Video Article

A Protocol to Infect *Caenorhabditis elegans* with *Salmonella typhimurium*

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Abstract

In the last decade, *C. elegans* has emerged as an invertebrate organism to study interactions between hosts and pathogens, including the host defense against gram-negative bacterium *Salmonella typhimurium*. *Salmonella* establishes persistent infection in the intestine of *C. elegans* and results in early death of infected animals. A number of immunity mechanisms have been identified in *C. elegans* to defend against *Salmonella* infections. Autophagy, an evolutionarily conserved lysosomal degradation pathway, has been shown to limit the *Salmonella* replication in *C. elegans* and in mammals. Here, a protocol is described to infect *C. elegans* with *Salmonella typhimurium*, in which the worms are exposed to *Salmonella* for a limited time, similar to *Salmonella* infection in humans. *Salmonella* infection significantly shortens the lifespan of *C. elegans*. Using the essential autophagy gene *bec-1* as an example, we combined this infection method with *C. elegans* RNAi feeding approach and showed this protocol can be used to examine the function of *C. elegans* host genes in defense against *Salmonella* infection. Since *C. elegans* whole genome RNAi libraries are available, this protocol makes it possible to comprehensively screen for *C. elegans* genes that protect against *Salmonella* and other intestinal pathogens using genome-wide RNAi libraries.

Video Link

The video component of this article can be found at https://www.jove.com/video/51703/

Introduction

The free-living soil nematode *Caenorhabditis elegans* is a simple and genetically tractable model organism used to study many biological questions. *C. elegans* dominantly exists as self-fertilizing hermaphrodites. Males are spontaneously generated by non-disjunction of the X chromosome during gametogenesis[1,2]. In the presence of abundant food, *C. elegans* continuously develop through four larval stages to adult. Temperature also influences *C. elegans* development; faster development is observed at higher temperatures. In the laboratory, *C. elegans* is cultured at a standard temperature of 20 °C on agar plates with seeded bacterium *Escherichia coli* (strain OP50) as food[1,2].

In the last decade, *C. elegans* has emerged as an invertebrate organism to study host-pathogen interactions[3-5]. In nature, *C. elegans* eats bacteria as its nutrient source[1,2]. Its normal bacterial laboratory food, OP50, can be easily substituted with other pathogens to examine the interactions between *C. elegans* and any chosen pathogen. Under these conditions, the intestine is the primary site of the infection. Indeed, a wide range of bacterial pathogens has been shown to lethally infect *C. elegans*[3-5].

The gram-negative bacterium *Salmonella* is a gastrointestinal pathogen that causes human food-borne illness worldwide[6,7]. *C. elegans* is a good model host for *Salmonella typhimurium* as this bacterium replicates and exhibits persistent intestinal infections[8-10]. *C. elegans* has been used to identify both novel and previously known *Salmonella* virulence factors[11]. Interestingly, the *C. elegans* immune system successfully limits *Salmonella* replication. It has been reported previously that inhibition of autophagy genes renders increased *Salmonella* replication in *C. elegans*, resulting in early death of infected worms[10]. Macroautophagy (herein referred to as autophagy) is a dynamic process involving the rearrangement of subcellular membranes to sequester cytoplasm and organelles for delivery to the lysosome for degradation[12]. Autophagy has been reported to limit the *Salmonella* replication in *C. elegans* and in mammals[10,13].

The *C. elegans* genome was the first multicellular eukaryotic genome sequenced; it is responsive to RNAi treatment[14-16]. Moreover, RNAi can be administrated effectively by subjecting worms to ingest bacteria containing the double-stranded RNA of the target gene, known as RNAi feeding[16,17]. Whole genome RNAi feeding libraries have been generated for genome-wide RNAi screening[16,18]. Herein, a *Salmonella* infection protocol is coupled with RNAi feeding to allow testing *C. elegans* genes of interest for their ability to protect against *Salmonella* infection.

Protocol

1. XLD (Xylose Lysine Desoxycholate) Agar Plates

XLD agar is a selective growth medium for *Salmonella,* which appears as black colonies on XLD agar plates. However, if there are no concerns of contamination, a regular LB plate can be substituted.

