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ORIGINAL ARTICLE

Physiological and Immunological Regulations in *Caenorhabditis elegans* Infected with *Salmonella enterica* serovar Typhi

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Abstract Studies pertaining to *Salmonella enterica* serovar Typhimurium infection by utilizing model systems failed to mimic the essential aspects of immunity induced by *Salmonella enterica* serovar Typhi, as the determinants of innate immunity are distinct. The present study investigated the physiological and innate immune responses of *S.* Typhi infected *Caenorhabditis elegans* and also explored the Ty21a mediated immune enhancement in *C. elegans*. Ty21a is a known live vaccine for typhoidal infection in human beings. Physiological responses of *C. elegans* infected with *S.* Typhi assessed by survival and behavioral assays revealed that *S.* Typhi caused host mortality by persistent infection. However, Ty21a exposure to *C. elegans* was not harmful. Ty21a pre-exposed *C. elegans*, exhibited significant resistance against *S.* Typhi infection. Elevated accumulation of *S.* Typhi inside the infected host was observed when compared to Ty21a exposures. Transcript analysis of candidate innate immune gene (*clec-60*, *clec-87*, *lys-7*, *ilys-3*, *scl-2*, *cpr-2*, F08G5.6, *atf-7*, *age-1*, *bec-1* and *daf-16*) regulations in the host during *S.* Typhi infection have been assessed through qPCR analysis to understand the activation of immune signaling pathways during *S.* Typhi infections. Gene silencing approaches confirmed that *clec-60* and *clec-87* has a major role in the defense system of *C. elegans* during *S.* Typhi infection. In conclusion, the study revealed that preconditioning of host with Ty21a protects against subsequent *S.* Typhi infection.

Keywords *Caenorhabditis elegans* · Innate immune response · Pre-exposed host · RNAi · *Salmonella enterica* serovar Typhi

Abbreviations

Ach	Acetylcholine
ASH	Amphied sensilla neuron of head
ATCC	American Type Culture Collection
CFU	Colony forming unit
CGC	Caenorhabditis Genetic Centre
DEPC	Diethylpyrocarbonate
IMTECH	Institute of Microbial Technology
LB	Luria–Bertani
LPS	Lipopolysaccharide
MAPK	Mitogen activated protein kinase
MTCC	Microbial type culture collection
NGM	Nematode growth medium
PAMP(s)	Pathogen associated molecular pattern(s)
PMK	p38 MAP kinase
SCP/TAPS	Sperm-coating protein/Tpx-1/Ag5/PR-1/Sc7

Introduction

Typhoid fever is a severe infection in the reticulo-endothelial system caused by the etiological agent *Salmonella enterica* serovar Typhi. Due to indiscriminate use of antibiotics to fight against the disease, it has led to the emergence of multidrug-resistant strains of *S.* Typhi. Invasive *S.* Typhi is able to overcome the stomach acid barrier and causes disease by initially colonizing the intestinal tract and entering the lymphatic system through the passage of

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lumen. However, the induction of a defensive immune response is reliant upon the bacteria possessing an entire lipopolysaccharide (LPS) [1]. In order to prevent and adopt immune to the disease, vaccines were developed [2, 3] like *S. enterica* serovar Typhi Ty21a, which is the licensed live attenuated oral bacterial vaccine against typhoid fever [4]. Ty21a is an attenuated strain of *S.* Typhi Ty2 isolated in the year 1970, which has been attenuated by an irreversible genetic defect, achieved by multiple mutations induced by chemical mutagenesis [1]. Multiple mutations made Ty21a as an avirulent strain and its inability of the reversion was confirmed through in vitro and in vivo studies [5].

