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Caenorhabditis elegans-based analysis of *Salmonella enterica*

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Abstract: *Caenorhabditis elegans* (*C. elegans*) have been widely used as an infection model for mammalian related pathogens with promising results. The bacterial factors required for virulence in non-mammalian host *C. elegans* play a role in mammalian systems. Previous reported that *Salmonella* found in vegetable and poultry meat could be potential health hazards to human. This study evaluated the pathogenicity of various serovars of *Salmonella enterica* (*S. enterica*) that recovered from local indigenous vegetables and poultry meat using *C. elegans* as a simple host model. Almost all *S. enterica* isolates were capable of colonizing the intestine of *C. elegans*, causing a significant reduction in the survival of nematodes. The colonization of *Salmonella* in *C. elegans* revealed that the ability of *S. enterica* in killing *C. elegans* correlates with its accumulation in the intestine to achieve full pathogenicity. Using this model, the virulence mechanisms of opportunistic pathogenic *S. enterica* were found to be not only relevant for the interactions of the bacteria with *C. elegans* but also with mammalian hosts including humans. Hence, *C. elegans* model could provide valuable insight into preliminary factors from the host that contributes to the environmental bacterial pathogenesis scenario.

Keywords: *Caenorhabditis elegans, Salmonella enterica*, pathogenicity

Introduction

Host pathogen interaction is a dynamic process, in which the infecting organism expresses a variety of virulence determinant in order to overcome host defense mechanisms and to establish infection (Dhakal *et al*., 2006). A variety of model host organisms has been use to illustrate the multi-factorial nature of microbial pathogenicity. *S. enterica*, a Gram-negative bacterium causes significant morbidity and mortality worldwide (Kaufmann *et al*., 2001; Learn-Han *et al*., 2009). In human diseases, *Salmonella* serovars can be divided into three groups that cause distinctive clinical syndromes, which are enteric fever (typhoid fever caused by *S.* Typhi), gastroenteritis (caused by most *Salmonella* strains such as *S.* Typhimurium), and bacteremia (caused by *S.* Choleraesuis) (Kaufmann *et al*., 2001; Santos *et al*. 2001). Animal models such as mice and calf are frequently used to study the virulence mechanisms of *Salmonella* serovars that are important for two of these human disease syndromes, typhoid fever, and enteritis (Santos *et al*. 2001). However, there are cost and ethical restraints on the use of laboratory mammals (Joshua *et al*., 2003).

In general, all animal models can be problematic because they often untruly represent disease condition in humans. The uses of a diverse group of animal models offer best alternative of full understanding about bacterial molecular pathogenesis. Thus, there is a great demand in the development of simple animal models of infection for bacterial pathogens (Finlay, 1999; Jay *et al*., 2006). Recent reports suggested that the soil nematode *C. elegans* can be used as an infection model for diverse groups of bacterial pathogens, including those affects both animals and plants (Tan *et al*., 1999; Couilault and Ewbank, 2002,). Moreover, the mechanisms of invasion and

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host responses may be paralleled in mammalian cells (Joshua *et al*., 2003).

C. elegans is emerging as a facile and economical model host for the study of evolutionary conserved mechanism of microbial pathogenesis and innate immunity (Santander *et al*., 2003). The self fertilizing nematode *C. elegans* lives in the soil, where it feeds on microorganisms has been evaluated as a model organism for over three decades, owing to its many advantages, which include small size, short life cycle, powerful genetics, simple growth conditions, invariant developmental lineage, and also first multicellular organism whose genome was completely sequenced (Dhakal *et al*., 2006).

Microbial genes known to be important for full virulence in mammalian models have been shown to be similarly required for maximum pathogenicity in nematodes (Alegado *et al*., 2003). *C. elegans* has been used in mutation-based screening system to identify novel virulence-related microbial genes and immune-related host genes, many of which have been validated in mammalian models disease (Dhakal *et al*., 2006). According to Aballay *et al.* (2000) and Labrousses *et al.* (2000), *C. elegans* has recently been identified as a good model in studies regarding pathogenesis of gram negative bacteria, which is *Salmonella* Typhimurium, a bacterium that persistently infects the *C. elegans* intestine and finally kills the nematode. Furthermore, *S.* Enteritidis and *S.* Dublin have also been shown to kill *C. elegans* (Aballay *et al.*, 2000).