1. Weigh out 5.5 g XLD agar and resuspend in 5 ml deionized water.
2. Mix thoroughly until all agar is wet. Add 95 ml deionized water until all lumps are gone and the medium is completely resuspended.
3. Boil the medium to dissolve completely (do not autoclave).
4. Cool the medium at room temperature to 50 °C.
5. Pour 25 ml agar in each 95 x 15 mm (diameter x height) plate (plates sealed with Parafilm can be stored at 4 °C for up to 1 month).

2. Nematode Growth Medium (NGM) RNAi Feeding Plates

Preparation of *C. elegans* NGM plates has been described previously[19]. Here a procedure is briefly described to add the antibiotic ampicillin and the RNAi chemical inducer isopropyl β-D-1-thiogalactopyranoside (IPTG) into the NGM media to make the RNAi feeding plates.

1. Dissolve 3 g NaCl and 2.5 g Bacto peptone in 1 L deionized water.
2. Add 17 g Bacto agar into the media.
3. Autoclave the media for 45 min and cool the media to 50 °C in a water bath.
4. Add the following solutions: 1 ml cholesterol (5 mg/ml in 95% ethanol), 1 ml 1 M $CaCl_2$, 1 ml 1 M $MgSO_4$, and 25 ml 1 M potassium phosphate buffer (pH 6.0). Mix well.
5. Add 1 ml 1 M IPTG and 500 µl ampicillin (100 mg/ml in sterile water).
6. Mix the solution well and pour into 60 x 15 mm (diameter x height) petri plates using a Pipet Aid and 25 ml serological pipette following sterile procedures. Fill each plate with 12 ml agar. Plates can be stored at 4 °C for up to 1 month.

3. Prepare RNAi-treated Animals for Infection

The essential autophagy gene *bec-1* is used as an example to examine the function of a host gene in defense against *Salmonella* infection. The experimental procedures are illustrated in **Figure 1** and **Table 1**. The protocol for preparing RNAi-treated animals for infection follows, with the day of each experimental step given in parentheses.

1. Inoculate *bec-1* RNAi feeding and control empty vector L4440 RNAi feeding bacteria by placing a flake of -80 °C frozen bacteria into 2 ml LB medium supplemented with 100 mg/ml ampicillin (**Day 1**). Repeat this step once a week during the entire experiment to have fresh RNAi bacteria. Store the culture in the 4 °C refrigerator when not used.
2. Seed 100 ml of overnight RNAi bacterial culture on RNAi plates. Prepare three *bec-1* RNAi and three control empty vector RNAi plates. Incubate the plates at 37 °C overnight (**Day 2**).
3. Remove the RNAi plates from the 37 °C incubator and allow them to cool down to room temperature on the bench. Pick up well-fed L4 wild type N2 hermaphrodites and transfer them to *bec-1* RNAi and control empty vector RNAi plates. Place three worms per plate, on triplicate plates. On the same day, prepare RNAi plates as described in step 3.2 (**Day 3**).
4. Incubate the RNAi plates with worms in the 20 °C incubator for 36-40 hr and transfer worms to fresh corresponding RNAi plates prepared in step 3.3. After worms are transferred, incubate the plates in the 20 °C incubator for 64 hr (**Day 4**).

4. Prepare *Salmonella* for Infection

1. Streak *Salmonella* -80 °C frozen stock on 1 XLD agar plate and incubate the plate at 37 °C overnight (**Day 5**).
2. Pick a well-isolated black *Salmonella* colony and inoculate it in 2 ml LB medium at 37 °C with shaking overnight (**Day 6**).
3. Seed 80 ml *Salmonella* overnight culture on 1 *C. elegans* 60 x 15 mm (diameter x height) NGM agar plate and prepare 6 plates in total. Incubate the plates at room temperature for 6 hr. The bacterial culture should dry and form a lawn on the plate (**Day 7**).

5. Infect RNAi-treated Worms with *Salmonella*

1. Transfer *bec-1* RNAi-treated and control empty vector RNAi-treated L4 N2 hermaphrodites (progeny of worms set up in Step 3) to *Salmonella* plates. Place 40 worms per plate on 3 plates for each group. Incubate the worm plates at 20 °C for 48 hr (**Day 7**).
2. Prepare one set of fresh RNAi plates as described in steps 3.1 and 3.2 (**Day 7** and **Day 8**).
3. After 48 hr infection, transfer *Salmonella*-infected worms to the corresponding RNAi plates prepared in step 5.2 and incubate at 20 °C (**Day 9**).