Earlier studies have demonstrated that certain genes were induced against *S.* Typhimurium infection in *Caenorhabditis elegans* [6, 7]. These studies fail to mimic the essential aspects of immunity induced by *S.* Typhi [8]. Moreover, the determinants of host defense system to *S.* Typhimurium and *S.* Typhi are basically different [9–12] and earlier studies designate that the host immune interaction in response to *S.* Typhimurium and *S.* Typhi are qualitatively diverse. The lack of a small model has been a foremost hindrance in understanding the virulence mechanisms of *S.* Typhi [8]. In contrast, there are no reports available on physiological and molecular response of host altered by *S.* Typhi and *S.* Typhi Ty21a. *Caenorhabditis elegans* as a suitable model for studying human pathogen due to its functional similarity with human [13]. Therefore this study aimed to evaluate the role of candidate innate immune genes against *S.* Typhi infection and also demonstrated the Ty21a mediated immune enhancement against *S.* Typhi using the simple tractable model system, *C. elegans*.

Materials and Methods

Bacterial Strains, Nematode, Media and Culture Conditions

Salmonella enterica serovar Typhi Ty21a was generously provided by Dr. S. Krishnaswamy (Madurai Kamaraj University, Madurai). The wild type strain of *S. enterica* Typhi (MTCC 733) was obtained from MTCC. *Escherichia coli* OP50 was provided by CGC. Bacterial cultures were maintained on Luria–Bertani (LB) medium at 37 °C, with aeration. Media was solidified by the addition of agar (18 g/l). *Caenorhabditis elegans* wild type strain N2 Bristol was provided by CGC, and maintained on nematode growth medium [14]. All the experiments were carried out using age-synchronized L4 stage worms. Synchronous populations were acquired as described by Sivamaruthi et al. [15].

Chemotaxis Assay

The cultures (0.5 OD) of *S.* Typhi or *S.* Typhi Ty21a and *E. coli* OP50 were spotted at a distance of 3 cm from the centre of NGM plates (90 mm) and denoted as zone A and zone B, respectively. Wild-type *C. elegans* were thoroughly washed from *E. coli* OP50 lawn and 25 worms were placed at the center of the culture spotted NGM plate. The number of worms in zone A and zone B were counted every 4 h. Similarly in control plates, both zones A and B were spotted with *E. coli* OP50.

Liquid Survival Assay

Immune conditioning of the host was done by treating the naive host with Ty21a for 12 h at 20 °C. Liquid killing assay was performed with naive or Ty21a pre-exposed (conditioned) *C. elegans* against *S.* Typhi and *S.* Typhi Ty21a as described by Sivamaruthi et al. [15]. Worms treated with *E. coli* OP50 served as a control and all the experiments were conducted at least three independent occasions. Statistical analysis was performed using SPSS Statistics 17.0. P values were generated by ANOVA followed by a Dunnett's T3 post hoc test. $P < 0.05$ was considered to be significant.

Short-Time Exposure Assays on *C. elegans*

L4 staged hermaphrodites were exposed to *S.* Typhi or *S.* Typhi Ty21a for short durations (1, 3, 5, and 14 h) and assessed the physiological changes as described by Sivamaruthi et al. [15].

Bacterial Accumulation

Accumulation of bacterial load (*S.* Typhi or *S.* Typhi Ty21a) inside the host system was assessed through the procedure prescribed by Sivamaruthi et al. [15]. *Salmonella Shigella* Agar (Himedia) medium was used for the plating purpose.

Brood Size and Pharyngeal Pumping Assay

To determine the pumping rate, naive and conditioned worms were placed on NGM plates seeded with *E. coli* OP50 (control) and *S.* Typhi. The number of progeny of the worms was counted and the pharyngeal pumping rate also was observed using a stereomicroscope (Nikon SMZ1000, Japan) for 30 consecutive seconds [15].

Aldicarb Sensitivity (Acetylcholine-Specific) Assay

Approximately, 20 N2 (control/*S*. Typhi infected) worms transferred onto 0.5 mM aldicarb plates. Every 10 min, worms were scored for paralysis for 2 h. The worms were allowed to stay on the aldicarb plates and the number of paralyzed worms was counted.

