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Cloning and Characterization of a Novel *cry2Ad5*-Type Gene from HD29 Strain of *Bacillus thuringiensis* subsp. *galleriae*

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ABSTRACT

Bacillus thuringiensis subsp. *galleriae* HD29 (*Bacillus* Genetic Stock Center ID 4G5; Serotype 5a5b) is known to harbor multiple Cry-type genes such as *cry1Cb*, *cry1Db*, *cry1Ga*, *cry2Ab*, *cry2Ac*, *cry9A* and *cry9B*, which produce crystals consisting of 65- and 130-kDa proteins as major constituents. A new *cry2Ad* gene coding for entomocidal crystal protein is being reported. The main objectives were (i) to clone, sequence and express the new *cry2Ad* gene coding for entomocidal crystal protein of *Bacillus thuringiensis* subsp. *galleriae* HD29 (ii) to determine the toxicity of this new toxin to insect pests and (iii) to determine and characterize the salient features of 3D structure of the protein. Routine methodology was adopted for cloning and sequencing of the gene. For expression analysis, *cry2Ad5* gene was sub-cloned in pET22b expression vector and transferred to *E. coli* BL21 (codon plus) for expression. Proteins expression was analyzed on 12% SDS-PAGE. The expressed protein was used for determining insecticidal activity against lepidopteran (*Helicoverpa armigera*, *Spodoptera litura*) and dipteran (*Aedes aegypti*, *Culex quinquefasciatus*) insects. *cry2Ad5* gene was cloned and sequenced (EMBL accession number AM765844). The protein, listed as Cry2Ad5 (https://www.bpprc-db.org/search_database), was expressed in the form of inclusion bodies, was found to be more toxic to *A. aegypti* than *C. quinquefasciatus* larvae. No significant effect was detected against *Helicoverpa armigera*. The deduced amino acid sequence with 633 amino acid residues shows only 96% identical residues when compared with other δ-endotoxins of the Cry2 family. The predicted 3-D model of Cry2Ad5, when compared with other Cry2Ad type proteins, revealed two semi-conserved and one non-conserved substitution in domain I, and only one semi-conserved substitution was found in the pore forming domain II. The receptor binding motif is present in the loops 1 and 2 of Domain II. A non-significant substitution existed in the interdomain region of the protein. The docking model of Cry2Ad5 was found to be forming 161 and 16 hydrogen bonds with Cadherin and APN of *Helicoverpa armigera,* respectively. The new Cry2Ad5 toxin cloned and expressed from *Bacillus thuringiensis* subsp. *galleriae* strain HD 29 is more toxic to *Aedes aegypti* larvae than to those of *Culex quinquefasciatus*.

KEY WORDS: *cry2Ad5* gene, *Helicoverpa armigera*, Genotyping, *Aedes aegypti* and *Culex quinquefasciatus* entomocidal crystal protein, *Bacillus thuringiensis* subsp. *galleriae* strain HD 29

INTRODUCTION

The HD strains of *Bacillus thuringiensis* harbor multiple *cry*-type genes and produces crystals consisting of 65- and 130-kDa proteins as major constituents. Commercial products containing the HD-1 isolate of *Bacillus thuringiensis* subsp. *kurstaki* have been used successfully to control certain lepidopterous pests since the early 1970s. At present, bio-insecticides are available for sale, which contain Btk HD-1 expressing Cry1Aa, Cry1Ab, Cry1Ac, and Cry2Aa proteins or Bt subsp. *aizawai* (Bta) producing Cry1Ab, Cry1C and Cry1D proteins, toxic to *Spodoptera* complex caterpillars including different populations of *Spodoptera frugiperda*, pest on cotton and corn, in the field[1]. Much of

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the insecticidal activity of HD-1 is attributed to the three 130-kDa proteins (CryIA) associated with the bipyramidal crystal[2,3]. However, another class of insecticidal protein(s), P2, also is present in HD-1[4]. The predominant protein that constitutes the P2 cuboidal crystal (now designated Cry2A) has a molecular mass of approximately 65 kDa, and is toxic to both lepidopteran and dipteran insects. Genes encoding Cry2A proteins have been cloned and sequenced from HD-1 and HD-263 of *B. thuringiensis* subsp. *kurstaki*[5,6,7]. Both genes and their deduced amino acid sequences are identical but share little nucleotide or amino acid homology with other Cry-type proteins[2,5]. HD37 and HD1 strain also show high pathogenicity against insect pests of irrigated rice[8].

