiments showed that vAh isolates are capable of growth in a M9 minimal medium containing inositol (M9I) as the sole carbon source (Hossain et al. 2013). Growth of vAh strains on M9I has been consistently observed for all strains isolated from diseased fish in ponds experiencing a vAh epizootic (n>129). By contrast, almost all *A. hydrophila* isolates taken from pond water, pond sediment or from fish in a processing plant (n=31) were not able to grow on M9I.

Furthermore, strains that were identified as positive on the M9I medium were also tested positive as vAh strains based on the vAh-specific quantitative polymerase chain reaction (qPCR) assay. The ability of vAh isolates to use *myo*-inositol as a sole carbon source may reflect pathogen adaptation to the catfish host. In 1989, it was discovered that catfish do not require *myo*-inositol as a dietary additive because catfish are capable of *de novo* synthesis of *myo*-inositol with high endogenous levels of *myo*-inositol in tissues (Burtle and Lovell 1989). However, the significance of *myo*-inositol catabolism as it pertains to virulence remains uncertain.

Virulent A. hydrophila Strains Have a Unique O-Antigen

The vAh strain ML09-119 and other sequenced catfish vAh strains from the United States of America contain a unique 26.5 kb O-antigen biosynthesis gene cluster that ATCC 7966 and Asian carp vAh isolates lack (Pang et al. 2015). There are 25 total predicted genes within the United States catfish cluster that are all organized in the same transcriptional orientation, which suggests that this is an O-antigen biosynthesis operon. The O-antigen biosynthesis gene clusters of other sequenced *A. hydrophila* strains vary in the number of genes in this cluster (22 in the Asian carp cluster and 34 in ATCC 7966), as well as in the relative organization of the genes and in the gene sequences.

There are 96 recognized serogroups within the motile mesophilic *Aeromonas* species (Thomas et al. 1990). Given the unique O-antigen biosynthesis operon in vAh, it is likely that this region has been introduced into vAh via lateral gene transfer, and that these isolates comprise a unique serotype. O-antigen contributes to the virulence of other *A. hydrophila* strains by increasing adhesion to host cells (Merino et al. 1996a, b); however, the contribution of the vAh O-antigen towards virulence needs to be investigated. In summary, our analysis of available genome sequence data strongly supports that Asian and American vAh strains share a common ancestor and that these vAh strains share many genotypic and phenotypic characteristics (e.g. use of *myo*-inositol as a sole carbon source). In addition, these data strongly suggest that lateral gene transfer has contributed to the pathogenicity of vAh strains.

Virulent A. hydrophila Strain-Specific Primer Design and Evaluation

The initial set of consensus sequences present in catfish-associated vAh strains was used to develop primer sets that were specific to these strains and be useful for diagnostic and epizootilogical studies. From the collective results of these studies, three primer sets were selected as providing the best specificity for known vAh strains while maintaining robust PCR conditions. The most promising primers (2986L and 2986R) were refined to provide better results in real-time PCR assays (Griffin et al. 2013). This has proven more useful and specific than the previous methods that were based on sequencing a portion of the *gyrB* gene. The assay is repeatable and reproducible with a linear dynamic range covering eight orders of magnitude and a sensitivity of approximately seven copies of target DNA in a 15 µl reaction.

In addition, the assay was able to detect and quantify the epizootic strain from catfish tissues (0.025 g), pond water (40 ml) and pond sediments (0.25 g) with a sensitivity limit of approximately 100 bacteria in a sample. Recent examination of 26 vAh isolates (now including Asian strains) found that many would not produce an amplicon. Touchdown PCR was performed on all isolates, and vAh specific primers were developed not only to differentiate vAh from tAh (targeting a serine protease), but to discriminate different vAh lineages (Table 1). A new vAh specific primer set (vAh-SerF and vAh-SerFR) has now been developed to include Asian and American isolates, while excluding non-vAh isolates (Rasmussen-Ivey et al. 2016b).

Table 1. Oligonucleotide primers specific to members of the vAh pathotype (vAh-SerF and vAh-SerR), previously described qPCR vAh primers (2986F and 2986R) and primers used to screen for unique isolates used in this study (ML09-119F, ML09-119R, S14-452F, S14-452R, ZC1F and ZC1R).