6. Survival Assay

1. Score the survival of worms daily and transfer worms to fresh corresponding RNAi plates during the egg-laying time. Prepare a set of fresh RNAi plates prior to each worm transfer as described in steps 3.1 and 3.2. Touch the worm body (head, middle part and tail) gently with an end-flattened platinum wire. A worm is scored as dead if no movement of the worm body is observed.

2. Score the survival of worms daily or every other day, and transfer worms to fresh corresponding RNAi plates twice a week after worms stop laying eggs.
3. After all worms die, pool the survival data from triplicate plates as one data set. Input the survival data of each group into appropriate statistical software such as GraphPad Prism to generate survival curves and to perform Kaplan-Meier survival analysis. The entire experiment is repeated at least once to confirm the conclusion.

Representative Results

At 20 °C, the median lifespan of wild type N2 worms is 17 days (**Figure 2A** and **Table 2**). *Salmonella* infection significantly decreases the median lifespan of N2 worms to 10.5 days (p = 0.0002, log-rank test) (**Figure 2A**).

If a *C. elegans* gene plays an important role in defense against *Salmonella* infection, it is predicted that its inhibition will impart susceptibility to *Salmonella* infection. In fact, compared to *Salmonella*-infected control RNAi-treated N2 animals, the median lifespan of *Salmonella*-infected *bec-1* RNAi-treated N2 worms is decreased from 10.5 days to 9 days (p < 0.0001, log-rank test) (**Figure 2B** and **Table 2**). The maximum lifespan is dramatically shortened by 14 days (from 24 days to 10 days, **Figure 2B** and **Table 2**). Moreover, the *bec-1* RNAi has no obvious effect on the lifespan of N2 worms that are not infected by *Salmonella* (p = 0.2593, log-rank test) (**Figure 2C** and **Table 2**), indicating that *Salmonella* infection, not *bec-1* RNAi treatment, decreases the lifespan of *Salmonella*-infected *bec-1* RNAi-treated worms. Also, *bec-1* is an essential gene in *C. elegans* defense against *Salmonella* infection.

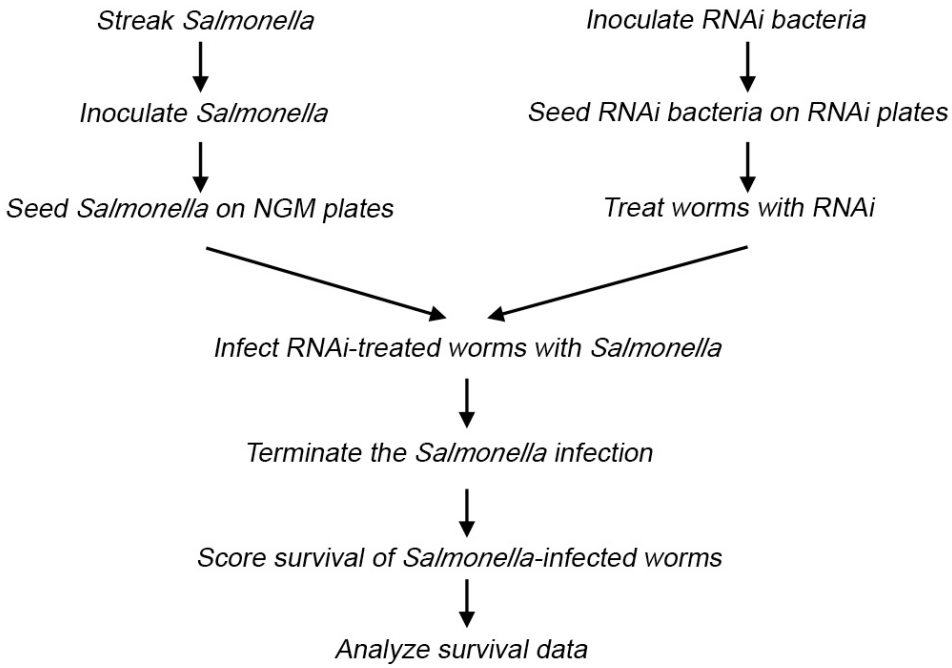

Figure 1. Flow chart of the experimental procedures.

