

Video Article

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

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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.

Video Link

The video component of this article can be found at <https://www.jove.com/video/58768/>

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*^{2,3,4,5}. 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 infection⁶. 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*^{6,7,8}. 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 fungus¹⁰ and enterohaemorrhagic *Escherichia coli* O157:H7¹¹. 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

NOTE: See Table 1 for solution preparation.

1. To prepare M9 medium¹², dissolve 1.5 g of KH₂PO₄, 5.66 g of Na₂HPO₄, 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 MgSO₄ before first use.
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 CaCl₂, 1 mL of 1 M MgSO₄, 1 mL of 5% cholesterol in ethanol, and 25 mL of phosphate buffer. Swirl to mix well.
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.
4. To prepare enriched NGM (ENG), 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 CaCl₂, 1 mL of 1 M MgSO₄, 1 mL of 5% cholesterol in ethanol, and 25 mL of phosphate buffer. Swirl to mix well.
5. Pour about 10 mL ENG 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.
6. To prepare S medium¹², mix 40 mL S Basal, 0.4 mL 1 M potassium citrate, 0.4 mL trace metals solution, 0.12 mL of 1 M CaCl₂, 0.12 mL of 1 M MgSO₄, 40 µL of 5% cholesterol in ethanol and 1 mL of 8 mM FudR before use.

2. Synchronization of *C. elegans*^{6,9,12}

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. 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.
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.
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.
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.
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.
7. Seed at most 10,000 incubated L1 stage worms with a pipet on a 9 cm ENG plate spread with *Escherichia coli* OP50.
 1. In this step, spread 0.5 mL overnight cultured *E. coli* OP50 with Luria-Bertani (LB) broth on the 9 cm ENG 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. Cool to room temperature before seeding L1 worms. Seed at most 10,000 incubated L1 stage worms on the ENG plate with *E. coli* OP50.
8. 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 ENG medium. A worm has a white dot in half-moon shape at the middle of body side at the L4 stage.
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}

1. On the day of seeding L1 worms on the ENG 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 *A. dhakensis* isolate. *A. hydrophila* A2-066, *A. veronii* A2-007, and *A. caviae* A2-9307121 are representatively clinical isolates from National Cheng Kung University Hospital.
2. Measure the OD₆₀₀ absorbance of bacterial broth and adjust the bacterial broth to OD₆₀₀ = 2.0 with LB broth.
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.
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.
5. Incubate the NGM plates at 20 °C until the assay is finished.
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).
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.

1. The day after seeding the L1 worms on the ENG 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 *A. dhakensis* isolate. *A. hydrophila* A2-066, *A. veronii* A2-007, and *A. caviae* A2-9307121 are representatively clinical isolates from National Cheng Kung University Hospital.

2. Measure the OD₆₀₀ absorbance of the bacteria broth.
3. Centrifuge the bacterial broth at 3,500 × *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. Wash off the L4 worms on the ENGM plate with *E. coli* OP50 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.
5. Incubate the 96-well plate on a shaker at 200 rpm at 25 °C.
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%.
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.

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 *A. dhakensis* isolate. *A. hydrophila* A2-066, *A. veronii* A2-007, and *A. caviae* A2-9307121 are representatively clinical isolates from National Cheng Kung University Hospital.
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.
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.
4. Take muscle images using a fluorescence microscope.
 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.
 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. 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

1. Perform all experiments a minimum of three times independently.
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.
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* infection⁷.

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.

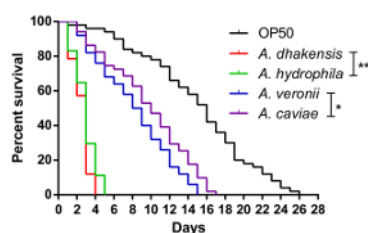


Figure 1: The survival curves of *C. elegans*. The survival assay shows the diverse toxicities of different *Aeromonas* species to *C. elegans* (**: $P < 0.01$; *: $P < 0.05$). [Please click here to view a larger version of this figure.](#)

