# Understanding the basis of specificity of *Bacillus thuringiensis* Cry2A toxins towards *Aedes aegypti*.

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# Work not submitted elsewhere for examination

**I hereby declare that this thesis has not been submitted, in whole or in part or in any other form, to this or any other University for the award of a degree.**

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# Abstract

*Bacillus thuringiensis* is a gram-positive spore forming soil bacterium and one of the most successful, environmentally friendly, intensively used and studied microbial insecticides. The major characteristic of Bt is the production of proteinaceous crystals containing toxins with specific activity against many insects including diptera, lepidoptera and coleoptera. Understanding the basis of specificity of Cry2A toxins of *Bacillus thuringiensis* is important for the risk assessment of novel insecticidal toxins from this bacterium to ensure that they are not detrimental to non-target organisms within the environment. Cry2A toxins are a group of three-domain proteins with highly similar sequences, and this project sought to understand the basis of the specificity of Cry2A toxins against the mosquito *Aedes aegypti*. This was investigated through finding out which domain(s) and /or amino acid motif(s) were crucial for activity. Cry2A toxins in our lab were characterised and expressed, after which bioassays were conducted against *Aedes aegypti*, and several hybrid toxins and mutants were created based on the bioassay results and were used to determine the relationship between amino acid sequence and toxicity through bioassay and bioinformatic analyses. Domain I was found to be responsible for the specificity of Cry2A toxins against *Aedes aegypti*, specifically the 49-amino acids comprising the N-terminal region, which folds back onto domain II. The specificity-determining region was further found to consist of four amino acids (E/RTD) within this N-terminal region. Finally, the mechanism of proteolytic activation of Cry2A by *Aedes aegypti* was studied *in vitro*, leading to a proposed model of proteolytic activation, which was contrary to previously published reports.

