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Convenient and reliable methods to evaluate the virulence and pathogenesis of Aeromonas infection in a Caenorhabditis elegans model

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Cover letter to JOVE

Dear Editor in chief:

We wish to thank the reviewers and the editorial board for the excellent reviews on the manuscript entitled "**Methods to study the virulence and pathogenesis of *Aeromonas* infection in a *Caenorhabditis elegans* model**". All authors have seen and agreed to the final version. There were no potential conflicts of interest and sources of financial support to be declared by all authors.

The human pathogen *Aeromonas* has been clinically shown to cause severe infection. Most human diseases have been reported to be associated with four species: *A. dhakensis*, *A. hydrophila*, *A. veronii*, and *A. caviae*. The model organism *Caenorhabditis elegans* is a bacterivore that provides an excellent infection model by which to study the bacterial pathogenesis of *Aeromonas*. Here, we introduce three different experiments to study *Aeromonas* infection using a *C. elegans* model, including survival, liquid toxicity, and muscle necrosis assays. The results of the three methods determining the virulence of *C. elegans* were consistent. These methods are shown to be a convenient way to clarify the toxicity among and within *Aeromonas* species and contribute to an understanding of the pathogenesis of *Aeromonas* infection.

We would appreciate it very much if you and the reviewers could review the manuscript to be published in your journal.

Sincerely yours,

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TITLE:

Evaluating Virulence and Pathogenesis of *Aeromonas* Infection in a *Caenorhabditis Elegans* Model

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KEYWORDS:

Aeromonas dhakensis, *Aeromonas hydrophila*, *Aeromonas veronii*, *Aeromonas caviae*, *Caenorhabditis elegans*, bacterial infection, Liquid toxicity assay, Survival assay, Muscle necrosis assay.

SUMMARY:

Here, we introduce three different experiments to study *Aeromonas* infection in *C. elegans*. Using these convenient methods, it is easy to evaluate the toxicity among and within *Aeromonas* species.

ABSTRACT:

The human pathogen *Aeromonas* has been clinically shown to cause gastroenteritis, wound infections, septicemia, and urinary tract infections. Most human diseases have been reported to be associated with four species of bacteria: *Aeromonas dhakensis*, *Aeromonas hydrophila*, *Aeromonas veronii*, and *Aeromonas caviae*. The model organism *Caenorhabditis elegans* is a bacterivore that provides an excellent infection model by which to study the bacterial pathogenesis of *Aeromonas*. Here, we introduce three different experiments to study *Aeromonas* infection using a *C. elegans* model, including survival, liquid toxicity, and muscle necrosis assays.

The results of the three methods determining the virulence of *Aeromonas* were consistent. *A. dhakensis* was shown to be the most toxic among the 4 major *Aeromonas* species causing clinical infections. These methods are shown to be a convenient way to evaluate the toxicity among and within *Aeromonas* species and contribute to our understanding of the pathogenesis of *Aeromonas* infection.

INTRODUCTION:

The human pathogen, *Aeromonas*, has been shown clinically to cause gastroenteritis, wound infections, septicemia, and urinary tract infections [1,2]. Most associated human diseases have been reported to be associated with four bacterial species: *Aeromonas dhakensis*, *Aeromonas hydrophila*, *Aeromonas veronii*, and *Aeromonas caviae* [2-5]. Among *Aeromonas* infectious diseases, soft-tissue infections can cause severe morbidity and mortality in humans. Of note, muscle necrosis is the most severe form of soft tissue infection[6]. Observation of the survival and muscle necrosis of *Caenorhabditis elegans* after infection is a convenient method by which to speculate the toxicity of *Aeromonas*.

Scientists have already developed numerous model organisms to study bacterial infections. In previous studies, mice, zebrafishes, and nematodes were used as animal models to study the pathogenesis and virulence of *Aeromonas* [6-8]. Every animal model has its' advantages and applications. The model organism, *Caenorhabditis elegans*, is a bacterivorous nematode which intakes bacteria as food naturally. *C. elegans* has developed a complicated innate immune system against bacterial infection over the course of its evolution. Under the stress of bacterial infection, *C. elegans* has been proven to be an excellent infection model to study the bacterial pathogenesis of *Aeromonas* [6,7,9] and other pathogens like fungus [10] and enterohaemorrhagic *Escherichia coli* O157:H7 [11]. However, there is still no publication that focuses on the methodology in using *C. elegans* as a model for studying the virulence of *Aeromonas*.

Here, we introduce three different experiments to study *Aeromonas* infection using *C. elegans* as an animal model: assays for survival, liquid toxicity, and muscle necrosis. These methods are a convenient way to evaluate the toxicity among and within *Aeromonas* species and improve the understanding of the pathogenesis of *Aeromonas*.

PROTOCOL:

1. Preparation of the Culture Medium

1.1 To prepare M9 medium[12], dissolve 1.5 g of KH_2PO_4, 5.66 g of Na_2HPO_4, and 2.5 g of NaCl in 500 mL of deionized water. Autoclave at 121 °C for 20 min. Wait until cooled to room temperature and then add 0.5 mL of 1 M $MgSO_4$ before first use.

1.2 To prepare nematode growth medium (NGM)[12], dissolve 3 g NaCl, 2.5 g bacterial peptone, and 20 g agar in 1 L of deionized water. Autoclave at 121 °C for 20 min. Wait until cooled to 55 °C in a water bath and then add 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1 mL of 5% cholesterol in ethanol, and 25 mL of phosphate buffer. Swirl to mix well.

1.3 Pour about 5 mL NGM into each 6 cm Petri dish. Avoid bubble production on the surface of plate. Leave plates at room temperature for 1 night and package to save at 4 °C. Bring back to room temperature before use.

1.4 To prepare enriched NGM (ENGM), dissolve 3 g NaCl, 5 g bacterial peptone, 1 g yeast extract, and 30 g agar in 1 L of deionized water. Autoclave at 121 °C for 20 min. Wait until cooled to 55 °C in a water bath, and add 1 mL of 1 M $CaCl_2$, 1 mL of 1 M $MgSO_4$, 1 mL of 5% cholesterol in ethanol, and 25 mL of phosphate buffer. Swirl to mix well.