Our recent studies indicate that vegetables and poultry can be sources of pathogenic microbes (Chai *et al*., 2009; Tang *et al*., 2009; Jeyaletchumi *et al*., 2010, Tunung *et al*., 2010; Usha *et al*., 2010). Virulotyping of *Salmonella* species isolated from local indigenous vegetables and poultry portrayed vast virulence component that might become the source of foodborne illness outbreak in Malaysia (Chai-Hoon *et al*., 2009). This study aimed to investigate the pathogenicity of *S. enterica* that recovered from local indigenous vegetables and poultry meat using *C. elegans* as a simple host model. This simple and economic host model can be greatly facilitating the study of the genetic background to the *in vivo* virulence of *S. enterica* strains that potentially causes disease in human.

Materials and Methods

Bacterial and nematode strains

A total of eight *S. enterica* isolates (including two reference strains and six food sources) associated with four different serovars were analyzed in this study. *C. elegans* strains glp-4 was kindly provided by the *Caenorhabditis* Genetics Center, University of Minnesota, Minneapolis, MN which is temperature sensitive that inability to reproduce when incubated at 25°C, this is to ensure a constant number of nematodes during the assays. The nematodes were maintained and propagated on nematode growth medium (NGM) (US Biological, Massachusetts, USA) with standard techniques (Stiernagle, 1999), using *Escherichia coli* (*E. coli*) OP50 as the internationally established feed. Table 1 listed the selected *S. enterica isolates* and nematode strain tested in this study. All *Salmonella* isolates were grown in tryptic soy agar (TSA) (Merck KGaA, Darmstadt, Germany) and *E. coli* OP50 was grown in Luria-Bertani (LB) agar (Merck KGaA, Darmstadt, Germany).

Preparation of synchronize C. elegans

All nematodes age were synchronized by a bleaching procedure prior to the killing assay (Stiernagle, 1999; Ikeda *et al*., 2007). The surface of 3 plates which full with eggs, were washed by 1 ml sterile M9 buffer (Stiernagle, 1999) across the plate several times to loosen eggs that are stuck in bacterial lawn. The suspension was aseptically transferred into a sterile 15 ml centrifuge tube and add sterile M9 buffer to total 3.5 ml. The suspension was treated with 0.5 ml 5N NaOH (Merck KGaA, Darmstadt, Germany) and 1 ml 5% bleach, sodium hypochlorite (NaOC1), then gently mixed intermittently for 10 min to kill all life cycle forms of the worm except the eggs. After a 30 s spin at RCF 1300 x g to pellet released eggs, the supernatant was discarded to 0.1 ml, and eggs were washed twice with 5 ml sterile M9 buffer as described above. After a final centrifugation, the eggs and dead worms were resuspended in 100 µl sterile M9 buffer, then the suspension was placed on NGM agar plate with food strain *E. coli* OP50, and incubated at 25°C for 3 days until maturation. Killing assay was begun with 3 days old young adult nematodes. This procedure to ensure that all worms used in assay were the same age. Adult worms were used in assay to confirm that they will ingest pathogenic bacteria, and persistence in the gut of ingested pathogens.

C. elegans killing assay

Bacterial lawns used for killing assays were prepared by plating 10 µl of the early log phase culture at OD_{600nm} of 0.4 on modified NGM agar in 35-mm dishes. Plates were grown overnight at 37°C and cooled to room temperature before introducing the worms. In each assay, 30 young adults (3 days old) nematodes were transferred from synchronized worms culture to fresh lawn per plate, and each

Table 1. *S. enterica* isolates and nematode strains used in this study

Strain (Code)	Relevant Genotype	Sources
S. Agona (Ag 1)	Wild type isolate	Vegetable: Kesum
S. Agona (Ag 2)	Wild type isolate	Vegetable: Selom
S. Agona (Ag 3)	Wild type isolate	Poultry Meat
S. Weltevreden (We 1)	Wild type isolate	Vegetable: Kangkong
S. Weltevreden (We 2)	Wild type isolate	Vegetable: Kesum
S. Weltevreden (We 3)	Wild type isolate	Vegetable: Selom
S. Typhimurium (ATCC 14028)	References strain	American Type Culture Collection
S. Paratyphi A (ATCC 9150)	References strain	American Type Culture Collection
E. coli OP50	Uracil auxotrophy	*Caenorhabditis* Genetics Center
C. elegans	glp-4	*Caenorhabditis* Genetics Center

