

**Israel Journal of Entomology Vol. XXXn (1998) pp. 129-133****IDENTIFICATION OF *BACILLUS THURINGIENSIS* SUBSP. *ISRAELENIS* ISOLATED FROM GERMANY BY THE USE OF POLYMERASE CHAIN REACTION (PCR) AND RESTRICTION ENZYME PROFILE**

THANIA V. GUAYCURUS,<sup>1</sup> ANA C.P. VICENTE<sup>2</sup> AND NORBERT BECKER<sup>3</sup><sup>1</sup> *Department of Bacteriology, Institute Oswaldo Cruz, Rio de Janeiro, Brazil* <sup>2</sup>*Department of Genetics, Institute Oswaldo Cruz, Rio de Janeiro, Brazil* <sup>3</sup>*German Mosquito Control Association, KABS, 67165 Waldsee, Germany*

**ABSTRACT**

This report refers to the identification of *Bacillus thuringiensis* subsp. *israelensis* isolated from areas along the River Rhine, by the application of the PCR technology using total DNAs extracted from a single bacterial colony as templates. The primers, which had homology to the regions inside the gene coding for the Diptera toxin, were used to amplify specific DNA fragments. The PCR conditions were 30 cycles which consisted of 94°C for 30s, 48°C for 30s and 72°C for 1 min. In a preliminary study 10 strains were analysed and 7 of them generated DNA fragments with 1 kb. The PCR product was digested with restriction endonuclease *Hae* HI and two bands of 670 and 330 bp were detected. The entomocidal activity was confirmed by bioassays against *Aedes cantans* (Meig.) larvae. By a serological test these 7 strains were classified as *B. thuringiensis* subsp. *israelensis*.

KEY WORDS: *Bacillus thuringiensis* subsp. *israelensis*, PCR, primers, restriction profile, bioassay.

**INTRODUCTION**

*Bacillus thuringiensis* subsp. *israelensis* is a Gram positive bacterium that produces crystals during the sporulation phase (De Barjac, 1978). This parasporal crystalline body is composed of polypeptides called delta-endotoxin which showed highly selective toxicity against larvae of different species of mosquitoes and black flies (Goldberg and Margalit, 1977).

The delta-endotoxins are solubilized in the alkaline digestive juice of the larval midgut, digested by the gut proteases to produce the activated toxins which bind to receptors localized in the midgut brush border, creating leakage channels in the cell membranes. This effect causes cellular lysis and the death of the larvae (Hoffmann et al., 1988a,b).

*B. thuringiensis* subsp. *israelensis* expresses a heterologous group of mosquitocidal crystal proteins (*cryIV* class) under the control of sporulation-dependent promoters (Hofte and Whiteley, 1989). *B. thuringiensis* subsp. *israelensis* *cryIVA* (Ward and Ellar, 1987), *cryIVB* (Chungjatupornchai et al., 1988), *cryJVC* (Thorne et al., 1986), *cryIVD* (Donovan et al., 1988), and *cytA* class (Waalwijk et al., 1989) are genes which encode proteins with the predicted molecular masses of 134.4, 127.8, 77.8, 72.4 and 27.4 kDa, respectively.

In this study we applied the PCR methodology, with the purpose to detect the presence of the *cryIVD* gene, which characterizes *B. thuringiensis* subsp. *israelensis* in the group of *B. thuringiensis* isolated from treated areas along the Upper Rhine Valley. For the confirmation of the DNA amplification specificity, the PCR product was digested with the restriction enzyme *Hae* III. Bioassays against *Aedes cantans* (Meig.) larvae were conducted, as well as serotyping.

## MATERIALS AND METHODS

### Sample collection

In each breeding area 25 soil samples were taken at distances of 10 meters. A total of 8 breeding areas was examined.

