Volume : 4 | Issue : 12 | December 2015 • ISSN No 2277 - 8179

Research Paper

Rapid and Quantitative Detection of Salmonella Harboring the Inva Gene in Indian Chironomid Larvae (Diptera: Chironomidae), Using Real-Time PCR

Molecular Biology

KEYWORDS : Salmonella typhimurium MTCC98, Real-Time PCR, InvA gene.

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ABSTRACT

The chironomid larvae inhabited in aquatic sediments act as potential vectors for pathogenic bacteria present in aquatic sediments. In our study, we identified the Salmonella species present in the gut of thirty south indian chironomids collected from randomly selected rural and urban aquatic habitats with Real-time PCR approach using fast SYBR green chemistry. We used specific primer for target gene invA located on the Salmonella pathogenecity island-1 (SPI-1), that encodes an inner membrane protein and provides the bacteria with the capability to invade epithelial cells. The detection assayed in 3 hours, the CT Values were used to determine the concentration of bacterial load in the larval gut. The CT value of samples inhabiting rural sites is 33.06±0.96 and of urban sites is 28.81±0.51 against the positive control 13.89. Our results show that the Rt-PCR assay was rapid, and could sensitively detect even a single copy of Salmonella in the samples studied.

Introduction:

Chironomids are found in wide range of aquatic habitats and many species are pollution tolerant (Moore et al., 2003) and are important nutrient source for both vertebrates and invertebrates (Armitage 1995; Bat and Akbulut 2001). The chironomids are used as indicators of environmental conditions (Saether 1979; Kuhlmann et al., 2001) and also they make an important link in the aquatic food chain.

Chironomid larvae feed on algae and detritus and represent a reservoir of zoonotic agents such as *Salmonella* (Rouf and Rigney 1993; Broza and Halpern 2001; Moore et al., 2003). *Salmonella*, as one of the most common pathogens of water and food borne diseases worldwide (Wang, 2011), is responsible for a great number of infections in both man and animals (Tirado and Schmidt, 2001).

With the addition of anthropogenic pollutants such as fecal excretion and domestic sewage into the natural waters, the persistence of pathogenic bacteria such as Salmonella has increased in sediments (Hendricks 1971; **LaLiberte and Grimes,** 1982; **Morinigo et al.,** 1986) with subsequent health hazard to humans. Since chironomids live in close contact with sediments, they ingest *Salmonella* present in higher concentrations in sediments of aquatic habitats as they feed. Chironomids potentially mobilize bacteria to different environments via the food chain, which is a major health concern for people and livestock. These bacteria are often washed downstream thereby contaminating new sediments. Avian species prey upon adult chironomids and pass on pathogens to terrestrial environments.

Various methods have been used for the detection of *Salmonella,* including conventional culture methods, Enzyme- linked immunosorbant assay (ELISA) and Real-time quantitative polymerase chain reaction (RT-PCR). Conventional culture methods are reliable but time consuming, laborious (Cocolin et al., 1998) and require final confirmation by biochemical and serological tests (Reed and Grivetti 2000). ELISA not only is time consuming, but also less sensitive to detect lower concentrations of pathogens (Dev das et al., 2009). Although conventional PCR method is advantageous, its use is limited due to its low resolution in the post PCR analysis by gel electrophoresis and often it gives false positive results. The Real-time quantitative PCR in contrast, has a large dynamic range, high level sensitivity and is highly sequence specific (Marisa and Juan 2005).

The Real-time PCR, which is recently developed for diagnostic purposes requires a DNA binding dye SYBR green for the detection of PCR products. This fluorescence based detection of PCR products (Ke et al., 2000) reduces the detection time and allows processing of large number of samples simultaneously with utmost accuracy and with an advantage of analysis of PCR products simultaneous to amplification. In this assay the target gene is quantified based on the exponential increase of the initial DNA amount with the number of PCR cycles performed (Mackay 2004). In recent years The PCR based detection methods have been reported as rapid, specific and sensitive alternatives to identify several pathogenic microbes from food (Burkhard Malorny et al., 2003a) and clinical samples (Hoorfar et al., 2000).