Alberola *et al*.[9] reported natural strains of *B. thuringiensis* which were toxic to larval and adult stages of *Bactrocera oleae*. The PCR analysis of HD12 strain with subfamily primers showed that that it harbors cry1Bb, *cry1Fb, cry1Ha, cry1J, cry2A* genes, while HD29 strain contained *cry1Cb, cry1Db, cry1Ga, cry2Ab, cry2Ac, cry9A* and *cry9B* genes[10]. These *B. thuringiensis* strains were found toxic to *B. oleae*. The observed toxicity in these strains may be due to these known genes singly, in combination or due to some other unknown genes[9]. Saleem and Shakoori[11] also demonstrated that spores of HD29 as well as some recombinant toxins cloned from this strain have shown high levels of toxicity against various lepidopteran as well as dipteran insects. Here we confirm the presence of an unrevealed *cry2Ad* gene in HD29 strain of *B. thuringiensis* subsp. *galleriae*. Zhang *et al*.[12] have cloned and described the insecticidal activity of *cry2A* gene from *B. thuringiensis*. The main objectives of this study were (i) to clone, sequence and express the new *cry2Ad* gene coding for entomocidal crystal protein of *Bacillus thuringiensis* subsp. *galleriae* HD29 (ii) to determine the toxicity of this new toxin to insects, and (iii) to determine and characterize the salient features of 3D structure of the protein. The present report deals with the cloning, characterization and insecticidal activity of *cry2Ad5* gene from HD29 strain of *B. thuringiensis* subsp. *galleriae*.

MATERIALS AND METHODS

Bacterial isolates

Bacillus thuringiensis subsp. *galleriae* HD29, isolated in Czechoslovakia from *Dendrolimus sibericus* (Bacillus Genetic Stock Center ID 4G5; Serotype 5a5b) was a generous gift from Professor David J. Ellar. This strain was used to clone novel *cry2Ad5* gene. However, nucleotide sequences of *cry2Ab* and *cry2Ac* in this strain have already been reported by Saleem and Shakoori[11].

Cloning and sequencing of cry2Ad *gene*

Total DNA of *Bacillus thuringiensis* subsp. *galleriae* HD29 was isolated according to method described by Kronstad *et al*.[13] To amplify full length *cry2Ad* gene from *Bacillus thuringiensis* subsp. *galleriae* HD29, Primer of *cy2Ac* are used as C and N terminal are same in *Cry2Ad* genes.

Cry2AcT F: 5′ ATGAATACTGTATTGAATAACGGAAG 3′ and Cry2AcT R: 5′ CCTTAATAAAGTGGTGGAAGATTAG 3′.

The gene was cloned and sequenced using protocols as described earlier[11,14]. The recombinant T/A clone containing amplified gene was transferred into *E. coli* DH5α. Recombinant plasmid pTZ/cry2Ad5 was extracted from recombinant colonies of DH5α and double restricted with *Nde*I and *Hin*dIII. Digested fragment of gene was ligated into digested vector pET22b at *Nde*I and *Hin*dIII restriction site. *E. coli* DH5α was then transformed with recombinant vectors pET22/Ad5. Integrity of recombinant vectors was further confirmed by nucleotide sequencing and restriction analysis.

Expression of cry2Ad5 *gene in Escherichia coli (BL21C⁺)*

A single recombinant colony containing the gene for the desired protein in BL21 (codon plus) strain of *E. coli* was then incubated in 50 ml LB broth containing ampicillin (100 µl/ml) and grown at 37 °C overnight with continuous shaking. Then primary culture was seed inoculated (2%) in 100 ml of LB broth (ampicillin100 µg/ml) with continuous shaking at 37 °C until OD_{600} was 0.6–0.8. It was then induced with 0.5mM IPTG for different time periods (2, 4, 6, 8 and 10 h). Afterwards, culture was centrifuged at optimum OD_{600} to extract the protein. Expression levels of the protein were analyzed on 12% SDS-PAGE. In all experiments, BL21C⁺ transformed with non-recombinant pET22b plasmid was preceded in parallel as a negative control.

Purification of Cry2Ad5

The recombinant *E. coli* cells, expressing the recombinant proteins were pelleted down, resuspended in wash buffer (Triton X-100 1.5%, Tris-Cl (pH 8.0) 50 mM, EDTA (pH 8.0) 10 mM, NaCl 200 mM, DTT 2 mM) and sonicated for 15 min at regular intervals (*i.e.* 30 seconds sonication and 2 min sonication round), then centrifuged at 9500 rpm (15,971 rcf) for 15 min at 4°C to harvest inclusion bodies. The pellets were then washed with wash buffer till the supernatant turned out to be clear. Afterward, pellet was suspended in 20ml distilled water and stored at -20°C[11].

Toxicity bioassays against dipteran and lepidopteron species

To evaluate the specificity of Cry protein, bioassays were performed on different species of Lepidoptera and Diptera. Controls were kept without any Cry protein application to compare the treated and untreated larvae.

Mosquito species *i.e. Aedes aegypti* and *Culex quinquefasciatus* as representative of dipteran insects were used for bioassays. All experiments were carried in three replicas. The 2nd-3rd instar larvae of both mosquito species were suspended in 20ml autoclaved distilled water containing various concentration of Cry2Ad5 protein in controlled temperature at 25-27°C. Mortality rate were observed after 48 h.

Lepidopteran species i.e. *Helicoverpa armigera* and *Spodoptera litura* were reared on an artificial diet[15] and after

pupation and adult emergence, moths were fed sugar solution. Neonates of these laboratory reared species were used for toxicity bioassays. Various concentration of Cry proteins used for bioassays for diet overlay and diet incorporation method, respectively[14]. In diet incorporation method, Cry proteins in the form of partially purified inclusion bodies were dissolved in liquid diet before its solidification.

