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Experimental induction of motile *Aeromonas* septicemia in channel catfish (*Ictalurus punctatus*) by waterborne challenge with virulent *Aeromonas hydrophila*

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ABSTRACT

Motile *Aeromonas* septicemia (MAS), caused by virulent clonal isolates of *Aeromonas hydrophila* (vAh), is emerging as a major disease in catfish (*Ictalurus punctatus*) aquaculture in the Southeastern United States. Predisposing conditions leading to vAh infection in catfish were however largely unknown. The objective of this study was to investigate factors that predispose catfish to vAh infection and establish a waterborne challenge model that mimics natural occurrence of MAS. Results of this study indicated that wounding on the fish body surface was one of the key factors that predisposed catfish to vAh infection via waterborne route. Relatively uniform wounds were created by clipping part of the fish adipose fin. Adipose fin clipped (Af-clipped) fish behaved normally in terms of swimming and feeding and no mortality occurred in the control treatment (a mock challenge). When subjected to challenge in vAh-infected water, Af-clipped fish were highly susceptible, showing typical symptoms of MAS observed in the field. The mortality rate of Af-clipped fish was significantly associated with vAh concentration, challenge time and water temperature. About 90% mortality occurred within 48 h when Af-clipped fish were challenged for 1 h with vAh at the concentration of 2×10^7 colony forming units per mL of water ($27 \pm 1\,^{\circ}C$). The waterborne challenge model was further tested using four field isolates including *A. hydrophila* and *A. veronii*. All vAh isolates caused about 90% mortality of Af-clipped fish and one isolate of *Aeromonas veronii* caused no mortality under the same challenge conditions. The waterborne challenge model described in this study would facilitate urgently-needed studies of MAS prevention (such as wound avoidance and healing) and control (such as prophylactic vaccination; antibiotics treatment and probiotics screening).

1. Introduction

Since the 2009 outbreak of motile *Aeromonas* septicemia (MAS) in West Alabama and East Mississippi, the disease has cost catfish aquaculture losses of about three million pounds of food-size fish annually (Hemstreet, 2010; Gresham, 2014). A new virulent clonal population of *Aeromonas hydrophila* (vAh) was etiologically determined to be responsible for the MAS outbreaks (Pridgeon et al., 2013, 2014; Hossain et al., 2013; Tekedar et al., 2013). To date, primary or obvious field conditions leading to the disease outbreaks were largely unknown (Hanson et al., 2014) and none of recommended management practices that have worked in the past seemed to be effective at limiting or preventing the outbreaks (Gresham, 2014).

Induction of MAS in laboratory trials can assist in evaluating virulence of field isolates of *A. hydrophila*, assessing predisposing factors and developing prevention methods against MAS. Currently, intraperitoneal (IP) injection (i.e. delivering bacterial cells into fish intraperitoneal cavity by a syringe) is the main method employed in laboratories to compare relative virulence of *A. hydrophila* isolates (Hossain et al., 2014) and to examine effect of prophylactic treatments on prevention of MAS (Zhang et al., 2014). The challenge method via IP injection is effective and reproducible, but is incongruous with the natural infection process (i.e. the waterborne route). Another challenge method is immersion of fish in water containing vAh cells. This practice, referred to waterborne or immersion challenge, is more natural, closely simulating infection route under aquatic conditions, but resulting mortality was low even at very high concentration of vAh cells (Xu et al., 2012). Crucial factors predisposing catfish to MAS have not yet be determined with the waterborne challenge method. The objective of this study was to investigate those factors that may result in an increased

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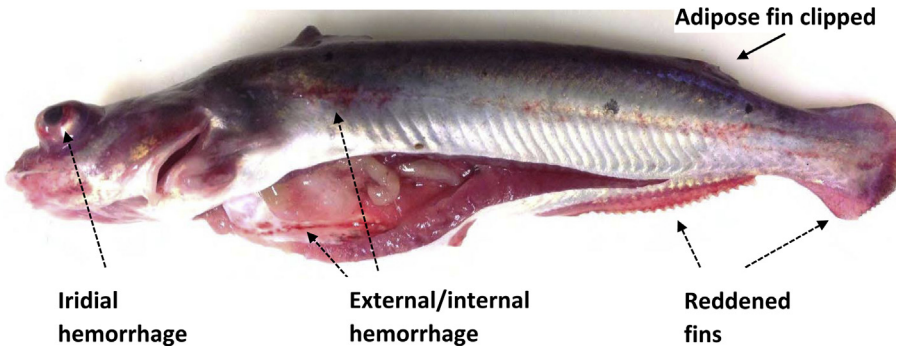

Fig 1. Typical symptoms of motile *Aeromonas* septicemia (MAS) shown in adipose fin-clipped catfish that were challenged by virulent *A. hydrophila* under waterborne conditions described.

susceptibility of catfish to vAh infection under laboratory conditions and establish a reproducible waterborne challenge model that mimics natural occurrence of MAS. The effectiveness of the waterborne challenge model was tested using four field isolates including *A. hydrophila* and *Aeromonas veronii*.