In the present study, we utilized the *inv*A gene of 617 bp as a marker for the detection and quantification of *Salmonella* species present in the gut of chironomids inhabiting in rural and urban water bodies, based on Real-time PCR approach using fast SYBR green chemistry.

Materials and Methods:

Sample collection and Identification of larvae:

Total thirty samples were collected from different collection sites as in table 1. The chironomid larvae collected by the conventional scooping method were brought to the laboratory. The samples were transferred to enamel trays for selection of larvae. The selected larvae were kept unfed for 24 hours in well aerated distilled water in order to clear gut from digesta and to eliminate transient microbiota. The larvae were then washed thoroughly with HPLC grade water and rinsed with 70% alcohol for surface sterilization. The larvae were beheaded and the head capsules were processed and mounted on the slides following Hazra et al., (2002) for species identification.

Genomic DNA preparation:

The surface sterilized, beheaded larvae were homogenized and collected in 1.5 mL Eppendorf tubes. DNA was extracted using Macherey-Nagel Nuceospin tissue kit. Genomic DNA concentration was measured using Thermo Scientific NanoDrop8000. The average concentration for every sample was about 100ng/ µL.

PCR Assays:

The two PCR assays (Conventional and Real-time PCR) were performed parallely for all samples. The same negative and positive controls were used for both the assays.

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Conventional PCR assay:
A Conventional PCR assay was performed following modified PCR analysis by Murugkar et al., (2003). To the MicroAmp® 96-Well reaction Plate (0.2mL), added 3µL buffer, 2µL dntps, 0.3µL *Taq* DNA polymerase (NEB, USA), 2µL 5M Betain, template 2µL, 10 picomoles concentration of Primer forward 2 µL, primer reverse 2 µL and HPLC water 6.7µL and sealed accordingly with the applicator. The *inv*A gene primer Forward - 5' TTG TGT CGC TAT CAC TGG CAA CC 3' and Reverse- 5' ATT GTT AAC CCG CTC TCG TCC 3' (Sigma genosys) was used. The Ambion Nuclease free water was used as negative control and *Salmonella typhimurium MTCC98* as positive control. Amplification was conducted in Applied Biosystems Veriti 96 well Thermal cycler system. The cycle conditions were 10 minutes of incubation at 95ºC, followed by 35 cycles of 30 seconds denaturation at 95ºC, 45 seconds annealing at 55°C, 1 minute extension at 72ºC and the final extension of 10 minutes at 72ºC.

PCR products were detected by using Agarose gel electrophoresis. Electrophoresis was performed with 2% Agarose gel (Himedia) prestained with 0.5 µL /100mL of Ethidium bromide (10mg/mL). Gels were run at 80V using 1X TAE buffer and then photographed under UV illumination by using a Gel documentation system (UVITEC Cambridge).

Real-time PCR assay:
The assay was performed using Fast SYBR® Green Master Mix kit following the standard kit protocol. Sample, primers and reaction mix were placed in MicroAmp® fast optical 96-well reaction plate (0.1 mL) and sealed accordingly with applicator. The reaction mixture includes Fast Sybr green master mix 5µL, primer forward 0.5µL, primer reverse 0.5µL, template 2µL, HPLC water 2µL. The primer sequence is Forward- 5' TTG TGT CGC TAT CAC TGG CAA CC 3'; and Reverse - 5' ATT GTT AAC CCG CTC TCG TCC 3' (Sigma genosys). Along with 30 samples Ambion nuclease free water was used as negative control and *Salmonella typhimurium MTCC98* as positive control for reproducibility and accuracy of the assay. Amplification was conducted in ABI PRISM7500 Fast Real-time PCR system. The cycle conditions were 2 minutes of incubation at 95ºC, followed by 40 cycles of 3 seconds denaturation at 95ºC and 30 seconds annealing and elongation at 60ºC.