Cry2Ad *gene analysis and clustal X analysis*

Sequences of known Cry2A toxins were retrieved. The amino acid sequences of toxins were aligned to see the level of homologies using Clustal W programme from DNA Databank of Japan (http://www.ddbj.nig.ac.jp/clustalW). The toxin sequences were aligned using CLUSTAL X ver. 1.83 software[16] and phylogenetic tree was produced.

Secondary structure prediction

The bioinformatics tool "Phyre" was used to predict secondary structure of protein as it provides a simple interface to results[17]. The structure was then predicted by giving submitting the FASTA sequence of the query protein in Phyre2 (www.sbg.bio.ic.ac.uk/phyre2). Using Pymol the predicted 3D structure was edited in order to highlight and label the mutations at significant sites *i.e* domains of the protein.

Protein-receptor docking

In-silico protein docking was performed using ClusPro[18] to analyze spatial and biochemical interaction of amino acids of each Cry2Ad5 protein with two Lepidopteran receptors *i.e.* Cadherin and APN of *Helicoverpa armigera* and one Dipteran receptor *i.e.* ALP of *Aedes aegypti*. Following docking, the models were evaluated for the number of bonds and the number of contacting amino acids between proteins and receptor molecules using PDBsum[19].

Statistical analysis

The mortality was determined using Probit analysis.

RESULTS

Cloning and sequencing of the cry2Ad5 *gene*

Full length *cry2Ad5* gene, cloned from *Bt* subsp. *galleriae* HD29 (Supplementary Fig. 1A), was sequenced from both strands and submitted in EMBL accession number AM765844. When compared the sequence of our novel gene from HD29 to all other *cry* genes simultaneously, it was found indeed a subclass of the *cry2Ad* gene. The toxin has been listed as novel gene *cry2Ad5* (https://www.bpprc-db.org/search_database/). The amplified gene of *cry2Ad5* were cloned in pET22b and then transformed into *E. coli* BL21 strain. Restriction analysis confirmed the integrity of recombinant vectors.

Protein production

Recombinant *E. coli* BL21 colonies containing the recombinant plasmid i.e. pET22/Ad5 was grown in LB broth at 37°C and 120 rpm. The OD_{600} of induced cultures with 0.5mM IPTG at 8 h was found to be optimum and used to harvest inclusion bodies of Cry proteins.

Expression of Cry2Ad5 protein and toxicity bioassays with lepidopteran and dipterans species

SDS-PAGE analysis showed that Cry2Ad5 (65kDa) express clearly (Supplementary Fig. 1B). The toxicity assays of Cry2Ad5 partially purified inclusion bodies were determined by bioassays employing dipteran *(Aedes aegypti* and *Culex quinquefasciatus*) and lepidopteran (*Helicoverpa armigera* and *Spodoptera litura*) insects. Cry2Ad5 proteins were almost equally toxic to the insects with an LC_{50} value of 1.2 µg/mL for *A. aegypti* and *C. quinquefasciatus* (Fig. 1A). Mortality started after 48 h. There was no significant toxicity data when Cry proteins were applied on the surface of the solidified diet as partially purified inclusion bodies on *H. armigera*. although diet over lay method is more effective than diet incorporation method (Fig. 1B).

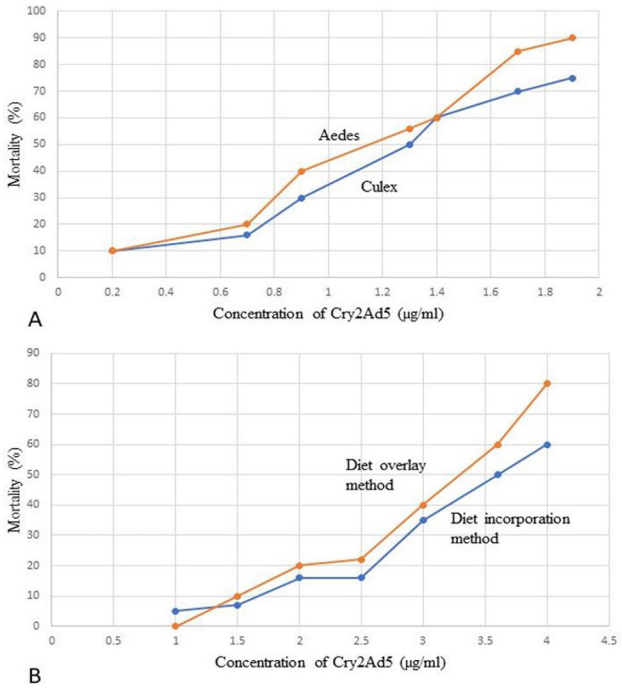

Fig. 1. Effect of different concentrations of Cry2Ad5 protein on mortality of *Aedes aegypti* and *Culex quinquefasciatus* (A) and *Spodoptera litura* (B).

Deduced amino acids sequence and phylogenetic analysis of Cry2Ad

The deduced amino acid sequence of Cry2Ad5 showed 96% of homology with other δ-endotoxins of the Cry2Ad subfamily (Fig. 2A). Figure 2B shows phylogenetic

tree created by CLUSTAL X ver. 1.83 software and TreeView programme. Cry2Ad has two subclusters, one cluster with Cry2Ad1 (Korean origin), Cry2Ad2 (Chinese origin), Cry2Ad3 and Cry2Ad4 (Pakistani origin) toxins, while other cluster has only Cry2Ad5 toxin. So Cry2Ad5 has least similarity with other toxins of same subfamily.