### Isolation of the crystal-spore forming strains

1 g of soil sample of each breeding area was dispersed in 10 mL distilled water and heated for 10 min to 80°C. Serial dilutions up to  $10^{-6}$  were prepared and 100  $\mu$ L of the dispersed material was spread on nutrient agar plates and incubated for 48 hr at 30°C. Plates containing 1 to 20 colonies were examined for the presence of parasporal bodies by phase contrast microscopy. Crystal-forming strains were purified by subculturing on nutrient agar plates until axenic cultures were indicated by visual and microscopic examination.

### Primers

The primers used and their sequences are listed in Table 1.

### DNA restriction profile

The PCR product was digested with the restriction enzyme *Hae* III in 30  $\mu$ L of reaction volume containing 5 to 10 U for each microgram of DNA. The digestion was incubated at 37°C for 2 hr.

### Preparation of DNA templates for PCR

The DNA templates were prepared from an 18 hr culture on nutrient broth medium. A 200  $\mu$ L sample of each bacterial culture (about  $2 \times 10^6$  bacteria per ml) was centrifuged for 10 min, the supernatants were removed and the pellets were resuspended in 200  $\mu$ L sterile deionized water. The cells were frozen for 5 min, boiled for 5 min, frozen and boiled again (Starnbach et al., 1989).

TABLE 1  
Primers used in PCR methodology

Primer	Sequence 5'-3'	Position	Target gene	Reference
DIP 2A	GGTGTCTTCCTATTCTTTGGC	740-760	<i>cryIVD</i>	Carozzi et al., 1991
DIP 2B	TGACCAGGTCCCTTGATTAC	2010-2030	<i>cryIVD</i>	Carozzi et al., 1991

### Amplification of target sequences

PCR amplifications were carried out in a 50  $\mu$ L reaction volume composed of 5  $\mu$ L DNA template, 1.25 U *Taq* polymerase, 3 mM MgCl<sub>2</sub>, and 20 pmol of each primer.

### PCR conditions

A DNA thermal cycler was used for the amplification and the parameters for amplification reaction consisted of 30s at 94°C (denaturation), 30s at 48°C (primer annealing) and 1 min at 72°C (primer extension-polymerization). A total of 30 cycles for the amplification was adopted. The PCR products were analyzed by electrophoresis in 1% agarose gel stained with ethidium bromide.

### Bioassay

At first a fast bioassay was conducted. A loop of *B. thuringiensis* colony incubated 72 h at 30°C was homogenized in 1 mL distilled water and added to 9 mL distilled water containing 10 4th instar larvae of *Aedes cantans*. Three cups were used for each colony. With the positive strains a 10<sup>-3</sup> dilution was prepared according to the protocol of the Unité des Bactéries Entomopathogènes, Institute Pasteur, Paris, France.

### Serological test

For the detection of the serovar *israelensis* the serotyping was performed in Unité des Bactéries Entomopathogènes, Institute Pasteur, Paris, France.

## RESULTS AND DISCUSSION

As shown in Fig. 1, 7 strains out of the 10 were positive in the PCR assay with a 1 kb DNA fragment.

The confirmation whether this fragment originates from the gene coding the dipteran crystal protein, was obtained by cutting of the 1 kb PCR product by *Hae* III. Two bands were obtained with the sizes of 670 and 330 bp, respectively (Fig. 2). These bands are well in agreement with the expected size for the *cryIVD* gene. In bioassays each of the 7 strains showed 100% mortality against *Aedes cantans* larvae up to the 10<sup>-3</sup> dilution. By serotyping the 7 mosquitocidal strains belonged to serotype H14 according to their flagellar antigen and were classified as subsp. *israelensis*. The remaining 3 strains which were not mosquitocidal were identified as subsp. *thompsoni* (H12).

As well known, the PCR methodology has many advantages in terms of speed, specificity and scale up.

In this work two points can be considered: first, it was not necessary to extract and purify the DNA, the lysing of the bacterial cells by freeze-boil was a rapid and inexpensive procedure and second, the results showed that the PCR technology using the specific primer for the dipteran group could be a fast and useful tool to determine one subspecies, in this case *israelensis*, of *B. thuringiensis*. The PCR result could be confirmed by bioassays and serotyping studies.