Osmosensation Assay

Infected worms were assessed for their ASH osmosensory function. High osmolarity ring with 1 cm radius was created on NGM plates using 4 M NaCl constitute of phenol-red (non-toxic dye to observe the solution and act as an indicator). Approximately, 20 worms (control/*S*. Typhi exposed) were washed with M9 buffer, allowed to crawl on an unseeded NGM agar plate, and then transferred to the center of high (4 M NaCl) osmolarity rings or normal NGM plate (control plate). The worms that escaped from the barrier ring were counted.

qPCR

Salmonella enterica serovar Typhi or *E. coli* OP50 infected worms were collected after each 24 h of post-infection and washed thrice with DEPC treated M9 buffer. Washed worms were incubated at −80 °C after treating with TRIzol (Invitrogen) reagent for 24–48 h. Total RNA was isolated according to the manufacturer's instructions. Total RNA was reverse-transcribed using Superscript III (Invitrogen) after the verification of RNA purity (BioSpec-nano). Real-time PCR was carried out to analyze the expression pattern of candidate antimicrobial genes (*clec-60*, *clec-87*, *lys-7*, *ilys-3*, *scl-2*, *cpr-2*, F08G5.6, *atf-7*, *age-1*, *bec-1* and *daf-16*) using gene specific primers (online resource Table S1). The conditions for qPCR were 94 °C for 30 s, annealing at 58 °C for 30 s, and extension at 72 °C for 40 s. The fold difference in the expression was calculated by comparative Ct value analysis with *act-2* (housekeeping gene) normalization.

RNAi

The mRNA sequences of target genes (*clec-60* and *clec-87*) were retrieved from Wormbase and the spliced sequences were used as input for the siRNA designing tool, Target Finder (Ambion^{R} life technologies). Two targets were selected for each gene from the software suggested silencing targets. The selection of the silencing target is based on the GC content and the position in the mRNA sequence. Specificity of selected targets was analyzed by NCBI–BLAST. The BLAST results revealed that the selected targets were 100 % specific to targeted mRNA. The siRNA duplexes were chemically synthesized from VBC biotech, Austria. The delivery of siRNA to the worms was done successfully by soaking method [16].

Results and Discussion

C. elegans Does Not Avoid *S*. Typhi and *S*. Typhi Ty21a

Bacterial pathogens are detected by *C. elegans* through its olfactory neurons and they respond against the pathogen by simply avoiding the bacteria. Hence, it is necessary to analyze the chemotaxis behavior of *C. elegans* against the bacteria to be studied intensively. In the present study, the chemotaxis assay results revealed that *C. elegans* does not avoid *S*. Typhi as well as *S*. Typhi Ty21a. *C. elegans* moved freely without any hindrance over the NGM plates spread with *S*. Typhi and *E. coli* OP50 or *S*. Typhi Ty21a and *E. coli* OP50. Both *S*. Typhi and *S*. Typhi Ty21a plates showed an equal distribution of worms (online resource Fig. S1).

S. Typhi Ty21a Enhanced the Survivability of Pre-exposed *C. elegans*

Even though the chemotaxis assay results indicated that *C. elegans* can uptake *S*. Typhi and *S*. Typhi Ty21a in solid condition, all the assays were performed in liquid medium to enhance the rate of intake of bacteria by the host. In liquid assays, *C. elegans* were exposed to *S*. Typhi or *S*. Typhi Ty21a continuously and monitored for their survival rate. Results of liquid assay revealed that the continuous exposure of *S*. Typhi to *C. elegans* caused host mortality with LT_{50} of 64 ± 3.4 h and complete death at 100 ± 6.9 h, whereas null mortality of the worms exposed to *E. coli* OP50 and reduced mortality (20 %) of *S*. Typhi Ty21a exposed worms were observed. *S*. Typhi Ty21a is a vaccine for typhoid. Typically, the function of a vaccine is stimulation of the host immune system against the respective infection. The previous study suggested that food and folate supplementation enhances the survivability of *S*. Typhi infected *C. elegans* [17]. The present study evaluated the Ty21a mediated induction of *C. elegans* immune system against *S*. Typhi infection. Interestingly, conditioned (12 h pre-exposed to *S*. Typhi Ty21a) worms showed an increased survival rate (LT_{50} of 132 ± 2.1 h) against *S*. Typhi, compared to naive *C. elegans* (LT_{50} of 64 ± 3.4 h), which revealed that the pre-exposed *C. elegans* survived longer (about ~54 %) than naive worms upon subsequent infection with *S*. Typhi. Neither heat-killed *S*. Typhi nor cell free supernatants caused significant mortality, suggesting that *S*. Typhi pathogenesis

required live infection (Fig. 1). The above results supported the notion that Ty21a can be used as an oral vaccine for a multicellular system infected with *S.* Typhi. In addition, it appears that Ty21a mediated enhancement of innate immunity is confined to *S.* Typhi mediated infection and appeared to be not significantly effective for other Gram-negative bacterial pathogens (Data not shown).