Primer name	Direction	Sequence	Amplicon size (bp)
vAh-SerF	Forward	5′-AG′CATCACCAGCGTTGGCCC-3′	502
vAh-SerR	Reverse	5′-GCCGGGCTGAACTTCCGCAT-3′	
2986F	Forward	5′-CTATTACTGCCCCCTCGTTC-3′	167
2986R	Reverse	5′-ATTGAGCGGTATGCTGTCG-3′	
ML09-119F	Forward	5′-GTTCCGTTCCATCTGTTCGTGA-3′	246
ML09-119R	Reverse	5′-CAACCATCTTGGTCGCAATC-3′	
S14-452F	Forward	5′-CAGAACGTGCTGCAGAGATTGA-3′	350
S14-452R	Reverse	5′-TCCGAGAATTCGATGACGAAGG-3′	
ZC1F	Forward	5′-GCAATTCTGCGGTCACTTCTCG-3′	400
ZC1R	Reverse	5′-AGCGTACCGTCTCGTCGATATG-3′	

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

Pathology of MASV

Natural Pond Outbreaks (Case Material from East Mississippi)

Catfish pond outbreaks were most often seen when temperatures were in the 30 to 32 ^{o}C range, i.e. in the summer months. Both channel and hybrid catfish ponds were affected equally. Externally, MASv pond catfish exhibited signs typical of motile aeromonad infection, with petechiation, periocular cellulitis, exophthalmia, panophthalmitis (Fig. 4A) progressing to ocular rupture, and endomeningitis with cellulitis overlying the brain (hole-in-the-head lesions, Figure 4A), as well as ulceration of the skin (particularly around the anus) and fins (Fig. 4B), (Baumgartner et al. 2016). Skeletal muscle was characteristically deep red throughout (Fig. 4C). Similar findings were seen in vAh challenge models (Rasmussen-Ivey et al. 2016b) (Fig. 5).

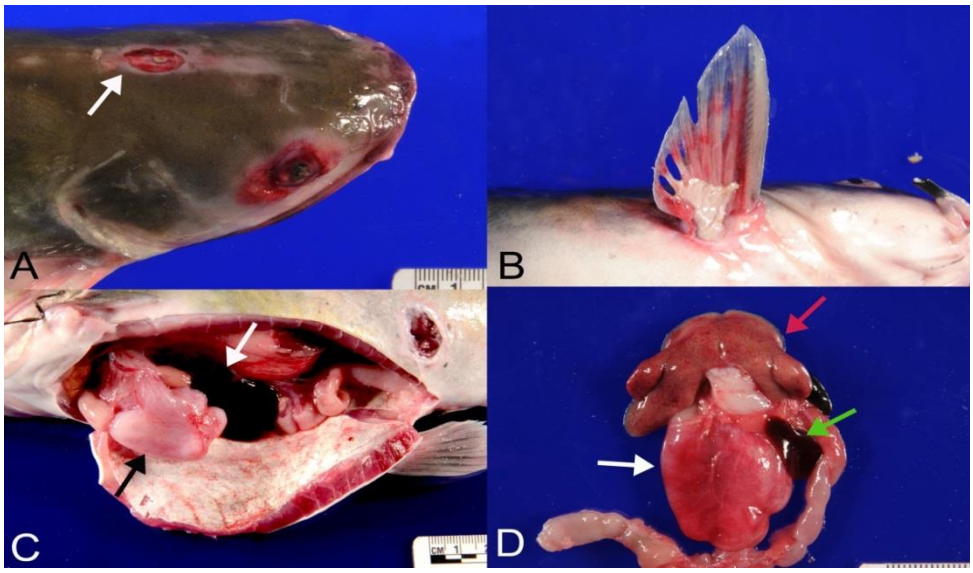

Fig. 4. Channel/hybrid Mississippi pond catfish naturally infected with virulent *Aeromonas hydrophila*. (A): The eye and surrounding soft tissues are swollen, haemorrhagic and ulcerated. The soft tissues overlying the brain are ulcerated with tan necrotic material (white arrow). (B): The right pectoral find exhibits severe necrosis of the skin and soft tissues between the fin rays. (C): Within the abdomen, tissues are hyperaemic and petechiated, with scant haemorrhagic fluid. Splenomegaly (white arrow) and gastric edema with petechiation (black arrow) are evident. The musculature is haemorrhagic and a deep ulcer is present at the right. (D): Viscera. The stomach (white arrow) is flabby with marked haemorrhage in the wall. The spleen (green arrow) is mildly enlarged and the liver (pink arrow) is petechiated with an enhanced reticular pattern. The intestine is hyperaemic.