1.5. Pour about 10 mL ENGM into each 9 cm Petri dish. Avoid bubble production on the surface of plate. Leave plates at room temperature for 1 night and package to save at 4 °C. Bring to room temperature before use.

1.6 To prepare S medium[12], mix 40 mL S Basal, 0.4 mL 1 M potassium citrate, 0.4 mL trace metals solution, 0.12 mL of 1 M $CaCl_2$, 0.12 mL of 1 M $MgSO_4$, 40 µL of 5% cholesterol in ethanol and 1 mL of 8 mM FudR before use.

Note: See **Table 1** for solution preparation.

2. Synchronization of *C. elegans*[6,9,12]

2.1 Wash about 2,000 worms in the gravid-adult stage with 10 mL of sterile deionized water in a 15 mL tube. Wash out the bacteria 3x, keeping the worms at the bottom of the tube.

2.2 Centrifuge the sample for 1 min at 500 x *g* to pull down the worms. Remove the supernatant, and keep the worms in 3.5 mL deionized water.

2.3 Add 1 mL NaOCl (10–15%) and 0.5 mL 5 M KOH into the tube. Mix evenly by shaking for <6 min to lyse the worm bodies.

2.4 After the eggs are released from the worms, add 10 mL deionized water to stop the lysis. Centrifuge for 1 min at 1,200 x *g* to pull down the eggs and remove the supernatant as much as possible. Wash with 15 mL M9 medium at least 3x.

Note: See step 1.1 for the composition of M9 medium.

2.5 Keep eggs in 1 mL M9 medium. Transport eggs to a 3.5 cm dish for incubation at 20 °C for one night (about 12–18 h).

Note: After the incubation, the eggs will hatch to worm larva which is called as first larva (L1) stage.

2.6 Pipette out 10 µL of L1 worms in M9 medium. Count the worm number and calculate the concentration of L1 worms in M9 medium.

2.7 Seed at most 10,000 incubated L1 stage worms with a pipet on a 9 cm ENGM plate spread with *Escherichia coli* OP50. In this step, spread 0.5 mL overnight cultured *E. coli* OP50 with Luria-Bertani (LB) broth on the 9 cm ENGM plate. Incubate the plate at 37 °C for 16–18 h

Note: To avoid contamination, the spreading step should be performed in a laminar flow hood.

2.8 Cool to room temperature before seeding L1 worms. Seed at most 10,000 incubated L1 stage worms on a 9 cm ENGM plate with *E. coli* OP50. Incubate the worms at 20 °C for 44 h or until they grow to the 4th larva (L4) stage.

Note: See step 1.3 for the composition of ENGM medium. A worm has a white dot in half-moon shape at the middle of body side at the L4 stage.

2.9 Use the synchronized L4 stage worms in the following assays. Use wild type N2 worms in the *C. elegans* survival assay with *Aeromonas dhakensis* and the *C. elegans* liquid toxicity assay with *Aeromonas dhakensis*. Use RW1596 worms (*myo-3(st386);stEx30[myo-3p::GFP::myo-3 + rol-6(su1006)]*) in the *C. elegans* muscle necrosis assay with *Aeromonas dhakensis*.

3. *C. elegans* Survival Assay with *Aeromonas*[6,9]

3.1 On the day of seeding L1 worms on the ENGM spreading with *E. coli* OP50, pick a single colony of each of the four *Aeromonas* strains or *E. coli* OP50 and culture with 2 mL LB broth respectively at 37 °C for 16 h.

Note: To avoid contamination, this step should be performed in a laminar flow hood. Here, the following bacterial strains were used: *E. coli* OP50, the normal food source of *C. elegans*. *A. dhakensis* AAK1, the first fully-sequenced pathogenic clinical isolated *A. dhakensis* clone. *A. hydrophila* A2-066, *A. veronii* A2-007, and *A. caviae* A2-9307121 are representatively clinical isolates from National Cheng Kung University Hospital.

3.2 Measure the OD600 absorbance of bacterial broth and adjust the bacterial broth to OD600 = 2.0 with LB broth.

3.3 Spot and spread 30 µL of bacterial broth each 6 cm NGM plate. Culture the plates at room temperature overnight.

Note: See step 1.2 for the composition of NGM medium.

3.4 On the day the L1 worms grow to the L4 stage, randomly pick and transfer 50 worms to an NGM plate with each of the four *Aeromonas* strains or *E. coli* OP50.

3.5 Incubate the NGM plates at 20 °C until the assay is finished.

3.6 Transfer all the living worms to a newly prepared NGM plate with bacteria and count the numbers of live, dead, and sensor worms every day until the last worm is dead.

Note: Transfer worms to a fresh-prepared NGM plate every day for the maintenance of infection, even if using sterile worms (*e.g., glp-4*, or with FudR).

3.7 Plot the survival curves based on the daily data calculation.

4. *C. elegans* Liquid Toxicity Assay with *Aeromonas*[7]

Note: To avoid contamination, steps should be performed in a laminar flow hood.

4.1 The day after seeding the L1 worms on the ENGM plate spread with *E. coli* OP50, pick a single colony of each of the four *Aeromonas* strains and *E. coli* OP50, and culture with 5 mL LB broth each at 37 °C for 16 h.

Note: In the protocol, the following bacteria strains were used: *E. coli* OP50, the normal food source of *C. elegans. A. dhakensis* AAK1, the first fully-sequenced pathogenic clinical isolated *A. dhakensis* clone. *A. hydrophila* A2-066, *A. veronii* A2-007, and *A. caviae* A2-9307121 are representatively clinical isolates from National Cheng Kung University Hospital.