Five-Hour Exposure of *S.* Typhi is Enough to Cause Infection

In short-time exposure study, the life span of *C. elegans* exposed to *S.* Typhi/*S.* Typhi Ty21a was kinetically monitored after placing them in *E. coli* OP50 seeded NGM plates. Ty21a exposed worms (for about 1–14 h) were active and produced progenies. Worms exposed to *S.* Typhi for 1–3 h were active and produced the next generation in the presence of a food source (*E. coli* OP50). However, worms exposed for 5 h were dead after 144 h with no progeny. Worms exposed to *S.* Typhi for 14 h were dead after 98 h even in the presence of a food source. These data indicated that *S.* Typhi required at-least 5 h of interaction to significantly infect and cause mortality in *C. elegans*, whereas Ty21a did not cause any lethal effect to the host system (Table 1).

Typhi Cause Persistent Infection in *C. elegans*

In order to demonstrate the presence and multiplication of *S.* Typhi inside the infected *C. elegans*, CFU assay was performed. Accumulation of *S.* Typhi or *S.* Typhi Ty21a in *C. elegans* after 24 h exposure was found to be 4.51×10^2 or 3.2×10^2 per worm, respectively. However, the multiplication of *S.* Typhi and *S.* Typhi Ty21a in exposed *C. elegans* was distinctly different in both the cases (after 24 h) (online resource Fig. S2). No live bacteria could be recovered from the control *C. elegans*. These results confirmed that accumulation of *S.* Typhi was gradually increased over the course of infection within the host compared to Ty21a (online resource Fig. S2). This result stated that wild-type *S.* Typhi is highly virulent to *C. elegans* when compared to Ty21a.

In egg laying assay, the wild-type worms exposed to *S.* Typhi produced 70 ± 9 progenies compared to the control worm (268 ± 10 progenies) which revealed that *S.* Typhi infection affects the reproductive mechanism of *C. elegans*. In pharyngeal pumping assay, the pumping rate of naive wild-type *C. elegans* infected with *S.* Typhi have been reduced in a time dependent manner compared to control (Fig. 2). This result indicated that the bacterial infection damages the pharyngeal region of the host.