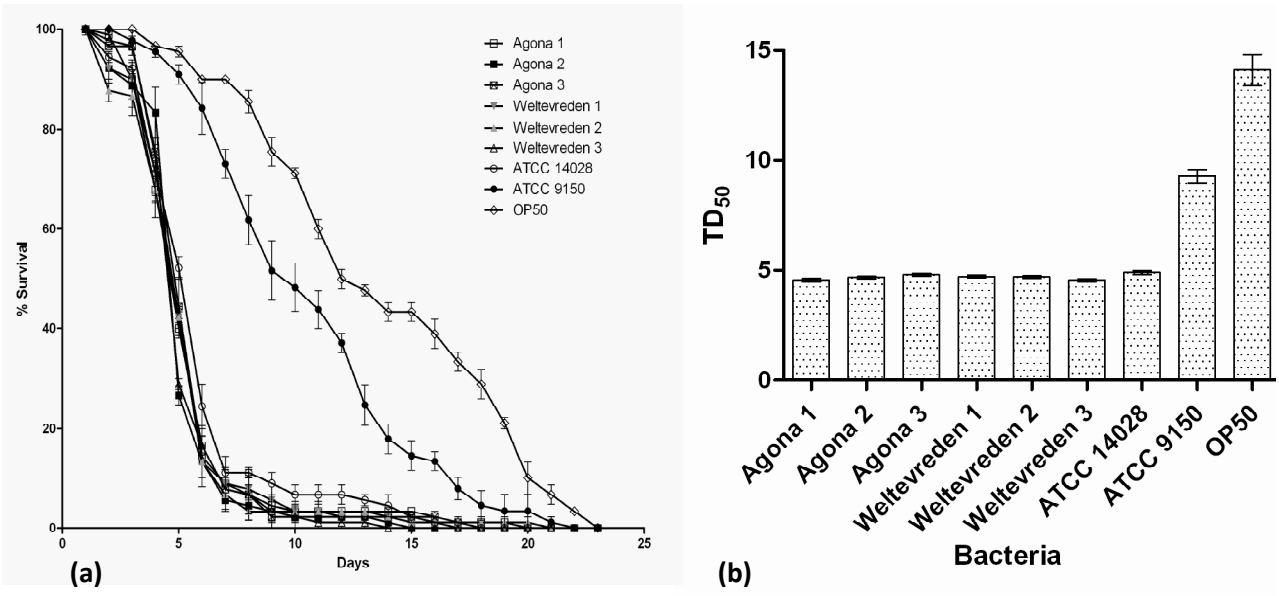

Figure 1. *C. elegans* killing by *S. enterica* (a) Survival rate of *C. elegans* on lawns of different *S. enterica* isolates and *E. coli* OP50 grown on NGM; (b) Time required for nematodes to die (TD50) after infected by different *S. enterica* isolates

assay was carry out in triplicate. The originally live worms were transferred to fresh lawns by every 2 days interval for first 6 days and incubated at 25°C. Worm mortality was scored over time every 24 h, and a worm was considered dead when it no longer responded to gentle touch by platinum wire. Worms that died as a result of getting stuck to the wall of the plate were excluded from analysis.

Bacterial accumulation assay

The numbers of *Salmonella* cells in the nematodes were determined according to the standard plate count assay method described by Garsin *et al* (2001) with slight modification. Three worms were picked (triplicate), and the surface bacterial were removed by washing the worms three times in 4 µl drops of sterile M9 buffer on NGM agar plates. Each nematode was placed in a 1.5 ml microtube containing 20 µl of sterile M9 buffer with 1% Triton X-100 (Merck KGaA, Darmstadt, Germany) and 1% saponin (Merck KGaA, Darmstadt, Germany), and subsequently mechanically disrupted using a microtube pestle. The volume was adjusted to 50 µl with sterile M9 buffer, and the number of *Salmonella* cells was determined by using xylose lysine deoxycholate (XLD) agar (Merck KGaA, Darmstadt, Germany). The treatment of infected nematodes ensured that only viable bacteria within the nematode gut were quantified in this assays.