2. Materials and methods

2.1. Pathogen culture

ML-10-51K, a virulent isolate of *A. hydrophila* (vAh) obtained from a moribund catfish with typical MAS symptoms in 2010 and verified by pathogenicity and gene analysis (Zhang et al., 2013, 2014), was used in this study. The bacterium was cultured in tryptic soy broth (TSB; Bacto™, Becton, Dickinson and Company, Sparks, MD, USA) at 28 °C with constant shaking at 200 rpm until the cell density reached to approximately 2×10^9 cells mL^{-1} based on optical density at 600 nm (OD_{600}). Ten percent of glycerol was then added to this culture and aliquots of 1 mL were frozen as stock culture at −80 °C. For pathogen challenge assays conducted in this study, bacterial cells were propagated in TSB using the same batch of stock culture at inoculation rate of 100 μL of stock culture per each 100 mL of medium. Upon about 15 h shaking-culture at 28 °C, the cell suspension had about $3.0 \pm 0.1 \times 10^9$ colony forming units (cfu) per mL of medium estimated using 6 × 6 drop plate method (Chen et al., 2003). This cell suspension was used throughout all experiments in this study unless otherwise specified.

2.2. Fish rearing

Channel catfish (*Ictalurus punctatus*) used in this study were purebred species (Delta Select) obtained from Warmwater Aquaculture Research Unit, USDA-ARS, Stoneville, Mississippi. Fingerlings, 31.8 ± 9.7 g in weight and 11.5 ± 0.6 cm in length, were maintained in 114-L tanks (about 100 fish $tank^{-1}$) or 57-L tanks (10 or 20 fish $tank^{-1}$). Tank water was supplied by heated (27 ± 1 °C; unless otherwise specified) and de-chlorinated city water at flow rate of 0.5–0.6 L min^{-1}. The typical parameters of the water were as follows: dissolved oxygen was 7.0 ± 0.4 mg L^{-1} (by Oxymenter, Sper Scientific), ammonia content was 0.67 ± 0.05 mg L^{-1}, nitrate concentration was 0.17 ± 0.09 mg L^{-1}, hardness was 96.9 ± 8.1 mg L^{-1} (as $CaCO_3$), alkalinity was 102 mg L^{-1} (as $CaCO_3$), and pH was 7.3 ± 0.1. Aeration was generated from air pump and constantly supplied via an air-stone. Fish were fed with Aquamax Grower 400 (crude protein ≥45% and crude fat ≥16%) at rate of about 3% of fish weight once daily. No feed was provided in the day when experimental challenge was performed.

2.3. Effect of handlings and wounding of fish on mortality

Based on the pilot experiments that fish mortality via waterborne challenge varied irregularly among replicates (unpublished data), it was noted that wounding resulting from fish to fish contact (physical colliding) during transfer was a potential variable. The following treatments were conducted to assess effect of various handlings and wounding of fish on mortality: (1) Light transfer and holding: Fish were transferred from the stock tank to the 50-L experimental tank (10 fish $tank^{-1}$) using an 8" (20 cm) commercial fish net (Nirox). No more than 2 fish were transferred at a time. Forty-eight hours post transfer, water flow was turned off and the tank water level was reduced to 15 L. Fish were challenged with vAh at concentration of $2.0 \pm 0.06 \times 10^7$ cfu per mL of tank water by adding 100 mL of TSB culture (described above) to the 15 L water. One hour post challenge, water flow was resumed. There were 6 replicates for *A. hydrophila* challenge and 3 for mock challenge (by adding 100 mL of uncultured TSB); (2) Light transfer: Ten fish were transferred to 50-L tanks filled with 15 L of water similar to Treatment 1 but were challenged immediately after transfer with the same amount of vAh and medium. Water flow was resumed 1 h post challenge. There were 6 replicates for *A. hydrophila* challenge and 6 for mock challenge: (3) Multiple transfer: About twenty fish were first transferred by a net from the stock tank to a bucket filled with about 20 L of tank water and with more than 3 fish were transferred at a time. Ten fish were then transferred from the bucket to 50-L tanks filled with 15 L of water and subjected to vAh challenge or mock challenge for 1 h as described above. There were five replicates for *A. hydrophila* challenge and four for mock challenge; (4) Skin abrasion: Fish were lightly transferred in the same way as described in Treatment 1 to a bucket filled with about 20 L of tank water containing 100 mg L^{-1} of tricaine methanesulfonate (MS-222). Individual immobilized fish were lightly abraded on one side of the tail part (crossing about 2.0–2.5 cm) using a 4-ct Scour Pad (Wilkesboro, NC, USA). Ten abraded fish were put to a 50-L tank filled with 15 L of water. There were six replicate tanks, three of which were subjected to challenge with vAh and the other three were mock challenge for 1 h; (5) Clipping of adipose fin: Fish were transferred to and anesthetized in a bucket as described in Treatment 4. Adipose fin located medially between the dorsal and caudal fin (Reimchen and Temple, 2004) was individually clipped with a pair of scissors (about 80–85% of the fin was trimmed off). A total of eighty adipose fin-clipped (Af-clipped) fish was equally dispensed into eight 50-L tanks filled with 15 L of water, of which four tanks were subjected to vAh challenge and the other four for mock challenge. Fish mortality for all above treatments and following experiments was monitored daily for two weeks.