# List of abbreviations

A- Ampere

*A. aegypti - Aedes aegypti*

AC -Adenylate cyclase

AMJ- *Aedes aegypti* mid gut juice

APS-Ammonium persulfate

ATP- Adenosine triphosphate

BSA -Bovine serum albumin

Bt - *Bacillus thuringiensis*

Bti- *Bacillus thuringiensis* subspecies *israelensis*

cAMP - 3', 5'- cyclic adenosine monophosphate

$^{0}$C- degree Celsius

Cry - Bt Crystal toxin

Cry2Ac (P) - Cry 2Ab Plasmid

Cry2AcNT - Cry2Ac N-terminus

Cry2Aa (P) - Cry2Aa Plasmid

Cry2AaNT- Cry2Aa N-terminus

Cry2Ab (P) - Cry2Ab Plasmid

Cry2AbNT- Cry2Ab N-terminus

Cry2ADI- Domain I of Cry2A toxin

Cry2AW/O DI- Cry2A toxin without domain I

Cry2AW/ONT-F- forward primer for Cry2A without N-terminus

Cry2AW/ONT-R- reverse primer for Cry2A without N-terminus

Cry2ANT-F- forward primer for Cry2A N-terminus

Cry2ANT-R - reverse primer for Cry2A N-terminus

Cyt- cytolytic

ddH2O - double distilled water

DDT- Dichlorodiphenyltrichloethane

DNA- Deoxyribonucleic acid

DTT- Dithiotretol

*E.coli- Escherichia coli*

EDTA - Ethylenediaminetetraacetic acid

g- Gram

GABA- Gamma amino butyric acid

IPTG- Isopropyl-β-D-1-thiogalactopyranoside

KDa- Kilo dalton

LB- Luria bertani

LC50- Half maximal lethal concentration

LD50- Half maximal lethal dose

M- Molar concentration

ml- Millilitre

mg/ml- Milligram per millilitre

MSA- Multiple sequence alignment

NT - N-Terminus

PBS- Phosphate buffer saline

PCR- Polymérase chain reaction

PKA- Protein kinase A

NT - N-Terminus

OD-Optical density

ORF- Open reading frame

PF - Pore forming

pGEM - pGEM-T Easy vector systems

PKA - Protein kinase A

RGB- Resolving gel buffer

RNA- Ribonucleic acid

SDS - PAGE - Sodium dodecyl sulphate - Polyacrylamide gel electrophoresis

SEM- Standard error of mean

SGB- Stacking gel buffer

TBE - Tris/Borate/EDTA

TEMED- Tetramethylethylenediamine

WHO- World Health Organisation

WT - Wild type

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# 1. General introduction

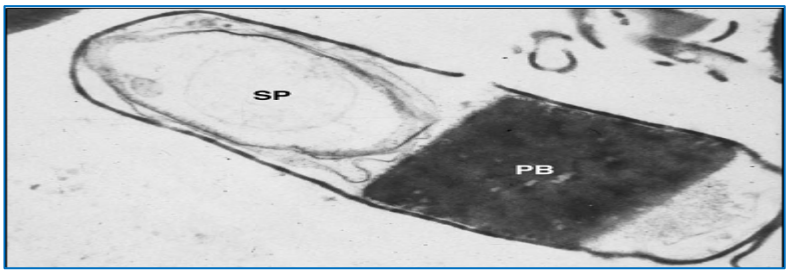
## 1.1 *Bacillus thuringiensis*

*Bacillus thuringiensis* is a gram-positive spore forming bacterium, which is classified in to the *Bacillus cereus* group of *Bacilli* and produces insecticidal toxins in the form of parasporal crystal proteins during its sporulation phase. This unique feature differentiates it from other members in the group (Read *et al*., 2003, Rasko *et al*., 2005).These are predominantly comprised of Cry and Cyt toxins, also referred to as δ-endotoxins (Figure 1.1.1) and are the major virulence factors for this pathogen.

*Bacillus thuringiensis* was originally discovered by a Japanese biologist named Shigetane Ishiwatari in 1902. He isolated it from a diseased silkworm, *Bombyx mori.* It was then formally characterised by Ernst Berliner of Germany in 1915 following its isolation from diseased larvae of *Ephestia kuhniella* (flour moth caterpillars) in Thuringia province, and it was then associated with the cause of a disease named Schlaffsucht (Milner, 1994).

*Bacillus thuringiensis* grows rapidly if the environmental conditions such as availability of nutrients and temperature appear to be favourable, whilst it has been shown that spores formation is activated by both internal and external factors, which include signals for cell density, nutrient starvation, and cell cycle progression (Hilbert and Piggot, 2004).

The toxins found in the crystals are classified into two major families referred to as Cry and Cyt toxins. The Cry (from crystal) toxins belong to a large family, which is currently composed of about 300 different members (Crickmore, 2018).The Cyt (from cytolytic) toxins are generally known to possess cytolytic activity in vitro, a property used in their characterisation, although they are also known to display a primarily dipteran specific activity in vivo (Soberon *et al.*, 2013). The third minor family of proteins are referred to as - the vegetative insecticidal proteins (Vips) because they are secreted from vegetative growing cells and not included in the crystals during sporulation, as such, they are not classified as crystal (de Maagd *et al.*, 2001).




*1.1.1* ***Transmission electron micrograph of a sporulating Bacillus thuringiensis (Bt) cell. PB stands for protein body while SP stands for the spore. Figure from de Maagd et al. (2001)***

## 1.2 The Cry Toxins

Cry toxins are officially defined as proteins that have remarkable sequence similarity to existing toxins within the Bt nomenclature or be a *B. thuringiensis* parasporal inclusion protein that shows pesticide activity, or some toxic effect to a particular organism that can be verified experimentally (Crickmore *et al.*, 1998).

Currently, there are around 75 primary subgroups of Cry toxins in the nomenclature having different primary ranks, such as (Cry1, Cry2, Cry3, etc.). Their lengths vary from, for instance, 369 in Cry34 to 1,344 amino acids found in Cry43 (Adang, 2014).

The naming of toxins is, solely, based on their amino acid sequence identity and does not consider their host specificity. Therefore, Cry toxins that showed activity against the same order of insect will not necessarily have similar names. The name of toxin consists of four different levels, e.g., Cry41Ab1, the first number is referred to as the primary level and toxins that share this number (41 in the above example) will share some significant sequence identity of at least 45%. The order of sequence identity will increase for toxins sharing secondary, tertiary and quaternary level respectively.

