

Isolation of Lepidopteran Active Native *Bacillus thuringiensis* Strains Through PCR Panning

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Received 28 July 2006 / Accepted 8 January 2007

Abstract. Screening the environment for new and highly potent strains of *Bacillus thuringiensis* (*B. thuringiensis*) has become inevitable as one of the strategies for insect resistance management. By adopting the modified acetate selection method, entomocidally potent *B. thuringiensis* isolates were obtained from grain samples and soil samples from sericulture environment. PCR was performed to determine the insecticidal potential of the isolates. SDS-PAGE analysis of PCR positive isolates exhibited typical Cry1 protein profiles with 130 to 140 kDa protoxin. Preliminary larvicidal assays against *Heliothis armigera* with spore-crystal mixture, showed that all 30 *B. thuringiensis* isolates were toxic to this species. Two isolates, namely BTRX24 (*B. thuringiensis* RathinamXavier 24) and BTRX 28 (*B. thuringiensis* RathinamXavier 28) showed higher mortality compared to other isolates. A dose response efficacy study was conducted in *Heliothis armigera*, *Plutella xylostella* with BTRX24 and BTRX28. BTRX28 showed elevated larvicidal activity, which may be attributed to the presence of multiple *cry* genes and possible synergistic activity among the cry proteins. Periodical introduction of such new strains will play a key role in insect resistance management against *B. thuringiensis* based biopesticides and in transgenic plants.

Keywords. *Bacillus thuringiensis*, biopesticides, PCR, bioassay

INTRODUCTION

Insect pests are a major limiting factor in successful crop production and cause about 15% loss of the total potential crop production annually (Boulter *et al.*, 1989). Overdependence, indiscriminate and uncontrolled use of chemical pesticides has resulted in an irreparable damage to humans. Continuous use of chemical insecticides has led to the emergence and spread of resistance in agricultural pests and vectors of human diseases (Georghiou, 1990) and environmental degradation. A major viable alternative to chemical control of insects is biological control, which is an integral part of Integrated Pest Management (IPM). Of all the microbial agents *Bacillus thuringiensis* has been successfully used as a biocontrol agent. *Bacillus thuringiensis*, is a ubiquitous gram-positive, spore-forming bacterium that forms a parasporal crystal during the stationary phase of its growth cycle. The insecticidal activity of *B. thuringiensis* is attributed to the parasporal crystals, also commonly known

as delta endotoxins or insecticidal crystal proteins (ICP), which are toxic to the larval forms of the insects belonging to the orders Lepidoptera, Diptera, and Coleoptera (Schnepp *et al.*, 1998); but they are harmless to most other organisms, including wildlife and beneficial insects (de Maagd *et al.*, 2001). The insecticidal crystal proteins are codified by *cry* genes and, up to date, more than 200 *cry* genes have been described and classified into a large number of groups and sub groups based on their insecticidal activity and amino acid homology (Hernandez and Ferre, 2005). The proteins toxic for lepidopteran insects belong to Cry1, Cry9, and Cry2 groups; toxins active against coleopteran insects are the Cry3, Cry7 and Cry8 proteins, which have dual activity. The Cry5, Cry12, Cry13, and Cry14 proteins are nematocidal,

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and the Cry2, Cry4, Cry10, Cry11, Cry16, Cry17, Cry19, and Cyt proteins are toxic for dipteran insects (Crickmore *et al.*, 1998). The mode of action of the insecticidal crystal proteins involves a complex and multistep process including, solubilization of the crystals to release cry proteins in their protoxin form, activation of the protoxin by midgut proteases to their active form, and binding of the toxin to the specific receptors on the midgut brush border membrane, followed by pore formation, membrane transport disruption, and cell lysis, ultimately leading to insect death (Herrero *et al.*, 2004). Insects that become resistant to *B. thuringiensis* do so by altering one or more steps of this process. Resistance to *B. thuringiensis* was first reported in *Plodia interpunctella* (Mc Gaughey and Johnson, 1992). Subsequently, resistance to *B. thuringiensis* was reported in field populations of *Plutella xylostella* and in laboratory-selected strains of *Heliothis virescens*, *Spodoptera exegua*, *Trichoplusia ni*, and other species (Van Rie and Ferre, 2000). The use of *B. thuringiensis* as a microbial insecticide offers several advantages over chemical control agents: *B. thuringiensis* has the remarkable ability to produce large quantities of larvicidal proteins, with high host specificity that renders them harmless to nontarget insects and vertebrate and the environment (Valadares *et al.*, 2001). In spite of the overwhelming advantages associated with this biological pesticide, preparations obtained from strains of *B. thuringiensis*, suffer from two major problems, which include narrow host range and development of insect resistance. These problems could be effectively overcome by the introduction of novel *B. thuringiensis* strains exhibiting broad host range and elevated larvicidal potency. Natural isolates of *B. thuringiensis* can produce several different crystal proteins, each of which may exhibit different target specificity (Lambert and Peferoen, 1992) and certain combination of cry proteins have been shown to exhibit synergistic effect (Lee *et al.*, 1996). In the present investigation an attempt has been made to isolate natural *B. thuringiensis* strains from diverse environments with increased potency and broad-spectrum insecticidal activity.