4.2 Measure the OD600 absorbance of the bacteria broth.

4.3 Centrifuge the bacterial broth at 3,500 x *g* for 15 min to remove the LB broth. Resuspend the bacteria and adjust the bacterial broth to OD600 = 3.0 with S medium. Add 195 µL of bacterial broth in S medium to at least 8 wells of a 96-well plate.

Note: See step 1.6 for the composition of S medium.

4.4 Wash off the L4 worms on the ENGM plate with *E. coli* OP5 using M9 medium. Adjust the worm solution to a concentration of 5 worms per µL and add 5 µL of the worm solution to each well of the 96-well plate. Ensure that there are approximately 25 worms per well.

4.5 Incubate the 96-well plate on a shaker at 200 rpm at 25 °C.

4.6 Count the number of live worms and dead worms after 24, 48, and 72 h. Calculate the survival rates of each well with the following formula: (Live worms/Total worms) × 100%.

4.7 Draw a scatter plot graph with the daily data calculation.

5. *C. elegans* Muscle Necrosis Assay with *Aeromonas*[6]

Note: To avoid contamination, these steps should be performed in a laminar flow hood.

5.1 On the day the L1 worms are seeded on the ENGM spread with *E. coli* OP50, pick a single colony of each of the four *Aeromonas* strains and *E. coli* OP50 and culture with 2 mL LB broth each at 37 °C for 16 h.

Note: Here, the following bacteria strains were used: *E. coli* OP50, the normal food source of *C. elegans. A. dhakensis* AAK1, the first fully-sequenced pathogenic clinical isolated *A. dhakensis* clone. *A. hydrophila* A2-066, *A. veronii* A2-007, and *A. caviae* A2-9307121 are representatively clinical isolates from National Cheng Kung University Hospital.

5.2 Measure the OD600 absorbance of the bacteria broth and adjust the bacteria broth to OD600 = 2.0 with LB broth. Spot and spread 30 µL of bacterial broth on 6 cm NGM plates. Culture the plates at room temperature overnight.

5.3 On the day the L1 worms grow to the L4 stage, transfer 50 worms to each NGM plate with each of the four *Aeromonas* strains or *E. coli* OP50. Incubate the NGM plates at 20 °C. Transfer worms every 24 h to newly prepared NGM plates with bacteria.

5.4 Take muscle images using a fluorescence microscope.

5.4.1. Randomly pick 10 worms onto a 2% agarose gel in M9 medium on a slide. Paralyze the worms using 2 µL of 1% sodium azide in M9 medium on agarose for less than 5 min before taking images.

5.4.2. Place a cover slip and capture the muscle images using a green fluorescent protein (GFP) filter on a fluorescent microscope with charge-coupled device camera. Capture the muscle images at 24, 48, and 72 h post the L4 stage.

5.5 Conduct image analysis and figure preparation with an image processing software.

Note: The definitions of the scores and the muscle damage levels are shown in **Figure 3**, for which the criteria are listed as follows: 3 points for missing muscle fibers; 2 points for ruptured or broken muscle fibers; 1 point for bent muscle fibers; 0 points for healthy muscle fibers.

6. Statistical Analyses

6.1 Perform all experiments a minimum of three times independently.

6.2 Use the Kaplan-Meier method to assess the *C. elegans* survival assays, and use the log-rank test in analyzing survival differences. Set statistical significance at 0.05.

6.3 Use Student's t-test to analyze the statistical results of the *C. elegans* liquid toxicity assays for the two different groups. Use one-way ANOVA test in analyzing differences among three or more values for one independent variable. Set statistical significance at 0.05.

REPRESENTATIVE RESULTS:

By following the protocols described above, it is easy to differentiate between the toxicities from the four *Aeromonas* strains. The survival assay of *C. elegans* is shown in **Figure 1**. The survival rates of *C. elegans* infected with *Aeromonas* species, shown in order from high to low were: *A. caviae, A. veronii, A. hydrophila,* and *A. dhakensis*. Although there is diversity in terms of toxicity among and within *Aeromonas* species, the survival rates of *C. elegans*, on average, showed highest mortality among the worms infected with *A. dhakensis*. The virulence of *A. hydrophila* was inferior to that of *A. dhakensis* ($p < 0.01$). In contrast to *A. dhakensis* and *A. hydrophila,* the attenuated virulences of both *A. veronii* and *A. caviae* were observed in the *C. elegans* survival assay. The diversity of the survival rates of *C. elegans* infected with different *Aeromonas* species is consistent with clinical observations of *Aeromonas* infection[7].

The results of the liquid toxicity assay of *C. elegans* are shown in **Figure 2**. With the infection time, the numbers of worms that survived decreased significantly when *C. elegans* was infected with either *A. dhakensis* or *A. hydrophila*. The survival rate of the worms infected with *A. dhakensis* was inferior to those infected with *A. hydrophila*. In contrast, the groups infected with *A. veronii* or *A. caviae* had higher survival rates at each time point as compared to *A. dhakensis* and *A. hydrophila*. The trends of survival rates of *C. elegans* in the liquid toxicity assay representing the virulence of different *Aeromonas* species are similar to those observed in the survival assay.

In the *C. elegans* muscle necrosis assay, we first classified the levels of muscle damage and assigned scores according to the corresponding levels. The definitions of the score and the muscle damage levels are provided in **Figure 3**. The scoring criteria for each muscle damage level is as follows: 0 points for healthy muscle fibers; 1 point for bent muscle fibers; 2 points for ruptured or broken muscle fibers; 3 points for loss of muscle fibers. The results of the *C. elegans* muscle necrosis assay in *Aeromonas* infected-*C. elegans* is shown in **Figure 4**. The degrees of muscle damage varied among the *Aeromonas* species. The levels of muscle damage ranged in order from mild to severe as follows: *A. caviae, A. veronii, A. hydrophila,* and *A. dhakensis* at each time point. In contrast with *A. hydrophila* and *A. dhakensis*, muscle necrosis was not obvious in worms infected with *A. caviae* and *A. veronii*. Most of the worms infected with *A. caviae* and *A. veronii* were recorded as 0 points. *C. elegans* infected with *A. hydrophila* or *A. dhakensis* exhibited severe muscle damage. Of note, most of the worms infected with *A. dhakensis* demonstrated muscle damage graded ≥ 2 points. The degree of muscle necrosis of *C. elegans* infected with *A. dhakensis* was more severe in terms of a higher percentage of scores ≥ 2 points compared with *A. hydrophila*. The severity of muscle necrosis in the 4 *Aeromonas* species correlated with the findings from the survival and liquid toxicity assays.