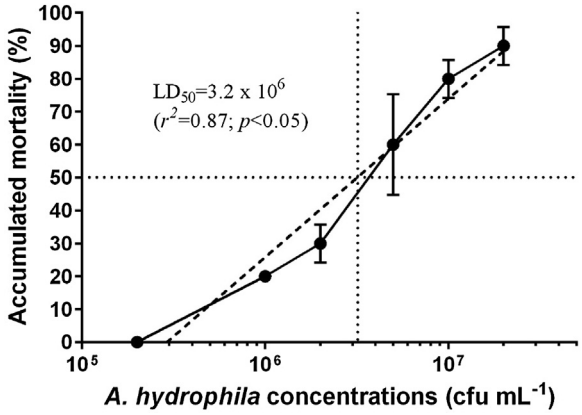

Fig. 2. Effect of *A. hydrophila* concentrations on adipose fin-clipped catfish mortality. Fish were challenged with 2×10^7 cfu mL^{-1} of water at 27 ± 1 °C. (LD_{50} was calculated from the semilog nonlinear regression by probit analysis, the broken line).

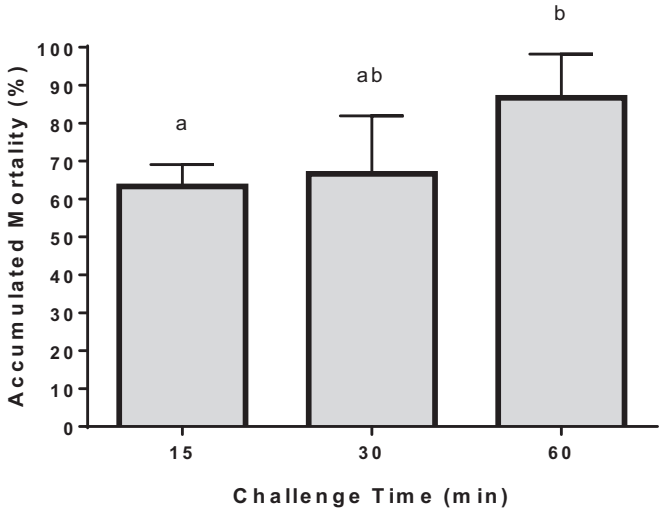

Fig. 3. Effect of *A. hydrophila* challenge time on adipose fin-clipped fish mortality. Fish were challenged with 2×10^7 cfu mL^{-1} of water at 27 ± 1 °C. Means of mortality with different letters above the columns are significantly different ($P < 0.05$).

2.4. *Effect of* A. hydrophila *concentration on fish mortality*

Individual 50-L tanks were filled with 15 L of water and received ten Af-clipped fish. Fish in tanks were challenged with six different concentrations of vAh cells in 100 mL of TSB, which resulted in 2×10^5, 1×10^6, 2×10^6, 5×10^6, 1×10^7, and 2×10^7 cfu per mL of tank water. There were three replicates for each concentration. Water flow was resumed one hour post challenge.

2.5. *Effect of* A. hydrophila *exposure time on fish mortality*

Af-clipped fish were added to tanks filled with 15 L of water containing 2×10^7 cfu per mL of water (from 100 mL of TSB culture). There were nine replicate tanks with each having 10 Af-clipped fish. At 15 min, 30 min and 60 min post exposure to vAh, water flow was resumed in three of the replicate tanks accordingly.

