IOSR Journal of Agriculture and Veterinary Science (IOSR-JAVS)
e-ISSN: 2319-2380, p-ISSN: 2319-2372. Volume 7, Issue 12 Ver. IV (Dec. 2014), PP 45-55
www.iosrjournals.org

Distribution of *cry*9 family members in local *Bacillus thuringiensis* isolates from Western India (Vidarbha region)

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Abstract: *The aim of present investigation was to screen the Bacillus thuringiensis (Bt) isolates obtained from local ecological niche of Vidarbha region for the presence of cry9 genes. PCR screening was carried out to determine the presence of cry9A, cry9B, cry9C, cry9D and cry9E genes using gene specific primers. In an attempt to obtain full length gene of cry9 gene family few primers were also designed by using Primer- BLAST software available on NCBI web portal.*

The cry gene content of local Bacillus thuringiensis isolates exhibited an appreciable diversity. The cry9A gene was found in abundant proportion in native Bt isolates. The cry9A gene is the largest clusters of the cry9 gene family showed specificity towards Lepidopteran pests and interestingly having different gut binding receptor than the existing Cry proteins. Considering this, cry9 gene was sequenced and cloned for further characterization. The sequence of cry9 gene family members obtained after sequencing was converted in to FASTA format and was BLAST with available non redundant sequences. The BLAST result confirmed the belonging of sequenced gene to the cry9 family. Similarly, the cry9Aa gene obtained from native Bt isolates showed maximum 88% to 97% similarity with existing cry9 genes, suggest the possibility of isolating new potent cry9 gene(s). These native isolates can be used for the production of more effective Bt based bio pesticides. Also, genes obtained can be explored for the production of next generation insect resistance transgenic plants.

Keywords: *Bacillus thuringiensis, Biological control, Characterization, Cry toxins, cry9, Isolates, PCR*

I. Introduction

Bacillus thuringiensis(Bt) is a gram-positive, spore-forming bacterium that produces parasporal inclusions during the sporulation phase. These inclusions are composed of δ-endotoxins (Cry proteins) which are highly toxic to a wide variety of insect pests and some invertebrates [6], [25]. These toxins show a highly selective spectrum of activity by killing a narrow range of insect species. Due to their high specificity, their safety to most non-target organisms and environment in general, Bt crystal proteins are preferred and as an alternative to chemical pesticides in pest management strategies against insect pests of agricultural crops [16] , [8]. Despite the wide spread use of the crystal protein in pest control it has led to the potential for development of resistance by target organisms to Bt toxins [21], [11], [13], [19]. Evolution of insect resistance to Bt toxins is a worldwide phenomenon with potential for great economic burden, and can reduce the long-term effectiveness of Bt crops, thus making Bt technology less effective [23]. Worldwide, there are several reports of field developing resistance in Heliothine against cry toxins used in first generation transgenic plants [23], [4].

In India also, the scenario of insect resistance against prevailing Bt toxin becomes grave. Pink ballworm, Pectinophora gossipiella has already evolved resistance against Cry1Ac [7]. The resistance allele frequency against Cry1Ac Bt toxin is also increasing in Helicoverpa armigera population collected from some pockets of Punjab [29]. The rapid increase in resistance to insecticides together with the potential adverse environmental effects produced by these chemicals has encouraged the development of alternative methods for Lepidoptera control. The cloning of insecticidal crystal proteins genes and their expression in plant associated micro-organisms or transgenic plants have provided potentially powerful alternative strategies for the protection of crops against insect pest damage. The variability of cry proteins described up to now, it is still necessary to search for more toxins, since a significant number of pests are not controlled with the available Cry proteins. It is also important to provide alternatives for coping with the problem of insect resistance especially with regard to the expression of Bt genes encoding insecticidal proteins in transgenic plants. Identification of new Bt isolates and the search for novel gene encoding new insecticidal proteins will remain a long term objective for pest control.

The cry 1, cry 2 and cry 9 gene groups shows strongest activity against Lepidoptera, the cry 3 and cry 7 gene groups are most toxic to coleopteran; whereas cry4 and cry11 gene groups are highly active against Dipterans. Some cry proteins display toxicity to more than one insect order, like Cry1 protein that is toxic to both Lepidoptera and Coleopteran [24]. and Cry1B protein with activity against Lepidoptera, Coleopteran and Dipterans [28]. cry9 genes are promising tool for effective control and resistance management of many agronomically important lepidopteran species of insect pest [10]. For example, expression of Cry9Ca in

transgenic corn protected the plant against the European corn borer (Ostrina nubilalis). Cry9Ca is significantly more toxic to budworm [2]. Another toxin belonging to the Cry9 group is Cry9Aa, the major crystal component of Bt subsp. Galleriae, which exhibits unique toxicity toward Galleria mellonella larvae. The cryptic gene cry9Ba was found to be localized upstream of cry9Aa. The fourth protein in this group, cry9Da, toxic to scarabaeid larvae of the order Coleoptera, was found in Bt subsp. Japonensis.A new gene cry9Ea, has very recently been discovered in Bt subsp. aizwai SSK-10 [2].

In this study we will summarize work regarding the identification of new insecticidal gene i.e. cry9 genes from local Bt isolates from Vidarbha region , their distribution, sequencing and cloning which might have higher toxicity , more specificity and adaptability than available cry genes against wide range of meets.

II. Material And Methods

2.1 Bacterial strains

Twenty eight local B. thuringiensis isolates (Bt PDKV-01 to 28), isolated from the premises of Dr. Panjabrao Deshmukh Krishi Vidyapeeth, Akola and Nagpur campus of Dr. PDKV, Akola were used in the present investigation. Reference strain HD1 was kindly obtained from Bacillus Genetic Stock Centre, Columbus, Ohio. Similarly nine different subspecies of Bt were obtained from NCIM (National Center for Industrially useful Microbes), NCL, Pune.

2.2 Oligonucleotide PCR primers

Novel cry9 family specific primers and cry9 gene specific primer for different cry9 genes were used for the screening of local Bt isolates for the presence of these genes. Considering the economic importance of lepidopteron insect pest, the present study was focused on lepidopteron specific cry9 genes i.e. cry9A, cry9B, cry9C, cry9D, cry9E (Table 1). Different primers were employed to screen the presence of various members of cry9 gene family by targeting the conserved domains of the respective genes.

