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# Virulence and recycling potential of entomopathogenic nematodes (Nematoda: Steinernematidae, Heterorhabditidae) from Saharanpur district, western Uttar Pradesh, India

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**Abstract**
Entomopathogenic nematodes have been developed as the new hope for insect pest management. Four isolates of entomopathogenic nematodes tagged as $CS_1$, $CS_{21}$, $CH_5$ and $CH_6$ were recovered from the soil of Saharanpur District of Uttar Pradesh, India, two of each genus *Steinernema* and *Heterorhabditis* respectively and were tested for their pathogenicity and recycling potential against *Galleria mellonella* larvae. Results revealed that isolate $CH_6$ was highly virulent and caused 100% mortality within 48 h followed by $CS_{21}$ and $CH_5$ within 60 hours. A positive correlation was found between $LC_{50}$ and $LT_{50}$ values where increment in $LT_{50}$ lead to the increment in $LC_{50}$ values. The recovery of IJs/larva was $3.1\pm0.13\times10^5$, $2.16\pm0.1\times10^5$, $1.68\pm0.08\times10^5$and $1.44\pm0.1\times10^5$ with $CS_1$, $CH_5$, $CH_6$ and $CS_{21}$ respectively. Results revealed that all the isolates are highly virulent with an excellent reproductive potential in laboratory condition and can be applied further for pest management and commercialization.

**Keywords:** Entomopathogenic nematodes, *Steinernema*, *Heterorhabditis*, reproductive potential, pest management.

## 1. Introduction
Development of resistance against chemical pesticides in agricultural insect pests is a common problem for their effective control. Hazardous nature and toxic environmental effects also lead for the development of efficacious bio-formulation with safe application and recycling nature within environment. Entomopathogenic nematodes (EPN), of the Families Steinernematidae and Heterorhabditidae, are free living soil dwelling insect parasitizing nematodes. These nematodes are in keen interest as they do not have detrimental effect on environment and yet to be safe for humans, flora and fauna, and can therefore be used as biocontrol representatives [1, 2]. They are ubiquitously distributed all over the world and most species are polyphagous, highly virulent, and kill their hosts rapidly. These traits have created extreme interest in the development of bio-pesticides against insect pests [1, 3]. Further, these nematodes have a unique mutualistic relationship with bacteria which reside in their alimentary canal and produce a large number of toxins which help nematodes to kill their hosts within very short time [4, 5]. For the development of a suitable formulation, the primary requirement is to use the appropriate indigenous EPN isolate, as they are more adapted to the local environment, high virulent and having a high recycling potential within the insect host. Keeping these concepts, the present study was planned in search of the locally available potential EPN isolates and to test them on larvae of *Galleria mellonella* because of their ideal susceptibility so that they would be applicable in Indian climatic conditions against agricultural pests.

## 2. Materials and Methods
**Site and Soil Sampling:** Sugarcane fields of district Saharanpur, Western Uttar Pradesh were selected for soil sampling. Fifty soil samples were collected from different sites and brought to the laboratory in well labelled polythene bags for isolation and extraction of nematodes.

**Insect Culture:** Larvae of *G. mellonella* were fed on semi-synthetic diet. 3rd to 4th stage larvae with approximate same size and weight were used in each bioassay experiments. Fully grown larvae were also used for isolation and mass production of nematodes for further implications.

**Nematode Isolation and Culture:** Entomopathogenic Nematodes were recovered from soil by adopting the method of Bedding and Akhurst [6]. Ten last instar larvae of *G. mellonella* were placed in polystyrene jar containing fine and moist soil and placed in BOD at 27±1 ºC. Samples were examined daily for insect mortality. The cadaver of *G. mellonella* were disinfected with 0.1% sodium hypochlorite, washed with double distilled water (DDW) and transferred on White trap [7] for isolation of Infective Juveniles (IJs). Isolated IJs were further disinfected with 0.1% sodium hypochlorite solution and washed properly before storing in culture flask using DDW.

**Bioassay:** Koch's postulate was performed for the confirmation of nematodes as entomopathogenic and they were identified upto genera level based on morphological characters. Well plate bioassay was performed for pathogenicity testing [8]. Four different concentrations of each isolate viz. 25, 50, 100 and 200 IJ were prepared in 350 µl DDW and were released in the wells of the well plates lined by double layer of filter paper. Ten replicates of larvae for each experiment along with control (only DDW) were placed in above prepared well plates. Observations were taken after each 12 hours post infection period (PIP) to check the mortality of insect larvae. Dead larvae of *G. mellonella* were placed to the modified White Trap [7] to observe the persistence of infection and emergence of IJ. Each bioassay was placed separately and all experiments were repeated thrice to reach the optimum authenticity.