Toxins that differ in the quaternary level descriptor only (e.g. Cry41Ab1 and Cry41Ab2) do show at least 95% sequence identity. All toxins that are newly characterised are given a different quaternary level descriptor, and thus some toxins that are identical to others in the nomenclature were assigned different names (Adang, 2014). In their 1989 review, Hofte and Whiteley identified five conserved sequence blocks in many of the Cry toxins, shown in Figure 1.2.1. The 3D-Cry toxins described in figure 1.2.1 share the following characteristics: they encode insecticidal proteins, either of 130 to 140 kDa or of ca. 70 kDa, which have a toxic fragment of 60+10 kDa. One exception is a member of the Cry4

toxins (Cry4D) protein, which has a ca. 30-kDa active core component (Chilcott and Ellar, 1988). For the 130- to140-kDa proteins, the toxic portion is localized in the N-terminal half of the protoxin. Whereas, the C-terminal part of the ca. 130-kDa proteins (Cry1, Cry4A, and Cry4B), extending from the five conserved blocks, is not required for activity, but it appears to be the domain that is highly conserved in these crystal proteins (Hofte and Whiteley, 1989). It is worthy of note to state that within these conserved blocks, no (or relatively few) gaps were required for the alignment of identical or related amino acids. These blocks were divided by highly variable sequences of varying lengths for the different crystal proteins. This property does not apply to Cry proteins which share homology only within block 1 region as can be seen in Figure 1.2.1. Another common characteristic for most crystal proteins is the presence of a stretch of hydrophobic amino acids at a similar position within the 120 N-terminal amino acids. This stretch of amino acids displays the properties of a predicted transmembrane sequence. Remarkably, it is the hydrophobic character of the amino acids and not their identity that is conserved in this, strongly supporting a functional significance (Hofte and Whiteley, 1989). It was proposed that the conserved hydrophobic region is required for interaction between the toxin and the membrane of mid gut epithelial cells (Schnepf *et al*., 1985), but there is no any direct experimental evidence for such interaction.