MATERIALS AND METHODS

Bacillus thuringiensis strains. All *B. thuringiensis* strains except *B. thuringiensis* subsp. *galleriae* and isolates used in this study were obtained from Institut Pasteur, Paris, France. These strains are stored as heat shocked spores in 50% sterile glycerol solution at -20°C .

Sample Collection. Soil samples were collected primarily from diverse agricultural fields including the fields in the sericulture environment (where *Bombyx mori* is reared for silk production). Soil samples (each weighing approximately 5 g) were collected, 2 cm below the surface by scraping off surface material with a sterile spatula. These samples were

stored in sterile plastic bags at ambient temperature. Samples (each weighing approximately 2 g) were also collected in sterile plastic vials from poultry feed mills and warehouses, where cereals and pulses are stored. The samples included infected broken grain dust, dead insects, insect webbings and rodent excreta.

Isolation of Bacillus thuringiensis. The selective process for isolation of *Bacillus* spp. involves inhibition of germination of *Bacillus* spores by sodium acetate (0.25 M), while allowing all other organisms to grow in a liquid medium. Subsequent heat treatment of the culture at 80°C for 3 min eliminates all of the nonsporulated microbes. The surviving spores were then plated on an agar plate to grow (Travers *et al.*, 1987). In the present study, as we had the problem of high background of other microorganisms, the selective process was slightly modified. The heat treatment was applied at 80°C for 10 min to eliminate other vegetative forms. Further, an aliquot of 1 ml from the thoroughly mixed, incubated sample was serially diluted to 10^{-9} and plated, to reduce the number of colonies per plate. Sodium acetate selectively inhibits the germination of *B. thuringiensis* spores, which was included in the Luria Bertani (LB) culture medium used for the incubation of samples for 4 h at 30°C on a shaker (200 rpm). Subsequent heat shock of 1 ml aliquot of the broth at 80°C for 10 min (Akiba and Katoh, 1986) to eliminate all vegetative forms. The serially diluted aliquots were plated on LB agar and incubated overnight at 30°C . Based on *B. thuringiensis* colony morphology, colonies were selected at random and inoculated in nutrient broth medium for sporulation. The cultures were then examined under phase contrast microscope for the presence of parasporal crystals.

Protein concentration was determined by Lowry's method using bovine serum albumin as the standard (Lowry *et al.*, 1951). Proteins present in cell extracts were analyzed by SDS-PAGE (Laemmli, 1970).

Polymerase Chain Reaction: Pathotyping PCR. A loopful of cells from a *B. thuringiensis* colony on an overnight nutrient agar plate was transferred to a 0.5 ml microfuge tube containing 100 μl of lysis buffer (10mM Tris- pH 8.0, 1mM EDTA, 1% Triton X-100) and kept on a boiling water bath for 10 min to burst the cells. The tubes were spun at 14,000 x g for 5 min and the supernatant solution containing the total DNA was used as PCR template. The *B. thuringiensis* isolates were prescreened by patho-typing PCR using the primer Lep1A and Lep2B (Carozzi *et al.*, 1991) (Table 1).