Statistically analysis

For killing assay, nematode survival was plotted as a nonlinear regression curve with the GraphPad Prism, version 5 Software. Survival curves are considered significantly different from the control when $P < 0.05$. The time required for 50% of the nematodes to die (TD_{50}) was calculated using GraphPad Prism software of following equation: $Y = \text{Bottom} + [\text{Top} - \text{Bottom}]/[1 + 10(\log EC_{50} - X)\text{(Hill slope)}]$, where X is the logarithm of days, Y is the average of dead worms, EC_{50} is the mid point of the curve.

Results and Discussion

Previous study has shown that the *S. enterica* isolated from the poultry and indigenous vegetable was found to possess highly virulence determinant by multiplex PCR (Chai-Hoon *et al*., 2009). According to Burton *et al.* (2006), *C. elegans* prevent microbial colonization of the intestine by peristalsis, low pH, lytic enzymes and antimicrobial substance as in mammals. However, pathogenic bacteria are capable of proliferating and killing *C. elegans* using different mechanism. To determine whether the ability to kill *C. elegans* is a common property of *S. enterica* strains, the six representative food isolates and two reference strains were tested to kill *C. elegans*. The virulence of *S. enterica* isolates in the *C. elegans* host model was evaluated by measuring the survival of worms fed on pure cultures of these strains. In Figure 1, the result shows that different serovars have different mortality rate. All studied pathogenic *S. enterica* isolates kill *C. elegans* faster than *E. coli* OP50, which is the standard laboratory food. According to Couillault and Ewbank (2002), worms that survived on *E. coli* OP50 cells can be taken as a baseline for healthy growth on live bacteria. As shown in Figure 1(a), most of *Salmonella* which recovered from food sources were able to killed *C. elegans* exponentially at fourth day of course of assay. The pathogenicity of the *Salmonella* against *C. elegans* that reported by Aballay *et al* (2000), Ikeda *et al* (2007) and Labrousses *et al* (2000) were reconfirmed that a decreased life span of worm after ingested *Salmonella*.

The time required for 50% nematode to die (TD_{50}) was calculated from the survival data in Figure 1(b), which ranged from 4.6 to 9.3 days after ingesting various serovars of *S. enterica* compared to 14.1 days after ingesting the positive control strain *E. coli* OP 50. As shown in Figure 1(b), the TD_{50} when feeding on *S.* Paratyphi A (ATCC 9150) (TD_{50} = 9.3±0.3040) was shorter than that for nematodes feeding on *E. coli* (TD_{50} = 14.1± 0.7084but greater life span than others *S. enterica* isolates. In addition, *S.* Agona (Ag1, TD_{50} = 4.6±0.0520; Ag2, TD_{50} = 4.7±0.0505; Ag3, TD_{50} = 4.8± 0.0638), *S.* Weltevreden (We1, TD_{50} = 4.7±0.0637; We2, TD_{50} = 4.7±0.0578; We3, TD_{50} = 4.6± 0.0533), and *S.* Typhimurium (ATCC 14028) (TD_{50} = 4.9 ± 0.0865) shows the similar TD_{50} which correlates with similar virulence pathogenicity pattern as reported in Chai-Hoon *et al* (2009).

C. elegans is susceptible to a number of bacterial pathogens, which kill the nematodes using a variety of mechanism. Aballay *et al* (2000) and Labrousses *et al* (2000) demonstrated that *S. enterica* established a persistent infection within the gut of the nematodes to achieve full pathogenicity. An important early step for the establishment of gastrointestinal infections is the attachment of bacterial pathogens to intestinal epithelial cells and proliferation within the intestinal tract (Dhakal *et al*., 2006). Abally *et al* (2000) and Garsin *et al* (2001), demonstrated that virulence mechanism of the pathogen which correlates to the gut accumulation can be measured by calculating the number of colony forming units (CFU) recovered from the host. The profile of bacterial accumulation in the gut was determined by scoring the number of