PCR products were detected by monitoring the increase in fluorescence of the reporter dye at each PCR cycle. The amplification plots for reporter signal against the number of amplification cycles were obtained using Applied Biosystems software. The threshold cycle (C_T) values for all the samples, negative control and positive controls were determined from the plots and the mean C_T values were determined.

Results and discussion:
Numerous molecular assays have been developed for the detection of *Salmonella* in different matrices (Drahovska et al., 2001; Fey et al., 2004; Krascsenicsova et al., 2008; Burkhard Malorny, et al., 2004; Oikonomou. et al., 2008). The Real time PCR method employed in the present study using fast SYBR green chemistry was relatively faster and accurate. In thirty south Indian chironomids collected from randomly selected rural and urban aquatic habitats the *Salmonella* harboring *inv*A gene could be detected without incubation. The detection and quantification of *inv*A gene could be completed within 3 hours.

The standard Conventional PCR assay that is carried out to establish the sensitivity of Real-time PCR, produced bright band of 617 bp for positive control and faint bands for urban samples, but no bands for rural samples due to a low copy number of bacteria present in the samples and its limitation of resolution in the post PCR analysis by gel electrophoresis (Fig: 5). The Real-Time PCR assay could sensitively detect single copy of *Salmonella* in the samples studied.

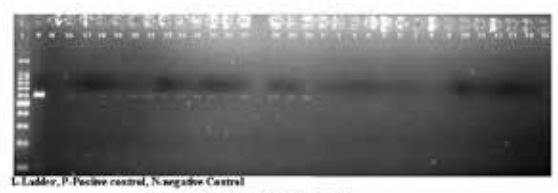

Figure 5: Conventional PCR Gel picture

In our work the C_T Values are used to determine the concentration of bacterial load in the larval gut. The C_T values (Fig: 3 and 4) for the detection of *Salmonella* in rural inhabiting larvae were much higher (33.06±0.96) than the C_T values observed in urban lakes (28.81 ±0. 51), indicate relatively lesser population in rural habitats. The copy numbers obtained based on a standard curve for rural samples are about 7 x 10^0 and for urban samples about 2.35 x $10^{2.}$ The standard curve showed a strict inverse correlation between the C_T and the concentration of Salmonella in the samples. In the negative control there is no detection of amplification and the C_T value is undetermined (Fig: 1), where as in positive control the initial molecules have undergone amplication and showed significant increase in fluorescence at C_T 13.89 (Fig: 2). The C_T value increases as the initial number of the available template molecules decreases. Thus, C_T values can potentially be used to quantify input target molecules. The strain *Salmonella typhimurium MTCC98,* which was used as positive control, co-amplified along with primers.

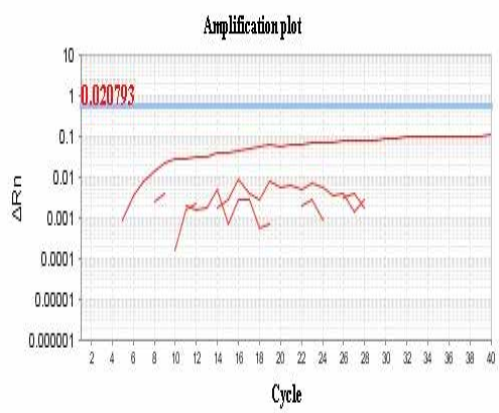

Figure 1: Negative control

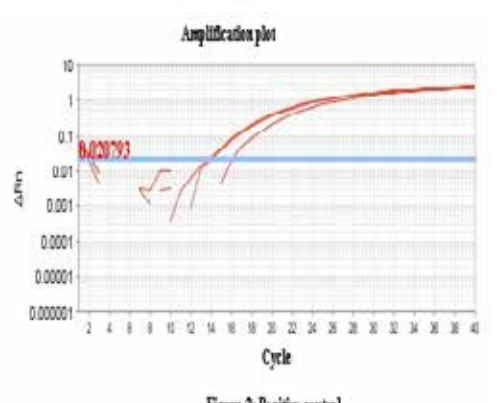

Figure 2: Positive control

The primary source of microbial pollution in rural water bodies is fecal matter generated from livestock production and cat-

tle feedlot runoff as well as anthropogenic sources. In the rural samples collected in the present study, in Real-time PCR after 33 PCR cycles the Salmonella could be identified, suggesting the presence of bacteria in minute quantities in the samples. This was confirmed by conventional PCR that showed no bands for these samples.