The methods described above offer a convenient way to differentiate virulence diversity among and within *Aeromonas* species. In addition, this is a reliable model by which to study the interaction between a pathogen and host.

FIGURE AND TABLE LEGENDS:

Figure 1: The survival curves of *C. elegans*. The survival assay shows the diverse toxicities of different *Aeromonas* species to *C. elegans* (**: P < 0.01; *: P < 0.05).

Figure 2: The survival rate of *C. elegans* from the liquid toxicity assay. The liquid toxicity assay shows the different toxicities of various *Aeromonas* species to *C. elegans*. At (**A**) 24 h, (**B)** 48 h, and (**C**) 72 hours post *Aeromonas* infection, *C. elegans* infected with *A. dhakensis* has the lowest survival rate. The result suggests that *A. dhakensis* is the most toxic *Aeromonas* species to *C. elegans* (***: P < 0.001; **: P < 0.01, Error bars: standard deviation, SD).

Figure 3: The corresponding scores for muscle necrosis in *C. elegans* induced by *Aeromonas* infection. The criteria for muscle necrosis and corresponding scores are as follows: 3 points for missing muscle fibers; 2 points for ruptured or broken muscle fibers; 1 point for bent muscle fibers; 0 points for healthy muscle fibers. Bent muscle fibers (arrows), ruptured muscle fibers (arrowheads), and missing muscle fibers (star) are indicated.

Figure 4: The *C. elegans* muscle necrosis scores. The muscle necrosis scores show the various toxicities of different *Aeromonas* species to *C. elegans*. At (**A**) 24 h, (**B**) 48 h, and (**C**) 72 h post *Aeromonas* infection, the *C. elegans* muscle damage is the severest from infection with *A. dhakensis.*

Table 1: Solution Preparation.

DISCUSSION:

C. elegans is a bacterivorous nematode that naturally intakes bacteria as food and has developed a complicated innate immunity to bacteria during its evolutionary process. Two of the major organs maintaining and supporting the immunity are the epidermis and intestine[9,13]. The epidermis and bands of muscle of *C. elegans* resemble the soft-tissue structures in mammals and humans[6]. Because of these characteristics, *C. elegans* is applicable as a model organism for studying the pathogenesis of *Aeromonas* infection.

Here, we present three methods for examining *Aeromonas* infection in *C. elegans* that are representative of the diverse toxicity among 4 *Aeromonas* species and are correlated with clinical observation [7]. These convenient methods distinguish the different degrees of toxicity among the *Aeromonas* species. Using these methods, researchers can study the virulence of different *Aeromonas* species and the defending mechanism of hosts upon *Aeromonas* infection.

In these three methods, *C. elegans* acts not only as a predator but also as a prey of *Aeromonas*. If the worms are not in healthy condition before test, the results of host-pathogen interaction will be different. Thus, it is important to be very careful when manipulating *C. elegans*. To avoid excessive damage to the eggs of *C. elegans* in the synchronizing step, be sure to observe the release of eggs continuously. Once the eggs are damaged, the hatching number of *C. elegans*

eggs will largely decrease. Even if the damaged eggs hatch successfully, the worms are likely to have developmental problems. Therefore, it is critical to complete step 2.3 within 6 min after the worms are exposed to NaOCl and KOH.

The three methods described above show cohesive results on the *Aeromonas* toxicities to *C. elegans*, including two different experiments measuring the survival rates of *C. elegans* under *Aeromonas* infection and one observing the morphological change of muscle. The virulence test using different designed methods may provide diverse results. For example, scientists have previously observed different outcomes in *C. elegans* plate and liquid assays[14]. In our research, we also found there was discrepancy existing between these two assays[9]. It is believed that the external environment will influence virulence of the pathogen, and the researchers need to find the optimal conditions to explore the virulence mechanism. From another perspective, it is worth studying and understanding the underlying mechanisms in specific condition of infection.