**Recycling Potential:** Ten larvae of *G. mellonella* (same size and weight), were infected with 100 IJ/larva and incubated in BOD at 27±1 ºC till they reach their mortality. They were then placed on white trap and IJ, which migrated into distilled water, were collected daily till the emergence stopped and stored in BOD at 15±1 ºC. The period of collection of IJ was 15-17 days. The IJ were counted in counting dish under stereomicroscope (Nikon SMZ 645).

**Statistical analysis:** The experimental data was analysed statistically through probit analysis to calculate $LC_{50}$ and $LT_{50}$ and the reproductive potential was presented in mean ± standard error of mean (SEM).

## 3. Results

### Bioassay

Four samples were found positive for the presence of nematodes and were named as isolate $CS_1$ and $CS_{21}$ for *Steinernema* species and isolate $CH_5$ and $CH_6$ for *Heterorhabditis* species. All the four different concentrations (25, 50, 100 and 200 IJ) were applied to infect the larvae and were believed to be equal to the rate of penetration of IJ /larva. All the isolates tested were strongly virulent (Fig 1). Both the isolates of *Heterorhabditis* viz. $CH_6$ and $CH_5$ killed all the larvae within 48 and 60 h respectively. *Steinernema* isolate, $CS_{21}$ also caused 100% mortality in 60 h but $CS_1$ was little bit less pathogenic and killed 50%, 60%, 60% and 90% larvae with all respective concentrations applied at this stage, but it was the only isolate observed which initiated mortality within 12 h in 200IJ /larva. Mortality initiation within 24 h with all the isolates with their respective concentrations showed that lowest dose of IJ (25IJ/larva) was sufficient to produce mortality. At 24 h PIP, the highest mortality was observed in $CH_6$ followed by $CH_5$, $CS_{21}$ and $CS_1$. After 36 h, 100% mortality was recorded in $CH_6$ following the penetration of 50, 100 and 200 IJ/larva, whereas $CH_5$ showed same mortality in 100 and 200 IJ/larva and $CS_{21}$ in 200 IJ/larva at 48 h PIP. In $CS_1$, 100% mortality was recorded at 72 h in 100 and 200 IJ/larva; lower dose caused 70% and 80%mortality up to this period of infection.

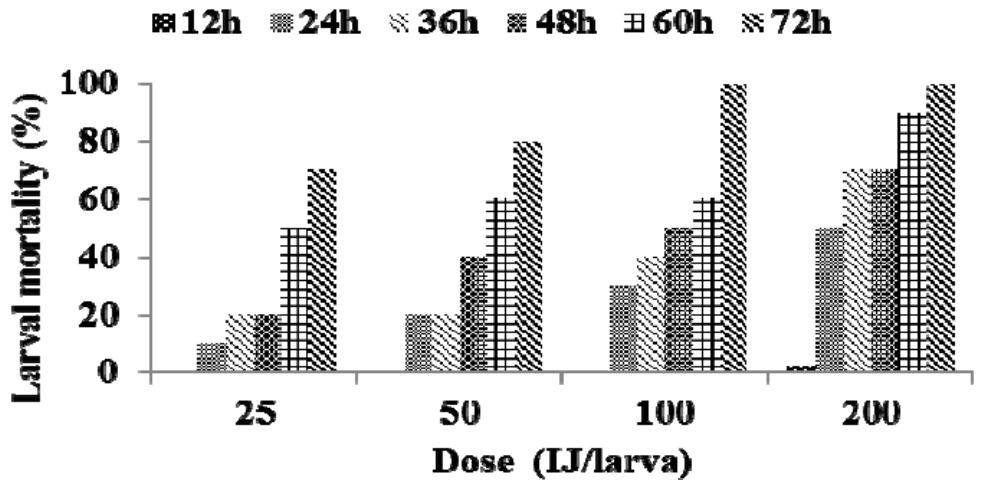
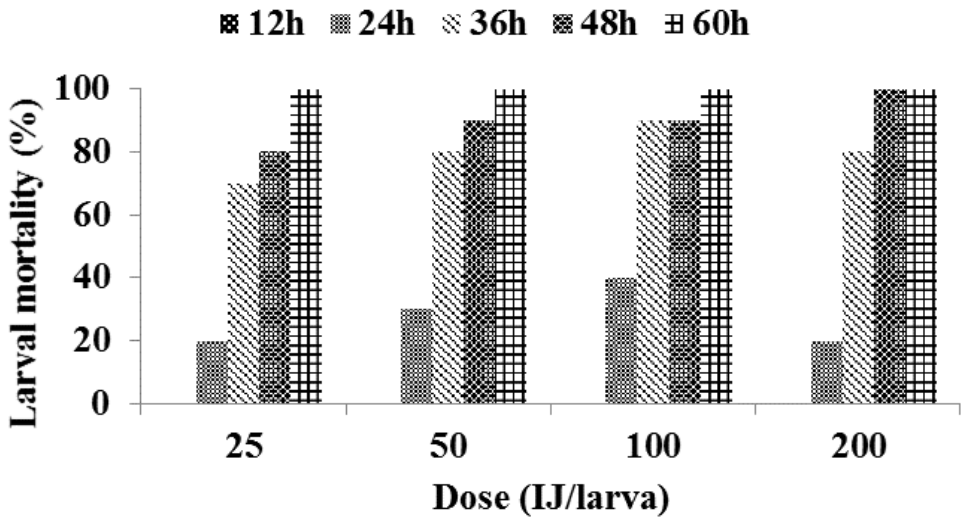