A Perkin-Elmer Gene Amp PCR system 9600 thermal cycler was used for DNA amplification. Ten μl of crude lysate was added to 90 μl of PCR mix, containing 1X PCR buffer, 2mM deoxy nucleotide triphosphates, primers, double distilled water and 2.5U Taq polymerase. The contents were mixed by gentle pipetting and overlaid with 30 μl of mineral oil. The step cycle program in the thermal cycler was set to

Table 1. Lepidopteran specific primers used in PCR screening

Primer	Sequence	Reference
Lep 1A	5'CCGGTGCCTGGATTGTGTTA3'	Carozzi <i>et al.</i> , 1991
Lep 2B	5'TACATGCCCTTTCACGTTCC3'	
CryI Specific Primers		Kalman <i>et al.</i> , 1993.
TYIAA	5'GAGCCAAGCAGCTGGAGCAGTTTACACC3'	
TYIAC	5'TCACATCCCATCGACATCTACC3'	
TYIC	5'CAACCTCTATTTTGGTGCAGGTTTC3'	
TYIE	5'CTTAGGGATAAATGTAGTACAG3'	
TYUN12	5'ATCACATGAGTCGCTTCGCATGTTTGACTTTTCTC3'	
TY6	5'GGTCGTGGCTATATCCCTTCGTGTCACAGC3'	
TY14	5'GAATTGCTTTCATAGGCTCCGTC3'	

denature at 95°C for 1min, anneals at 48°C for 1 min and extended at 72°C for 3 min for a total of 35 cycles. Following amplification, the PCR products were electrophoresed in 0.7% agarose gel with ethidium bromide (Sambrook *et al.*, 1989).

CryI sub-typing PCR. The total DNA prepared from the selected isolates was used for PCR analysis. To identify CryI sub type genes, five forward oligonucleotide primers (TYIAA, TYIAC, TYIC, TYIE and TY6) and two reverse primers (TY1UN12 and TY14) were used in separate reaction to amplify *CryIA(a)*, *CryIA(b)*, *CryIA(c)*, *CryIC* and *CryIE* genes. Oligonucleotide primer TYUN 12 is a universal reverse primer that binds to specific sequences of various *CryI*-type gene except the *CryIA(b)*-type gene. TY6 and TY14 were specific to *CryIA(b)* (Kalman *et al.*, 1993) (Table 1).

For PCR amplification 10 µl of the diluted DNA was mixed with the 90 µl of the PCR mix containing 1X PCR buffer, 2 mM deoxy nucleotide triphosphate, distilled water, primer and 2.5 U of Taq polymerase. PCR was performed for 35 cycles with a denaturation temperature of 94°C for 1 min, annealing at 52°C for 1 min and extension at 72°C for 2 min. PCR products were analyzed by 1.2% agarose gel electrophoresis in Tris borate buffer, stained with ethidium bromide.

Bioassay. The larvicidal activity of *B. thuringiensis* isolates was determined using second instar larvae of *Heliothis armigera*, and third instar larvae of *Plutella xylostella* using surface contamination method (Beegle, 1989). The spore crystal suspension in sterile distilled water containing 0.02% TritonX-100 was smeared on the natural diet (Bhendi vegetable) cabbage respectively, and air dried at room temperature. The larvae were fed on this contaminated food at 25°C. In each assay, a standard *B. thuringiensis* strain was included and a negative control treated with sterile water containing Triton X-100 with no bacterial addition. Each assay consisted of six concentrations with 30 larvae tested per concentration. To remove exotoxin, the broth was centrifuged and pellets were resuspended in distilled water. This spore crystal mixture was used for bioassay. Bioassay results were obtained by scoring

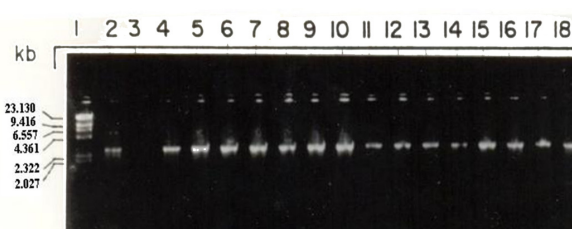


Figure 1. Screening of *B. thuringiensis* strains from environmental samples (Grain storage facilities) through PCR (Patho-type PCR) using CryIgroup specific oligonucleotide primers. Lane 1. Molecular weight standard λ DNA digested with *Hind* III; Lane 2. *B. thuringiensis* subsp. *kurstaki* HD1; Lane 3. PCR mix without template DNA; Lane 4-18. Unknown *B. thuringiensis* isolates (BTRX1 – BTRX15) from grain samples.