In this work, we established an infection model in *C. elegans* to study the virulence of *Aeromonas* and the corresponding host response after infection. These three methods could be applied to study the other *Aeromonas* species beyond the 4 species addressed in the present study. In addition, these approaches will provide a platform to study the underlying mechanism responsible for muscle necrosis induced by bacterial infection and to develop potential therapies to improve the clinical outcomes of severe soft tissue infections.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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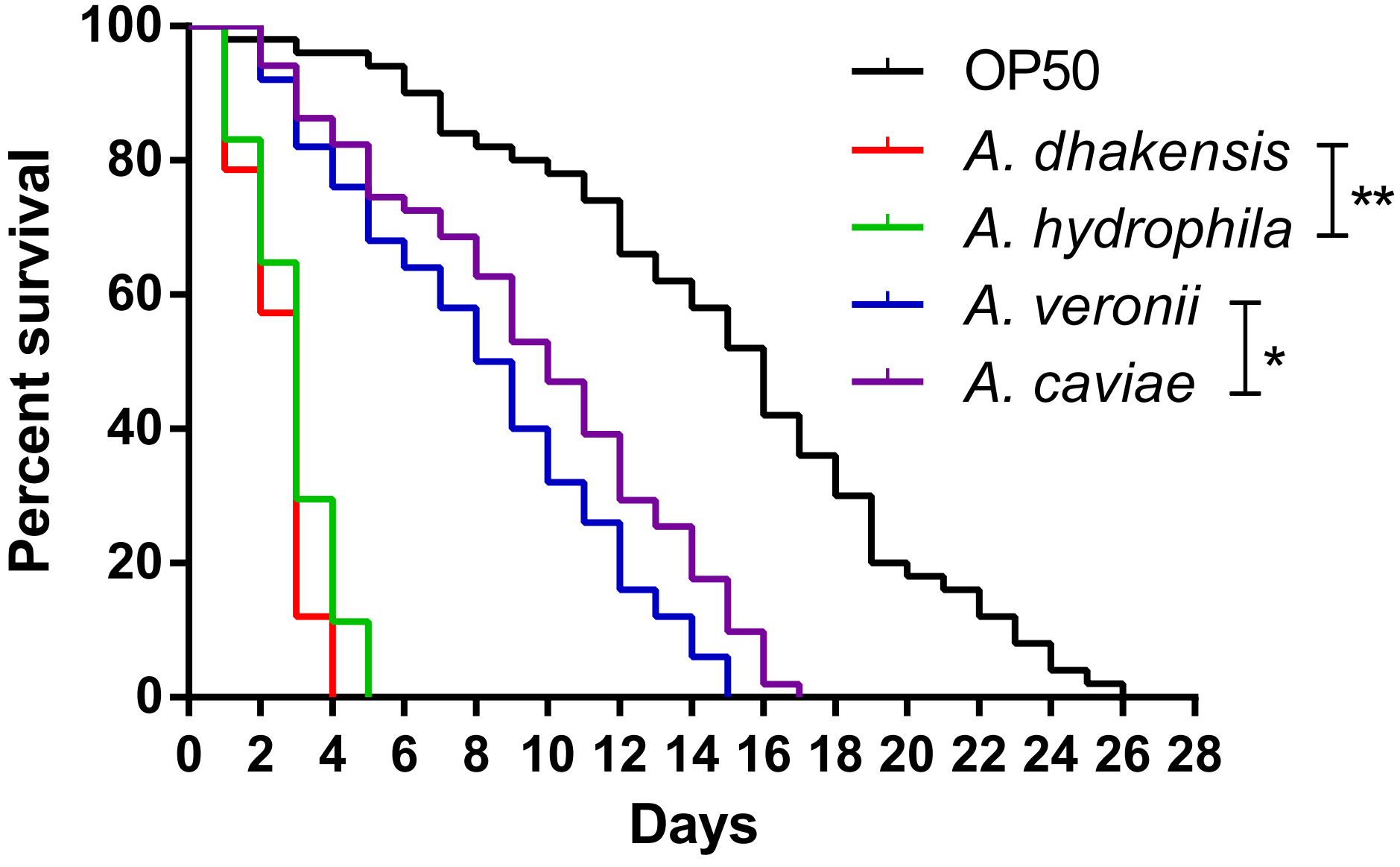

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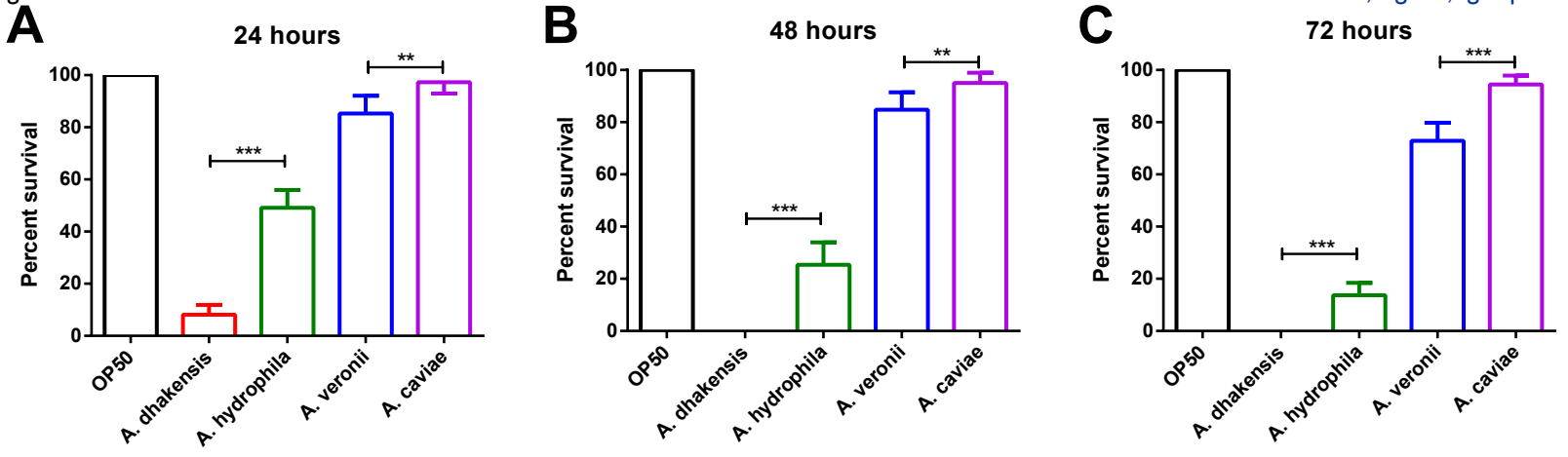