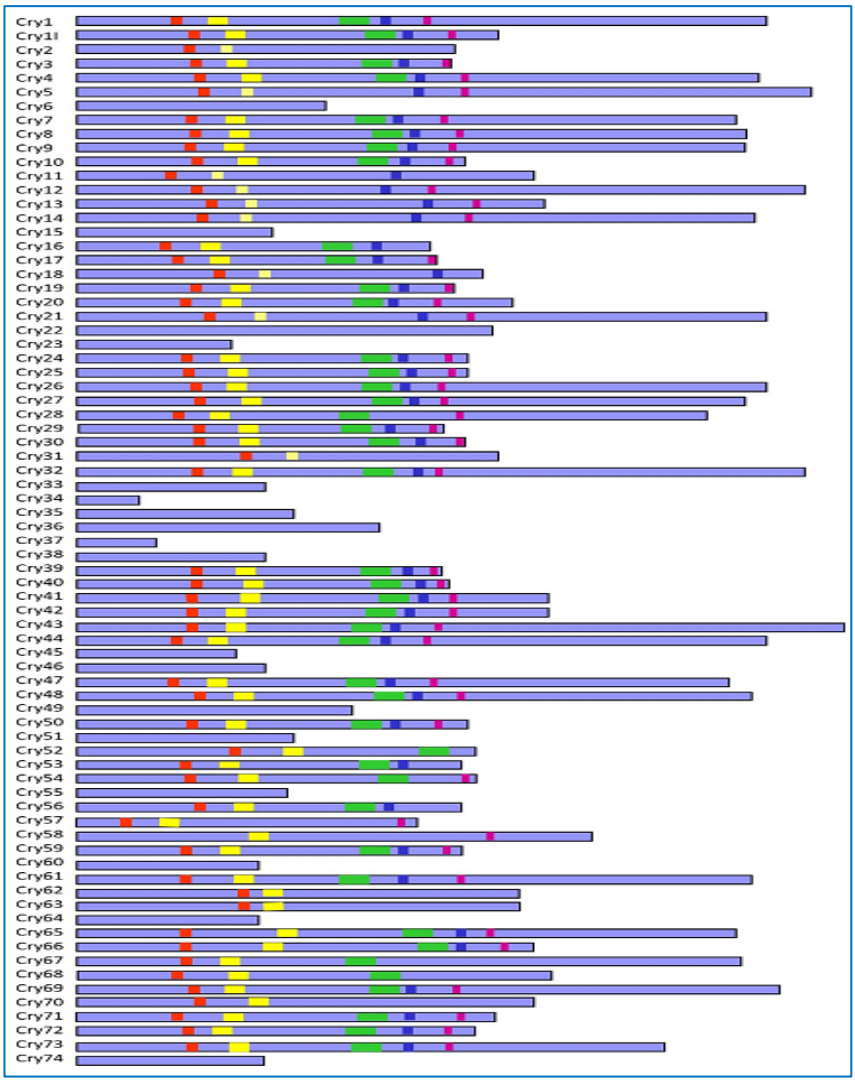



***Figure 1.2.1 Graphical representation of the diversity of Bt Cry toxins. The length of each toxin is drawn to scale and the five conserved blocks described by Hofte and Whiteley (1989) are shown as coloured inserts. Figure taken from Adang (2014).***

Most Cry toxins have three structural domains and share a high level of topological similarity. Domain I is made up of a bundle of seven α-helices connected by loops. There is a central amphipathic α-helix in this α-helical bundle, which is well conserved among all the toxins. Different mutations in domain I appear to affect toxicity but not its ability to bind to cellular receptors. It is not known if these mutations affect the overall conformation of the toxin molecule, thus compromising toxicity. Based on the observation that there are similarities between domain I of Bt and pore forming domains from other bacterial toxins, and the fact that most of the α-helices of domain I are long enough to span a hydrophobic cellular membrane, the involvement of domain I in membrane insertion and pore formation was hypothesized and later proven (Li *et al.*, 1991). Domain II is made up of three sets of antiparallel β-sheets, each of which

terminates with a loop. The beta sheets are packed around a central hydrophobic core, thereby forming a structure called beta-prism. Domain III consists of a sandwich of two antiparallel β-sheets, which form a "jelly-roll" topology. Experimental results from site-directed mutagenesis and truncation analysis provided strong evidence for the involvement of Domain II in receptor binding and oligomerisation (Liu and Dean, 2006), while domain III is also shown to be involved in receptor binding and toxicity (Ibrahim *et al*., 2010).

## 1.3 Mechanism of action of Cry toxins

There are many hypotheses in the literature, which seek to explain how Cry toxins exert their killing activity. These have now given rise to two current models, which describe the mechanisms of action of Cry toxins. The first one is the sequential binding model, which involves pore formation (PF), (Haider and Ellar, 1989, Grochulski *et al*., 1995, Schnepf *et al*., 1998, Bravo *et al*., 2004, Rausell *et al*., 2004, Knowles, 1987). This model, which is for three domain toxins with C-terminal extensions postulates that, on ingestion, a crystal toxin is solubilised by the alkaline environment of the insect's mid gut leading to the release of protoxins initially processed by mid gut proteases. The C-terminal half and about 30 amino acid residues from the N-terminal of CryIA protoxin are removed as a result of the initial cleavage of a Cry1A protoxin by the gut proteases. This is followed by the release of the active toxin monomers, which bind to different receptors such as cadherin, Aminopeptidase N etc. (Atsumi *et al*., 2008, Bel *et al*., 2009, Chen *et al*., 2009, Fabrick *et al*., 2009, Munoz-Garay *et al*., 2009, Obata *et al*., 2009, Pacheco *et al*., 2009, Arenas *et al*., 2010). It is proposed that the initial binding of the activated toxins to the receptors results in some conformational changes, which facilitate a second cleavage by a membrane bound protease leading to the removal of the N-terminal helix α-1. The removal of helix α-1 results in the formation of oligomers, which possess membrane insertion ability (Bravo *et al*., 2004). It was shown that the binding of Cry toxins to the cadherin-like receptors involved some specific interactions of the variable loop regions within domain II and III with cadherin epitopes (Soberon *et al*., 2013).

The oligomerised activated toxin gets bound to the membrane receptors, and is inserted into the apical membrane of mid gut cells, thus causing osmotic shock, bursting of cells within the mid gut and ultimately ending in the death of the insect (Haider and Ellar,

1989, Grochulski *et al*., 1995, Schnepf *et al*., 1998, Bravo *et al*., 2004, Rausell *et al*., 2004, Knowles, 1987). The sequential binding model as proposed by Bravo *et al*. (2004) for Cry1A toxins is presented in Figure 1.3.1 below.