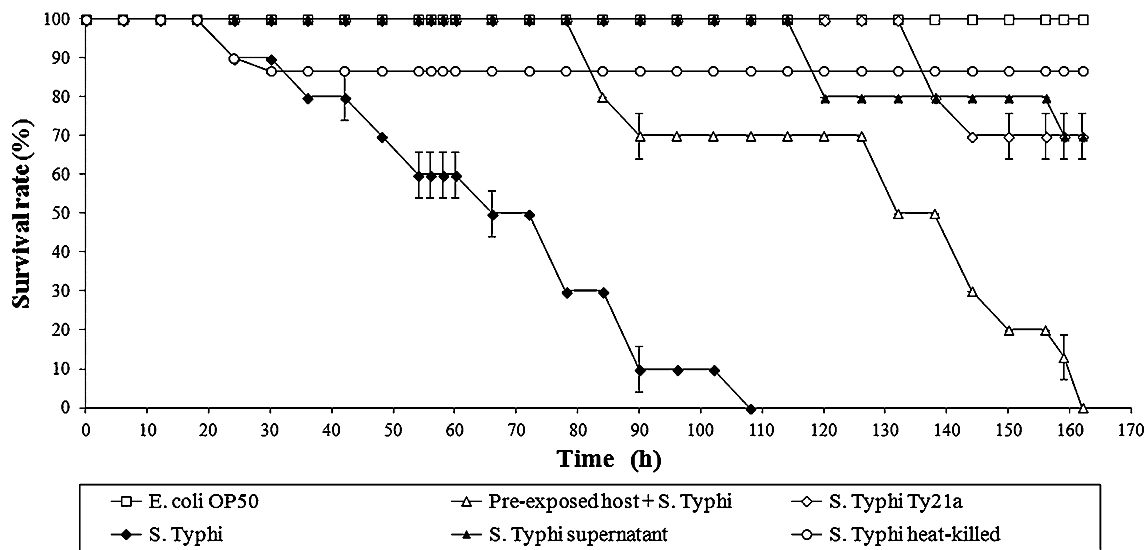

Fig. 1 Survival of *C. elegans* in liquid assays. Pre-exposed *C. elegans* (wild-type) exhibited resistance against *S.* Typhi infection during liquid infection assay. Age synchronized young adult worms were pre-exposed with *S.* Typhi Ty21a (pre-treatment for 12 h) as described in "Materials and Methods" section. *Escherichia coli* OP50 exposed worms were used as a control (*open square*). The naive *C. elegans* exposed to pathogenic *S.* Typhi (*closed rhombus*) showed a significant difference in their survival rate when compared with the pre-exposed *C. elegans* exposed to pathogenic *S.* Typhi (*open triangle*). The differences between the survival rate of *E. coli* OP50 exposed naive *C. elegans* and *S.* Typhi challenged pre-exposed *C. elegans* ($P = 0.006$) or naive *C. elegans* ($P = 0.004$), were significant. Ty21a exposed worms survived significantly different from naive *C. elegans* exposed to *S.* Typhi ($P = 0.012$). *P* values were generated by ANOVA (Dunnett's T3 post hoc test). $P < 0.05$ was considered to be significant

Table 1 Status of *C. elegans* placed on *E. coli* OP50 seeded medium during the short time exposure assay

Time (h)	*S.* Typhi	*S.* Typhi Ty21a
1	*C. elegans* were active and able to produce next generation	*C. elegans* were active and able to produce next generation
3	*C. elegans* were active and able to produce next generation	*C. elegans* were active and able to produce next generation
5	*C. elegans* were active for 72 h without producing progeny and died after 144 h	*C. elegans* were active and able to produce next generation
14	*C. elegans* were active for 72 h without producing progeny and died after 98 h	*C. elegans* were active and able to produce next generation

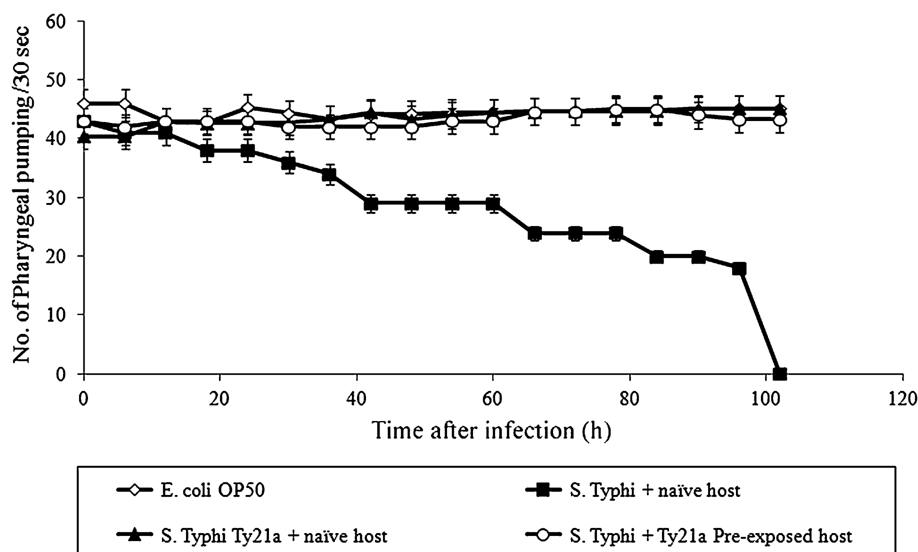

Fig. 2 Impact of infection in pharyngeal region of the host. Pharyngeal pumping rate (number of flings per 30 s) of control (*open rhombus*), *S.* Typhi infected naive (*closed square*), pre-exposed *C. elegans* (*open circle*) and *S.* Typhi Ty21a (*closed triangle*) infected naive host during infection