2.3 Primer designing

In an attempt to obtain full length cry9gene of given Bt isolates primers were designed from the sequences deposited in Gene Bank, NCBI. Primers were designed by primer design software Primer-BLAST which flanks the cry9 genes at open reading frame (ORF). These primers were custom synthesized from private technical support laboratories, GeneOmebio technologies. The detail is given in TABLE 2.

2.4 Total genomic DNA isolation

Genomic DNA was isolated from the Bt isolates as per the method given by [17], [18]. All PCR amplifications were performed by using the gradient thermal cycler (EP gradient, Eppendorf). Each PCR was performed with 25µl of (final volume) mixture containg 100ng of genomic DNA (2µl), 2.5µl of 2mM of deoxynucleoside triphosphate mixture, 2.5µl of 25mM Mgcl2 solution, 1 unit of Taq DNA polymerase, 10µM of 1µl of reverse primer and forward primer. To obtain full length gene primers were designed to amplify conserved regions of cry9 genes in order to amplify all possible cry9 genes. The PCR conditions were as follows for screening, denaturation for 5min at 95^0C, 30 cycles of denaturation 94^0C for 1 min, annealing 52^0C for 45 sec, extension 72^0C 1.5 min and final extension at 72^0C for 15 min. The PCR conditions for the PCR done with other primers were similar, except annealing temperature were set at 52^0C for cry9 gene , cry9A, cry9B and cry9C, 59 for cry9D, 50 for cry9E, 64 and 52 for cry9 full length and cry9A full length gene. Amplified products were confirmed on 1% agarose gel.

2.5 Sequencing of cry9 genes

The PCR product was purified by QIAquick TM minielute PCR purification kit (Quigen make). Purified PCR product i.e. cry9, cry9 like protein gene and cry9Aa gene were sent for the sequencing to the Genomebio Technologies Pvt. Ltd., Pune. Sequence obtained was BLAST against the non redundant nucleotide database by using the nucleotide BLAST programme available on NCBI web portal. Highly similar sequences with lowest expect value were considered for assigning the putative class to the newly sequenced gene.

2.6 Cloning of cry9 genes

cry9 genes were cloned in the cloning vector pTZ57R/T (InsTA clone kit, MBI fermentas). The 3.1 kb PCR product into pTZ57R/T by using standard procedures from Sambrook and Rusell 2007. The vector having gene of interest was transformed into a E.coli host strain (JM109). The clone was confirmed by Blue-White screening and colony PCR with cry9 specific primer which yielded 506 bp product.

2.7 SDS-PAGE analysis

Proteins were analysed by Sodium dodecyl sulphate- polyacrylamide gel electrophoresis with 10% gels and stained with comassive Brilliant Blue (R-250). Prestained molecular marker (#SM 0441) and unstained molecular marker (#SM0431) from MBI fermentas were used as standards.

III. Results

3.1 Screening of Bt native isolates

3.1.1 cry9 gene

Screening of Bt isolates with primer cry9 gene specific primer revealed the presence of 506 bp PCR product. Twelve isolates viz. PDKV-01, PDKV-02, PDKV-05, PDKV-06, PDKV-08, PDKV-09, PDKV-11, PDKV-13, PDKV-15, PDKV-16, PDKV-18 and PDKV-19 showed amplification for cry9 gene with expected size of 506 bp. (Fig.2)

3.1.2 *cry*9Agene

Screening of Bt isolates with primer cry9A gene specific primer revealed the presence of 571 bp PCR product. thirteen isolates viz., PDKV-01, PDKV-05, PDKV-06, PDKV-08 PDKV-09, PDKV-10, PDKV-11, PDKV-13, PDKV-14, PDKV-15, PDKV-16, PDKV-17 and NCIM-2159. Percent abundance data showed more appreciable of cry9A gene i.e. 35.13%.(Fig.3)

3.1.3 *cry*9B gene

For profiling of cry9B gene specific primer was used in the present investigation by PCR method given by Bravo et al., and Silva-Werneck[5], [20,. The strains showing amplification for this primer in Four isolates viz. PDKV-05, PDKV-06, PDKV-08, and PDKV-15, showed amplification for cry9B gene with expected size of 402bp. (Fig.4)

3.1.4 *cry*9C gene

Out of 37, only three isolates viz. PDKV-03, PDKV-04, and PDKV-13 showed amplification of 306bp expected size for cry9C gene. (fig.5)

3.1.5 *cry*9D gene

Method given by Ben-Dove et al for cry9D gene by using specific primer, which amplified at 938bp amplicon was carried out of 37 local Bt isolates and standard Bt strains none showed amplification for cry9D [1]. The PCR profiling was carried out many times by altering annealing temperature, melting temperature, final extension step and stringency condition, however, results remained same. This might be due to difference in habitat, geographical location and other environmental conditions.

3.1.6 *cry*9E gene

The method given by Silva-Werneck [5] for cry9E gene amplification by using specific primer was carried out during PCR Profiling of cry9E gene primer, showed only one positive result of expected size 452bp. Out of 37 Bt isolates only one isolate showed amplification for cry9E viz. PDKV-28. (Fig.6)

3.2 Determination of the *cry*9 gene content of B. thuringiensis local isolates.

PCR screeening of cry9 genes in local Bt isolates showed considerable diversity of cry9 gene content. Overall, cry9 gene was found to be in 32.43% isolates. Among gene specific screening abundance range from 0% to 35.13%. cry9A,cry9B, cry9C, cry9D and cry9E showed the abundance up to 35.13, 10.8, 8.10, 0.0 and 2.7 percent, respectively(Fig.1)

3.3 PCR amplification of *cry*9 full length genes from native Bt isolates

Molecular characterization of 20 Bt isolates with Btk HD-1 as standard reference strain was carried out in order to obtain full length genes. A method given by Safiudin [17] for cry9 gene was used for amplifying cry9 full length gene with designed primers. Out of 20 isolates four isolates viz. PDKV-03, PDKV-04, PDKV-08, and PDKV-20 showed amplification with expected size of 3000bp (fig.7) For obtaining cry9 full length gene specially designed primer cry9 like protein gene was employed for PCR amplification. The cry9 like protein gene primer gave amplification in range of 2500bp to 3000bp (fig no.8). The expected fragment size of PCR product is approximately 3000bp as given by Primer-BLAST software. In PCR amplification, only two isolates viz., PDKV-04 and PDKV-08 showed amplification of 3000 bp and 2900bp respectively (fig no.9). Remaining isolates either did not showed amplification or yielded non-specific amplification. The designed cry9Aa primer

expected to give fragment size of approximately 3500bp. In PCR amplification just two isolates viz. PDKV-04 and PDKV-08 amplified at 3300bp and 3200bp respectively.