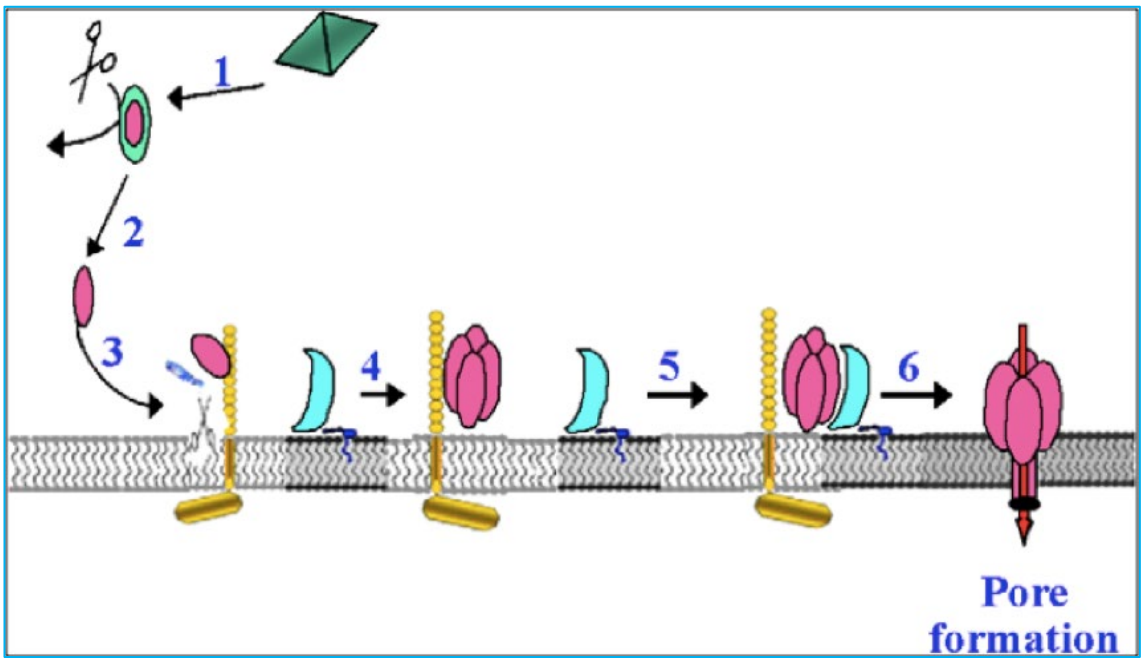



***Figure 1.3.1 Bravo model of the mode of action of Cry1A toxins. 1 Solubilisation of the crystal toxin, 2 Initial cleavage by gut proteases, 3 Binding of toxin monomer to the receptors and its second cleavage by membrane bound protease, 4 Formation of membrane insertion-competent oligomer, 5 Binding of oligomers to receptors, 6 Formation of Lytic pores. Taken from Bravo et al. (2004).***

The second model known as the signalling pathway model, proposed by Zhang *et al*. (2005), which is based on a single system, Cry1Ab, critiques the notion that 'Cry toxins kill cells exclusively by osmotic lyses'. However, they proposed that the binding of Cry1Ab toxin monomer to the cadherin receptor BT-R1 activates a $Mg^{2+}$-dependent signal-transduction pathway, which leads to cell death. It was shown in this model that Cry1Ab oligomers integrated into the cell membrane of living cells do not associate with cytotoxicity (Fig. 1.3.2). They propose that the mechanism of action of Cry toxin is much more complex than the toxin-induced osmotic lysis earlier proposed. The action of Cry toxin is a complex and dynamic process involving the univalent binding of toxin to the highly conserved structural motif in the cadherin receptor BT-R1, which triggers a series of events leading to a programmed cell death known as oncosis.

Molecular signal is induced by the binding of Cry1Ab toxin to the BT-R1 receptor, which stimulates heterotrimeric G protein and adenylyl cyclase leading to a marked increase in cAMP production (Zhang *et al*., 2005). The cAMP activates protein kinase A, resulting

in several kinds of cellular alterations including cytoskeletal rearrangement and ion fluxing. The chemistry of the cell is altered by the acceleration of this second messenger pathway, which results in cell death. In addition, the killing process involves promotion of exocytotic translocation of BT-R1 from intracellular membrane vesicles to the cell membrane by the toxin (Zhang *et al.*, 2005), movement of the receptor is brought about by the toxin-induced signal-transduction, the amplification of which signalling pathway is directly linked to the execution of cell death.