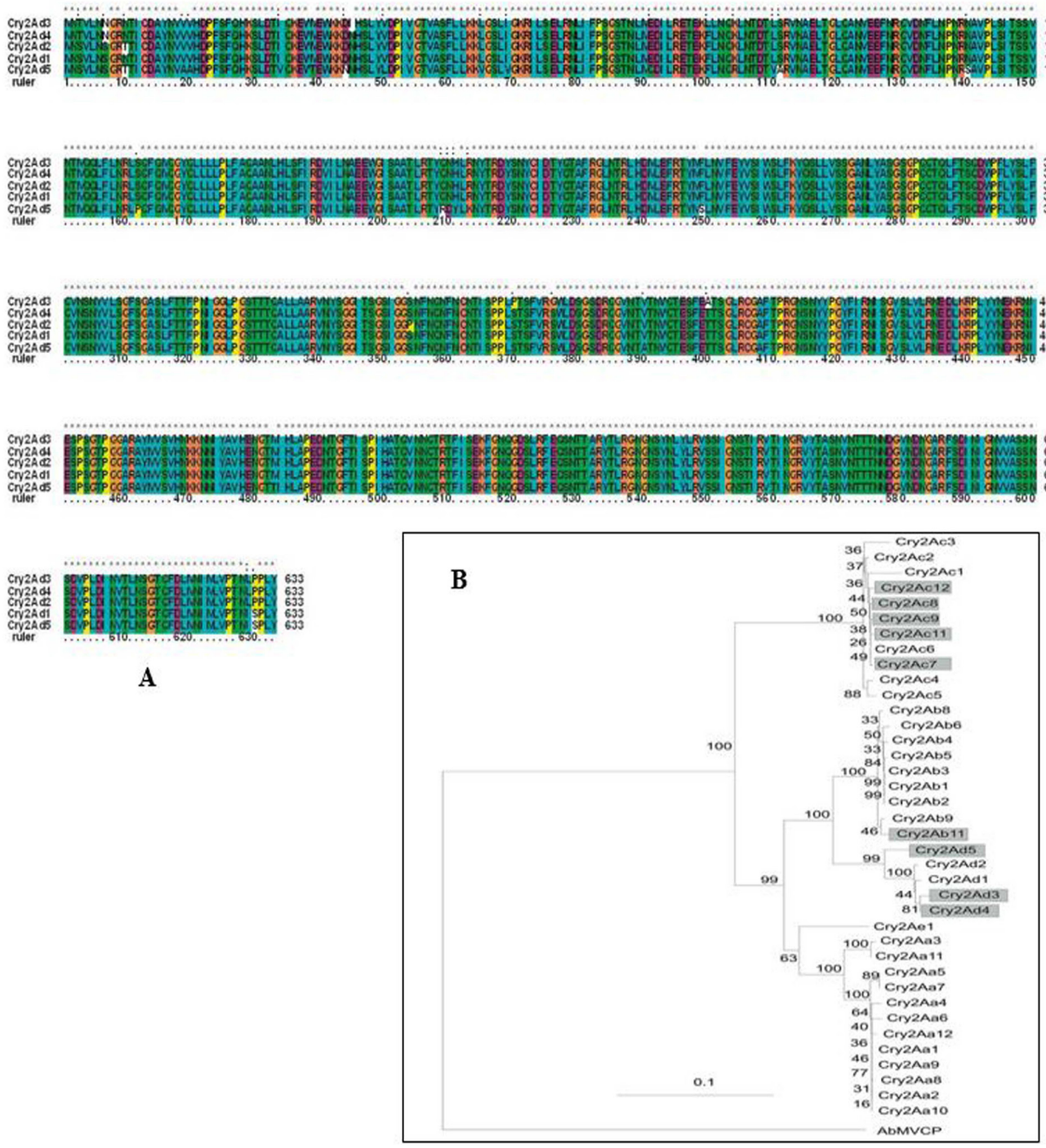

Fig. 2. A) Deduced amino acid sequence homology of Cry2Ad5 with other -endotoxins of the Cry2Ad subfamily. B) Phylogram demonstrating amino acid sequence identity among Cry 2A-type toxins. Toxins reported in the present study (*Cry2Ad5*) and those from Pakistani isolates are highlighted[11]. The Tree is rooted against AbMVCP sequence.