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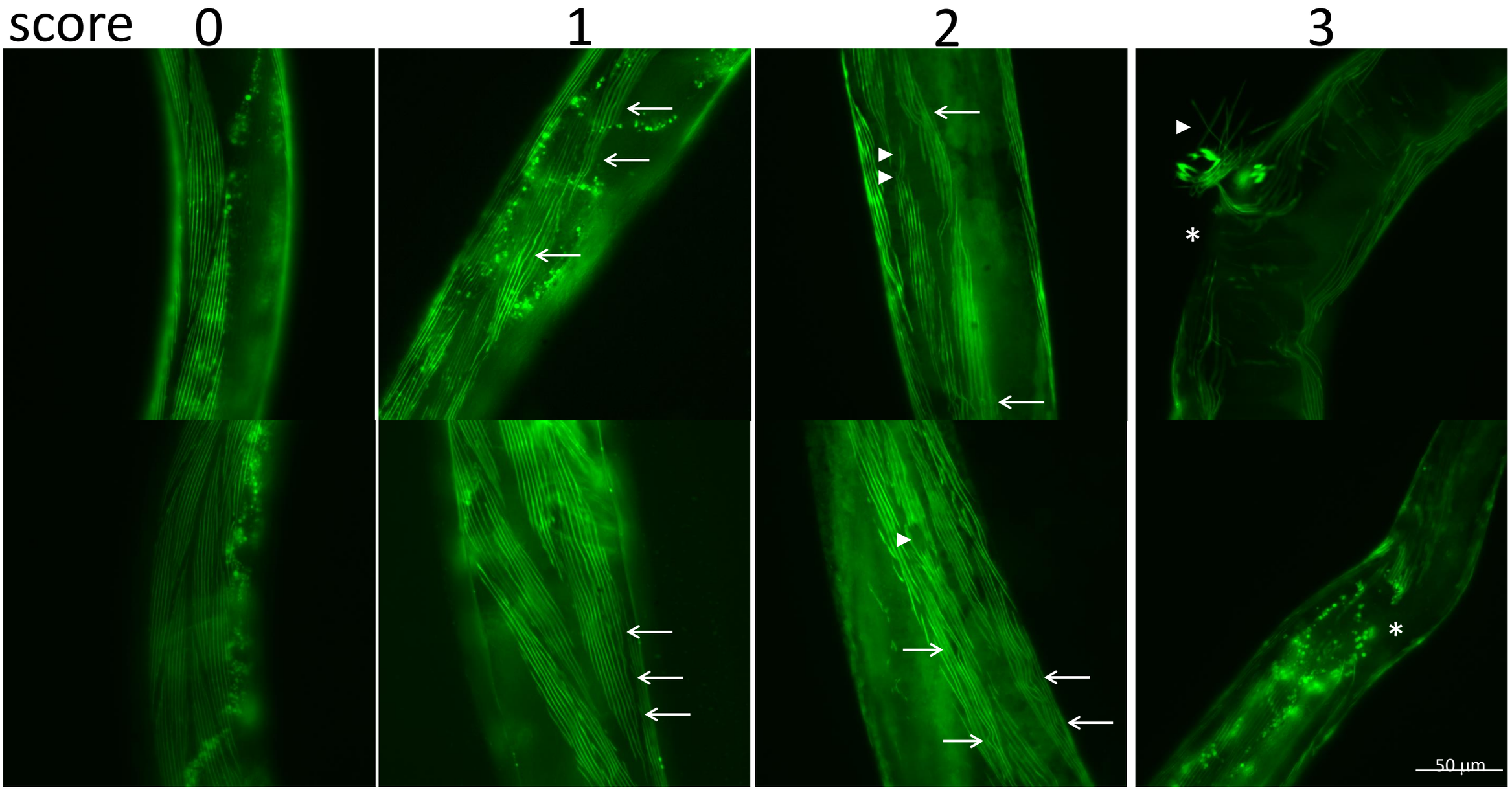

Figure 4

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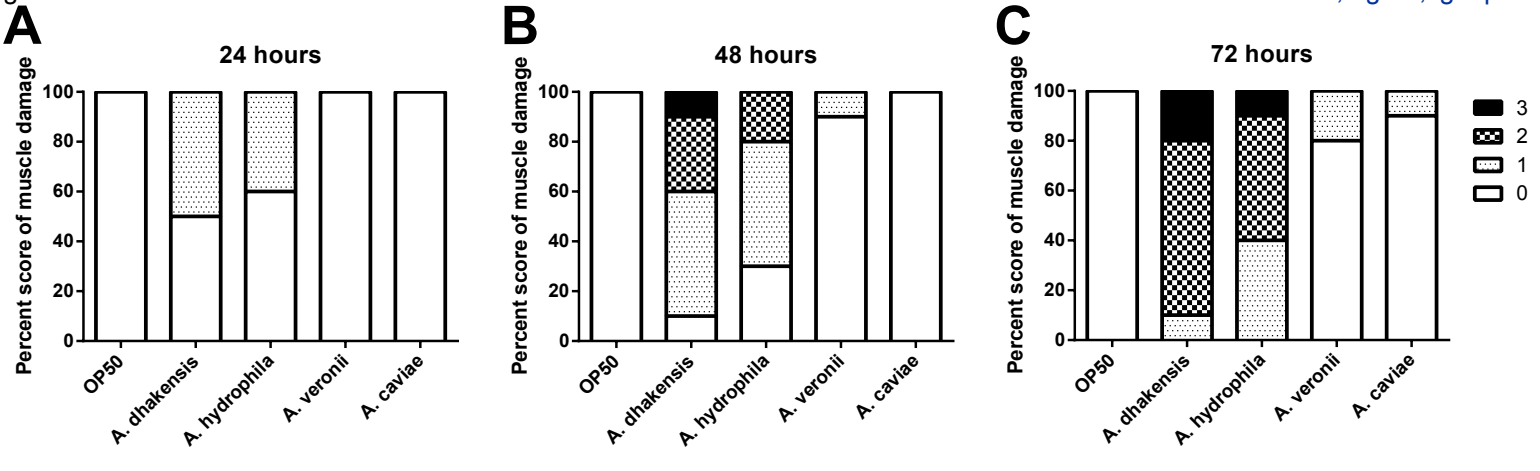