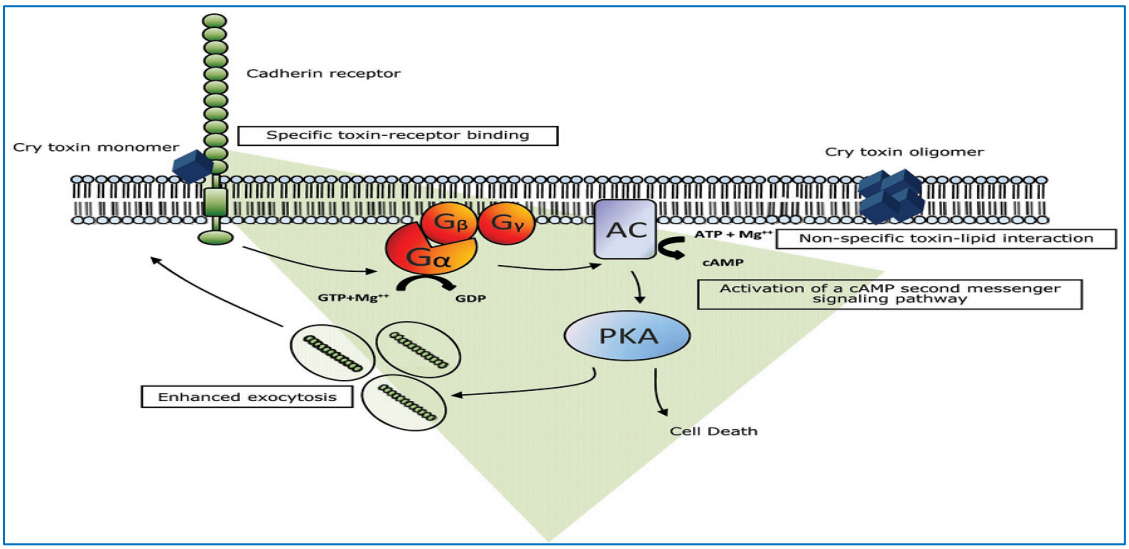



***Figure 1.3.2 The Zhang model for Cry toxin action. The univalent binding of Cry toxin monomer to BT-R initiates cell death by transmitting a death signal into the cell. A signal transduction pathway, involving G protein (Gα) adenylyl cyclase (AC) and protein kinase A (PKA) is activated. Activation of this signalling pathway brings about exocytosis of the BT-R receptor from intracellular vesicles to the cell membrane. The resulting enhanced display of BT-R on the cell surface facilitates recruitment of additional toxin molecules, which, in turn, amplifies the original signal in a cascade-like fashion. The signalling kinase PKA modifies downstream molecules that promote the biochemical activities that destroy the cell. Toxin oligomers incorporated into the plasma membrane of living cells do not form lytic pores and are not toxic (Ibrahim et al., 2010).***

## 1.4 Mode of action of Cry toxins in Mosquitoes

Bt subsp. *israelensis* (Bti) is reported to be highly toxic to different *Aedes*, *Culex* and *Anopheles* mosquito species, which are vectors that transmit human diseases (Margalith and Ben-Dov, 2000). This bacterium produces crystal inclusions comprising of four major toxins namely: Cry4Aa, Cry4Ba, Cry11Aa, Cyt1Aa, and to a minor extent two toxins namely Cry10Aa and Cyt2Ba (Berry *et al*., 2002). (Cry11Aa, Cry4Aa and Cry4Ba, which are mosquitocidal active toxins share structural similarities with the lepidopteran active Cry1Aa, suggesting a similar mode of action of these Cry proteins in mosquitoes as well, just that the receptors may differ. The various receptors of Cry toxins for mosquitoes are shown in Table 1.4-1 below.