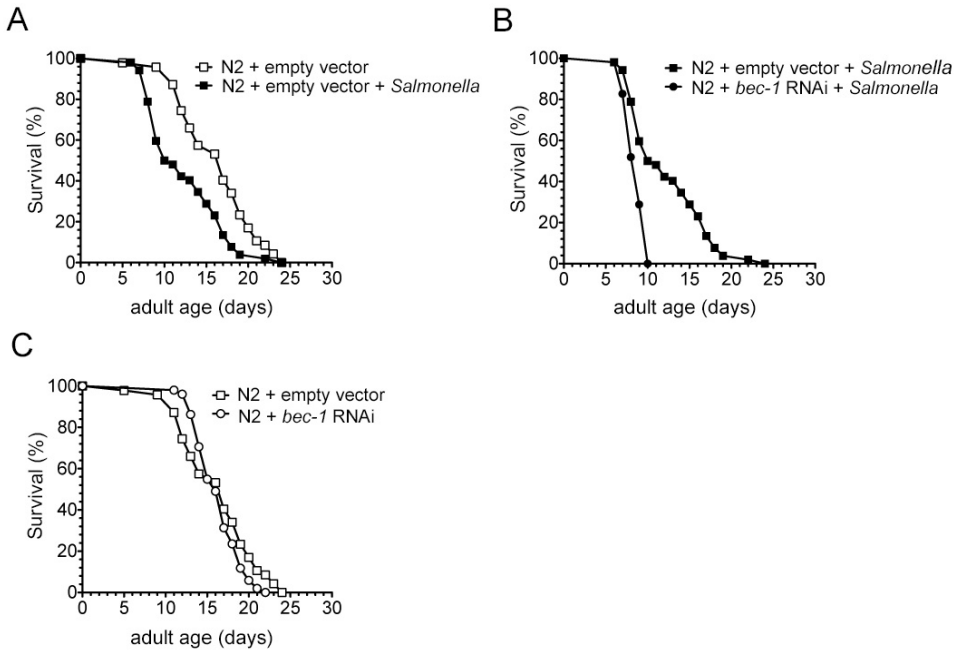

Figure 2. Inhibition of *bec-1* gene by RNAi confers susceptibility to *Salmonella* infection in *C. elegans*. A-C) Survival curves of wild type N2 animals treated with either control empty vector RNAi or *bec-1* gene RNAi following a 2 day exposure to *Salmonella typhimurium* or nonpathogenic *Escherichia coli* at 20 °C. Please click here to view a larger version of this figure.

Time		Experiments
Week 1		
Tuesday	5:00pm	Inoculate RNAi bacteria
Wednesday	9:00am	Remove RNAi bacterial culture from the incubator
	5:00pm	Seed RNAi bacteria on RNAi plates
Thursday	9:00am	Put L4 hermaphrodites on RNAi bacteria plates
	5:00pm	Seed RNAi bacteria on RNAi plates
Friday	9:00am	Remove RNAi bacterial plates from the incubator
	5:00pm	Transfer worms to fresh RNAi bacterial plates
Saturday	5:00pm	Streak *Salmonella* on XLD plate
Sunday	9:00am	Remove *Salmonella* XLD plate from the incubator
	5:00pm	1.) Seed RNAi bacteria on RNAi plates 2.) Inoculate *Salmonella*
Week 2		
Monday	9:00am	1.) Seed *Salmonella* on NGM plates 2.) Pick up control L4 hermaphrodites
	3:00pm	Transfer RNAi-treated L4 hermaphrodites to *Salmonella* plates to initiate the infection
	5:00pm	Seed RNAi bacteria on RNAi plates
Tuesday	9:00am	Transfer control worms to fresh RNAi bacterial plates
	5:00pm	Seed RNAi bacteria on RNAi plates
Wednesday	9:00am	Transfer control worms to fresh RNAi bacterial plates
	3:00pm	Transfer *Salmonella*-infected worms to RNAi bacterial plates to stop the infection

Table 1. *Salmonella* infection protocol timeframe.

Strain	Median (days)	Maximum (days)	N[1]	P value[2]
No *Salmonella* infection				
N2 + empty vector	17	24	47	
N2 + *bec-1* RNAi	16	22	51	= 0.2593
Salmonella infected				
N2 + empty vector	10.5	24	52	
N2 + *bec-1* RNAi	9	10	52	< 0.0001

[1]N = population size. [2]P values (log-rank test) for median life span of *bec-1* RNAi-treated worms compared to empty vector RNAi-treated control animals.