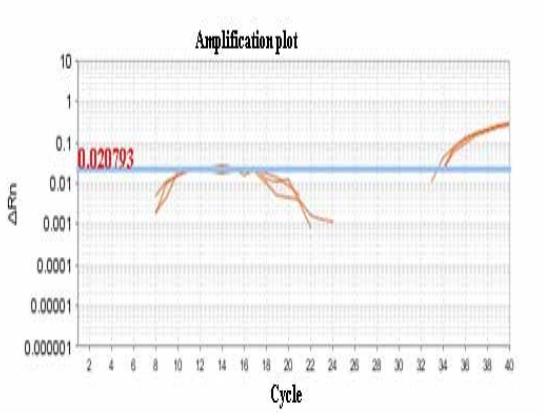

Figure 3: Average C_T Value of Rural samples

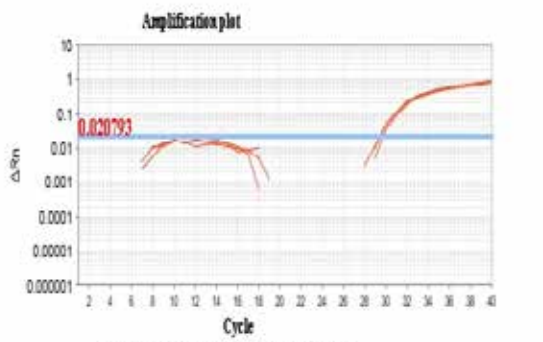

Figure 4: Average C_T value of Urban samples

Conclusions:

In this study, the quantitative determination of *Salmonella* in the Chironomids was done using the *inv*A marker gene present in *Salmonella*. The C_T values are used to determine the concentration of bacterial load in the larval gut. The standard curve analysis shows strict inverse correlation between the C_T and the concentration of *Salmonella* in the samples indicating relatively lesser population in rural habitats. The amount of microbial load in water bodies depends on the amount of anthropogenic sources. The bacterial load in the gut of Chironomid larvae is a potential indicator of the environment health. The Real time PCR technique using fast SYBR green chemistry employed in the present study was relatively faster and accurate to determine even the minute loads of bacteria in the environment compared to the conventional PCR method.

Competing interests: The authors have no competing interests.

Authors contributions:

T T involved in designing the experiment, analysis of results. R K participated in collection of samples from different sampling sites, performed the experiment that involves extraction of genomic DNA, conventional PCR and RT-PCR. G V S R participated in the analysis of experimental results, manuscript preparation, english grammer revision to prepare the final version of the manuscript.

Acknowledments:

The authors are grateful to Mr. Maddi Reddy, for helping in the collection of samples, preparation of manuscript, and to Dr Venu G for his help in revising the manuscript.