3.4 Sequencing of *cry*9 gene and its analysis

The successfully purified PCR product of cry9 gene (PDKV-08), cry9 like protein (PDKV-04) and cry9Aa (PDKV-08) were sent for the sequencing to GenOmebio Technologies Pvt. Ltd., Pune. Single pass sequencing of cry9 gene, cry9 like protein and cry9Aa by using forward primer yielded 470 bp, 480bp, and 470bp sequence respectively. However, it's sequencing through reverse primer yielded 480bp, 470bp, and 170bp sequences respectively for cry9, cry9 like protein and cry9Aa. The accession numbers of these sequences are KF620117 KF620118 & KF620119(TABLE. 4 & 5)

The gene sequence obtained through reverse primer was converted firstly in to complement then reversed by using online search engine http://bioinformatics.org/sms2/rev_comp.html. Thus, complete sequence obtained by clubbing the forward and reverse primer. The sequence of cry9 gene, cry9 like protein gene and cry9Aa genes in FASTA format was BLAST with the non redundant sequences available in NCBI, by employing all default parameters. Top 5 sequences showing similarity. The BLAST result clearly indicates that the new cry9 gene amplicons belongs to the cry9 family genes. On the other hand it showed significant difference than the all existing cry9 genes. This indicates that obtained cry9 gene having different motifs than the available Cry9 toxins which will be useful in developing transgenic technology.

3.5 Cloning of *cry*9 genes

Amplified fragments were purified by using PCR product purification kit (Quigen make) and purified fragments of cry9 from PDKV-04 (2900bp) and cry9Aa from PDKV-08 (3100bp) were cloned in pTZ57R/T by using InsTAclone kit (MBI Fermentas). Successful clones of cry9 genes in pTZ57R/T plasmid were confirmed by Blue-White screening method. Recombinant plasmid containing colonies yielded white colour, while non-recombinant plasmid containing colonies were identified by their specific blue colour. Successful cloning of cry9 gene was also confirmed by specific amplification of clone cry9 amplicon (506bp) by using primer Cry9 gene (5'CACCATCATAAAGTCCATCTTGTG3'(f)

5'GACAAGATTTTGAGCGTCCATAAT3'(r) (fig no.10). Amplification of inserted cry9 amplicon was carried out by using cry9 family specific primer. Further attempts for the full length gene sequencing by primer walking and cloning of open reading frame of cry9 genes are underway.

3.6 SDS-PAGE analysis of different Cry9 proteins

SDS PAGE was carried out to study the presence of different Cry proteins harboured by Bt isolates under study which gave the positive results for cry9 genes in molecular screening. Earlier reports suggest that molecular weight of Cry9protein ranges from 120 kD to 140 kD. SDS-PAGE was performed for the total protein extracted from all 18 local Bt isolates showed several multiple banding due to the partial purification of Cry proteins was detected among strains. The SDS-PAGE electrophoretogram represents that there is much variation amongst the local Bt isolates. It is relevant that a dark band position of the putative PDKV-04, PDKV-05, PDKV-06, PDKV-08, PDKV-09, PDKV-10, PDKV-11, PDKV-17, PDKV-18, PDKV-19, (130kD) appeared much more intense than in all others lanes. Also, remaining isolates showed bands between the ranges of 100 kD-140kD. (Fig.11)

IV. Discussions

The characterization of the native Bt isolates is presented. These isolates have great value, since Vidarbha region has very different climatic conditions with high diversity of insects. We found appreciable genetic diversity amongst local Bt isolates. We determined the presence of different cry9 family member genes within the population of local isolates (TABLE 3.). The cry9A gene was the most frequently found in local Bt isolates i.e. 35.13% (fig no.3). The second most abundant genes in local Bt isolates was cry9B, percent abundance was 10.08% (fig no.4). This distribution of cry9 genes was different from distribution reported for other Bt isolates collection. Ben-Dove et al. and Bravo et al .presented an interesting PCR analysis of 215 Bt strains they found that strains containing cry1 genes were the most abundant; however, strains harbouring cry4 genes were the second most abundant, while strains with cry3 genes were absent. On the other hand, Chak et al. presented a PCR characterization of 225 Bt strains isolated from soil samples from Taiwan that showed a different cry gene distribution. They reported five different profiles of cry genes in their collection. The cry1A genes were the most abundant, followed by the cry1C and cry1D genes; only four strains harboured cry4 genes, and no strains harboured cry3, cry1B, cry1E, or cry1F genes. It is surprising that no cry3 genes were found in any of the Asian Bt strain collections (Bravo et al., Ben-Dove et al. and Chak et al.). The third most abundant gene in the local isolates was cry9C8.1% abundance found in local isolates (fig.5). We did not find any single isolate which harboured the cry9D genes. Seinfinjad et al., (2007) showed that cry9 family gene gave positive

results with universal primer but negative after screening with gene specific. We also got the result in sameline as that of Seinfinjad et al. The cry9E gene was found in only one single isolatei.e. NI VIII(Fig.6). These isolates are good candidates in the search of biocontrol agents with a wide spectrum of host range. Also it is important to mention that number of the isolates harboured more than one cry9 family member suggesting that Bt isolates have high frequency of genetic information and adoptability

Overall, cry9 gene was found to be in 32.43% isolates. The total abundance of cry9 genes in local Bt isolates was moderate and the important thing i.e. PDKV-08 Bt isolate harboured highest cry9 genes, it contains almost 6 genes of cry9 family. Wang et al. (2002) studied the presence of cry9 in Bt obtained from different ecological regions and sources of China and showed only 15.5% of the isolates contained cry9 gene. Fuping et al., (2003) screened 310 Bt isolates for the presence of cry9 genes and obtained only 15.5% of the isolates containing cry9 gene. Seifinjad et al., (2007) characterized the 70 Bt strains isolated from different agro-ecological regions of Iran. In that study cry9 gene abundance was 30%. These reports are in same line with our results of cry9 profiling.