Table 2. Statistical analysis of lifespan data in Figure 2.

Discussion

C. elegans is a simple genetic model organism that eats bacteria as its nutrient source. Thus, it is easy to substitute its normal bacterial food with an intestinal pathogen to investigate the interactions between *C. elegans* and the chosen pathogen. Herein a protocol is described to combine *Salmonella* infection and *C. elegans* RNAi feeding treatment to examine the role of host genes in defense against *Salmonella* infection. Previous infection protocols expose *C. elegans* worms to pathogenic bacteria including *Salmonella* during their lifetime[20]. In the present

protocol, *Salmonella* infects the worms in a two-day period. After that, the worms are no longer exposed to *Salmonella*. This *Salmonella* infection significantly decreases the lifespan of *C. elegans* wild type animals. Thus, invaded *Salmonella* replicate inside worms and kill the animals[10]. This short period of exposure to *Salmonella* mimics human *Salmonella* infection, which should help to uncover useful information to understand human food-borne illness caused by *Salmonella* infection. Moreover, this protocol combines the RNAi feeding treatment with *Salmonella* infection, making it possible to test any candidate genes that might be involved in host defense against *Salmonella* infection, especially when the genetic mutants are not available. The autophagy gene *bec-1* known to be involved in defense against *Salmonella* infection is used as an example in the present study. *bec-1* mutations are lethal[21], which prevents testing its role in defense against *Salmonella* infection in adults. Using the current protocol, it was shown that inhibition of *bec-1* by RNAi yields susceptibility to *Salmonella* infection in *C. elegans*. In the present study, N2 wild type worms fed with L4440 bacteria have a similar lifespan as animals fed with OP50. The animals start to die around day 6 and the maximum lifespan is around four weeks. N2 worms infected by *Salmonella* live a few days shorter. By contrast, *bec-1* RNAi-treated worms infected with *Salmonella* die about two times faster than control animals although the beginning date for animals to die in both groups is only a few days apart (**Figure 2**). The entire experiment lasts about 1 month.

In this protocol, the coordination of RNAi feeding and *Salmonella* preparation is required so that RNAi-treated L4 stage hermaphrodites are subjected to *Salmonella* infection. A typical timeframe of the protocol used in the authors' lab is described in **Table 1**. RNAi feeding bacteria are prepared weekly and the bacterial culture is stored at 4 °C when not used. Of note, on day 7, the infection will start 6 hr after *Salmonella* overnight cultures are placed on NGM plates. During this 6 hr period, RNAi-treated L4 N2 hermaphrodites are picked from corresponding *bec-1* and control empty vector RNAi plates. The non-infected worms are used as controls to ascertain if *Salmonella* infection shortens the lifespan of infected worms and if *bec-1* RNAi treatment has any influence on worm lifespan.

Currently, survival of *C. elegans* after infection is commonly used to measure the pathogen virulence[3-5]. However, RNAi inhibition of certain *C. elegans* genes result in decreased lifespan. Therefore, one should be careful when interpreting the data. When this situation is encountered, different concentrations of the RNAi inducer, IPTG, can be tested to identify the desired concentration that only influences the host response to the pathogen infections without impact on the animal lifespan. As reported previously[10], the 1 nM IPTG concentration was successfully used to examine the role of autophagy in IGF signaling-mediated pathogen resistance in *C. elegans*.

Given that the *C. elegans* genome has been sequenced and *C. elegans* RNAi feeding libraries have been generated[16,18], it is possible to revise the described protocol to perform a genome-wide RNAi screening to identify all host genes involved in defense against *Salmonella* infection. For example, instead of using the median survival to measure the virulence of *Salmonella*, the maximum survival is used. Moreover, infected worms can be sterilized by supplementing plates with fluorodeoxyuridine, a DNA synthesis inhibitor. Thus, transferring of infected worms is unnecessary as long as food is supplied to prevent worms from starvation. These modifications will reduce the workload for a high-throughput screen tremendously. This type of large-scale study may shed light on understanding the human response to *Salmonella* infection as many biological pathways in *C. elegans* are evolutionarily conserved in humans.

Disclosures

The authors declare that they have no competing financial interests.

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