Secondary structure prediction

According to "Phyre" Cry2Ad5 has regular local structure of linear segments of polypeptides chains. It contains 33% alpha helix and 25% beta strands while 26% of the region is disordered (Fig. 3). The predicted model of Cry2Ad5 type protein and labeled at substitutions in comparison with other Cry2Ad type proteins is shown in Figure 4A. Nature of the major substitutions seen in Cry2ad5 in comparison with other subtypes and level of conservation of a substitution are explained in the Table I. The labeled Cry2Ad5 type protein at substitutions at domain levels was described below:

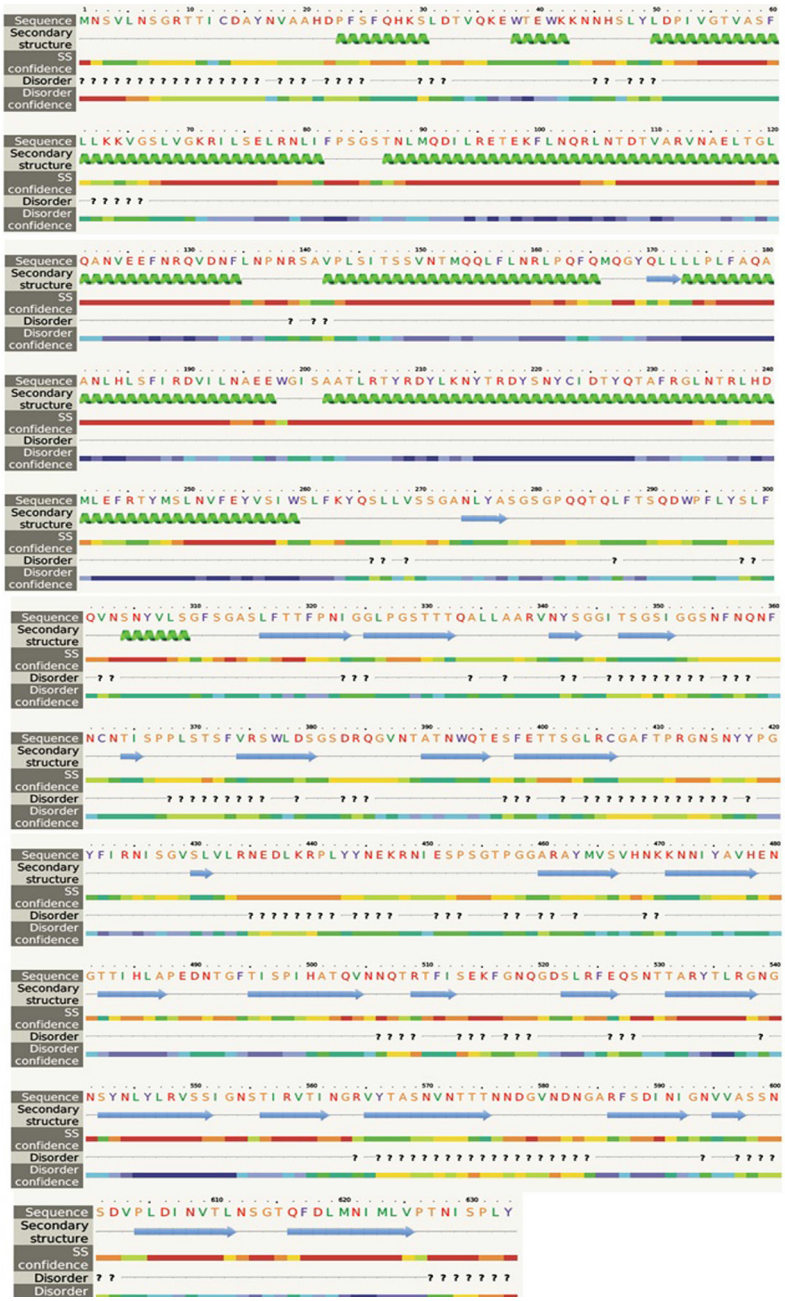

Fig. 3. Deduced secondary structure of Cry2Ad5 using "Phyre[17].

Table I. Comparison of nature of the major substitutions in Cry2Ad5 in comparison with other subtypes.

Sr. No.	Amino acid position	Cry-2Ad1	Cry-2Ad2	Cry-2Ad3	Cry-2Ad4	Cry-2Ad5	Level of conservation of a substitution	Explanation
1	3	S	S	T	T	S	Conserved	
2	7	S	S	N	N	S	Semi-conserved	
3	10	T	N	N	N	T	Semi-conserved	Asparagine and Threonine both are polar amino acids, hence more or less performs the same function[23]
4	19	V	V	V	V	A	Semi-conserved	Being non-polar and uncritical Alanine does not cause any drastic change by substituting Valine which is hydrophobic in nature and both tends to reside in the backbone structures of the protein but alanine is a smaller amino acid while valine is not hence the substitution is semi-conserved.
5	20	V	V	V	V	A	Semi-conserved	As above
6	34	I	I	I	I	V	Conserved	Ile and Val are closely related amino acids both having aliphatic side chains hence perform the same functions. So the substitution is marked as completely conserved.
7	39	M	M	M	M	T	Not conserved	Methionine has an aliphatic functional group and hence is not that reactive while Threonine contains a free hydroxyl group making it fairly reactive. Hence this substitution can have significant effect in protein's structural conformation.
8	44	D	D	D	D	N	Conserved	Substitution of aspartate by asparagine is a conserved one as both are polar and both are very closely related in their structures.
9	45	N	N	I	N	N	Not conserved	
10	50	V	V	V	V	L	Conserved	Valine and leucine share same aliphatic properties and hence the substitution of a Valine residue by a leucine will not cause any major change in the protein
Domain I- Membrane insertion domain								
11	65	L	L	L	L	V	Conserved	As above
12	69	I	I	I	I	V	Conserved	Isoleucine and valine both tend to reside in hydrophobic cores of the protein so performs the same functions causing the substitution to be conserved
13	91	E	E	E	E	Q	Conserved	Glutamate and glutamine are almost perfect substitutions for each other as both are polar and share very similar properties.
14	100	F	F	L	F	F	Conserved	
15	104	K	K	K	K	R	Conserved	Both arginine and lysine are positively charged residues and can replace each other in a peptide chain, conserving each other's function.
16	110	L	L	L	L	V	Conserved	
17	111	S	S	S	S	A	Conserved	Serine and alanine are similar in the aspect that they both are smaller in size, serine is a relatively indifferent amino acid hence its replacement by alanine does not cause major changes in the protein
18	140	N	N	N	N	S	Semi-conserved	Substitution of Asparagine by any amino acid which is not closely related to it can be significant as lying in a particular domain it can have important functions to perform which cannot be replaced by serine as it is a non-polar and relatively non-reactive when compared with Asn. The substitution is though semi-conserved.