The characterization of the Bt strain collection is also valuable because it may help in the understanding of the role of Bt in the environment. The distribution of Bt strains is ubiquitous, and their direct relationship with specific insects has been questioned by Martin et al. A study aimed to find a correlation between the distribution of cry genes and specific targets is proposed, and in order to draw clear conclusions on a worldwide scale, it would be desirable to analyze the cry gene content of Bt strains from other regions. A correlation between the frequency of active strains and the geographical origin of the samples was presented by Bernhard et al.

New cry9 genes obtained from local Bt isolates showed 88% to 99% similarity with existing cry9 family gene(s). So that these cry9 gene amplicon from PDKV-08 isolate were successfully cloned into pTZ57R/T cloning vector and then transformed into E.coli. host strain (JM-109). The chimeric plasmids were confirmed by Blue-White screening and colony pcr with cry9 family specific primer. We also successfully did protein analysis in which appreciable cry toxin diversity was obtained. This study has also showed the presence of cry9Aa which showed 12% significant difference with existing cry9 genes . It is useful to isolate a new novel cry9 gene for future transgenic programme. This kind of studies yield us a new Bt strain and novel cry genes with higher toxicity, more specificity and adoptability than available cry genes against wide range of insects.

V. Tables& Figures

Table 1.Details of the primers used for PCR screening of the local Bt isolates

Primer name	Gene identified	Product size (bp)	Sequence	Ref.	Annealing temp (^oC)
Cry9 gene	cry9	506bp	5'CACCATCATAAAGTCCATCTTGTG3'(f) 5'GACAAGATTTTGAGCGTCCATAAT3'(r)	Silva-Werneck (2008)	52
Spe cry9A	cry 9A	571bp	5'GTTGATACCCGAGGCACA3'(f) 5'CCGCTTCCAATAACATCTTTT3'(r)	Silva-Werneck (2008)	52
Spe cry9B	cry9B	402bp	5'TCATTGGTATAAGAGTTGGTGATAGAC3'(f) 5'CCGCTTCCAATAACATCTTTT3'(r)	Silva-Werneck (2008)	52
Spe cry9C	cry9C	306bp	5'CTGGTCCGTTCAATCC3'(f) 5'CCGCTTCCAATAACATCTTTT3'(r)	Silva-Werneck (2008)	52
EB-9D	cry9D	938bp	5'GCAATAAGGGTGTCGGTCACTGG3'(f) 5'GTTTGAGCCGCTTCACAGCAATCC3'(r)	Ben-Dove et al. (1999)	59
Cry9E	cry9Ea	452bp	5'ACAGCTCCAACAACTAATAGC 3'(f) 5'CTATCCGCAGTAATTGTGTTC 3'(r)	Silva-Werneck (2008)	50
Cry9 full length	cry9	~3200bp	5'TCTAGACCTATTGTGGGTGTCCGTCAG3'(f) 5''GGATCCTTGCTATACGTTAGGATCCCCTC3'(r)	Safiudin M.(2009)	64

Table 2. Primer specifically designed for present investigation

Sr. no.	Accession number	Primer name	Primer sequence	Annealing temp (^oC)	Amplicon size (kb)
1	AB011496	cry 9 like protein	5'TTGTGGGTGTCCGTCAGATG3'(f) 5' TTGTGGGTGTCCGTCAGATG 3'(r)	54	~3.0
2	GQ2249294	cry9Aa	5'AGAGGCTCTGGATAGCTGGA3'(f) 5' AGAGGCTCTGGATAGCTGGA 3'(r)	54	~3.5

Table 3. Distribution of cry9 genes in local Bt isolates

Sr. No.	Isolate name	Cry9 gene	Cry9A	Cry9B	Cry9C	Cry9D	Cry9E	Total
1	PDKV1	+	+	-	-	-	-	02
2	PDKV2	+	-	-	-	-	-	01
3	PDKV3	-	-	-	+	-	-	01
4	PDKV4	-	-	-	+	-	-	01
5	PDKV5	+	+	+	-	-	-	03
6	PDKV6	+	+	+	-	-	-	03
7	PDKV7	-	-	-	-	-	-	00
8	PDKV8	+	+	+	-	-	-	03
9	PDKV9	+	+	-	-	-	-	02
10	PDKV10	-	+	-	-	-	-	01
11	PDKV11	+	+	-	-	-	-	02
12	PDKV12	-	-	-	-	-	-	00
13	PDKV13	+	+	-	+	-	-	03
14	PDKV14	-	+	-	-	-	-	01
15	PDKV15	+	+	+	-	-	-	03
16	PDKV16	+	+	-	-	-	-	02
17	PDKV17	-	+	-	-	-	-	01
18	PDKV18	+	-	-	-	-	-	01
19	PDKV19	+	-	-	-	-	-	01
20	PDKV20	-	-	-	-	-	-	00
21	NI II	-	-	-	-	-	-	00
22	NI III	-	-	-	-	-	-	00
23	NI IV	-	-	-	-	-	-	00
24	NI V	-	-	-	-	-	-	00
25	NI VI	-	-	-	-	-	-	00
26	NIVII	-	-	-	-	-	-	00
27	NI VIII	-	-	-	-	-	+	01
28	2130	-	-	-	-	-	-	00
29	2513	-	-	-	-	-	-	00
30	2976	-	-	-	-	-	-	00
31	5112	-	-	-	-	-	-	00
32	5110	-	-	-	-	-	-	00
33	2977	-	-	-	-	-	-	00
34	2514	-	-	-	-	-	-	00
35	2979	-	-	-	-	-	-	00
36	2159	-	+	-	-	-	-	01
37	HD-1	-	-	-	-	-	-	00
TOTAL		12	13	04	03	00	01	33

Table 4. Significant alignments of cry9 gene sequence with existing cry9 genes by using nucleotide BLAST