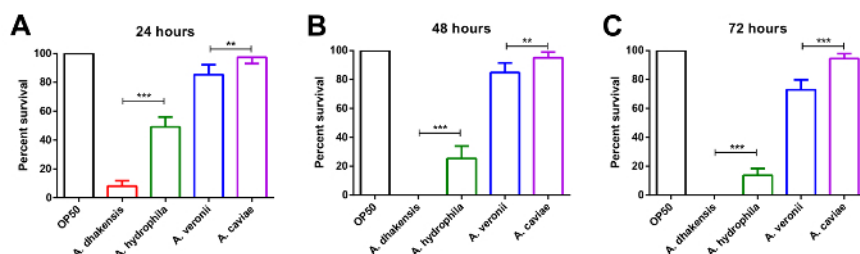


Figure 2: The survival rate of *C. elegans* from the liquid toxicity assay. The liquid toxicity assay shows the different toxicities of various *Aeromonas* species to *C. elegans*. At (A) 24 h, (B) 48 h, and (C) 72 h post *Aeromonas* infection, *C. elegans* infected with *A. dhakensis* has the lowest 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). [Please click here to view a larger version of this figure.](#)

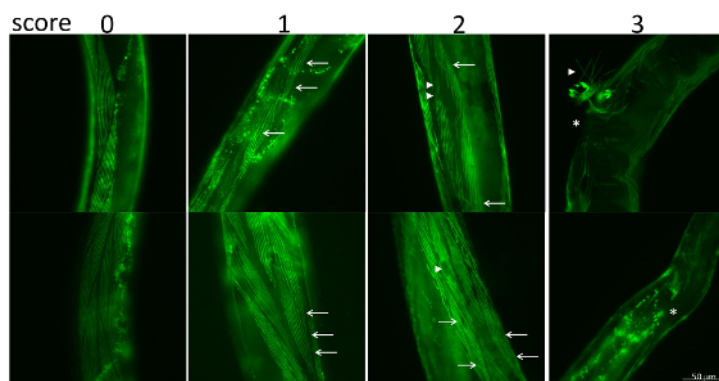


Figure 3: The corresponding scores for muscle necrosis in *C. elegans* induced by *Aeromonas* infection. The criteria for muscle necrosis and corresponding scores are as follows: 3 points for missing muscle fibers; 2 points for ruptured or broken muscle fibers; 1 point for bent muscle fibers; 0 points for healthy muscle fibers. Bent muscle fibers (arrows), ruptured muscle fibers (arrowheads), and missing muscle fibers (star) are indicated. [Please click here to view a larger version of this figure.](#)

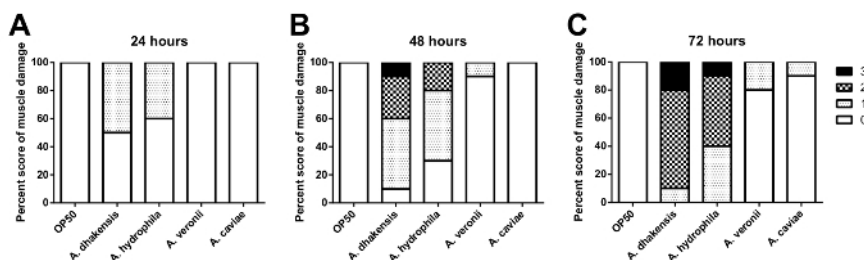


Figure 4: The *C. elegans* muscle necrosis scores. The muscle necrosis scores show the various toxicities of different *Aeromonas* species to *C. elegans*. At (A) 24 h, (B) 48 h, and (C) 72 h post *Aeromonas* infection, the *C. elegans* muscle damage is the severest from infection with *A. dhakensis*. [Please click here to view a larger version of this figure.](#)

Solution	Preparation
Phosphate Buffer	Dissolve 119.35 g of KH ₂ PO ₄ and 21.43 g of K ₂ HPO ₄ , 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 ₂ PO ₄ , and 1 g K ₂ HPO 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 ₂ O and 58.7 g tri-potassium citrate•H ₂ O 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 ₂ O, 0.2 g MnCl ₂ •4 H ₂ O, 0.29 g ZnSO ₄ •7 H ₂ O, 0.025 g CuSO ₄ •5 H ₂ O 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.

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 humans⁶. 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 observation⁷. 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 assays¹⁴. In our research, we also found there was discrepancy existing between these two assays⁹. 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.

Disclosures

The authors have nothing to disclose.

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