Scientific Research Publishing

Advances in Microbiology, 2017, 7, 689-695
http://www.scirp.org/journal/aim
ISSN Online: 2165-3410
ISSN Print: 2165-3402

# Age Does Not Affect the Induction of Mortality by the Foodborne Pathogen *Salmonella enterica* in *Caenorhabditis elegans*

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**How to cite this paper:** Burns, D.M.F., Harper, J.M. and Lynne, A.M. (2017) Age Does Not Affect the Induction of Mortality by the Foodborne Pathogen *Salmonella enterica* in *Caenorhabditis elegans*. *Advances in Microbiology*, **7**, 689-695.
https://doi.org/10.4236/aim.2017.710054

**Received:** August 23, 2017
**Accepted:** October 28, 2017
**Published:** October 31, 2017



## Abstract

*Salmonella* is a common cause of foodborne illness within the United States with the severity of the infection being a factor of both the age and overall health of the infected individual. The nematode worm *Caenorhabditis elegans* has proven to be a useful model to study infection dynamics of pathogenic bacteria, including *Salmonella enterica,* and its short lifespan makes it a powerful model system to assess the effect of organismal age on infection severity. In this study, we infected *C. elegans* with each of 6 serovars of *S. enterica* at 1, 3 or 5 days of worm age and monitored their survival. Worms infected with *E. coli* OP50 were used as a control. Infection with *S. enterica* resulted in a significant reduction in mean longevity relative to OP50 ($p < 0.05$); however, there was no significant effect of age on mean survival time regardless of the strain of bacteria used.

## Keywords

*Salmonella enterica*, *Caenorhabditis elegans*, Aging, Infection

## 1. Introduction

*Salmonella enterica* is a significant cause of foodborne disease, leading to thousands of hospitalizations, and hundreds of deaths, annually [1]. Importantly, infection severity within vertebrates is a function of both age and immunocompetence [2] [3]. The genus Salmonella is divided into two species: *Salmonella enterica* and *Salmonella bongori*. *S. enterica* is further divided into six subspecies. There are more than 2500 serovars of *Salmonella* that are classified based on differences in the LPS (O-antigen) and the flagellar (H-antigen) surface antigens, as outlined by the Kauffmann-White scheme with around 99% of these serovars

belonging to the species *S. enterica* [4] [5]. Furthermore, of the serovars that are pathogenic to humans, approximately 99% of these are *S. enterica* [6]. These different serovars present a variety of host ranges, some of which are narrow and highly specific to certain species (e.g., Paratyphi and Typhi); while other serovars have broad host ranges, but usually a preferred host. Serovars with broad host ranges can typically infect multiple species and are referred to as being host-adapted (e.g., Typhimurium). In addition to varying host preferences, *Salmonella* serovars differ in disease outcomes within a specific host. For example, while serovar Typhimurium causes enteritis in humans, it causes salmonellosis in cattle and typhoid-like fever in mice. The difference in host range and disease outcome among the serovars is most likely due to the loss of gene function through deletions and point mutations, as well as acquisition of new virulence genes by horizontal gene transfer events [7].

*Caenorbabditis elegans* is a soil-dwelling nematode that feeds on bacteria. Its life cycle is comprised of an embryonic stage, followed by four larval stages (L1 - L4), and finally adulthood where the worm is capable of laying eggs. Several features of the worm render it a useful model organism [8] [9] [10] [11]. First, the generation time and lifespan is short: 3 days and 3 weeks, respectively. Moreover, its growth rate can be manipulated by varying the rearing temperature with worms growing faster at warmer temperatures (*i.e.* 20˚C - 25˚C). Second, the worms can be fed bacterial lawns, so the cost of feeding and maintaining the worms is low, and the worms can be frozen and stored indefinitely. Third, the adult worm grows to a length of about 1 - 1.5 mm allowing for easy propagation with minimal space needs. Fourth, they undergo self-fertilization which reduces time, and otherwise spend crossing sexes to propagate the worms. Furthermore, hermaphrodites can be crossed with males to produce large brood sizes [8]. Fifth, the worm has a transparent body that allows for visualization of cells by fluorescence microscopy. Sixth, the genome is small (~100 Mb) and is completely sequenced.

With regard to infection studies themselves, *C. elegans* is a simple, yet powerful, tool for the investigation of the innate immune function. First, *C. elegans* naturally feeds on bacteria providing a simple and effective delivery system. An added benefit of this feeding lifestyle is that the activity of individual genes can readily be knocked-down by RNAi through bacterial feeding. Second, the transparent body allows for the direct visualization of anatomical features in addition to more advanced methods such as immunohistochemistry of intact worms. Third, several virulence genes essential for mammalian infection are also essential for pathogenicity in the worm [12], and host genes involved in pathogen defenses are also similar between *C. elegans* and mammals [13]. Finally, the innate immune system is highly conserved between vertebrates and invertebrates [14].