Accession number	Description	Total score	Query cover	E.Value	Max. identity
GQ479198.1	Bt strain SC5(D4) cry9Ba like protein gene , partial CDS	1501	94%	0.0	99%
GQ249293.1	Bt strain SC5(D2) cry9Aa like protein gene complete CDS	1501	94%	0.0	99%
GU299522.1	Bt insecticidal crystal protein cry9Ba gene complete CDS	1501	94%	0.0	99%
GQ249298.1	Bt strain T23001 Cry9Eb like protein gene complete CDS	1213	94%	0.0	93%
GQ249294.1	Bt strain T03C001 cry9Aa like protein gene complete CDS	1102	94%	1e-161	90%

Table 5. Significant alignments of cry9 like protein gene sequence with existing cry9 genes by using nucleotide BLAST

Accession number	Description	Total score	Query cover	E.value	Max.identity
GQ479198.1	Bt strain SC5(D4) cry9Ba like protein gene , partial CDS	1438	81	0.0	99%
GQ249293.1	Bt strain SC5(D2) cry9Aa like protein gene complete CDS	1438	81	0.0	99%
GU299522.1	Bt insecticidal crystal protein cry9Ba gene complete CDS	1438	81	0.0	99%
GQ249297.1	Bt strain T03B001 cry9Eb like protein gene complete CDS	938	81	2e-141	89%
GQ249295.1	Bt strain T03B001 cry9Da like protein gene complete CDS	938	81	2e-141	89%