Table 1: List of Species of Chironomidae and collection sites

Sample ID	Species	Collection site, Locality	Family, Type locality
1	Clinotanypus spp.	Sulikere Lake, Bangalore rural	Tanypodinae, Clean water bodies (Rural)
2	Clinotanypus spp.	Attirala Lake, Andhra Pradesh	
3	Clinotanypus spp.	Maddur canal, Maddur, Karnataka	
4	Clinotanypus spp.	Upparavari Palli wet lands, Andhra Pradesh	
5	Procladius spp.	Maddur canal, Maddur, Karnataka	
6	Procladius spp.	Ammanakere Lake, Karnataka.	
7	Tanypus kraatzi	Sulikere Lake, Bangalore rural	
8	Tanypus kraatzi	Maddur canal, Maddur, Karnataka	
9	Tanypus kraatzi	Srirangapatna canal, Mysore	
10	Tanypus kraatzi	Upparavari Palli wet lands, Andhra Pradesh	
11	Zavrelimyia spp.	Sulikere Lake, Bangalore rural	
12	Zavrelimyia spp.	Maddur canal, Maddur, Karnataka	
13	Polypedilum spp.	Srirangapatna canal, Mysore	
14	Polypedilum spp.	Sulikere Lake, Bangalore rural	
15	Polypedilum spp.	Upparavari Palli wet lands, Andhra Pradesh	
16	Camptochironomus spp.	Hebbal Lake, Bangalore	Chironominae, Polluted water bodies (Urban)
17	Camptochironomus spp.	Pondichery sewage canal, Pondichery	
18	Chironomous circumdatus	Nagadevana halli Lake, Bangalore	
19	Chironomous circumdatus	Ooty Lake, Ooty	
20	Chironomous circumdatus	Hebbal Lake, Bangalore	
21	Chironomous circumdatus	Bellandur Lake, Bangalore	
22	Chironomous circumdatus	Varthur Lake, Bangalore	
23	Chironomous circumdatus	Chidambaram canal, Tamilnadu	
24	Chironomous circumdatus	Yellamallappa chetty Lake, Bangalore	
25	Glyptotendipes spp.	Ulsoor Lake, Bangalore	
26	Glyptotendipes spp.	Hebbal Lake, Bangalore	
27	Glyptotendipes spp.	Varthur Lake, Bangalore	
28	Nilodorum biroi	Hebbal Lake, Bangalore	
29	Nilodorum biroi	Varthur Lake, Bangalore	
30	Nilodorum biroi	Jnanabharathi sewage pond, Bangalore	

Volume : 4 | Issue : 12 | December 2015 • ISSN No 2277 - 8179

Table 2: C_T values of Samples, Negative and Positive controls

Sample Name	Target Name	Task	Reporter	Quencher	C_T Value
Negative Control	*invA* gene	NTC	SYBR	None	Undetermined
Positive control	*invA* gene	IPC	SYBR	None	13.89
1	*invA* gene	UNKNOWN	SYBR	None	33.82
2	*invA* gene	UNKNOWN	SYBR	None	33.91
3	*invA* gene	UNKNOWN	SYBR	None	33.34
4	*invA* gene	UNKNOWN	SYBR	None	34.01
5	*invA* gene	UNKNOWN	SYBR	None	33.06
6	*invA* gene	UNKNOWN	SYBR	None	33.83
7	*invA* gene	UNKNOWN	SYBR	None	34.02
8	*invA* gene	UNKNOWN	SYBR	None	33.13
9	*invA* gene	UNKNOWN	SYBR	None	33.09
10	*invA* gene	UNKNOWN	SYBR	None	34.01
11	*invA* gene	UNKNOWN	SYBR	None	33.18
12	*invA* gene	UNKNOWN	SYBR	None	33.65
13	*invA* gene	UNKNOWN	SYBR	None	34.02
14	*invA* gene	UNKNOWN	SYBR	None	33.81
15	*invA* gene	UNKNOWN	SYBR	None	33.12
16	*invA* gene	UNKNOWN	SYBR	None	28.91
17	*invA* gene	UNKNOWN	SYBR	None	29.09
18	*invA* gene	UNKNOWN	SYBR	None	29.1
19	*invA* gene	UNKNOWN	SYBR	None	29.08
20	*invA* gene	UNKNOWN	SYBR	None	29.14
21	*invA* gene	UNKNOWN	SYBR	None	28.81
22	*invA* gene	UNKNOWN	SYBR	None	29.01
23	*invA* gene	UNKNOWN	SYBR	None	29.11
24	*invA* gene	UNKNOWN	SYBR	None	29.02
25	*invA* gene	UNKNOWN	SYBR	None	28.98
26	*invA* gene	UNKNOWN	SYBR	None	28.89
27	*invA* gene	UNKNOWN	SYBR	None	29.32
28	*invA* gene	UNKNOWN	SYBR	None	29.08
29	*invA* gene	UNKNOWN	SYBR	None	29.12

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