During the past 15 years, there has been a dramatic upswing in the number of studies using *C. elegans* as a model of innate immune function, especially in the context of human bacterial disease. Importantly, despite the fact that individual worms are unlikely to encounter human bacterial pathogens such as *Pseudo-*

*monas, Staphylococcus* or *Salmonella* under normal conditions, each is capable of establishing an infection in *C. elegans*. Consequently, worms maintained in the presence of these pathogens die more rapidly than worms maintained on the non-pathogenic OP50 strain of *E. coli*. Typically, worms die subsequent to the establishment of a persistent infection in the gastrointestinal tract; although in some cases killing is a consequence of endotoxin activity. Not surprisingly, there is marked variation in the degree of pathogenicity among bacterial species with the time it takes to kill 50% of a cohort (TD50) differing by as much as 3-fold or more.

The *Salmonella C. elegans* infection model began when two independent groups [15] [16] showed that *S. enterica* serovar Typhimurium establishes a persistent infection within the gut of *C. elegans*. This causes the intestine to become distended and leads to an accelerated onset of mortality. Since then, many studies have examined both the bacterial virulence factors and the *C. elegans* host immune response that contribute to the pathogenesis of a *S. enterica* infection. For example, antimicrobial peptides (AMP) are a significant component of innate immune defenses among eukaryotes that evolved to combat diverse infectious agents, including bacteria, fungi, and viruses [17]. In *C. elegans* infected with *S. enterica*, Alegado and Tan [13] demonstrated that the AMPs ABF-2 and SPP-1 are differentially induced during an infection, with an absence of these peptides resulting in an increased bacterial load within the intestine. Similarly, Porter-Celhay and Blaser [18] demonstrated that strains of *C. elegans* defective in a wide range of immune regulatory factors suffer from shortened longevities coupled with increased bacterial loads.

While many studies have looked at the effect of bacterial infection on *C. elegans,* few studies have looked at the effect of age of *C. elegans* with regard to worm mortality during infection. The purpose of this study was to assess if host age influences the infection severity of different *S. enterica* serovars in *C. elegans.*

## 2. Materials and Methods

Each *S. enterica* serovar was obtained from Michael McClelland at the San Diego Institute of Biological Research including: Typhimurium LT2, Paratyphi A SARB 42, Heidelberg SGSC 4915, Enteritidis 125109, Saintpaul SC-B67, and Muenchen SARB 32. The *C. elegans* N2 stain was obtained from the *Caenorhabditis* Genetics Center (University of Minnesota) and was maintained on Nematode Growth Media (0.25% Tryptone, 0.3% NaCl, 1.5% Afar, 1 mM $CaCl_2$, 1 mM $MgSO_4$, 25 mM $KPO_4$, 5 μg/ml Cholesterol) seeded with *Escherichia coli* OP50. In the laboratory, *C. elegans* is typically maintained on lawns of *E. coli* OP50 [19]. This strain also served as the negative control strain for this study.

To generate known-age adult worms, cohorts of worms were age-synchronized using an established protocol generating a cohort of worms at the first (L1) larval stage [20]. These L1 worms were maintained as indicated above until adult-

hood, then incubated an additional 1 - 5 days on OP50 as needed. When they reached the age of either 1, 3 or 5 days the adults were transferred to plates containing lawns of the individual *S. enterica* serovars and incubated at 25˚C. In each case, 10 adult worms were transferred and their survival was monitored thereafter until all the individuals had died. In total, the survival of 2100 individual worms was monitored in each of 10 cohorts (*i.e.* n = 210 worms per cohort). The number of worms alive was assessed twice daily. Worms that failed to respond to prodding with a sterile platinum wire were scored as dead. Finally, adult worms were moved to fresh plates every two days to ensure only known age individuals were being monitored.

An initial screening of the survival data for each cohort (Cox proportional hazards regression) [21] indicated marked variation in the risk ratios among the individual cohorts for each age and strain (including the OP50 control). Consequently, a Kaplan-Meier estimate of the median survival time for each cohort (n = 10 per age*serovar combination) was used in the final statistical model. In particular, the mean of the median survival time for each cohort was used to construct a two-way ANOVA model with age and serovar as the independent variables. Tukey-Kramer post hoc tests were conducted as appropriate. All statistical analyses were conducted using NCSS 11.