**Fig 1.1**

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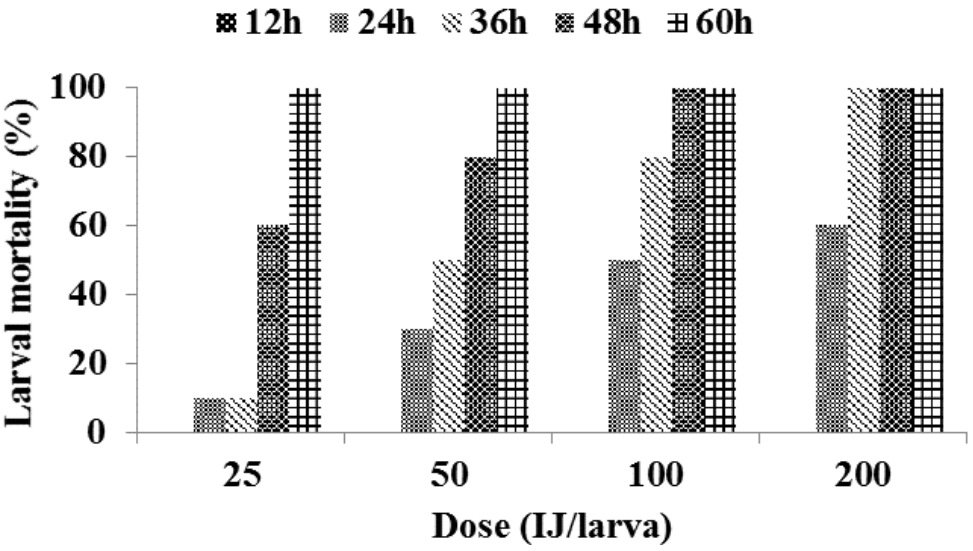


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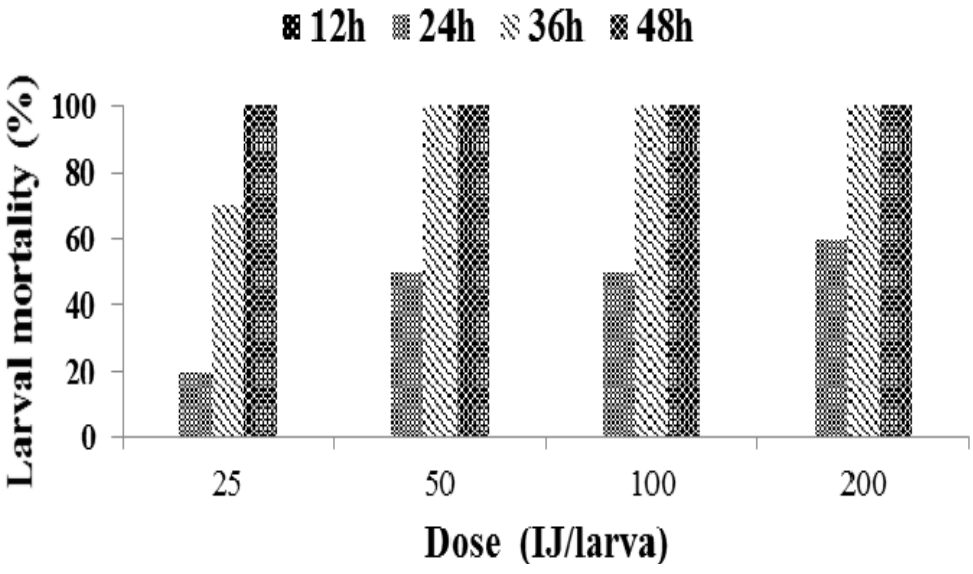


**Fig 1.4**

**Fig 1:** The percentage mortality of *G. mellonella* larvae at the exposure to different doses of infective juveniles (IJ) of nematodes. **1.1**-$CS_1$, **1.2**-$CS_{21}$, 1.3-$CH_5$, 1.4-$CH_6$.