Figure 2. Screening of *B. thuringiensis* strains from environmental samples (Sericulture environment) through PCR (Patho-type PCR) using CryIgroup specific oligonucleotide primers. Lane 1. Molecular weight standard λDNA digested with *Hind* III; Lane 2. *B. thuringiensis* subsp. *kurstaki* HD1; Lane 3-17. Unknown *B. thuringiensis* isolates (BTRX16 – BTRX30) from soil samples from sericulture environment; Lane 18. PCR mix without template DNA

larval mortality after 72 h. for *Heliothis armigera* and 48 h. for *Plutella xylostella*. The results of the bioassays were evaluated using probit analysis (Finney, 1971).

RESULTS AND DISCUSSION

Polymerase Chain Reaction. By adopting the specific primed PCR method, unknown isolates were screened for the presence of *B. thuringiensis* ICP genes. The insecticidal activity could be further predicted on the basis of the electrophoretic patterns of the PCR products. The forward primer (Lep1A) and the reverse primer (Lep2B) were designed to probe the most conserved regions of all known *CryI* type gene sequences present in the *B. thuringiensis* isolates. The electrophoretic pattern of the PCR products indicated that all the thirty isolates harbor *CryI* specific genes. The predicted size of the PCR product of 2.7kb indicating the presence of a potentially active anti lepidopteron ICPs (Figure 1 and 2).

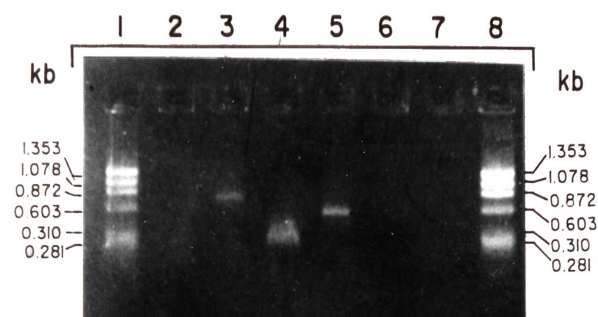


Figure 3. Determination of cry gene content of the entomocidally potent *B. thuringiensis* isolate BTRX-28. CryI subgroup specific oligonucleotide primers corresponding to the genes cryIA(a), cryIA(b), cryIA(c), cryIC and cryIE were used. PCR product profile of *B. thuringiensis* isolate BTRX-28 reacted with CryI subgroup specific oligonucleotide primers. Lane 1. Molecular weight standards Φ X174 DNA digested with *Hae* III; Lane 2. PCR mix without DNA; Lane 3. cryIA(a) primer; Lane 4. cryIA(b) primer; Lane 5. cryIA(c) primer; Lane 6. cryIC primer; Lane 7. cryIE primer; Lane 8. Molecular weight standards Φ X174 DNA digested with *Hae* III.

The insecticidal potential of a *B. thuringiensis* isolate can be better understood at the molecular level by analyzing the gene content rather than by serological or immunological methods. Different PCR primers were used to determine the insecticidal putative crystal protein gene content of previously uncharacterized

B. thuringiensis strains. The patho-typing PCR determines whether a given strain contains genes which are likely to be active against Lepidopterans or Coleopterans. For this purpose *cryI* subtyping PCR was performed and the result suggests the presence of different *cry* genes including *cry IA(a)*, *cry IA(b)* and *cry IA(c)* (Figure 3).

SDS-PAGE. The total protein profile of all the 30 PCR positive isolates were studied using SDS-PAGE. The results (Figure 4) showed that most of the *B. thuringiensis* isolates produced a protein profile similar to that of lepidopteron specific *B. thuringiensis* subsp. *galleriae*. However a few isolates showed varying protein pattern. Isolate BTRX4 from grain samples for example showed a unique protein profile with a major protein of approximately 80-85 kDa (Lane 6, Figure 4). In contrast the protein profile of all the *B. thuringiensis* isolates from the sericulture environment showed typical Cry1 protein profile having approximately 130kDa and 68kDa proteins (Figure 5).