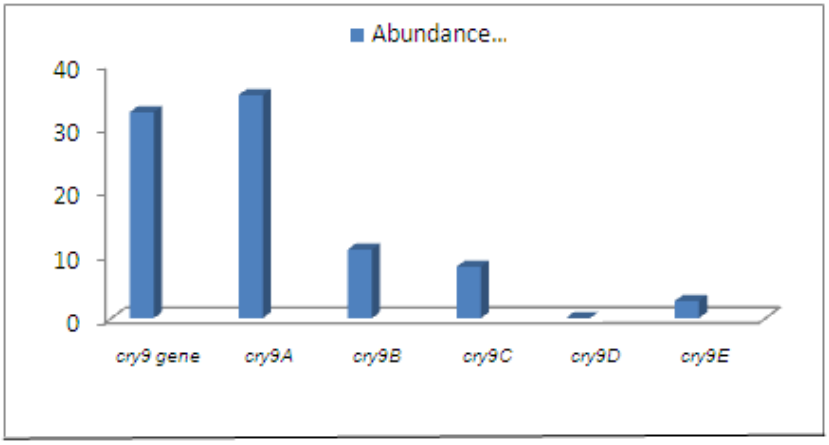

Fig.1 Distribution of cry9 genes obtained from 37 local Bt isolates

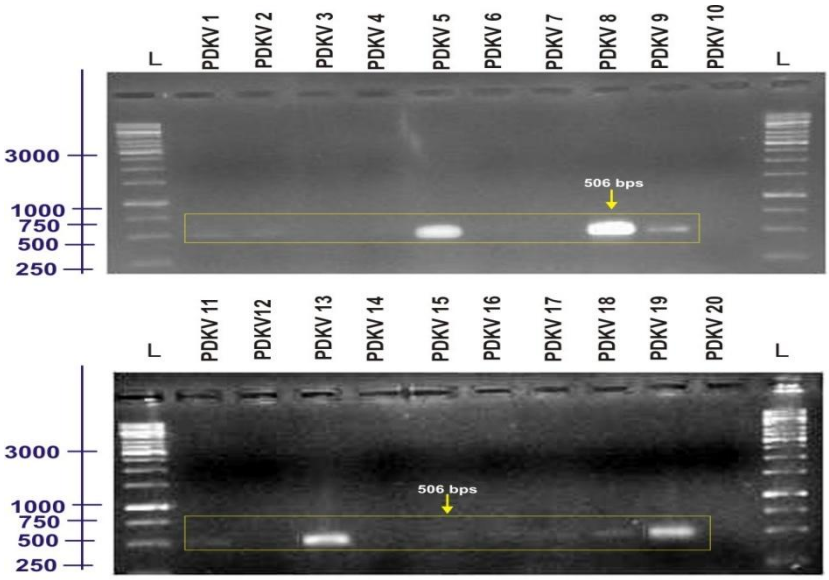

Fig.2 Screening of Bt isolaes for the presence of cry9 gene

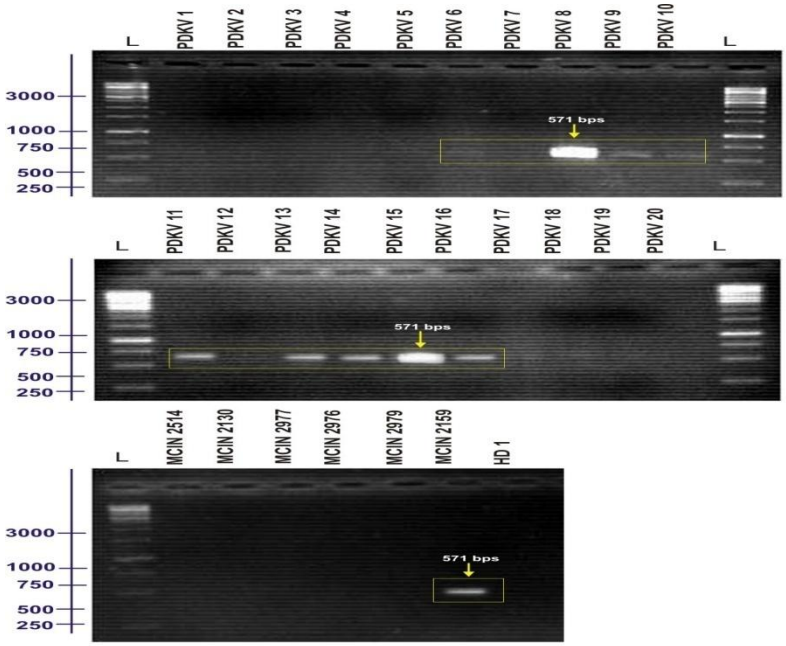

Fig.3 Screening of Bt isolates for the presence of cry9A gene

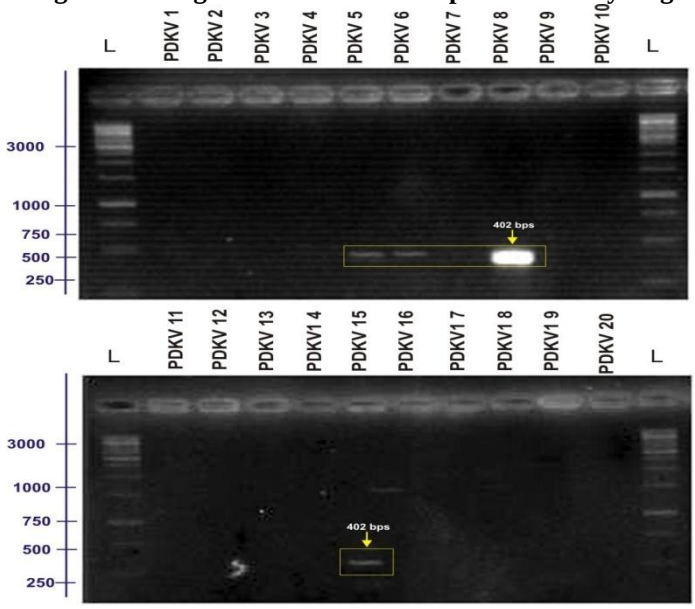

Fig.4 Screening of Bt isolates for the presence of cry9B gene

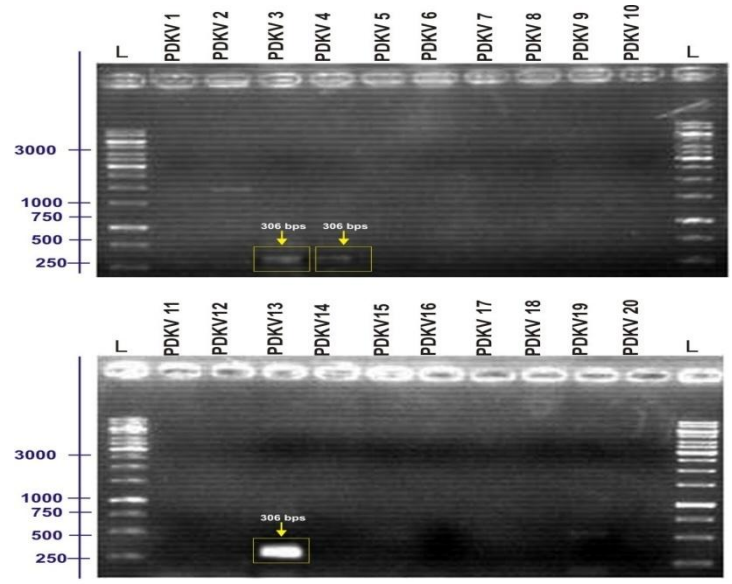

Fig.5 Screening of Bt isolates for the presence of cry9C gene

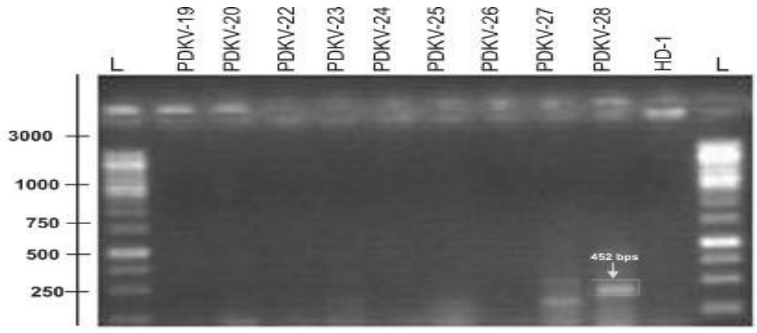

Fig.6 Screening of Bt isolates for the presence of cry9E gene

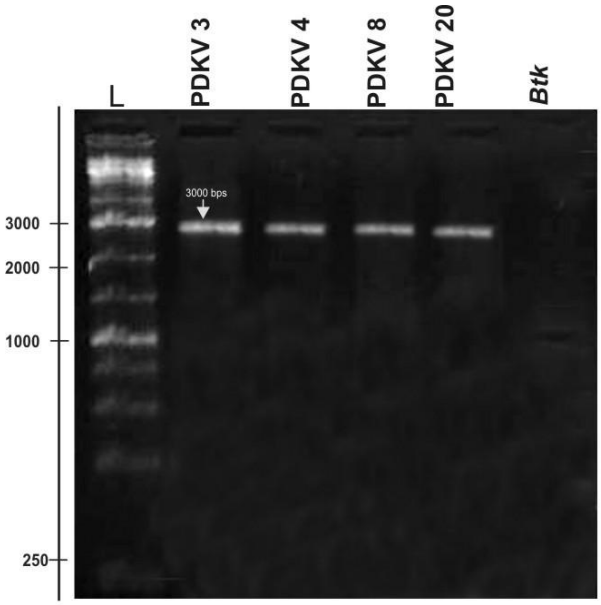

Fig.7 PCR amplification for cry9 full length gene

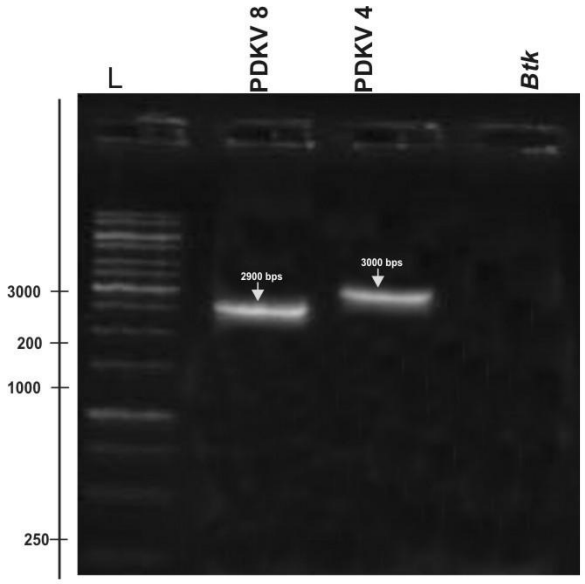

Fig.8 PCR amplification for cry9 like protein full length gene

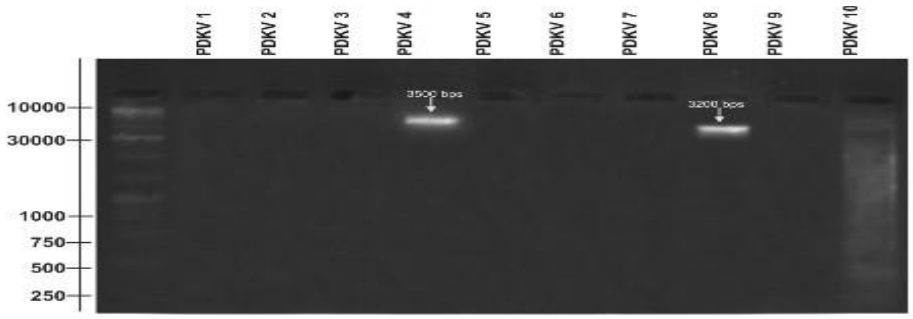

Fig.9 PCR amplification for cry9Aa gene

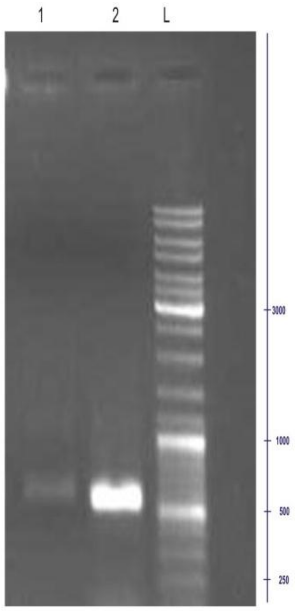

Fig.9 Clone confirmation by PCR

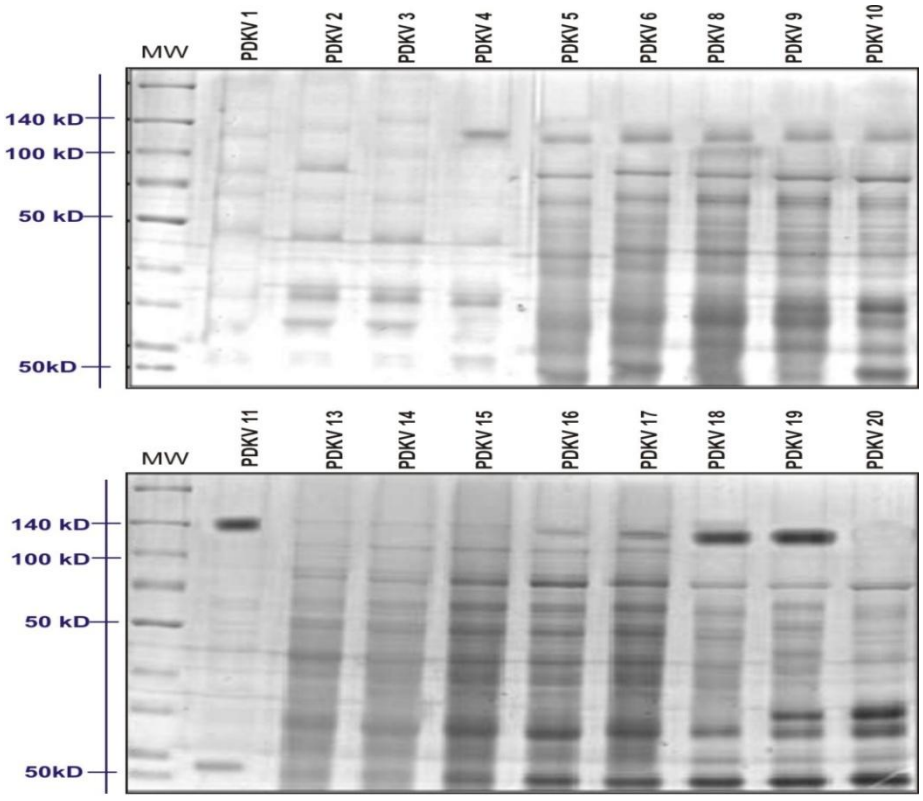

Fig.10 Protein profiling of local Bt isolates

References

[1]. Ben-Dov, E. A. Zaritsky, E. Dahan, Z. Barak,R. Sinai, R. Manasherob, A. Khamraev, E. Troitskaya, A. Dubitsky, N. Berezina and Y. Margalith, Extended screening by PCR for seven cry-group genes from field collectedstrains of Bacillus thuringiensis, Applied and Environmental Microbiology, 63,1997, 4883–4890.

