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Research article

Bacillus thuringiensis strains from Western Ghats of India possess nematocidal property against *Haemonchus contortus* larvae of goats

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ABSTRACT

Nematocidal properties of spore crystal mixtures of six *Bacillus thuringiensis* (Bt) strains (KAU 49, 50, 52, 61, 99 and 424) collected from Western Ghats, a biodiversity hot spot of India, were analysed against *Haemonchus contortus* larvae isolated from goats. One dose nematocidal assay dose response to lyophilised spore-crystal mixtures (SCM) of the six Bt strains were determined by adding 200 μg/mL of each SCMs to culture plate wells containing aqueous suspension of *H. contortus* larvae. Out of the strains screened, KAU 50 and 424 were found to possess nematocidal properties. Maximum nematocidal properties were exhibited 7 days post-inoculation of the lyophilised SCMs. The 50 per cent lethal concentrations deduced by log probit analysis for KAU 50 was found to be 130.59 μg/mL, whereas that of KAU 424 was found to be 144.536 μg/mL at 95 per cent confidence level. This is the first report on the nematocidal propery of Bt strains against *Haemonchus contortus* larvae isolated from goats. Further studies are needed for identification and characterisation of the toxin.

1. Introduction

The prevalence of anthelmintic resistance in nematode parasites of livestock necessitates the need to develop new control methods with lesser chance of developing resistance. In this regard, *Bacillus thuringiensis* (Bt), a naturally occurring, Gram-positive spore forming bacterium had gained attention as early as in 1985, when Bottjer et al., 1985 found that toxins from Bt were lethal towards *Trichostrongylus colubriformis* eggs. The drawback of current chemotherapeutic approaches to animal parasite nematode control is that it is aimed almost exclusively at the parasitic stages. Meadows et al. (1989) reported that the toxin from *B. thuringiensis* serovar *kurstaki* was lethal towards eggs, first and second stage larvae, as well as ensheathed or exsheathed third stage larvae of *T. colubriformis*. Lack of chemical residues, target species specificity and low mammalian toxicity are the advantages of bio control agents like Bt over traditional chemotherapeutic agents (Schnepf et al., 1998; Siegel, 2001).

According to Kotze et al. (2005), two Bt isolates were highly toxic to larvae and adult of *Haemonchus contortus*, *T. colubriformis* and *Ostertagia circumcincta in vitro*, one of which Bt strain expressed Cry 5A and Cry 5B proteins and the other expressed Cry 13A. They also reported that LC_{50}

values for the parasporal crystal inclusion of Bt strain L366 in larval development assays for *H. contortus* was 41 ng/mL. Studies using *in vitro* larval development and migration assays have also shown that the toxicity of Bt (L366) crystal protein significantly decreased if *H. contortus* larvae reached the late L_2 stage before exposure to the bacterium, while it reduced the migration of fully developed Bt exposed larvae compared with controls (O'Grady et al., 2007).

In the present study six Bt isolates from Western Ghats of India, were screened for their nematocidal properties against *H. contortus* larvae of goats. Reports on the nematocidal Bt strains from India are unavailable. So this study was undertaken with the objective of screening six *Bacillus thuringiensis* strains (KAU 49, KAU 50, KAU 52, KAU 61, KAU 99 and KAU 424) collected from Western Ghats of India, a biodiversity hot spot of the world, for nematocidal activity against the larvae of *H. contortus*, a pathogenic parasite of small ruminants. Western Ghats, being a biodiversity hot spot in terms of natural flora and fauna, would be the most potent natural habitat for novel strains with varying biological properties.

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2. Results and discussion

On monitoring larval mortality from 1st to 10th day in larval suspensions treated with lyophilised SCM of all the six strains (KAU 49, 50, 52, 61, 99 and 424), mortality could be detected from 3rd day onwards in the wells to which aqueous suspensions of SCMs of KAU 50 and 424 were added. In these wells there was a gradual increase in mortality up to 7th day, which remained at that level till the 10th day. The 50 per cent lethal concentration (LC$_{50}$) calculated from the dose response curves for KAU 50 and 424 were 130.59 µg/mL and 144.536 µg/mL, respectively (Fig. 1). Out of the 6 haemolytic Bt strains, screened for nematocidal properties, the strains KAU 50 and 424 were found to be most effective.