$LC_{50}$ values in number of IJ/larva were calculated at three different time intervals viz., 24, 48 and 72 h, through probit analysis which decreased with time increment (Table. 1). At 24 h, the $LC_{50}$ values were 95.19 IJ/larva, 119.22 IJ/larva, 211.89 IJ/larva and 357.51 IJ/larva in $CH_6$, $CH_5$, $CS_1$ and $CS_{21}$ respectively. $LC_{50}$ value at 48 h was not calculated in $CH_6$ due to 100% mortality achievement. The lowest $LC_{50}$ value was calculated in $CS_{21}$with 6 IJ/larva followed by $CH_5$ (22.29 IJ/larva) and $CS_1$ (88.67 IJ/larva). At 72 h PIP, the $LC_{50}$ value was 17.73 IJ/larva in $CS_1$ and was not calculated in other isolates due to 100% mortality within 60 h. $LT_{50}$ values (in h) of all the isolates were also calculated followed by combined mortality in all the doses (Fig. 2). The pattern of $LT_{50}$ values from highest to lowest were as $CH_6$> $CS_{21}$>$CH_5$>$CS_1$ with 24.91, 28.94, 29.87 and 42.39 h respectively.

**Table 1:** $LC_{50}$ values (in terms of number of IJ/larva) of all the isolates at different time intervals

| Isolate | $LC_{50}$ @ 24h | $LC_{50}$ @ 48h | $LC_{50}$ @ 72h |
|---|---|---|---|
| $CS_1$ | 211.89 | 88.67 | 17.726 |
| $CS_{21}$ | 357.51 | 6.00 | * |
| $CH_5$ | 119.22 | 22.29 | * |
| $CH_6$ | 95.19 | * | * |

* 100% mortality achieved upto this time hence, statistics was not computed

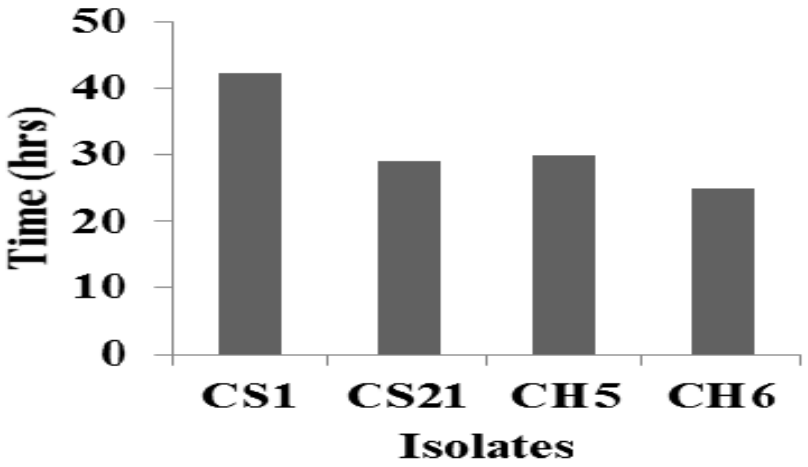


**Fig 2:** $LT_{50}$ values (in hrs) following the total mortality with all the respective concentrations**.**

**Recycling potential**

The descriptive statistics of number of IJ/larva was performed for all the isolates and presented in mean ± SEM. The data revealed that the Infective Juveniles production was maximum in $CS_1$ with $3.1\pm0.13\times10^5$ IJ/larva (260000-372000) followed by $CH_5$ with $2.16\pm0.1\times10^5$ IJ/larva (159000-269000), $CH_6$ with $1.68\pm0.08\times10^5$ IJ/larva (108000-198000)and $CS_{21}$ with $1.44.\pm0.1\times10^5$ IJ/larva (90000-193000) (Fig. 3).