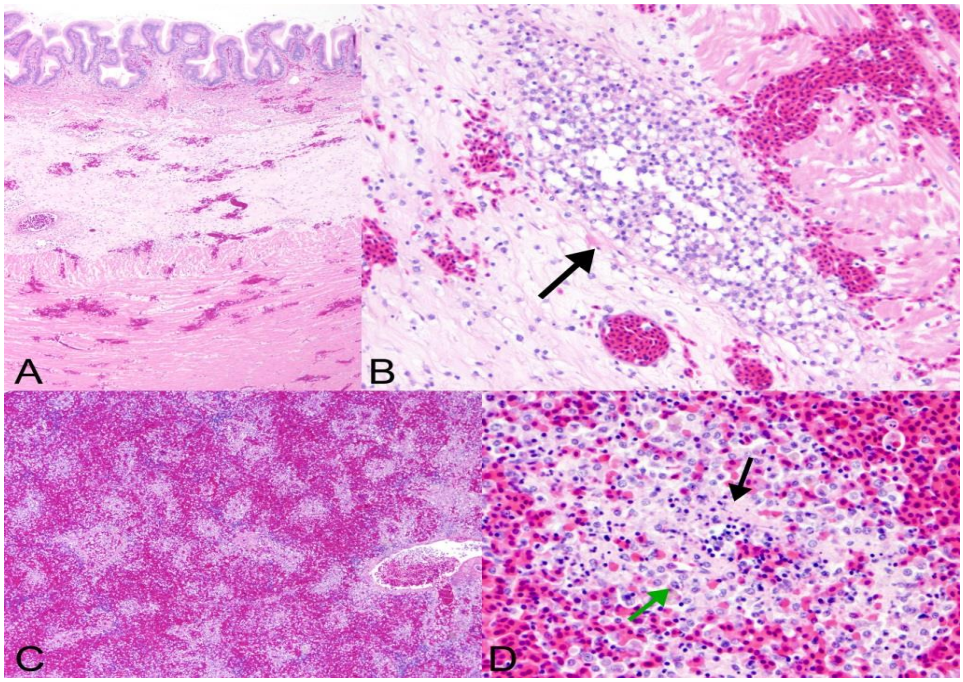

Fig. 5. Channel/hybrid Mississippi pond catfish naturally infected with virulent *Aeromonas hydrophila*. Photomicrographs, haematoxylin and eosin stain. (A): Spleen. Ellipsoid sheaths are enlarged (pink areas) and the red pulp is expanded by myriad erythrocytes. 40x. (B): Spleen. An ellipsoid sheath wall (black arrow) is expanded and obscured by pink fluid, fibrin, pyknotic cell debris and macrophages (green arrow). 400x. (C): Stomach, mucosa at the top, muscularis at the bottom. Multifocal haemorrhage spans the lamina propria, submucosa and muscularis. Edema expands the submucosa, with evenly dispersed leucocytes. 40x. D: Stomach, submucosa on the left, muscularis at the right. Edema and haemorrhage expand the submucosa. A dilated lymphatic (black arrow) is effaced by macrophages and karyorrhectic cells. 200x.

Internal tissues were hyperaemic and petechiated, with small amounts of haemorrhagic fluid in the coelom (Fig. 4C). Large bloody spleens were characteristic, occasionally with large infarctions. Microscopically, splenic ellipsoids exhibited severe necrosis with karyorrhectic debris, very few bacteria and variable numbers of large macrophages peripherally (Fig. 5A, B). The red pulp was markedly expanded by erythrocytes in the majority of cases. Thrombosis of large blood vessels was not uncommon. In cases where splenic infarctions had progressed beyond the early stages, the rarefied, friable tissues were filled with bacteria; aeromonads are facultatively anaerobic, and thus may thrive in devitalized tissue. Also, stomachs were often edematous with petechiation or mural haemorrhage in a paintbrush pattern (Fig. 4D). Microscopically, changes were largely confined to the submucosa and muscularis, where edema and acute haemorrhage were abundant (Fig. 5C). Often, lymphatics were filled with macrophages, neutrophils, karyorrhectic debris and small numbers of bacteria. Frequently, lymphatic walls were obscured by inflammation (lymphangitis and gastritis, Fig. 5D). The mucosa exhibited mild to moderate gland atrophy/apoptosis, with occasional erosion and inflammation. Changes in the spleen and stomach were often some of the first to appear grossly, occasionally prior to external signs of disease. The intestines often had mild, occasionally moderate, random ecchymosis. Microscopically, haemorrhage was common in the lamina propria and submucosa, with variable epithelial necrosis and inflammation. Livers had scant (Fig. 5D) to abundant petechiation but often lacked appreciable inflammation, and in general had only mild hepatocellular necrosis. Perivascular hepatic pancreatic tissue frequently exhibited mild to moderate necrosis, which can be seen in bacterial sepsis from various causes. Renomegaly was inconsistent; in severely diseased fish (possibly in a later stage of infection), mild granulomatous inflammation and haematopoietic hyperplasia were seen, particularly in the pronephros (head kidney). Mild random acute haemorrhage was often seen in the brain (Baumgartner et al. 2016).