2.6. *Effect of water temperature on fish mortality*

For this specific assay, unheated de-chlorinated city water (about 16 ± 1 °C) was used and fish were first acclimated for one week in two 114-L tanks with water temperature of 18 °C and 27 °C, respectively. The targeted temperature was adjusted using Hydor aquarium heaters (Hydor USA Inc, Sacramento, CA, USA). Four sets of 50-L tanks (3 tanks per set) were filled with 15 L of unheated water and water temperature was adjusted with heaters to 17 ± 1 °C, 20 ± 1 °C, 25 ± 1 °C, and 30 ± 1 °C, respectively. Ten Af-clipped fish from 18 °C stock tank were added to each of 17 °C and 20 °C tanks and ten Af-clipped fish from 27 °C stock tank were added to each of 25 °C and 30 °C tanks. Fish in all tanks were subjected to vAh challenge for 1 h at concentration of 2.0×107 cfu mL^{-1} of water. Water temperatures in individual sets of tanks were monitored twice daily during a 2-week observation.

2.7. *Effect of water salinity on mortality*

Different amounts of sodium chloride (NaCl) were added to individual 50-L tanks filled with 15 L of water, resulting in following four concentrations: 0, 0.01, 0.1, and 0.5%. There were three replicates for each concentration. Ten Af-clipped fish were added to individual tanks and challenged with vAh at concentration of 2×10^7 cfu per mL of water for 1 h. In this specific trial, culture of vAh in TSB was centrifuged at $5000 \times g$ for 20 min and the supernatant medium was discarded. The bacterial cell pellet was resuspended in 100 mL of fish-rearing water and used for challenge.

2.8. *Assessing the effectiveness of the waterborne challenge model with Af-clipped fish*

With ML-10-51K, used in this study, as positive control, three other characterized or partially characterized *Aeromonas* species (Table 2) were used to assess the effectiveness of the waterborne challenge model. Among the three test isolates, AL09-71 and ML-10-208K were known to be virulent *A. hydrophila* following IP injection challenge (Pridgeon et al., 2013, 2014) and ALG-10-089, though isolated from a diseased fish, was likely an isolate of *A. veronii* based on partial genomic DNA sequences (Zhang et al., 2014; unpublished data). The pathogenicity of ALG-10-089 was unknown. In this trial, 20 Af-clipped fish were added to 50-L tanks filled with 15 L of water (27 ± 1 °C). There was no additional salt added. The fish were challenged by adding 100 mL of TSB culture (with approximately 3×10^9 cfu mL^{-1}) of each isolate. Water flow was resumed following 1 h challenge. There were three replicate tanks for each isolate. Mortalities caused by individual isolates were recorded and compared in following timeframes: 8 h, 12 h, 24 h, 32 h, 48 h, 72 h, and 14 days post challenge.

2.9. *Re-isolation of* A. hydrophila *from diseased fish and bacterial cell agglutination assay*

Moribund fish were aseptically dissected to check the presence of *A. hydrophila* in tissues of internal organs (kidney and spleen). Bacteria isolated from issues were subjected to agglutination assay, using catfish anti-serum raised against extracellular products (ECP) of ML-10-51K in accordance with methods described previously (Zhang et al., 2014). Briefly, the anti-serum was serially diluted in phosphate buffered saline (PBS, Ph 7.4; Sigma, St. Louis, MO, USA) in wells of a 96-well microtiter plate. Bacterial cells of test isolates cultured on TSA at 28 °C for 24–48 h were suspended in PBS with concentration of $7.5 \pm 0.2 \times 10^8$ mL^{-1}. Aliquots of 50 μl of the bacterial cell suspension were then mixed into individual wells containing 50 μL of diluted anti-serum. The microtiter plate was kept still at room temperature for about 4 h. The titer of cell agglutination was determined by the reciprocal of the highest anti-serum dilution factor that resulted in visible clumping of bacterial cells. Agglutination titers of individual test isolates were compared with the titer of the original isolate of ML-10-51K.

Table 1
Effect of handlings and wounding of fish on mortality caused by *A. hydrophila* infection.

Treatment of fish	Challenge*	Number of replicate	Number of fish	Accumulated mortality (% ± SD)**
Light transfer & holding	TSB medium	3	30	0[a]
for 2 days	*A. hydrophila*	6	60	0[a]
Light transfer	TSB medium	6	60	0[a]
	A. hydrophila	6	60	5.0 ± 7.5[a]
Multiple transfer	TSB medium	4	40	0[a]
	A. hydrophila	5	50	32.0 ± 7.5[b]
Skin scratching	TSB medium	3	30	0[a]
	A. hydrophila	3	30	20.0 ± 0.0[b]
Clipping of adipose fin	TSB medium	4	40	0[a]
	A. hydrophila	4	40	90.0 ± 7.1[c]

* *A. hydrophila* cultured in 100 mL of TSB medium (28 °C & shaking at 200 rpm for 15 h) with concentration of approximately $3.0 \pm 0.08 \times 109$ cfu mL^{-1} was added in 15 L of water (resulting in $2.0 \pm 0.06 \times 107$ cfu per mL of water). For control, 100 mL TSB medium was added to 15 L of water.
** Total mortality at 2 weeks post challenge. Numbers with different superscripted letters were different significantly ($p < 0.05$).