Table 1 Click here to access/download;Table;Table 1.xlsx

Solution	Preparation
Phosphate Buffer	Dissolve 119.35 g of KH2PO4 and 21.43 g of K2HPO4, in 1 L deionized water. Autoclave at 121 °C for 20 min Adjust the pH value to pH = 6.0 with KOH. Wait until cooled to room temperature
S Basal	Dissolve 5.85 g NaCl, 6 g KH_2PO_4, and 1 g K_2HPO_4 in 1 L of deionized water. Autoclave at 121 °C for 20 min. Wait until cooled to room temperature.
1 M Potassium citrate	Dissolve 4 g citric acid•H_2O and 58.7 g tri-potassium citrate•H_2O in 0.2 mL of deionized water. Adjust the pH value to pH=6.0. Autoclave at 121 °C for 20 min. Wait until cooled to room temperature.
Trace metals solution	Dissolve 1.86 g disodium EDTA, 0.69 g $FeSO_4$ •7 H_2O, 0.2 g $MnCl_2$•4 H_2O, 0.29 g $ZnSO_4$•7 H_2O, 0.025 g $CuSO_4$•5 H_2O in 1 L deionized water. Autoclave at 121 °C for 20 min. Wait until cooled to room temperature Keep the solution in the dark.
LB broth	Dissolve 10 g NaCl, 10 g tryptone, and 5 g yeast extract in 1 L of deionized water. Autoclave at 121 °C for 20 min. Wait until cooled to room temperature.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Shaker incubator	YIH DER	LM-570R	Bacteria incubation
K2HPO4	J.T.Baker	MP021519455	Culture medium preparation
KH2PO4	J.T.Baker	3246-05	Culture medium preparation
Na2HPO4	J.T.Baker	MP021914405	Culture medium preparation
NaCl	SIGMA	31434	Culture medium preparation
MgSO4	SIGMA	M7506	Culture medium preparation
agar	Difco	214530	Culture medium preparation
CaCl2	SIGMA	C1016	Culture medium preparation
cholesterol	SIGMA	C8503	Culture medium preparation
ethanol	SIGMA	32205	Culture medium preparation
KOH	SIGMA	P5958	Culture medium preparation
6 cm petri plate	ALPHA PLUS	46	agar plate preparation
96-well plate	FALCON	353072	liquid assay
bacterial peptone	Affymetrix/USB	AAJ20048P2	Culture medium preparation
yeast extract	SIGMA	92144	Culture medium preparation
citric acid•H2O	SIGMA	C1909	Culture medium preparation
tri-potassium citrate•H2O	SIGMA	104956	Culture medium preparation
FudR	SIGMA	1271008	Culture medium preparation
disodium EDTA	SIGMA	E1644	Culture medium preparation
FeSO4•7 H2O	SIGMA	215422	Culture medium preparation
MnCl2•4 H2O	SIGMA	221279	Culture medium preparation
ZnSO4•7 H2O	SIGMA	204986	Culture medium preparation
CuSO4•5 H2O	SIGMA	C8027	Culture medium preparation
tryptone	SIGMA	16922	Culture medium preparation
Microscope system	Nikon	Eclipase Ti inverted	microscope imaging
Scientific CCD Camera	QImaging	Retiga-2000R Fast 1394	microscope imaging

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Author(s):	Yi-Wei Chen, Wen-Chien Ko, Chang-Shi Chen, and Po-Lin Chen*

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Editor of JoVE August 20th, 2018

Dr. Alisha DSouza

Dear Dr. Alisha DSouza:

We wish to thank the reviewers and the editorial board for the excellent reviews on the manuscript entitled "**Convenient and reliable methods to evaluate the virulence and pathogenesis of *Aeromonas* infection in a *Caenorhabditis elegans* model**" (Manuscript ID: JoVE58768). We have revised the manuscript according to the valuable recommendations. The revisions we made are summarized in the following context and are marked with underlines. The draft has been edited by a native English speaker before re-submission. We hope our revised manuscript will be suitable for publication in Froniters in Immunology.

Yours Sincerely,

Yi-Wei Chen

Editorial comments:

Changes to be made by the Author(s) regarding the written manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Ans: Many thanks for the suggestion. We have re-edited the English writing of manuscript with the help from Miss Savana Moore, an English proofreading editor with 15-years experience in English editing of the language center of National Chung-Kung University.

2. Please revise the title to be more concise and clear.

Ans: Many thanks for the suggestion. We have revised the title as follow: "Convenient and reliable methods to evaluate the virulence and pathogenesis of *Aeromonas* infection in a *Caenorhabditis elegans* model".

3. Please spell out each abbreviation the first time it is used.

Ans: Many thanks for the suggestion. We have revised to spell out each abbreviation the first time it is used. (lines 39-40; 53-54)

4. Please use centrifugal force (x g) for centrifuge speeds.

Ans: Many thanks for the suggestion. We have revised it. (lines 139)

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Ans: Many thanks for the suggestion. We have removed these commercial languages. (lines 193, 219, and 238-248)

6. Please revise the protocol (1.11-1.13, 6.1-6.3, etc.) to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used

sparingly and actions should be described in the imperative tense wherever possible.
Ans: Many thanks for the suggestion. We have revised the protocol to contain only action items. (lines 154-170 and 251-258)

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
Ans: Many thanks for the suggestion. We have revised and added more details and references of these protocols. (lines 75-258)

1.1: What is ddwater? What volume of ddwater is used to wash?
Ans: We have revised ddwater to deionized water and added the volume of usage. (line 136)

1.5: What eggs?
Ans: We have revised eggs to "eggs are released from the worms". (line 143)

1.7: Please provide composition of M9 medium. What volume of M9 medium is used to wash?
Ans: Many thanks for the suggestion. The composition of M9 was described at lines 76-81. And we have revised and added the volume of usage. (lines 146-147)

1.10: How is L4 stage defined?
Ans: We have added the description of the definition of L4 stage of *C. elegans* as follow: "Note: Worms has a white dot in half-moon shape on the middle of body side at L4 stage." (line 165)

3.5: Please specify incubation time.
Ans: Many thanks for the suggestion. We have added the incubation time as follow "Incubate the NGM plates at 20^{o}C until the assay is finished." (line 188)

3.6: How many worms are transferred?
Ans: All the living worms are needed to be transferred. We have revised at lines 189-192.

4.4: Please provide composition of S medium.
Ans: Many thanks for the suggestion. The composition of S medium was described at lines 108-129.