The pond outbreak descriptions are taken from case materials at the MSU-CVM aquatic diagnostic laboratory in Starkville, Mississippi; descriptions of MASv from Alabama, Arkansas or the Mississippi delta (Stoneville, Mississippi) catfish may be different, depending on the infectious strain in those areas. There are some differences in pond outbreak lesions versus experimental disease; the gastritis, remarkably severe splenic necrosis and relatively mild involvement of the liver and kidneys in the case materials are interesting and may be significant. The natural route of infection for MAS and MASv is not well understood; skin, gill and gastrointestinal tract are all possible routes, and all may potentially contribute to disease in a pond outbreak. The stomach lesions seen in natural MASv outbreaks are not typical of Gram-negative septicaemia in catfish, including aeromonad infections; the significance of this finding is uncertain. The pathology in the stomach is largely that of vasculitis in the submucosa and muscular tunics. There are various possible explanations for this, some of which include: the stomach may be an early entry point for the organism, other disease mechanisms may cause an accumulation of bacteria/toxin-laden fluids into the stomach tissues, or that ligands for the bacteria are present within the stomach. Aeromonad bacteria have numerous virulence factors, and can produce a toxaemia in fish that gives rise to multi-organ necrosis.

When considering the amount of organ damage, extent of haemorrhage and relatively few bacteria seen microscopically in catfish cases, it seems probable that toxaemic tissue damage plays a large role in the progression of disease. Further understanding of the additional virulence factors acquired by vAh strains may, in time, explain these changes.

Experimental Challenge

Virulent MAS strain ML09-119 demonstrated high mortality (greater than 90 %) within 24 hr in channel catfish after intraperitoneal injection of 10^5 colony forming units (cfu), in contrast to lower mortality observed by tAh strain AL06-06, which had 10 % mortality after 1week post-intraperitoneal injection of an equivalent bacterial inoculum (Fig. 6). In contrast, much lower mortality was observed for ML09-119 in grass carp (Hossain et al. 2014).

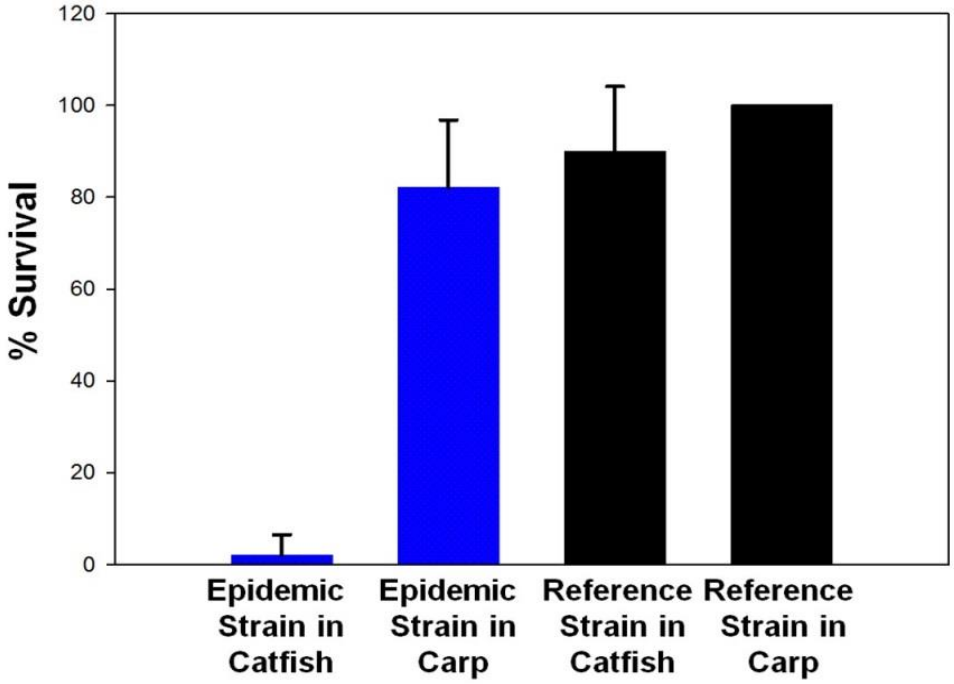

Fig. 6. Percent survival of channel catfish or grass carp after interperitoneal injection of approximately 10^5 cfu of the vAh (epizootic) strain ML09-119 or reference strain AL06-06 (n=5 tanks containing 20 fish per treatment group).