2.10. Data analysis

Fish mortality data among challenges and mock-challenges were analyzed using one-way ANOVA and Tukey's multiple comparisons test with the aid of software GraphPad Prism version 6.0 (GraphPad Software Inc. San Diego, CA, USA). *P*-values of 0.05 or less were considered statistically significant.

3. Results

3.1. Effect of fish handling and wounding on mortality

Routinely-performed transfer practice by netting had significant impact ($p < 0.05$) on fish susceptibility to *A. hydrophila* infection. As shown in Table 1, light transfer from tank to tank resulted in 5% mortality while multiple transfer had 20–40% mortality (mean ± SD = 32.0 ± 7.5). Notably, vAh caused no mortality for fish that were lightly transferred and held in tanks for 48 h before challenge. Artificial wounding of fish skin by light abrasion, though no apparent injury was seen, significantly increased mortality (20%). Wounding created by clipping of adipose fin was fatal in fish susceptibility to vAh infection, resulting in about 90% mortality, although Af-clipped fish showed normal in terms of swimming and feeding behaviors and had no inflammation in clipping site and no mortality in mock challenge.

Diseased and moribund fish all showed reddened fins, external/internal septicemia, and iridial hemorrhage (Fig. 1), which were typical symptoms of motile *Aeromonas* septicemia (MAS) observed in field diseased fish (Hanson et al., 2014). Samples of moribund fish were all positive for the presence of the challenging bacteria in internal tissues; the agglutination titers were the same as the titer of ML-10-51K original culture.

3.2. Dose-dependent mortality

Af-clipped fish mortality was significantly associated with vAh challenge concentrations (Fig. 2). At 2×10^5 cfu mL^{-1}, no mortality was observed while mortality elevated from 20 ± 0% to 90 ± 10% as concentration of vAH increased from 1×10^6 to 2×10^7 cfu mL^{-1}. The median lethal dose (LD_{50} by probit analysis) was approximately 3.2×10^6 cfu mL^{-1} with 95% confidence interval ranging from 2.3×10^6 to 4.4×10^6 cfu mL^{-1}.

3.3. Effect of exposure time on mortality

Challenge time had significantly effect ($p < 0.05$) on mortality of Af-clipped fish (Fig. 3). The mortalities at challenge time 15 and 60 min were 63.3 ± 5.8% and 86.7 ± 11.5%, respectively. For challenge time at 30 min, the mortality was 66.7 ± 15.2%, which was higher than mortality at 15 min and lower than mortality at 60 min, but statistically was not different from them.

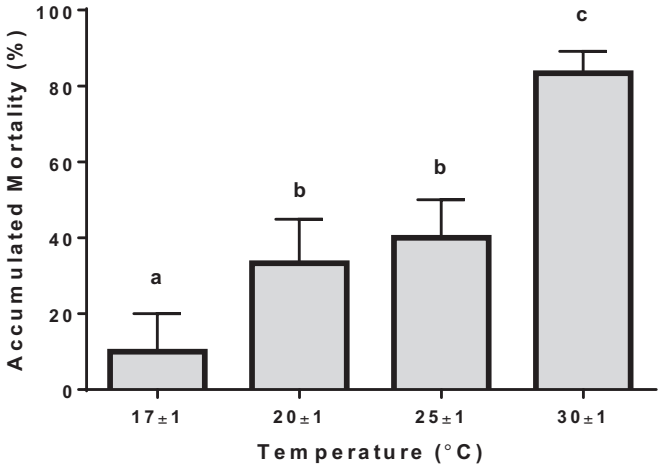

Fig. 4. Effect of various water temperature on adipose fin-clipped fish mortality caused by *A. hydrophila* infection. Fish were challenged with 2×10^7 cfu mL^{-1} of water at water temperature indicated. Means of mortality with different letters above the columns are significantly different at $p < 0.05$).

3.4. Effect of water temperature on mortality

Water temperature also had significant effect on Af-clipped fish mortality following challenge of vAh (Fig. 4). The highest mortality (83.3 ± 5.8%) was seen in 30 ± 1 °C water while the lowest (10.0 ± 10.0%) in 17 ± 1 °C. There was no significant difference of mortality ($p > 0.05$) in temperature settings between 20 ± 1 °C and 25 ± 1 °C, in which the mortalities were 33.3 ± 11.6% and 40.0 ± 10.0%, respectively, although they were significantly different from those in 17 ± 1 °C and 30 ± 1 °C water.

3.5. Effect of water salinity on mortality

Mortalities of Af-clipped fish in 4 different salt (NaCl) concentrations post vAh challenge were 83.3 ± 15.3%, 80.0 ± 10.0%, 83.3 ± 5.8%, and 86.7 ± 11.6%, respectively (Fig. 5). There was no significant difference among the means of mortality ($p > 0.05$).