5.7: Please describe how to paralyze the worms. For instance, for how long are the worms placed in M9 medium with 1% sodium azide?
Ans: We have revised the protocol of how to paralyze the worms at lines 238-244.

8. 2.2-2.4: Please break up into sub-steps.
Ans: Many thanks for the suggestion. We have broken up these steps into sub-steps. (lines 76-133)

9. Figure 2: Please define error bars in the figure legend.
Ans: Many thanks for the suggestion. We have revised as follow: "(Error bars: standard deviation, SD)". (line 309)

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:
a) Critical steps within the protocol
b) Any modifications and troubleshooting of the technique
c) Any limitations of the technique
d) The significance with respect to existing methods
e) Any future applications of the technique
Ans: Many thanks for the suggestion. We have revised the discussion at lines 336-353.

11. Please combine Finding into the Acknowledgements section.
Ans: Many thanks for the suggestion. We have revised and combined at lines 363-370.

12. References: Please do not abbreviate journal titles.
Ans: Many thanks for the suggestion. We have revised at lines 376-410.

Reviewers' comments:

Reviewer #1:

The paper entitled "Methods to study the virulence and pathogenesis of Aeromonas infection in a Caenorhabditis elegans model" describes the three approaches to evaluate virulence of Aeromonas spp. The authors have tried well to indicate that the adopted methods are convenient and useful. However it is not clear which method is more convenient and reliable to evaluate virulence? Or all three methods should be tested.

It is also not indicated the need and significance of this work if methods are already exist.

The authors have successfully able to generate some useful information and may be accepted for publication provided that the above points and points mentioned below should be considered.

English grammar and scientific language need to be improved.
Ans: Many thanks for the suggestion. We have re-edited the English writing of manuscript with the help from Miss Savana Moore, an English proofreading editor with 15-years experience in English editing of the language center of National Chung-Kung University.

Abstract:
All scientific names should be in italics
* A. dhakensis, A. hydrophila, A. veronii, and A. caviae
Caenorhabditis elegans
Ans: Many thanks for the suggestion. We have revised to spell out each abbreviation the first time it is used. (lines 39-40; 53-54)

* The results of the three methods determining the virulence of C. elegans were consistent.(This sentence is not clear, As you are determining the virulence of bacteria and not C elegans Pls correct it.)
Ans: Many thanks for the suggestion. We have revised at line 44.

* Clarify change to evaluate
Ans: Many thanks for the suggestion. We have revised and changed “clarify” to “evaluate” at line 46.

* an understanding change to Our understanding
Ans: Many thanks for the suggestion. We have revised and changed “an” to “our” at line 47.

Protocols:
* References of Protocol used should be given if standard methods were adopted. If modification was done then it should be indicated that what modification done in the standard was done.
Ans: Many thanks for the suggestion. We have revised and added references at lines 76, 82, and 108.

* Source of Bacterial cultures and C. elegans should be mentioned clearly.
Ans: Many thanks for the suggestion. We have revised and added description at lines 76-258.

REPRESENTATIVE RESULTS:
The methods described above offer a convenient way to differentiate virulence diversity among and within Aeromonas species. In addition, this is also a reliable model by which to study the interaction between a pathogen and host.

Reviewer #2:

Manuscript Summary:
This manuscript describes the use of C. elegans as a host to assess virulence determinants for four different species of Aeromonas known to cause clinical infections. Understanding the mechanisms of virulence and toxicity that are responsible for Aeromonas pathogenicity will be helpful for developing new strategies for combatting infection. This new C. elegans - Aeromonas infection model should make it simpler to evaluate host survival and tissue pathology. I think that the procedure is appropriate for publication in JoVE.

Major Concerns:
None

Minor Concerns:

1) Add more information on C. elegans as a host for various kinds of bacterial and fungal infections.
Ans: Many thanks for the suggestion. We have revised and added description at lines 64-67.

2) Define all abbreviations (e.g., ddwater). JoVE is not always read by specialists (e.g., some high school teachers use JoVE protocols for their biology labs).
Ans: Many thanks for the suggestion. We have revised to spell out each abbreviation the first time it is used. (lines 39-40; 53-54)

3) How do you seed ENGM with OP50? What is the appropriate amount of bacteria?
Ans: Many thanks for the suggestion. We have revised and added the description of seeding step. (lines 152-162)

4) Line 133 (step 3.1). Here and elsewhere (e.g. 4.1), what "respectively" refers to? Looks like all of the bacteria were cultured in LB.
Ans: Yes. All the bacteria are cultured separately.

5) Line 134 (step 3.4). How do you transfer worms? Picking? Washing off and dropping?
Ans: Many thanks for the suggestion. We have revised and added the description of picking to transfer worms. (lines 186-187)

6) Line 141 (step 3.6) Is the reason for the transfer to avoid progeny of non-sterile N2 worms that will interfere with the assay outcome? What happens if sterile worms (e.g. fer-15;fem-1, glp-1, glp-4, or FuDR are used?)
Ans: Yes. Transfer worms every day can avoid the effect of progeny. Besides, transfer every day can make sure the worms are fed with fresh-prepared bacteria. If using sterile worms, we still suggest to transfer worms every day. (lines 189-192)

7) Line 4.10. Given that assay is run for up to 72h, there is likely some egg-laying happening. Being in liquid condition strongly enhances internal

hatching, bagging and death of the mother (e.g. PMID: 10545455). Van Voorhie in his manuscript, entitled "The influence of metabolic rate on longevity in the nematode Caenorhabditis elegans" states that "In liquid as compared to solid medium, C. elegans grows more slowly, has an altered morphology, cannot mate, has reduced fertility and adult worms are subject to up to 90% mortality rates from internal egg hatching (Braeckman et al., 2000; Mitchell et al., 1979; Gandhi et al., 1980)." Was this artifact taken into account in this assay?

Ans: No. The “internal egg hatching” phenotype does not happen because of the use of FudR in the S medium in the *C. elegans* liquid toxicity assay. (lines 108-129)