Table continued on next page.....

Sr. No.	Amino acid position	Cry-2Ad1	Cry-2Ad2	Cry-2Ad3	Cry-2Ad4	Cry-2Ad5	Level of conservation of a substitution	Explanation
19	162	S	S	S	S	P	Semi-conserved	Proline's unique properties show that it does not substitute well but it may replace small amino acid as in serine here hence the substitution is labeled as semi-conserved.
20	209	Q	Q	Q	Q	R	Conserved	
21	210	N	N	N	N	D	Conserved	
22	211	H	H	H	H	Y	Conserved	
23	213	R	R	R	R	K	Conserved	
24	249	F	F	F	F	S	not conserved	Phenylalanine has an aromatic side chain and hence is a bigger molecule when compared to a small molecule like serine so the replacement can be significant as phenylalanine might have some roles in substrate binding[23] and its substitution by serine may affect the protein's function.
Domain II- Receptor bonding domain								
25	354	S	P	S	S	S	Semi-conserved	
26	370	S	S	P	S	S	Semi-conserved	
27	376	S	S	G	S	S	Semi-conserved	
28	390	V	V	V	V	A	Semi-conserved	Being non-polar and uncritical Alanine does not cause any drastic change by substituting Valine which is hydrophobic in nature and both tends to reside in the backbone structures of the protein but alanine is a smaller amino acid while valine is not hence the substitution is semi-conserved[23]
29	400	T	T	A	T	T	conserved	
Inter domain region								
30	482	M	M	M	M	T	Not conserved	Methionine has an aliphatic functional group and hence is not that reactive while Threonine contains a free hydroxyl group making it fairly reactive[23]
Domain III								
31	629	I	L	L	L	I	conserved	
32	630	S	P	P	P	S	Semi-conserved	Proline's unique properties show that it does not substitute well but it may replace small amino acid as in serine here hence the substitution is labeled as semi-conserved.

Domain I: In the first domain comprising of 51-266th residue[20] there are two semi conserved substitutions N140S, S162P (Fig. 4B) and one non-conserved substitution F249S (Fig. 4C). These substitutions can be significant since they lie in the functional domain of the protein. The positions 140 and 162 can be especially crucial as they lie in the region α4-α5 which are reported to play key role in membrane insertion mechanism[21].

Domain II: In the pore forming domain (267-472) comprising of 3 β-sheets a semi conserved substitution i.e. V390A can be observed (Fig. 4D). The receptor binding motifs lie in the inter loops of β-sheets i.e. 310-313, 367-379, 438-456 are the residues lying in the loop 1, 2 and 3, respectively[22]. The detected substitution does not fall in any of these motifs hence it can be predicted that it might not have any significant effect on the function of the protein. A non-conserved substitution M482T was found in the inter domain region of the protein (Fig. 4E). This may not have any effect on the functional aspects of the protein but can play a role in determining is physical properties as methionine is fairly non-reactive while on the other hand threonine comes under the category of polar and reactive amino acids. But threonine is also known as a fairly indifferent amino acid[23] hence it may not influence any of the properties of protein.

Domain III: No important substitution was found in the third domain i.e. 494-628. But there is a semi conserved substitution in C-terminal extension P630S.