Behavioral Changes in *C. elegans* Exposed to *S.* Typhi

Additionally, behavioral assays (Ach-mediated behavioral assay and osmosensation assay) were performed to study the possible defects in specific classes of neurons within the control and pathogen exposed worms. Aldicarb blocks acetylcholine esterase (AchE) and that leads to paralysis. Infection mediated alteration in the expression of AchE has an impact on the time duration required for the aldicarb induced paralysis in naive *C. elegans* [18]. Uninfected wild-type *C. elegans* in aldicarb incorporated plate showed paralysis after 2 h from the time of transfer and its pharyngeal pumping rate (no. of flings per 30 s) decreased from about 51 to 39. The wild-type worms exposed to *S.* Typhi for 24 h were also paralyzed after 2 h with a decrease in pharyngeal pumping (online resource Table S2). This result suggested that *S.* Typhi infection appears to not affect the expression of AchE in *C. elegans.*

Worms avoid the high osmotic strength areas and this behavior is controlled by several neurons located in the head of the worm. The defects associated with specific neurons of worms exposed with OP50 and the pathogen was analyzed based on this avoidance behavior of high osmolarity barrier. When N2 control worms are placed at the midpoint of a high osmolarity ring, they tend to withdraw from the boundary and are trapped inside the ring. However, wild-type *C. elegans* exposed to *S.* Typhi for 48 h crossed the high osmolarity ring and the uninfected *C. elegans* sensed the boundary of the high osmolarity ring remains trapped inside the ring (online resource Fig. S3).

Regulation of Candidate Innate Immune Genes in *C. elegans* Upon *S.* Typhi Infection

Regulations of candidate innate immune genes (*clec-60*, *clec-87*, *lys-7*, *ilys-3*, *scl-2*, *cpr-2*, F08G5.6, *atf-7*, *age-1*, *bec-1* and *daf-16*) were analyzed in *C. elegans* during *S.* Typhi infection. C-type lectin-like domain containing proteins (CTLD protein) are secreted as transmembrane proteins in the intestine and as soluble proteins. In *C. elegans*, the potential contribution of CTLD proteins to immune specificity is demonstrated by their differential up-regulation towards pathogens [19–24].

In the present study, the level of expression of *clec-87* and *clec-60* was evaluated. During the initial hours of infection, both *clec-60* and *clec-87* were up-regulated compared to the control. After 48 h of post-infection, reduction in the expression of *clec-60* was observed, whereas a higher level of expression of *clec-87* was

observed after 72 h of infection. The reason for the elevated expression of *clec-87* is may be the necessity to recognize the proliferating bacterial cells within the host, which suggested that *clec-60* and *clec-87* are required for the recognition of invading pathogen, thus host can provoke the active defense mechanism against infection (online resource Fig. S4).

Lysozymes play a significant role in both vertebrate and invertebrate immunity. In *C. elegans*, *lys-7* expression was up-regulated in response to infection with the Gram-negative bacterium *Serratia marcescens*, indicating that *lys-7* likely plays a role in the innate immune response [20]. Invertebrate lysozyme (*ilys-3*) and *lys-7* were gradually up-regulated upon infection, which indicated the degradation of intracellular *S*. Typhi in *C. elegans*. After 96 h of post-infection, *lys-7* and *ilys-3* expression was observed to the maximum level. This elevation may be the resultant of survived worms even after 72 h of continuous exposure to *S*. Typhi (online resource Fig. S5).

Gradual reduction of *daf-16* was observed in infected N2 worms. *daf-16* was up-regulated in *C. elegans* after 72 h of *S*. Typhi infection (up-regulation of *daf-16* leads to the longevity in *C. elegans*) indicating its involvement in extending the life of the host. However, the virulence mechanism of bacterial pathogen masked the expression of *daf-16* by suppressing the expression during the later hours of infection (online resource Fig. S4).