3.6. Assessment of waterborne challenge model with Af-clipped fish

Among the four isolates tested, ML-10-51K, AL09-71 and ML-10-208K had the same agglutination titers to catfish anti-serum raised against ECP of ML-10-51K (Table 2). They were likely clonal isolates. All of the three isolates caused more than 88% mortality under the

Table 2
Mortality of adipose fin clipped catfish after challenge with isolates of *Aeromonas hydrophila and A. veronii.* Percentage of mortality (average ± SD) showed was accumulated at two weeks post challenge. Catfish anti serum was raised against extracellular products (ECP) of isolate ML-10-51K and used for agglutination assay.

Isolates	Mortality (%)	Agglutination titer	Identification	Reference
ML-10-51K	90 ± 5	64	*A. hydrophila*	Zhang et al. (2014)
AL-09-71	88 ± 3	64	*A. hydrophila*	Xu et al. (2012); Pridgeon et al. (2014)
ML-10-208K	90 ± 5	64	*A. hydrophila*	Pridgeon et al. (2013)
ALG-10-089	0	<2	*A. veronii*	Zhang et al. (2014)

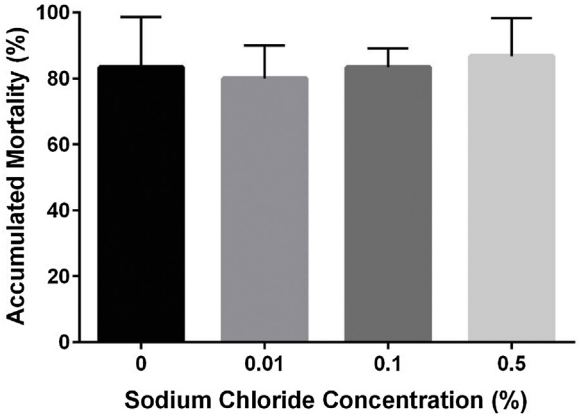

Fig. 5. Effect of water salinity on adipose fin-clipped fish mortality caused by *A. hydrophila*. Fish were challenged with 2×10^7 cfu mL^{-1} of water at 27 ± 1 °C (In this specific assay, the culture medium, TSB, was removed by centrifugation; the bacterial cell pellet was resuspended in tank water). There were no significant difference among means of mortality in different concentrations of NaCl ($p > 0.05$).

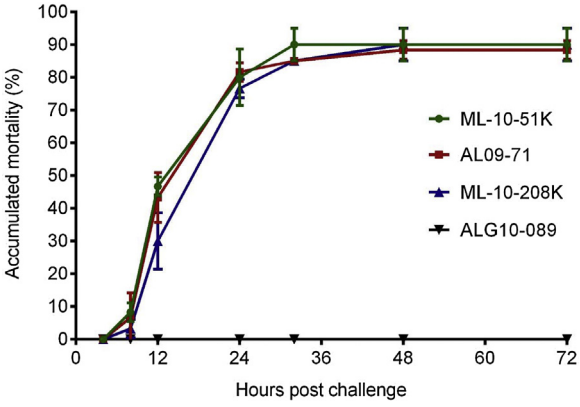

Fig. 6. Progress trends of mortality caused by four *Aeromonas* sp. within 72 h post challenge (hpc). Adipose fin-clipped fish were challenged with 2×10^7 cfu per mL of water at 27 ± 1 °C. Mortality was observed as early as 8 hpc, plateauing at 48 hpc (see Table 2 for additional data).

waterborne challenge conditions (Table 2 and Fig. 6). There was no significant difference among the three means of accumulated mortalities ($p > 0.05$). The isolate, ALG-10-089, showed no agglutination reaction and caused no mortality during the two week observation. The mortality progress trend within 72 h was similar for the three virulent isolates (Fig. 6). About 10% mortality were observed as early as 8 h post challenge; peak mortality occurred within 24 h; and mortality reached to plateau at 48 h post challenge. There was no more mortality for fish that survived at day 3 (72 h) post challenge (during the two week observation period).

4. Discussion

Results of this study showed that fish body surface wounding was one of the most important factors that predisposed fish to vAh infection. The wounding incurred during net transfer may be minor but had significant impact on fish susceptibility under liable conditions as observed in other related studies (Bader et al., 2006). Artificial abrasion of fish skin, mimicking fish physical colliding during net transfer, was evidently shown to cause 20% mortality. The association of skin wounds or abrasion with *A. hydrophila* infection was reported in a human clinic case, in which an outbreak of *A. hydrophila* infection in "mud football" participants was due to foot cuts and scratches incurred in infected abrasive mud (Vally et al., 2004). Another observation in this study showed that transferred fish were resistant to infection if they were held for 2 days (for possible wound healing) before challenge, suggesting that only fresh wounds may be vulnerable. Effect of wound healing on vAh infection has yet to be verified in the future investigation. A field survey of risk factors for *A. hydrophila* outbreaks showed that catfish ponds that were seined more than twice a year had a significantly greater odds of MAS outbreaks (Bebak et al., 2015). This observation implies that fish may suffer injuries or wounds from seining practice and become vulnerable to *A. hydrophila* infection. Furthermore, microscopic wounds resulted from parasitism of ciliated protozoans were also demonstrated to enhance fish susceptibility to *A. hydrophila* infection (Xu et al., 2012).