[2]. Ben-Dove, E. Q. Wang, A. Zaritsky, R. Manasherob, Z. Barak, B. Schneider, A. Khamraev, M. Baizhanov, V. Glupov, and Y. Margalith, Multiplex PCR Screening To Detect cry9 Genes in Bacillus thuringiensis Strains,Applied and EnviromentalMicrobiology, 65,1999, 3714-3716.

[3]. K. Bernhard, P. Jarett, M. Meadows, J. Butt, D.J. Ellis and G. M. Roberts, Natural isolates of Bacillus thuringiensis Worldwide distribution, characterization and activity against insect pests,Journal ofInvertebrate Pathology, 70,1997, 59-68.

[4]. A. Bravo and M. Soberón, How to cope with insect resistance to Bt toxins? Trends Biotechnology, 26(10),2008, 573-579.

[5]. A. Bravo, S. Sarabio, L. Lopez, H. Ontiveros, C. Abarca, A. Ortiz, M. Ortiz, L Lina, F. J. Villalobos, G. Pena, M. Munez-Valdez, M. Soberon and R. Uintero,Characterization of cry genes in a Mexican Bacillus thuringiensis strain collection,Applied and Enviromental Microbiology, 64, 1998,4965-4972.

[6]. K. F.Chak, D. C. Chao, M. Y. Tseng, S. S. Kao, S. J. Tuan and T. Y. Feng,Determination and distribution of cry-type genes of Bacillus thuringiensisisolates from Taiwan, Applied Environmental Microbiology,60,1994, 2415–2420.