Previous reports revealed that decreased level of *age-1* increases the lifespan in *C. elegans* [25, 26]. During the initial hour of exposure, *age-1* was found to be up-regulated to lead the normal life cycle, whereas after 72 h of post-infection, *age-1* expression was suppressed to facilitate the host to extend the lifespan to tackle the pathogenic condition. *Salmonella enterica* serovar Typhi infected host illustrated gradual up-regulation of *bec-1* except 96 h. Previous findings revealed that BEC-1 has role in *C. elegans* dauer formation and plays a role in autophagy pathway. The higher expression of this gene during *S*. Typhi infection revealed that host struggled to resist the infection by attaining the dauer phenomena and lysosomal degradation of the pathogen.

Level of *cpr-2* was up-regulated with higher fold induction at 24 h and sudden reduction at the latter hours of *S*. Typhi infection. Drastic reduction of *cpr-2* (CPR-2 is a Cysteine protease related family member known to involve in development and apoptosis) might have favoured the multiplication of pathogen within the host system. F08G5.6 is an unnamed CUB domain containing protein involved in both MAPK and *daf-16* regulated insulin signaling pathway of *C. elegans*. Expression of this gene is greatly regulated in *C. elegans* during *S*. Typhi infection. The previous study revealed that F08G5.6 has been involved in defense mechanism and its expression can be altered by the pesticides. ATF-7 plays a key role in the innate immune response by regulating the expression of immune genes downstream of PMK-1/p38 MAPK. During *S*. Typhi infection, F08G5.6 and *atf-7* was up-regulated with gradual reduction except 96 h of post-infection, respectively (online resource Fig. S5). These results suggested that *S*. Typhi infection alters the defense mechanism of host by altering the regulatory genes of aging, defense and apoptotic process.

C-type Lectins (*clec-60*, *clec-87*) are the Essential Players in the Defense System of *C. elegans*

Physiological observation of silenced *C. elegans* indicated that RNAi of *clec-60* affects the normal development of worm tail. Tail swelling was observed in notable number of *clec-60* silenced worms (online resource Fig. S6) whereas, there is no physiological alternations observed in *clec-87* silenced worms. Both *clec-60* and *clec-87* silenced worms exhibited normal life span and silencing was confirmed through semi-qPCR analysis (online resource Fig. S7).

Survival assays were performed using silenced *C. elegans* to analyze the role of *clec-60* and *clec-87*. *Salmonella enterica* serovar Typhi infected *clec-60* and *clec-87* RNAi worms died with complete mortality at 41 ± 1.4 and 50 ± 1.4 h, respectively (online resource Figs. S8, S9). Compared to the mortality of wild type worms during *S*. Typhi infection (Fig. 1), the silenced worms showed a huge difference by causing infection followed by earlier death, which revealed that *clec-60* and *clec-87* are necessary players of *C. elegans* immunity during *S*. Typhi infection. Although, the qPCR data indicated that *clec-87* is up-regulated than *clec-60*, the silencing study results indicated that *clec-60* is more important than *clec-87*. It appears that though the level of regulation of *clec-60* is comparatively lower than that of *clec-87*, the siRNA data suggested that among the pattern recognition receptors analyzed, *clec-60* appeared to have more regulatory role during the immune response against *S*. Typhi infection. These results revealed that the pattern recognition receptors of a host system probably have major impact in *C. elegans* immunity during *S*. Typhi infection.

In conclusion, the present study confirmed that *S*. Typhi causes infection and leads *C. elegans* mortality by an active process that required live infection, which correlated with the bacterial accumulation and persistent infection in the intestine. Merely 5-h exposure of *S*. Typhi is enough to cause infection in *C. elegans* and the *S*. Typhi infection affects the neuron system of the host. Surprisingly, Ty21a pre-exposed host exhibited enhanced immunity merely specific against subsequent *S*. Typhi infection. This findings have supported the theories [27] of special adaptive immune response in *C. elegans*. The immune system of the

host was altered in response to *S.* Typhi infection. The C-type lectins, *clec-60* and *clec-87* are the major immune players essential for the dynamic defense system of *C. elegans* against *S.* Typhi infection. Finally, the present study explores the simple nematode model, *C. elegans* for the study of human specific pathogen *S.* Typhi.

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