To verify the effect of wounding with relative uniformity, an injury was artificially created by clipping part of the adipose fin. The adipose fin, a fleshy and non-rayed fin, is generally considered to have no essential function (Vander Haegen et al., 2005) although it helps, acting as a precaudal flow sensor, for fish living in turbulent waters like streams and rivers (Temple and Reimchen, 2008; Buckland-Nicks et al., 2011). Removal of the adipose fin of rainbow trout was reported to enhance mortality in *Flavobacterium psychrophilum* waterborne challenge (Long et al., 2014). Results in this study showed that adipose fin clipped (Af-clipped) catfish behaved normally in aquarium tanks in terms of swimming and feeding and no mortality was observed in control treatments (i.e., mock challenge). However, Af-clipped fish were highly susceptible to vAh infection and diseased fish showed typical clinical signs of MAS (Fig. 1).

With Af-clipped fish, the rate of mortality caused by vAh was found to be dose dependent (Fig. 2). An average of 90% mortality occurred at 2×10^7 cfu mL^{-1} of water; this *A. hydrophila* concentration was 15 times lower than what had been used (Xu et al., 2012), in which about 23% of mortality was observed when fish (with adipose fin intact) were challenged with the same exposure time in water containing 3×10^8 cfu mL^{-1} (The virulence of *A. hydrophila* isolate used, AL09-71, was similar to that of ML-10-51K shown in Table 2 and Fig. 6). Additionally, the rate of mortality of Af-clipped fish was correlated with the time exposed to vAh (Fig. 3); there was significant difference between challenge times of 15 min and 1 h. There may be a mechanism involved in interaction between the pathogen and the host, which is currently unknown. Water temperature was also significantly affecting the rate of mortality (Fig. 4). About 10% mortality occurred in lower temperature (17 °C), 33–40% in mild temperature (20–25 °C) and 83% in high temperature (30 °C). Water temperatures fluctuating from 20 to 30 °C prevailed in spring and fall in catfish ponds where most MAS outbreaks occurred (Camus et al., 1998; Hanson et al., 2014). According to the survey of Bebak et al. (2015), sodium chloride (NaCl) was routinely used in some

catfish farms at levels of 0.014–0.016% but correlation of MAS outbreak with the use of NaCl was inconclusive. Results of the present studies indicated that NaCl concentrations ranging from 0 to 0.5% had no significant effect on the rate of mortality (Fig. 5).

Assessment of waterborne challenge model with four field isolates of *Aeromonas* (Table 2) showed that vAh isolates (with high titers of cell agglutination) caused about 90% mortality within 48 h post challenge; the isolate of *A. veronii* (without agglutination reaction) caused no mortality or adverse effect under the same challenge conditions. These results indicate that the waterborne challenge model developed in this study can effectively mimic field conditions in laboratory to reproduce MAS caused by vAh infection in catfish.

In summary, fresh body surface wounds appeared to be a prerequisite for vAh infection via the waterborne route. Various types of surface wounds, possibly resulting in injuries of cutaneous mucus (Bader et al., 2006), were vulnerable to the opportunistic pathogen to some extent. Clipping of the adipose fin to create relatively uniform wounds was shown in this study to be an effective method to mimic natural infection route and reproduce clinically MAS disease under waterborne condition. The waterborne challenge model described in this study will facilitate urgently-needed studies of MAS prevention (such as wound avoidance and healing) and control (such as prophylactic vaccination and probiotics screening).

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References

Bebak, J., Wagner, B., Burnes, B., Hanson, T., 2015. Farm size, seining practices, and salt use: risk factors for *Aeromonas hydrophila* outbreaks in farm-raised catfish, Alabama, USA. Prev. Vet. Med. 118, 161–168.

Bader, J.A., Moore, S.A., Nusbaum, K.E., 2006. The effect of cutaneous injury on a reproducible immersion challenge model for *Flavobacterium columnare* infection in channel catfish (*Ictalurus punctatus*). Aquaculture 253, 1–9.

Buckland-Nicks, J.A., Gills, M., Reimchen, T.E., 2011. Neural network detected in a presumed vestigial trait: ultrastructure of the salmonid adipose fin. Proc. Biol. Sci. 279, 553–563.