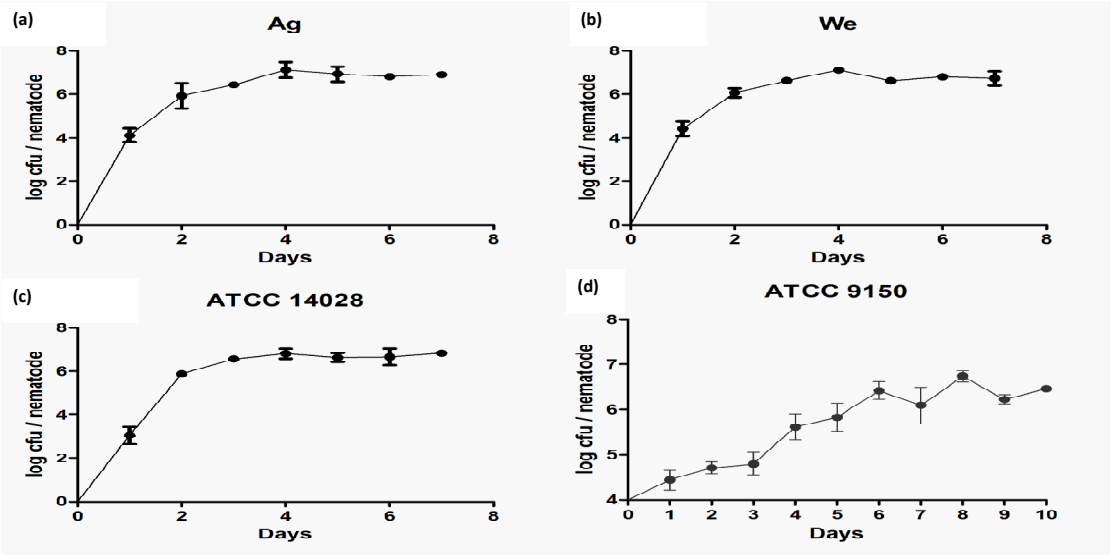

Figure 2. The numbers of *Salmonella* cells recovered from nematodes on the first day to seven days after the infection. (a) Nematodes infected by *S*. Agona serovar; (b) Nematodes infected by *S*. Weltevreden serovar; (c) Nematodes infected by *S*. Typhimurium (ATCC 14028)

live bacteria in the gut according standard plate count method as shown in Figure 2.

In addition, *S*. Agona serovar, *S*. Weltevreden serovar, and *S.* Typhimurium (ATCC 14028) shows the similar persistency after 4 days of the infection which correlated to their TD_{50} which were ranged from 4.6 to 4.9 days. Similar results were reported with *P. aeruginosa* (Tan *et al*., 1999), *S. typhimurium* (Abally *et al*., 2000, Labrousses *et al*., 2000), *Enterococcus faecalis* (Garsin *et al*., 2001, Kurz *et al*., 2003), *S. marcescens* (Kurz *et al*., 2003) and *V. vulnificus* (Dhakal *et al*., 2006) which suggested that the proliferation and accumulation of *S. enterica* isolates (except *S*. Paratyphi A, ATCC 9150) in the intestine of *C. elegans* may constitute the major cause of the earlier deaths of the worm. *S*. Paratyphi A (ATCC 9150), which exhibit an decrease of infection cells number at 9 days of the infection does not correlated to the TD_{50} =9.3 days. This might be due to different pathogenesis mechanism of *S*. Paratyphi A towards the infected worms. Furthermore, the obtained results review that there is enormous variation in the virulence and epidemiology of different serovar of *S. enterica* despite they are genetically closely related. For instance, most *S. enterica* serovars (*S.* Agona, *S*. Weltevreden, and *S.* Typhimurium) cause only gastroenteritis (Bangtrakulnonth *et al*., 2003; Sahilah *et al*., 2003; Reen *et al*., 2005; Emberland *et al*., 2007), while specific serovars (*S*. Paratyphi A) cause enteric fever (Fierer and Guiney, 2001). As reported by Chai-Hoon *et al.* (2009), *S*. Paratyphi A possesses different virulence pattern compare to *S.* Agona, *S*. Weltevreden, and *S.* Typhimurium.

The findings demonstrated that the virulence factors essential to mammalian pathogenesis also required for full pathogenicity in *C. elegans*. These observations provide evidence that *S. enterica* kill *C. elegans* using virulence mechanisms important for pathogenesis in mammalians systems and that nematodes may be used to rapidly analyze host factors involved in early stages of the pathogenic processes. This study also reviewed that the isolated *S. enterica* serovars from food sources can cause similar mortality rate in *C. elegans* as comparing with pathogenic references strain *S.* Typhimurium.

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