Bioassay. On the basis of PCR amplification and protein analysis, 30 *B. thuringiensis* isolates were selected and subjected to preliminary larvicidal assays against 2nd instar larvae of *Heliothis armigera* at a fixed concentration of 300 μ g/ml of spore-crystal mixture. The results showed that all 30 isolates were toxic to *H. armigera* and mortality ranged from

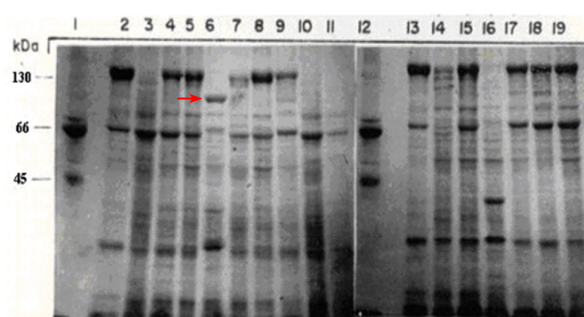


Figure 4. SDS-PAGE analysis of isolates of *B. thuringiensis* strains from stored grain samples. Lane 1. Molecular weight standard; Lane 2. *B. thuringiensis* subsp. *galleriae*; Lane 3-11. *B. thuringiensis* isolates (BTRX1 – BTRX9) from stored grain samples; Lane 12. Molecular weight standard; Lane 13. *B. thuringiensis* subsp. *galleriae*; Lane 14-19. *B. thuringiensis* isolates (BTRX 10 – BTRX15) from stored grain samples.

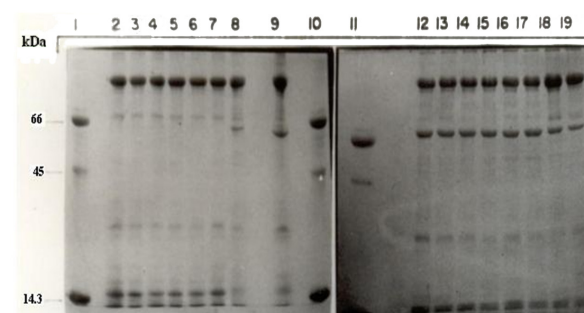


Figure 5. SDS-PAGE analysis of *B. thuringiensis* isolates from soil samples from sericulture environment. Lane 1. Molecular weight standard; Lane 2-8. *B. thuringiensis* isolates (BTRX16 – BTRX22) from soil samples from sericulture environment; Lane 9&13. *B. thuringiensis* subsp. *galleriae*; Lane 10-11. Molecular weight standard; Lane 12-19. *B. thuringiensis* isolates (BTRX23 – BTRX30) from soil samples from sericulture environment

40 to 100%. Interestingly, the *B. thuringiensis* isolates namely BTRX24 and BTRX28 showed higher mortality when compared to other isolates. Again a concentration response efficacy study was carried out to evaluate the larvicidal potency of the two isolates against the second instar larvae of *H. armigera*, and third instar larvae of *Plutella xylostella*, keeping *Bacillus thuringiensis* subsp. *galleriae* as the standard. The bioassay results showed that the isolate BTRX28 exhibited nearly four times higher larvicidal activity against *H. armigera* when compared to *Bacillus thuringiensis* subsp. *galleriae* (Table 2). The results of the bioassay conducted against *Plutella xylostella* revealed that BTRX-24 and BTRX-28 exhibited approximately 12 times and 20 times higher mortality compared to *Bacillus thuringiensis* sub sp *galleriae* (Table 3). The maximum mortality of *Plutella xylostella* occurred within 24 h, whereas for *Heliothis armigera* it was

at 48 h. Further, it was clearly demonstrated that a definite correlation exists between the group specific PCR and larvicidal activity.