Immersion challenges in catfish infected with Asian and United States isolates using a fin-clip model found that the grass carp isolate (ZC1) was significantly less virulent in catfish (Fig. 7), with only 26.7 % mortality versus that in United States isolates, which was greater than 60 % in 48 hr. Necropsy of these challenges found large haemorrhagic spleens with prominent ellipsoid necrosis, similar to that in pond outbreaks. However, unlike the pond cases, experimentally diseased fish had prominent liver and kidney changes, relatively mild intestinal disease and no stomach pathology; this is typical of descriptions of the pathology of *A. hydrophila* in the literature (Ventura and Grizzle 1987, 1988; Rasmussen-Ivey et al. 2016b).

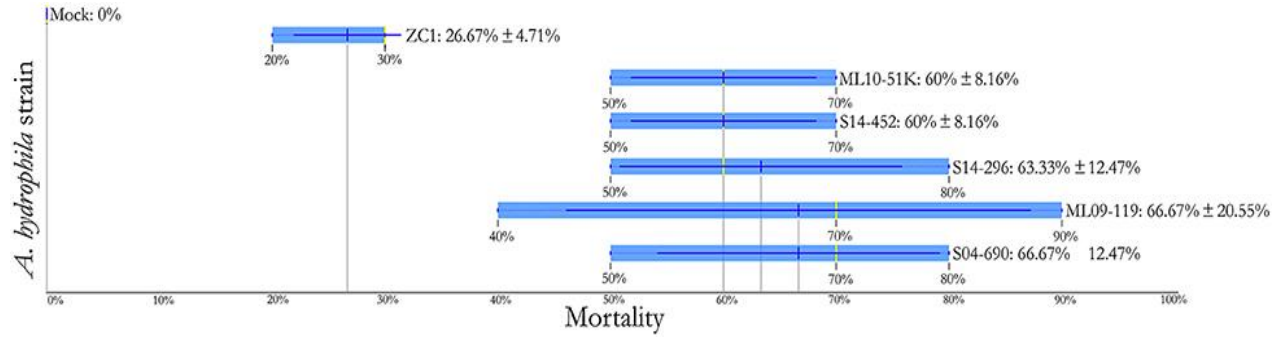

Fig. 7. Comparative assessment of the relative virulence of vAh isolates (ZC1 from Chinese grass carp, all others from American channel catfish) in channel catfish using 1 hr immersion exposure with fin clip (ANOVA = 7.628, p-value = 0.001).[1]

[1]Source: Rasmussen-Ivey et al. (2016b), used under Creative Commons Attribution License (CC BY).

Conclusion

In the last few years, a hypervirulent pathotype of *A. hydrophila* has emerged in the United States catfish industry, with severe consequences. Evidence indicates that catfish isolates share a recent common ancestor with Asian carp strains, giving this pathotype an international distribution associated with economically important disease (Fig. 8). This pathotype has unique metabolic activities, an expanded suite of virulence factors, and characteristic lesions that distinguish it from traditional *A. hydrophila* strains.