## 3. Results

Figure 1 depicts the survival time for worms infected with each of the strains/serovars at each of the three ages. Consistent with previous studies, we found that worms maintained on *E. coli* OP50 lived significantly longer than those infected *S. enterica* [15] [16]. In addition, worms maintained on either the Saintpaul or Meunchen serovars were the shortest-lived when the data were pooled across age and were significantly shorter-lived than worms infected with Parathyphi A only. Neither of these serovars had previously been tested in the *C. elegans* model. Although older worms tended to die sooner after infection, there was no significant main effect of age on worm survival regardless of the strain/serovar used; the interaction term (age*strain/serovar) was also insignificant.

## 4. Discussion

The effect of worm age on infection outcome has been little studied. Previous studies have shown that many human pathogens can infect and kill *C. elegans* including *Pseudomonas, Staphylococci* and multiple serovars of *Salmonella* [13]. Similar to *S. enterica*, *Pseudomonas aeruginosa* is a gram-negative bacterium that can infect a wide range of hosts and whose course of infection mirrors that seen with *S. enterica* [22]. Notably, mortality within these models is dependent on bacterial accumulation within the gut and differs from other, toxin based models described in the literature [21]. Gram-positive bacteria, such as *Staphylococci aureus* cause mortality in a similar manner [23].

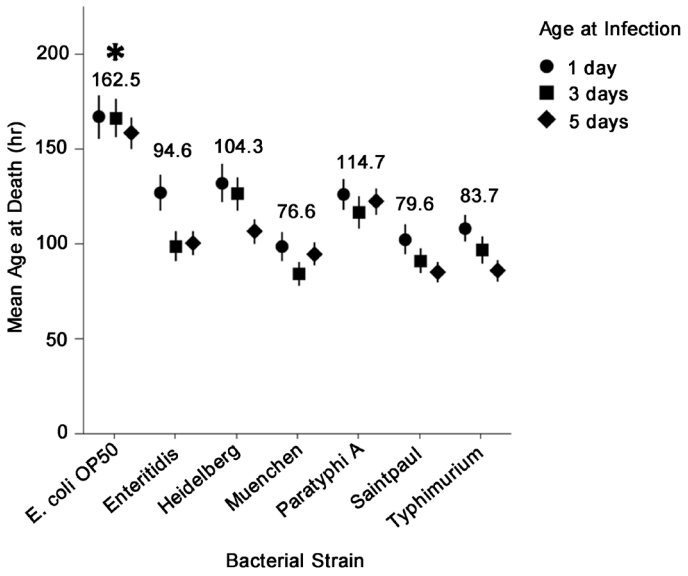


**Figure 1.** Mean (±SEM) survival of *C. elegans* at each of three adult ages (*i.e.* 1, 3 or 5 days old) maintained on lawns of different bacterial strains. Each point represents the mean time to death (in hours) of the median survival time for each of 10 individual cohorts per age and strain. Numbers above the points indicates the mean survival after infection pooled across age. *indicates a significant main effect of strain on survival time via two-way ANOVA ($p < 0.05$). See text for details.

In the case of *P. aeruginosa* PA14 and *Yersinia pseudotuberculosis* YP3, worms that were infected at older ages died more rapidly than younger worms, but whether this has to do with the specific species, or strain, of bacteria remains unknown [24]. The effect of worm age on bacterial induced mortality is limited. Specifically, Laws *et al.* [24] found that 7-day-old worms (approximately 80% of the LT50 of N2 worms maintained on OP50) infected with a variety of pathogens, including *S. enterica* serovar Typhimurium, died more rapidly than individuals infected at the late L4 larval stage. A more detailed analysis of the pathogens *Pseudomonas aeruginosa* strain PA14 and *Yersinia pseudotuberculosis* strain YP3 also showed differences in post-infection mortality, even in worms separated by as little as 1 - 2 days of age. Age-related increases in mortality post-infection has also been noted with the human opportunistic pathogen *Serratia marcesens* [25], in addition to a second study with *P. aeruginosa* [26] and another using *Legionella sp.* [27]. At least in the case of *P. aeruginosa* the increased mortality is associated with an increased degree of infection severity such that newly infected 9-day-old worms had a higher bacterial burden than did newly infected 6-day-old worms.

## 5. Conclusion

We found that *C. elegans* infected with *S. enterica* die more rapidly than worms maintained on *E. coli* OP50 and that the age of the worm at infection had no ef-

fect on survival time regardless of the bacterial strain/serovar used. However, the ages tested are only 10% - 50% of the LT50 of N2 worms maintained on OP50. The possibility remains that for bacterial pathogens, the short post-infection period leading up to mortality is a reflection of an overall decline in the health of the worms, and not a decline in immune function *per se*. To document a decline in immune function, it is necessary for future studies to quantify the degree of infection at individual ages.

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