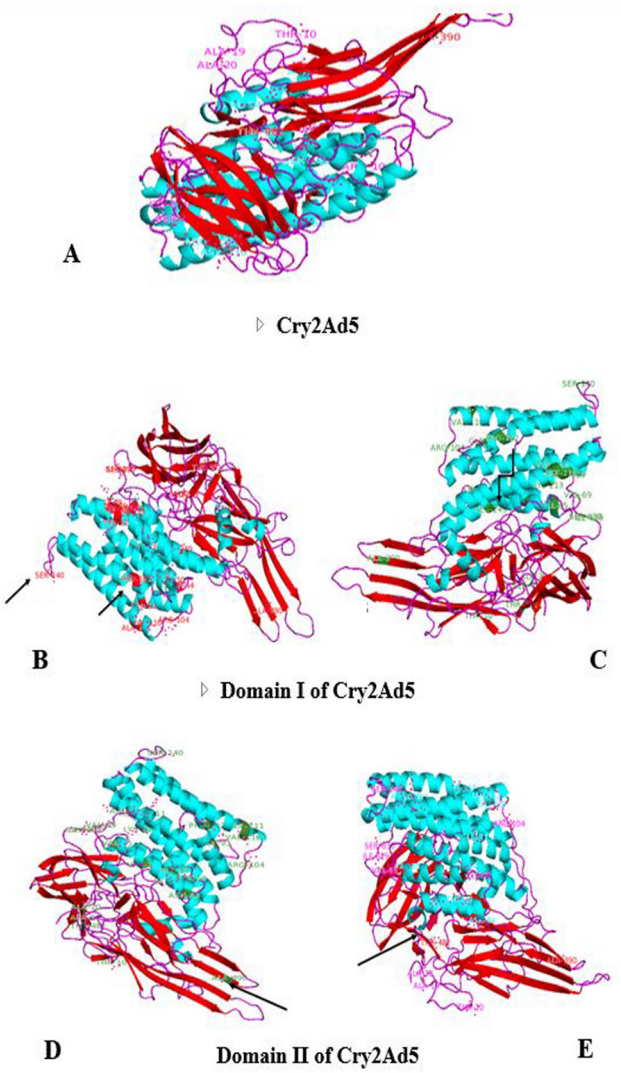

Fig. 4. Predicted models of Cry2Ad5 protein and labeled at substitutions in comparison with other cry2Ad type proteins (predicted through Phyre2 edited through Pymol) (A) showing semi-conserved substitutions N140S and S162P (B) and non-conserved substitution F249S (C) in domain 1, and semi conserved substitution V390A (D) and non-conserved inter-domain substitution M482T (E) in domain II of Cry2Ad5.

Comparison of Cry2Aa protein with Cry2Ad5

Figure 5 shows alignment of cry2Ad5 sequence with the specificity determining regions[24]. Three non-conserved substitutions in block D (307-340) can be recognized (yellow color) that determine dipteran specificity[24] while four non-conserved substitutions shown in blue color are found in the L block (341-412) which determine the dipteran specificity. The non-matching substitutions are shown in magenta color. The changes in the highly conserved block I are highlighted in yellow color (Fig. 5).

Docking of Cry2Ad5 toxins with Helicoverpa armigera *and* Aedes aegypti *receptors*

Using ClusPro online software all the proteins under study were docked with Cadherin (CADR) of *Helicoverpa armigera*. Docking models obtained were then selected on the basis of highest score to analyze them further through PDBsum tool of Expasy. None of the docked models showed the presence of any salt bridges and disulphide bonds. Cry2Ad5 showed that 9 hydrogen bonds forming amino acid residues predicted docked model with Cadherin of *Helicoverpa armigera* (Supplementary Fig. 2). Moreover, Cry2Ad5 toxin was found to be forming 16 and 31 hydrogen bonds with APN of *Helicoverpa armigera* (Supplementary Fig. 3). Cry2Ad4 toxin was found to be forming 19 and 15 hydrogen bonds with ALP correspondingly with alkaline phosphatase of *Aedes aegypti*.

Non-Conserved Blocks

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Cry2Aa:  QVNSNYILSGISGTRLSITFPNIGGLPGSTTTHSLNSARVNYSGGVSSGL 350
Cry2Ad5:QVNSNYVLSGFSGASLFTTFPNIGGLPGSTTTQALLAARVNYSGGITSGS 350
         * * * * *.***.**:.*  **************:.* .*********:.**
Cry2Aa   IGATNLNHNFNCSTVLPPLSTPFVRSWLDSGTDREGVATSTNWQTESFQT 400
Cry2Ad5 IGGSNFNQNFNCNTISPPLSTSFVRSWLDSGSDRQGVNTATNWQTESFET 400
         **.:*.*.****.*. *****.*********.**.** *.********.*
Cry2Aa   TLSLRCGAFSARGNSNYFPDYFIRNISGVPLVIRNEDLTRPLHYNQIRNI 450
Cry2Ad5 TSGLRCGAFTPRGNSNYYPGYFIRNISGVSLVLRNEDLKRPLYYNEKRNI 450
         * .******.:******.*.*********.**.*****.***.**. ***
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Conserved Block

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Cry2A     169 YQLLLLPLFAQAANMHLSFIRDVILNADEW
Cry2Ad5   169 YQLLLLPLFAQAANLHLSFIRDVILNAEEW
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Fig. 5. Structure-based alignment of Cry2A and Cry2Ad5: A, three non-conserved substitutions in block D (307-340) determines dipteran specificity[24] which has been colored yellow while four non-conserved substitutions were found in the L block (341-412) which determines the dipteran specificity and is colored blue. The non-matching substitutions are colored magenta. B, shows the sequence alignment of highly conserved block I of Cry2A and Cry2Ad5.

DISCUSSION

The Cry proteins have been used as bio-pesticide for more than 50 years, and their safety has been demonstrated[25].