Molecular characterisation of Cry genes in 496 Mexican Bt strains had not revealed any of the so far identified nematode active Cry genes *ie.,* Cry 5, Cry 12, Cry 13 and Cry 14 (Bravo et al., 1998). The nematocidal Bt strains identified in the present study were previously reported to possess different sets of *Cry* genes from those reported so far. KAU 50 was reported to possess *Cry6, Cry16, Cry20* and KAU 424 was also reported to have *Cry1* and *Cry14* (Krishnaraj et al., 2009). Among the Cry genes reported from KAU 424, the protein of *Cry 14* was already reported to have nematocidal property but Cry 1 protein was reported to be toxic for lepidopteran insects. So the nematocidal properties of this particular Bt strain might be attributed to Cry 14 protein. Whereas in case of KAU 50, the genes reported to be possessed were not so far identified to have nematocidal properties. It is therefore interesting to note that newer nematocidal proteins might also exist in nature among different Bt strains. Further protein profiling, separation and characterisation have to be done to identify the exact protein responsible for nematocidal properties of this particular Bt strain. The different Cry proteins reported might be due to their wide spectrum of toxicity and species specificity. The identification of nematocidal Bt strains from the native strains of Western Ghats, clearly pointed out the richness of diversity in the Bt strains in different geographical locations.

In the present study, maximum nematocidal property for *H. contortus* was observed on 7th day post-inoculation. Leyns et al. (1995) reported the maximum mortality of *Caenorhabditis elegans* larvae from 8 h to 24 h post-inoculation of Bt spore crystal mixture. However, in the present study the targeted nematode species was different and the longer incubation time to achieve the mortality might be due to the difference in the larval development stages. Kotze et al. (2005) could observe complete cessation of movement of the adult *H. contortus* worms within 2 and 4 days of treatment with Bt, which is a timeframe more commensurate with the present study.

The LC$_{50}$ value from the dose-response curve of the two nematocidal Bt strains KAU 50 and 424 were found to be 130.59 µg/mL, respectively (Fig. 1) by probit analysis. Beyond 3rd dilution no difference in larval mortality could be detected. Kotze et al. (2005) had reported LC$_{50}$ value of 41 ng/mL when the purified parasporal inclusion protein of Bt strain L366 was fed to *H. contortus* larvae. In the present study, the use of SCM instead of purified protein could have resulted in the higher LC$_{50}$ values of both the strains. It is noteworthy that Bt spores are able to pass through the gastro intestinal tract of a large number of animal species and germinate in faecal cultures to form colonies producing parasporal inclusions (Lee et al., 2002). However, purified proteins when fed to the animal might digest in the stomach itself (Kotze et al., 2005). So further research is needed to deduce alternative options like direct delivery of the protein to the mucosal surface of intestinal tract using chitosan (Senel and McClure, 2004) thus protecting the bioactive molecules from acid digestion in the gut. Lyophilised SCM is a better option to be fed orally to animals so that when the spores get excreted through the faeces they would serve as effective bio control agent against the nematode.

3. Conclusion

The SCM of the Bt strains, KAU 50 and 424, identified to possess nematocidal activity, could serve as prospective bio control agents when fed orally to goats as the spores would resist the acid digestion in the stomach of the animal. The present study points to the fact that nematocidal Bt strains may be widely distributed in nature with variations in their nematocidal properties. Extensive screening of native Bt strains from different geographical locations of the world may help to identify novel strains capable of synthesizing more specific and powerful nematocidal proteins. Studies need be directed towards analysing the efficacy of spraying of lyophilised SCM on the pasture lands for the control of developing larvae in faecal pats, thus reducing the number of infective larval uptake by the grazing animals. The scope of application of newer target specific drug delivery systems could also be sought for protecting the bioactive molecules from acid digestion in the gut. Further gene level characterisation, purification and identification of the nematocidal proteins of the two strains would be of great help in categorising the novel nematocidal proteins with promising therapeutic potential.

4. Materials and methods

Nematocidal properties of Bt strains (KAU 49, 50, 52, 61, 99 and 424) were assessed with the *H. contortus* larvae obtained from a goat.