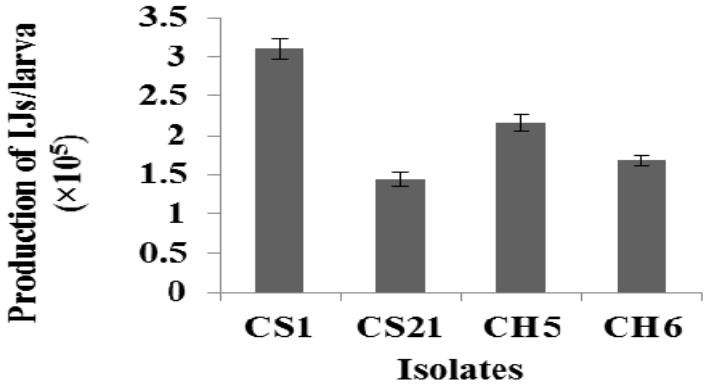


**Fig 3:** Mean infective juveniles production in larvae of *G. mellonella* exposing with 100 IJ/larva**.**

**4. Discussion**

In the present study, all the four isolates tested were found enough virulent to kill the larvae within short duration with high progeny productions. The pathogenicity of nematodes was varied within isolates which was previously demonstrated by different workers [9, 10]. A positive correlation was found in rate of penetration and mortality which was not initially observed by Caroli *et al.* [11] and Ricci *et al.* [12] but was clearly indicated by several other workers [8, 13, 14, 15]. This theory suggested that high infection rate increases the production of toxins produced by developing bacteria associated with nematodes [5]. *Heterorhabditis* isolate $CH_6$ took least time to cause 100% mortality and killed all the larvae within 48 h following all the concentrations whereas isolate $CH_5$ of the same genus killed all the larvae in only 100 and 200 IJ/larva doses. In *Steinernema* isolate $CS_{21}$ only 200 IJ/larva dose

killed all the larvae and the result can be corroborated with the findings of Phan *et al.* [15] and Caroli *et al.* [11] with other *Steinernema* species/strains. Except $CS_1$, all the nematode isolates were able to kill all the larvae within 60 h duration following all the concentrations. Isolate $CS_{21}$ of *Steinernema* sp. showed lowest $LC_{50}$ values at 48 h followed by least pathogenic *Steinernema* isolate $CS_1$ but $CH_6$ was judged to be most pathogenic isolate among all, as at 48 h $LC_{50}$ value was unpredictable because of its high mortality aptitude. $LT_{50}$ values ranges from 25.09-42.39 h where highly virulent *Heterorhabditis* isolate $CH_6$ took least time followed by ceiling time taken by *Steinernema* $CS_{21}$ isolate. Decrease in $LC_{50}$ with time increment suggested that longer time lead to the multiplication of more bacteria result in more production of toxins to kill insect host by septicaemia. Positive correlation was found between $LC_{50}$ and $LT_{50}$ values where increment in $LT_{50}$ lead to the increment in the $LC_{50}$ values, this also indicated the higher virulence of $CH_6$ with minimal time and IJs required.

Entomopathogenic nematodes have numerous attributes that make them an admirable bio-control driving force. The genera *Steinernema* and *Heterorhabditis* of these nematodes have gained much importance for commercialization and their application [14, 16]. *In vivo* mass production of EPNs is easy where *G. mellonella* is a most widely used insect species. In present study, the IJs production rate from larvae of *G. mellonella* was very high as was proposed by Elawad *et al.* [17]. The least pathogenic isolate $CS_1$ showed highest recycling potential whereas the highest pathogenic isolate $CH_6$ produced lower number of Infective juveniles. This could be because of longer residing time within the host for production of more generations. The same result was obtained with $CH_5$ as it also showed less progeny count as compared to $CS_{21}$ but more than $CH_6$. However, recovery of IJs from $CS_{21}$was lowest which was strong virulent after $CH_6$, could lead a controversy in above said hypothesis. The IJs production of *Steinernema* isolates was comparable with the results of some other workers with different *Steinernema* species [17, 18]. Shapiro-Ilan *et al.* [19] reported the production of *H. bacteriophora* from single *G. mellonella* upto 300,000. Comparatively to the author, the production of IJs of studied unknown *Heterorhabditis* isolates was similar.

## 5. Conclusion

Based on the findings of present study it can be concluded that all the four isolates of EPN were virulent to larvae of *G. mellonella* and produced good number of IJ. However *Steinernema* isolate $CS_1$ was less pathogenic to *Steinernema* isolate $CS_{21}$ and *Heterorhabditis* isolate $CH_6$ and $CH_5$, but its progeny production rate was high in comparison to all. Further work is required to identify the isolates and study the host range, as well as the virulence of its symbiotic bacteria. It may therefore, be concluded that these all EPN isolates have good bio-control potential for future applications on insect pests and commercialization against them.

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