Camus, A.C., Durborow, R.M., Hemstreet, W.G., Thune, R.L., Hawke, J.P., 1998. *Aeromonas* bacterial infections-motile aeromonad septicemia. SRAC Publ. 478, 1–4.

Chen, C.-Y., Nace, G.W., Irwin, P.L., 2003. A 6 × 6 drop plate method for simultaneous colony counting and MPN enumeration of *Campylobacter jejuni*, *Listeria monocytogenes*, and *Escherichia coli*. J. Microbiol. Methods 55, 475–479.

Gresham, J., 2014. Producers, researchers will ramp up *Aeromonas* efforts in 2015. Catfish J. 27, 10.

Hanson, L., Liles, M.R., Hossain, M.J., Griffin, M., Hemstreet, W., 2014. Motile Aeromonas Septicemia In: Fish Health Section Blue Book Edition 2014, Section 1.2.9. American Fisheries Society-Fish Health Section. Bethesda, Maryland.

Hemstreet, W.B., 2010. An update on *Aeromonas hydrophila* from a fish health specialist for summer 2010. Catfish J. 24, 4.

Hossain, M.J., Waldbieser, G.C., Sun, D., Capps, N.K., Hemstreet, W.B., Carlisle, K., Griffin, M.J., Khoo, L., Goodwin, A.E., Sonstegard, T.S., Schroeder, S., Hayden, K., Newton, J.C., Terhune, J.S., Liles, M.R., 2013. Implication of lateral genetic transfer in the emergence of *Aeromonas hydrophila* isolates of epidemic outbreaks in channel catfish. PLoS One 8 (11), e80943, http://dx.doi.org/10.1371/journal.pone.0080943.

Long, A., Fehringer, T.R., LaFrentz, B.R., Call, D.R., Cain, K.D., 2014. Development of a waterborne challenge model for *Flavobacterium psychrophilum*. FEMS Microbiol. Lett. 359, 154–160.

Pridgeon, J.W., Klesius, P.H., Song, L., Zhang, D., Kojima, K., Mobley, J.A., 2010. Identification, virulence, and mass spectrometry of toxic ECP fractions of west Alabama isolates of *Aeromonas hydrophila* obtained from a 2010 disease outbreak. Vet. Microbiol. 164, 336–343.

Pridgeon, J.W., Zhang, D., Zhang, L., 2014. Complete genome sequence of the highly virulent *Aeromonas hydrophila* AL09-71 isolated from diseased channel catfish in west Alabama. Genome Announc. 2 (3), 00450–514, http://dx.doi.org/10.1128/genomeA.00450-14.

Reimchen, T.E., Temple, N.F., 2004. Hydrodynamic and phylogenetic aspects of the adipose fin in fishes. Can. J. Zool. 82, 910–916.

Tekedar, H.C., Waldbieser, G.C., Karsi, A., Liles, M.R., Griffin, M.J., Vamenta, S., Sonstegard, T., Hossain, M., Schroeder, S.G., Khoo, L., Lawrence, M.L., 2013. Complete genome sequence of a channel catfish epidemic isolate, *Aeromonas hydrophila* strain ML09-119. Genome Announc. 1 (5), 13–e00755, http://dx.doi.org/10.1128/genomeA00755-13.

Temple, N.F., Reimchen, T.E., 2008. Adipose fin condition and flow regime in catfish. Can. J. Zool. 86, 1079–1082.

Vally, H., Whittle, A., Cameron, S., Dowse, G.K., Watson, T., 2004. Outbreak of *Aeromonas hydrophila* wound infections associated with mud football. Clin. Infect. Dis. 38, 1084–1089.

Vander Haegen, G.E., Blankenship, H.L., Hoffmann, A., Thompson, D.A., 2005. The effects of adipose fin clipping and coded wire tagging on the survival and growth of spring Chinook salmon. North Am. J. Fish. Manage. 25, 1161–1170.

Xu, D.-H., Pridgeon, J.W., Klesius, P.H., Shoemaker, C.A., 2012. Parasitism by protozoan Ichthyophthirius multifiliis enhanced invasion of *Aeromonas hydrophila* in tissues of channel catfish. Vet. Parasitol. 184, 101–107.

Zhang, D., Pridgeon, J.W., Klesius, P.H., 2013. Expression and activity of recombinant proaerolysin derived from *Aeromonas hydrophila* cultured from diseased channel catfish. Vet. Microbiol. 165, 478–482.

Zhang, D., Pridgeon, J.W., Klesius, P.H., 2014. Vaccination of channel catfish with extracellular products of *Aeromonas hydrophila* provides protection against infection by the pathogen. Fish Shellfish Immunol. 36, 270–275.