B. thuringiensis has been found to occur naturally in diverse habitats (De Luca *et al.*, 1981), such as soil (Martin and Travers, 1989), grain dust (Meadows *et al.*, 1992), sericulture environment, phylloplane of several deciduous and coniferous tree species (Smith and Couche, 1991) and dried tobacco leaves (Kaelin *et al.*, 1994). The selective process to isolate *B. thuringiensis* strains from environmental samples was slightly modified to reduce the occurrence of contaminating organisms and to increase the recovery of *B. thuringiensis* from the samples. In our investigation no *B. thuringiensis* strain could be detected by PCR screening procedure in soil samples from non-sericulture environment. Adverse environmental and climatic factors or the problem of adherence to soil particles may probably be the reasons for the lack of *B. thuringiensis* spores in non-sericulture regions. In contrast, the high frequency of recovery of *B. thuringiensis* in sericulture environment showed the persistent association of this insect pathogen with *Bombyx mori*, over a period of time. Lambart and Peferoen (1992) observed that *B. thuringiensis* is not well adapted to soil environments. In general, the granaries and sericulture environments are rich sources of *B. thuringiensis* and the present study also confirms the above hypothesis. Although there are a number of characterization techniques available for *B. thuringiensis*, such as crystal morphology, flagellar antigens (De Barjac and Bonnefoi, 1962), rapid biochemical tests (Martin *et al.*, 1985), use of monoclonal antibodies (Hofte *et al.*, 1988), DNA probes (Thorne *et al.*, 1986, Prefontaine *et al.*, 1987) none is comprehensive. However, PCR analysis has been proven to be a powerful tool to detect the presence of insecticidal crystal protein genes. Accordingly, a logical combination of different methods at appropriate stages of screening and characterization was adapted to improve the efficiency of isolation and to minimize the chance of losing a novel strain. A four-stage characterization strategy was followed.

- Initial screening by modified acetate protocol
- PCR
- SDS-PAGE analysis
- Insect Bioassay

Although PCR has been an effective method to detect the presence of a specific gene, no single PCR screening method would be adequate to characterize *B. thuringiensis* strains. However, PCR with group specific primers can be used for the preliminary screening. The PCR profile of a majority of the samples from sericulture environments showed, in addition to the *Cry1* specific PCR product, other small molecular weight PCR product(s).

The limitations of the PCR screening, is that if the novel gene did not have any of the primer sequences, no PCR product would be produced and the strain/gene would

Table 2. Insecticidal activity of cry1 *B. thuringiensis* isolates against second instar larvae of *Heliothis armigera*

NO.	<i>Bacillus thuringiensis</i> isolates	LC ₅₀ (µg/ml)	Fiducial Limits (95%) (µg/ml)		Regression equation*
			Lower	Upper	
1.	BTRX-24	12.49	10.17	15.34	Y=3.625+1.254 log X
2.	BTRX-28	7.69	6.07	9.76	Y=3.912+1.228 log X
3.	<i>Bt.g</i> (Standard)	30.72	25.04	37.68	Y=3.305+1.140 log X

*Y=a+bx; where Y=probit; X=dose(µg/ml); a=intercept; b=slope;
Bt.g - *B. thuringiensis* subsp. *galleriae*

Table 3. Insecticidal activity of cry1 *B. thuringiensis* isolates against third instar larvae of *Plutella xylostella*

NO.	<i>Bacillus thuringiensis</i> isolates	LC ₅₀ (µg/ml)	Fiducial Limits (95%) (µg/ml)		Regression equation*
			Lower	Upper	
1.	BTRX-24	0.785	0.569	1.083	Y=5.102+0.968 log X
2.	BTRX-28	0.458	0.297	0.697	Y=5.319+0.931 log X
3.	<i>Bt.g</i> (Standard)	8.958	7.375	10.881	Y=3.774+1.288 log X

*Y=a+bx; where Y=probit; X=dose(µg/ml); a=intercept; b=slope;
Bt.g - *B. thuringiensis* subsp. *galleriae*

be missed. However, PCR with group specific primers has proved to be a reliable tool to specifically screen for entomocidal *B. thuringiensis* isolates. Further characterization by SDS-PAGE can reveal the protein profile and ultimately the insecticidal activity of the selected isolates can be established by bioassay. Future work can include the sequence analysis of the amplified genes to further confirm their identity.

In the present study two entomocidally potent *B. thuringiensis* isolates BTRX24 and BTRX28 have been isolated from diverse environments from Indian soils by adopting a four-step strategy.

The *B. thuringiensis* isolate BTRX28, which showed elevated larvicidal activity can be a promising candidate as a component in integrated pest management (IPM). Moreover, such periodical introduction of new *B. thuringiensis* stains can potentially reduce the incidence of insect resistance. In addition, the full potential of this BTRX28 can be assessed by extending the bioassay with other lepidopteran insect pests.

ACKNOWLEDGEMENTS

We thank Dr.S.Sreeramanan and Ms. Kavitha for their comments and assistance in manuscript preparation.

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