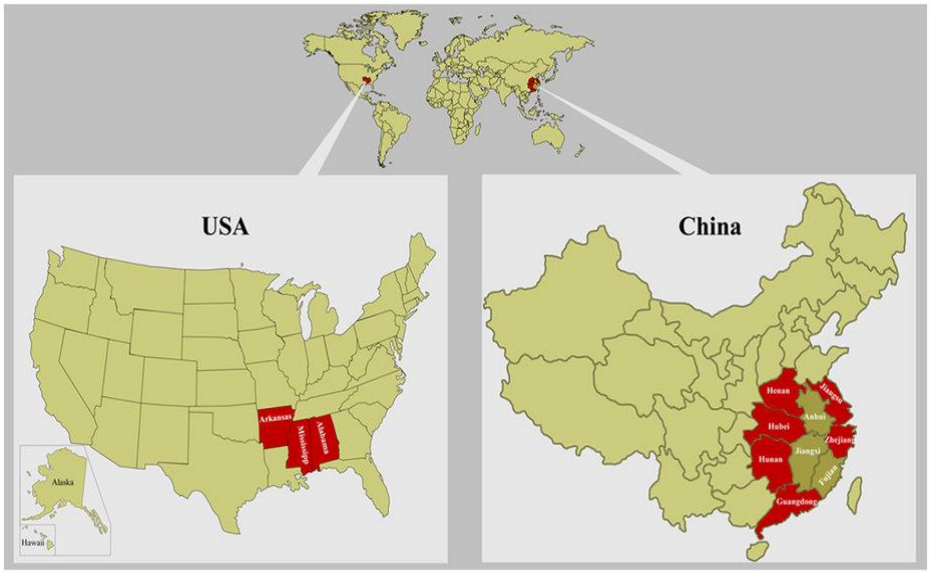

Fig. 8. Geographical distribution of the ST251 clonal group *Aeromonas hydrophila*. The regions filled in with red represent the distribution of the ST251 clonal group.[1]

[1]Source: This map was modified based on the maps obtained from PowerPoint Toolkit (http://ppt-toolkit.com/); Pang et al. (2015), used under Creative Commons Attribution 4.0 International License.

References

Abu-Elala, N., M. Abdelsalam, Sh. Marouf and A. Setta. 2015. Comparative analysis of virulence genes, antibiotic resistance and gyrB-based phylogeny of motile *Aeromonas* species isolates from Nile tilapia and domestic fowl. Letters in Applied Microbiology 61:429–436.

Baumgartner, W., L. Ford and L. Hanson. 2016. Lesions caused by virulent *Aeromonas hydrophila* in farmed catfish (*Ictalurus punctatus* and *I. punctatus x I. furcatus*) in Mississippi. Journal of Veterinary Diagnostic Investigation 29:747–751.

Brandi, G., M. Sisti, G.F. Schiavano, L. Salvaggio and A. Albano. 1996. Survival of *Aeromonas hydrophila*, *Aeromonas caviae* and *Aeromonas sobria* in soil. Journal of Applied Microbiology 81:439–444.

Burtle, G.J. and R.T. Lovell. 1989. Lack of response of channel catfish (*Ictalurus punctatus*) to dietary myo-inositol. Canadian Journal of Fisheries and Aquatic Sciences 46:218–222.

Chen, H.Q. and C.P. Lu. 1991. Study on the pathogen of epidemic septicemia occurred in cultured cyprinoid fishes in southern China. Journal of Nanjing Agriculture University 14:87–91.

Cipriano, R.C. and B. Austin. 2011. Furunculosis and other aeromonad diseases. In: Fish disease and disorders, Volume 3: viral, bacterial, and fungal infections, 2nd edn. (eds. P.T.K. Woo and D.W. Bruno), pp. 424–483. CABI International, Oxfordshire.

Cipriano, R.C, G. Bullock and S. Pyle. 2001. *Aeromonas hydrophila* and motile aeromonad septicemias of fish. Fish Disease Leaflet 68, Washington, DC, United States Department of the Interior, 24 pp.

Deng, G.C., X.Y. Jiang, X. Ye, M.Z. Liu, S.Y. Xu, L. Liu, Y.Q. Bai and X. Luo. 2009. Isolation, identification and characterization of *Aeromonas hydrophila* from hemorrhagic grass carp. Microbiology China 36:1170–1177.

Euzéby, J.P. 1997. List of bacterial names with standing in nomenclature: a folder available on the Internet. International Journal of Systematic Bacteriology 47: 590–592.

Ghatak, S., J. Blom, S. Das, R. Sanjukta, K. Puro, M. Mawlong, I. Shakuntala, A. Sen, A. Goesmann, A. Kumar and S.V. Ngachan. 2016. Pan-genome analysis of *Aeromonas hydrophila*, *Aeromonas veronii* and *Aeromonas caviae* indicates phylogenomic diversity and greater pathogenic potential for *Aeromonas hydrophila*. Antonie van Leeuwenhoek 109:945–956.

Grant, J.R. and P. Stothard. 2008. The CGViwer server: a comparative genomics tool for circular genomes. Nucleic Acids Research 36:181–184.

Griffin, M.J., A.E. Goodwin, G.E. Merry, M.R. Liles, M.A. Williams, C.W. Ware and G.C. Waldbieser. 2013. Rapid quantitative detection of *Aeromonas hydrophila* strains associated with disease outbreaks in catfish aquaculture. Journal of Veterinary Diagnostic Investigation 25:473–481.