[7]. A.Chattopadhyay, N.B.Bhatnagar and R.Bhatnagar, Bacterial insecticidal toxins,Crit Rev Microbiol, 30, 2004,33-54.

[8]. S. Dhurua and G. T. Gujar, Field-evolved resistance to Bt toxin Cry1Ac in the pink bollworm, Pectinophora gossypiella (Saunders) (Lepidoptera: Gelechiidae), from India,Pest Management Science, 67(8),2011, 898-903.

[9]. V. K.Frankenhuyzen, The challenge of Bt. pp 1-35. In P. F. Entwistle, J. S. Cory, M. J. Baily and S. R. Higgs (ed.), Bt, an environmental biopesticide: theory and practice. John Wiley and Sons Ltd., Chichester, UK.

[10]. S. Fuping, J. Zhang, G. Aixing, H. Lanlan, H.Kanglai, C. Zhongyi and H. Dafang, Identification of cry1I-Type Genes from Bacillus thuringiensis strains and characterization of a novel cry1I-type gene, Applied and Environmental Microbiology, 69,2003, 155–169.

[11]. C.Guoffon, A. V. Vilet, J.V. Rie, S. Jansens, J. L. Jurat-Fuentes, Binding site for Bacillus thuringiensis Cry2Ac toxin on Heliothine brush border membrane vesicles are not shared with cry1A, cry1F and vip3A toxin,Applied and Environmental Microbiology, 77,2011, 3182-3188.

[12]. S. Herrero,B. Oppert and J. Ferré, Different mechanisms of resistance to Bacillus thuringiensis toxins in the indianmeal moth,Applied Environmental Microbiology, 67,2001, 1085-1089.

[13]. K.van Frankenhuyzen, Insecticidal activity of Bacillus thuringiensis crystal proteins,Journal of Invertebrate Pathology 101, 2009, 1 -16.

[14]. Y. B.Liu, B. E. Tabashnik, B. E. Meyer and N. Crickmore, Cross-resistance and stability of resistance to Bacillus thuringiensis toxin Cry1C in diamondback moth,Applied Environmental Microbiology, 67, 2001, 3216-3219.

[15]. J.Liu, F. Song, J. Zhang, R. Liu, K. He, J. Tan and D. Huang, Identification of vip3A-type genes from Bacillusthuringiensis strains and characterization of a novel vip3A-type gene, Lett. Appl. Microbiology, 45(4), 2007, 432-438.

[16]. P. A. W. Martin and R. S. Travers, Worldwide abundance and distribution of Bacillus thuringiensis isolates, Applied Environmental Microbiology,55, 1989,2437–2442.

[17]. J. Y. Roh, J. Y.Choi, M. S. Li, B. R. Jin, Y. H.Je,Bacillus thuringiensis as a specific, safe and effective tool for insect pest,Journal of Microbial Biotechnology, 17,2007, 547-559.

[18]. M. Safiudin, Cloning and Expression of cry1, cry2 and cry9 Genes From Native Bacillus thuringiensis Isolates, M.Sc. Thesis (Pub), University of Agricultural Sciences, Dharwad, 2009.

[19]. Sambrook, and Russell, Molecular cloning: a laboratory manual, 3rd edition, (Cold Spring Harbor Laboratory Press, New York,2007) pp. 1.31-1.162.

[20]. A. H.Sayyed, B. Raymond, M. S. Ibiza-Palacios, B. Escriche, D. J. Wright, Genetic and biochemical characterization of field-evolved resistance to Bacillus thuringiensis toxin Cry1Ac in the diamondback moth, Plutella xylostella,Applied Environmental Microbiology, 70,2004,7010-7017.

[21]. J. O.Silva-Werneck and D. J. Ellar, Characterization of a novel cry9Bb δ-endotoxin from Bacillus thuringiensis, Journal of Invertebrate Pathology, 98, 2008, 320-328.

[22]. B. E,Tabashnik,Y. B. Liu, D. E. Maagd, T. B. Dennehy, Cross-resistance of pink bollworm (Pectinophora gossypiella) to Bacillus thuringiensis toxins,Applied Environmental Microbiology, 66,2000, 4582-4584.

[23]. B. E Tabashnik and Y. Carriere, Field evolved resistance to Bt Cotton bollworm in the U.S. and pink bollworm in India,Southwestern Entomologist, 35(3),2010,417-424.

[24]. B.E.Tabashnik, A.J. Gassmann, D.W. Crowder and Y. Carriere, Insect resistance to Bt crops: evidence versus theory,Nature Biotechnology, 26(2),2008, 199-202.

[25]. Tailor, R., J. Tippett, G. Gibb, S. Pells, D. Pike, L. Jordan and S. Ely, Identification and characterization of a novel Bacillus thuringiensis d-endotoxin entomocidal to coleopteran and lepidopteron larvae, Mol. Microbiology, 6, 1992, 1211–1217.

[26]. G. T. Vilas-Bôas, A.P. S.Peruca, O.M.N. Arantes, Biology and taxonomy of Bacillus cereus, Bacillus anthracis and Bacillus thuringiensis, Can J Microbiology, 53,2007,673-687.

[27]. K. Wilson, Preparation of Genomic DNA from Bacteria,Current Protocols in Molecular Biology, 2.4.1-2.4.5,(1997) ,http://www.peds.ufl.edu/divisions/cellular/zolotukhin

[28]. J. Wang, A. Boets, J. V. Rie and G. Ren, Charcterization of cry1, cry2, andcry9 genes in Bacillus thuringiensis isolates from China,Journal of Invertebrate Pathology, 82,2002,63-71.

[29]. C. Zhong, D. J. Ellar, A. Bishop, C. Johnson, S. Lin and E. R. Hart, Characterization of a Bacillus thuringiensisendotoxin which is toxic to insects in three orders, Journal of Invertibrate Pathology, 76, 2000,131–139.

[30]. Kaur, P. and V. K. Dilawari, 2011. Inheritance of resistance to Bacillus thuringiensis Cry1Ac toxin in Helicoverpa armigera (Hubner) (Lepidoptera: Noctuidae) from India. Pest Man. Science, 67(10):1294-1302.