4.1. Larval culture

The modified Veglia's method (Sathianesan and Peter, 1970) was followed for copro-culture. About 25 g of the faecal sample was transferred into the bottle without soiling the sides. In order to provide optimum moisture for the faecal culture sufficient quantity of water was added till adequate consistency was achieved. Then the bottle was closed
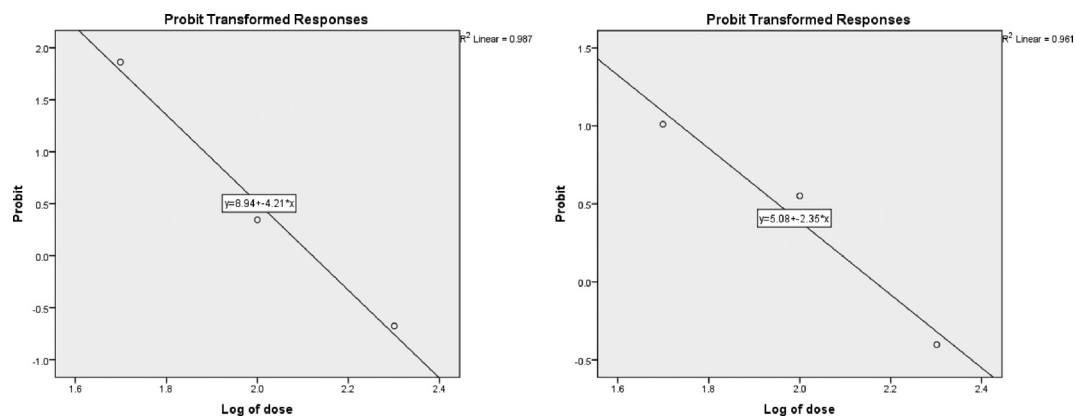

Fig. 1. Dose-response curve of nematocidal activity of lyophilised spore crystal mixture Bt strains against *Haemonchus contortus* larvae obtained from goats 7 days post-inoculation.

and kept in dark at room temperature. The presence of larvae on the sides of the bottle was detected microscopically after five to seven days. The larvae were then examined under a light microscope and morphologically identified as per Van Wyk et al. (2004).

4.2. Preparation of lyophilised spore crystal mixture (SCM)

A loopful of six non haemolytic Bt strain cultures (KAU 49, 50, 52, 61, 99 and 424) were inoculated in to 50 ml each of T3 (Traver's et al., 1987) broth and shaken at 200 rpm, 37 °C for 4 days. On the 5th day the culture was examined for sporulation. The broth was centrifuged at 11,000 x g for 10 min at 4 °C, followed by removal of supernatant and two washings with milli Q water. The pellets were suspended in one mL of milli Q water each and kept for lyophilisation at -70 °C (Operon, Korea). Lyophilised powder was kept in the refrigerator.

4.3. One dose nematocidal assay

One dose nematocidal assay responses to the lyophilised SCMs of six Bt strains (KAU 49, 50, 52, 61, 99 and 424) were determined by adding a 100 µL aliquot of the aqueous suspensions of 200 µg SCMs each to culture plate well containing 900 µL of aqueous suspension of 30–40 *H. contortus* larvae. Then the final concentration of SCM became 200 µg/mL. The nematode suspension also contained kanamycin sulphate (Hemedia, Mumbai) 30 µg/mL to prevent bacterial growth. An aqueous suspension (500µL) of 30–40 larvae was kept as control. All the treatments and controls were replicated three times. The culture plates were incubated at 28 °C for 10 days. Larval mortality was monitored from 1st to 10th day of incubation through stereo-zoom microscope (Zeiss, Germany).

4.4. Dose-response study

The two nematocidal strains KAU 50 and 424 were assayed in six serial dilutions, each in triplicate. The aqueous larval suspensions were prepared in the same concentration as mentioned for the one dose nematocidal assay. Larval mortality was estimated on the 7th through stereo-zoom microscopy. The 50 per cent lethal concentration (LC$_{50}$) was deduced from the dose response curve drawn by probit analysis.

4.5. Statistical analysis

Statistical analysis was performed using Statistical Product and Service Solutions (SPSS) version 21.0.

Declarations

Author contribution statement

V Beena:conceived and designed the experiments; performed the experiments; analyzed and interpreted the data; contributed reagents, materials, analysis tools or data; wrote the paper.

V Ramnath: conceived and designed the experiments; contributed reagents, materials, analysis tools or data; wrote the paper.

G Devaki: conceived and designed the experiments; performed the experiments.

K Kakkattuparambil, K.P. Sreekumar: conceived and designed the experiments.

B Lakshmanan, R Radhika: contributed reagents, materials, analysis tools or data.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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