Grizzle, J.M. and Y. Kiryu. 1993. Histopathology of gill, liver, and pancreas, and serum enzyme levels of channel catfish infected with *Aeromonas hydrophila* complex. Journal of Aquatic Animal Health 5:36–50.

Hawke, J.P. and R.L. Thune. 1992. Systemic isolation and antimicrobial susceptibility of *Cytophaga columnaris* from commercially reared channel catfish. Journal of Aquatic Animal Health 4:109–113.

Hazen, T.C., C.B. Fliermans, R.P. Hirsch and G.W. Esch. 1978. Prevalence and distribution of *Aeromonas hydrophila* in the United States. Applied and Environmental Microbiology 36:731–738.

Hossain, M.J., D. Sun, D.J. McGarey, S. Wrenn, L.M. Alexander, M.E. Martino, Y. Xing, J.S. Terhune and M.R. Liles. 2014. An Asian origin of virulent *Aeromonas hydrophila* responsible for disease epidemics in United States-farmed catfish. mBio 5:e00848–00814.

Hossain, M.J., G.C. Waldbieser, D. Sun, N.K. Capps, W.B. Hemstreet, K. Carlisle, M.J. Griffin, L. Khoo, A.E. Goodwin, T.S. Sonstegard, S. Schroeder,K. Hayden, J.C. Newton J.S. Terhune and M.R. Liles. 2013. Implication of lateral genetic transfer in the emergence of *Aeromonas hydrophila* isolates of epidemic outbreaks in channel catfish. PloS One 8:e80943.

Janda, J.M. 1991. Recent advances in the study of the taxonomy, pathogenicity, and infectious syndromes associated with the genus *Aeromonas*. Clinical Microbiology Reviews 4:397–410.

Janda, J.M. and S.L. Abbott. 1998. Evolving concepts regarding the genus *Aeromonas*: an expanding panorama of species, disease presentations, and unanswered questions. Clinical Infectious Diseases 27:332–344.

Janda, J.M. and S.L. Abbott. 2010. The genus *Aeromonas*: taxonomy, pathogenicity, and infection. Clinical Microbiology Reviews 23:35–73.

Lee, C.-T., I.T. Chen, Y.-T. Yang, T.-P. Ko, Y.-T. Huang, J.-Y. Huang, M.F. Huang S.-J. Lin, C.-Y. Chen, S.-S. Lin, D.V. Lightner, H.-C. Wang, A. H.-J. Wang, H.-C. Wang, L.-I. Hor and C.-F. Lo. 2015. The opportunistic marine pathogen *Vibrio parahaemolyticus* becomes virulent by acquiring a plasmid that expresses a deadly toxin. Proceedings of the National Academy of Sciences of the United States of America 112:10798–10803.

MacMillan, J. and T. Santucci. 1990. Seasonal trends in intestinal bacterial flora of farm-raised channel catfish. Journal of Aquatic Animal Health 2:217–222.

Merino, S., X. Rubires, A. Aguilar and J.M. Tomás. 1996a. The O:34-antigen lipopolysaccharide as an adhesin in *Aeromonas hydrophila*. FEMS Microbiology Letters 139:97–101.

Merino, S., X. Rubires, A. Aguillar, J.F. Guillot and J.M. Tomás. 1996b. The role of the O-antigen lipopolysaccharide on the colonization *in vivo* of the germfree chicken gut by *Aeromonas hydrophila* serogroup O:34. Microbial Pathogenesis 20:325–333.

Meyer, F.P. 1975. The pathology of the major diseases of catfish. In: The pathology of fishes (eds. W.E. Ribelin and G. Migaki), pp. 275–286. The University of Wisconsin Press, Madison, WI, USA.

Miller, R.W. and W.R. Chapman. 1976. *Epistylis* and *Aeromonas hydrophila* infections in fishes from North Carolina reservoirs. Progressive Fish-Culturist 38:165–168.

Pang, M., J. Jiang, X. Xie, Y. Wu, Y. Dong, A.H.Y. Kwok, W. Zhang, H. Yao, C. Lu, F.C. Leung and Y. Liu. 2015. Novel insights into the pathogenesis of epidemic *Aeromonas hydrophila* ST251 clones from comparative genomics. Science Report 5:9833.

Piotrowska, M. and M. Popowska. 2015. Insight into the mobilome of *Aeromonas* strains. Frontiers in Microbiology 6: 494.

Plumb, J.A. 1999. Catfish bacterial diseases. In: Health maintenance and principal microbial diseases of cultured fish (eds. J.A. Plumb), pp. 181–204. Iowa State University Press, Ames, IA, USA.