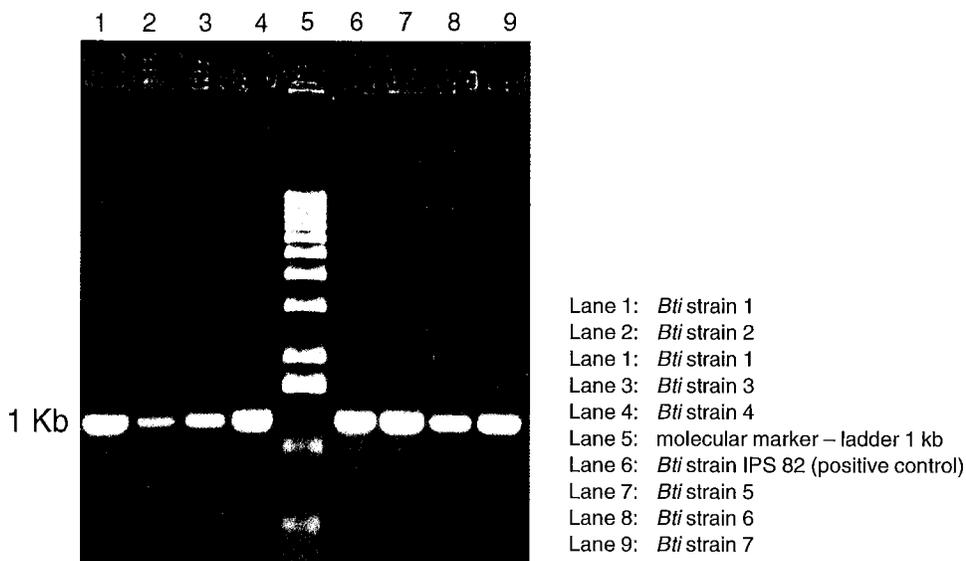


Fig. 1. PCR amplification product of *Bacillus thuringiensis* subsp. *israelensis* (*Bti*) strains.

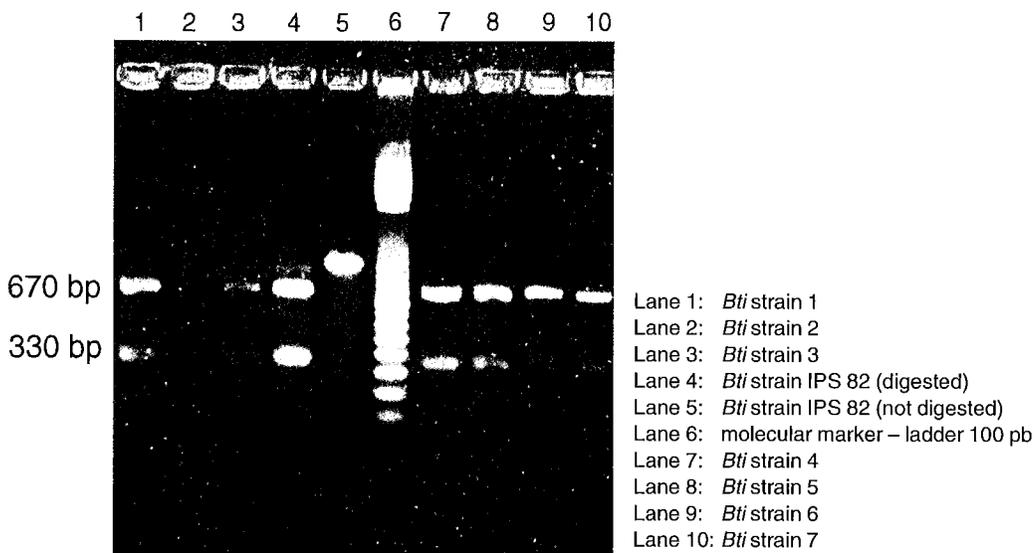


Fig. 2. Restriction profile of PCR product digested with *Hae* III.

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