In this study *Bacillus thuringiensis* subsp. *galleriae* HD29 (Bacillus Genetic Stock Center ID 4G5; Serotype 5a5b) were used to clone novel *cry2Ad* gene. Identification of *cry* gene content by PCR is the most effective technique in screening large native collections when predicting insecticidal activities of individual strains[26].

The comparison of novel gene sequence from HD29 to all other *cry2A* genes took place with the help of phylogenetic tree. The amino acid sequence identity among Cry2A-type toxins and toxins reported in the present study (Cry2Ad5) and those from Pakistani isolates[11] are highlighted. As it is of great interest to investigate the different *cry2* gene profiles of native *B. thuringiensis* isolates in order to define their distribution, predict their insecticidal activity and detect novel genes or combinations thereof among Pakistani isolates. There are two subclusters of Cry2Ad, one cluster with Cry2Ad1 (Korean origin) and Cry2Ad2 (China origin) toxins, while other cluster has Cry2Ad3 and Cry2Ad4 (Pakistan origin). It is evident from the tree that each of the Cry2 toxins has close amino acid sequence identities and grouped together. While Cry2 toxin isolated from China showed variation and produce two clusters. Cry2Ac2 and Cry2Ac6 formed a separate cluster from Cry2Ac4 and Cry2Ac5. Similarly Cry2Ab3, 4, 5, 6 and Cry2Ab8 produce a different group from Cry2Ab9. *Bacillus thuringiensis* strains are characterized by using a number of different methods to identify their toxicity against different insect orders[27, 28,29]. However, information about the distribution of *cry* genes is still limited and does not cover many distinct geographic areas. There is, therefore, the need to search for novel and more potent strains with new pathogenic spectra and wider host ranges, especially in parts of the world that have not been adequately sampled. Pakistan constitutes one such area which needs to be explored.

Protein function is key to any understanding of the consequences of amino acids substitution. According to "Phyre" disordered region is found in Cry2Ad5. These disordered regions have often been found to be involved in protein function and should be taken into account when analyzing predicted functional sites. "Phyre" used a consensus prediction to obtain a confidence value for the predicted secondary structures. Confidence values are displayed from 0 (low confidence) to 9 (high confidence). Two-state disorder prediction score discovered that whether regions of the query are structurally ordered or disordered. The Cry2A type proteins are known to be toxic for lepidopteran and dipteran classes of insect where Cry2Ab and Cry2Ac are specific for lepidopteran class of insects while Cry2Aa is specific for both lepidopteran and dipteran classes. When protein under study is compared with Cry2A by using bioinformatics approaches, it was predicted that Cry2Ad5 type also contained conserved block 1 which is present in all Cry2A proteins[8,25]. The two substitutions seem to be conserved as methionine and leucine are closely related while glutamate and aspartate can also substitute each other well.

The proteins were docked with the receptor proteins of these insects which are involved in the mode of action of these toxins and hence play a vital role in the resistance gaining mechanism against biopesticides[30]. The docking results of all the models were analyzed and the bonding predicted among the query toxins and receptor proteins contained either hydrogen bonds or non-bonded contact regions among residues. Docking studies on two Lepidopteran receptors *i.e.* Cadherin and APN of *Helicoverpa armigera* and one Dipteran receptor *i.e.* ALP of *Aedes aegypti* helps out to predict experimental proteins had strong tendency to bond to all these receptors[31].

Saleem and Shakoori [11]demonstrated that high toxicity of sporulated *Bacillus thuringiensis* subsp. *galleriae* HD29. Lethal concentrations at 50% (LC_{50}) of HD29 harbor *cry2* gene against *Helicoverpa armigera* (American bollworms) and *Musca domestica* as 62.9 and 479 µg/gm, respectively. It appears that strain HD29 contains a diversity of crystal toxins. Nicholls *et al*. [32]isolated a 49-kDa P2 (CryII) crystal toxin from HD29 and showed that it was active against *Pieris brassicae*, *Aedes aegypti*, and *Anopheles gambiae*[32]. Thus, it appears that strain HD29 contains a diversity of crystal toxins and used as effective bio-pesticides.

CONCLUSION

The amino acid sequence deduced from nucleotide sequence of *cry2Ad5* clone shows 633 amino acid residues having 96% homology with δ endotoxins of Cry2Ad subfamily. The predicted 3-D model of Cry2Ad5, when compared with other Cry2Ad type proteins, show two semi-conserved and one non-conserved substitution in domain I, and one semi-conserved substitution in the pore forming domain II and 19 and 15 hydrogen bonds with ALP of *Aedes aegypti*. The receptor binding motif is present in the loops 1 and 2 of Domain II. A non-significant substitution existed in the interdomain region of the protein. The docking model of Cry2Ad5 was found to be forming 11and 16 hydrogen bonds with Cadherin and APN of *Helicoverpa armigera* respectively. Potential of *Bacillus thuringiensis* HD29 strain as biological control agent should be investigated against wide variety of insect pests.

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Authors' contribution

FS, MSA: Investigation, methodology, validation, writing-original draft. ARS: Conceptualization, investigation, funding acquisition, project administration, resources, supervision, writing-review and editing.

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Statement of conflict of interest

The authors have declared no conflict of interest.

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