Pridgeon, J.W., D. Zhang and L. Zhang. 2014a. Complete genome sequence of a moderately virulent *Aeromonas hydrophila* strain, pc104A, isolated from soil of a catfish pond in West Alabama. Genome Announcements 2: DOI:10.1128/genomeA.00554-14.

Pridgeon, J.W., D. Zhang and L. Zhang. 2014b. Complete genome sequence of the highly virulent *Aeromonas hydrophila* AL09-71 isolated from diseased channel catfish in west Alabama. Genome Announcements 2: DOI:10.1128/genomeA.00450-14.

Rasmussen-Ivey, C., M.J. Figueras, D. McGarey and M.R. Liles. 2016a. Virulence factors of *Aeromonas hydrophila*: in the wake of reclassification. Frontiers in Microbiology 7:1337.

Rasmussen-Ivey, C., M.J. Hossain, S.E. Odon, J.S. Terhune, W.G. Hemstreet, C.A. Shoemaker, D. Zhang, D. Xu, M.J. Griffin, Y. Liu, M.J. Figueras, S.R. Santos, J.C. Newton and M.R. Liles. 2016b. Classification of a hypervirulent *Aeromonas hydrophila* pathotype responsible for epidemic outbreaks in warm-water fishes. Frontiers in Microbiology 7:1615.

Rock, L.F. and H.M. Nelson. 1965. Channel catfish and gizzard shad mortality caused by *Aeromonas liquefaciens*. Progressive Fish-Culturist 27:138–141.

Seshadri, R., S.W. Joseph, A.K. Chopra, J. Sha, J. Shaw,J. Graf, D. Haft, M. Wu, Q. Ren, M.J. Rosovitz, R. Madupu, L. Tallon, M. Kim, S. Jin, H. Vuong, O.C. Stine, A. Ali, A.J. Horneman and J.F. Heidelberg. 2006. Genome sequence of *Aeromonas hydrophila* ATCC 7966T: jack of all trades. Journal of Bacteriology 188:8272–8282.

Suarez, G., B.K. Khajanchi, J.C. Sierra, T.E. Erova, J. Sha and A.K. Chopra. 2012. Actin cross-linking domain of *Aeromonas hydrophila* repeat in toxin A (RtxA) induces host cell rounding and apoptosis. Gene 506**:**369–376.

Tekedar, H.C., A. Karsi, A. Akgul, S. Kalindamar, G.C. Waldbieser, T. Sonstegard, S.G. Schroeder and M.L. Lawrence. 2015. Complete genome sequence of fish pathogen *Aeromonas hydrophila* AL06-06. Genome Announcements 3: DOI:10.1128/genomeA.00368-15.

Tekedar, H.C., G.C. Waldbieser, A. Karsi, M.R. Liles, M.J. Griffin, S. Vamenta, T. Sonstegard, M. Hossain, S.G. Schroeder, L. Khoo and M.L. Lawrence. 2013. Complete genome sequence of a channel catfish epidemic isolate, *Aeromonas hydrophila* strain ML09-119. Genome Announcements 1: DOI:10.1128/genomeA.00755-13.

Thomas, L.V., R.J. Gross, T. Cheasty and B. Rowe. 1990. Extended serogrouping scheme for motile, mesophilic *Aeromonas* species. Journal of Clinical Microbiology 28:980–984.

Ventura, M.T. and J.M. Grizzle. 1987. Evaluation of portals of entry of *Aeromonas hydrophila* in channel catfish. Aquaculture 65:205–214.

Ventura M.T. and J.M. Grizzle. 1988. Lesions associated with natural and experimental infections of *Aeromonas hydrophila* in channel catfish *Ictalurus punctatus* (Rafinesque). Journal of Fish Diseases 11:397–407.

Walters, G.R. and J.A. Plumb. 1980. Environmental stress and bacterial infection in channel catfish, *Ictalurus punctatus* Rafinesque. Journal of Fish Biology 17:177–185.

Yanez, M.A., V. Catalan, D. Apraiz, M.J. Figueras and A.J. Martinez-Murcia. 2003. Phylogenetic analysis of members of the genus *Aeromonas* based on *gyrB* gene sequences. International Journal of Systematic and Evolutionary Microbiology 53:875–883.

Yang, W., N. Li, M. Li, D. Zhang and G. An. 2016. Complete genome sequence of fish pathogen *Aeromonas hydrophila* JBN2301. Genome Announcements 4: DOI: 10.1128/genomeA.01615-15.