| | Glycosylphosphatidylinositol-anchored proteins | | | |
|---|---|---|---|---|
| | Cadherin | Aminopeptidase N | Alkaline phosphatase | α-Amylase |
| *A. aegypti* | | **Cry4Ba** (Bayyareddy *et al.*, 2009) | **Cry4Ba** (Bayyareddy *et al.*, 2009; Thammasittirong *et al.*, 2011; Jimenez *et al.*, 2012) | |
| | **Cry11Aa** (Chen *et al.*, 2009a; Rodriguez-Almazan *et al.*, 2012) | **Cry11Aa** (Chen *et al.*, 2009b) | **Cry11Aa**(Fernandez *et al.*, 2006) | |
| | **Cry11Ba** (Likitvivatanavong *et al.*, 2011) | **Cry11Ba** (Likitvivatanavong *et al.*, 2011) | **Cry11Ba** (Likitvivatanavong *et al.*, 2011) | |
| *A. gambiae* | **Cry4Ba** (Hua *et al.*, 2008) | | | |
| | **Cry11Ba** (Hua *et al.*, 2013) | **Cry11Ba** (Zhang *et al.*, 2008) | **Cry11Ba** (Hua *et al.*, 2009) | **Cry11Ba** (Zhang *et al.*, 2013) |
| *A. quadrimaculatus* | | **Cry11Ba** (Abdullah *et al.*, 2006) | | |
| *A. albimanus* | | | | **Cry4Ba** **Cry11Aa** (Fernandez-Luna *et al.*, 2010a) |

***Table 1.4-1 Cry protein receptors in mosquitoes. Table taken from Zhang et al. (2017).***

## 1.5 Aedes *aegypti* Mosquito

Mosquitoes are major pests in human health because they transmit pathogens such as viruses and parasites through blood feeding, causing serious human diseases including but not limited to malaria, dengue fever, west Nile fever, zika fever, lymphatic filariasis, yellow fever, Japanese encephalitis other forms of encephalitis. These diseases

outbreaks are very frequent in tropical and subtropical regions, where environmental conditions are ideal for mosquito breeding, and result in billions of disease cases and millions of deaths worldwide annually (WHO, 2006).

Among mosquitoes, *Aedes aegypti* is a primary disease vector in urban areas transmitting viruses that cause chikungunya, yellow fever, dengue fever and zika fever (WHO, 2005a, Tomori, 2004, Ligon, 2006, Faucon *et al*., 2017). Yellow fever, for example, is a serious disease in Africa and South America: with 200,000 infections annually resulting in 30,000 deaths despite vaccine usage (WHO, 1998). Dengue fever is a serious arboviral disease of the Americas, Asia, and Africa and causes 1,000 million infections and 25,000 deaths worldwide annually (WHO, 1997). Moreover, there is no effective vaccine for dengue fever and the incidence of dengue fever is on the increase. Therefore, control of its vector, *Aedes aegypti*, is the only reasonable preventive option.

For a long time, attempts to manage *Aedes* mosquitoes have used chemical, biological, and physical methods. Chemical insecticides such as DDT, Malathion, or pyrethroids have been used, the world over, since the 1940s. Physical methods were also attempted in many sites where breeding sites were eliminated or predators were added to remove larvae. This strategy, a combination of insecticide treatment and breeding site elimination, seemed to contribute to the successful control of *Aedes aegypti* (Gomez-Dantes and Willoquet, 2009). Unfortunately, the widespread use of insecticide has resulted in outbreaks of insecticide-resistant *Aedes* mosquitoes in the Americas (Rodriguez *et al*., 2007, Harris *et al*., 2010).Insecticides often kill even non-target organisms and contribute to environmental pollution, while physical methods have limitations in their applications. Therefore, biological methods are now considered an alternative, including the introduction of parasites and predators, or use of pathogens to target mosquitoes. Among the pathogens used to control mosquito larvae are various bacterial strains, including *Bacillus thuringiensis* and *Lysinibacillus sphaericus*. *B. thuringiensis* has high insecticidal activity, low toxicity against non-target organisms, and is environmentally friendly. Thus, strains of this bacterium are used worldwide for the control of *Aedes aegypti* and other mosquito species (Alphey *et al*., 2013).

### 1.5.1 The Life Cycle of Aedes *aegypti* Mosquito.

*Aedes* mosquitoes are container-inhabiting mosquitoes; they often breed in spare tyres, untreated swimming pools, unused flowerpots, and drainage ditches. They grow well in urban areas in close contact with people, which make them to be an exceptionally successful vector. They are very common in areas that lack piped water systems and, which relied mainly on stored water. Both the adult male and female feed on the nectar of plants; however, females feed on blood-meal obtained mainly from humans to produce eggs, and are active during the daytime. Eggs could easily be spread to new locations because they possess the capacity to survive desiccation for long periods.

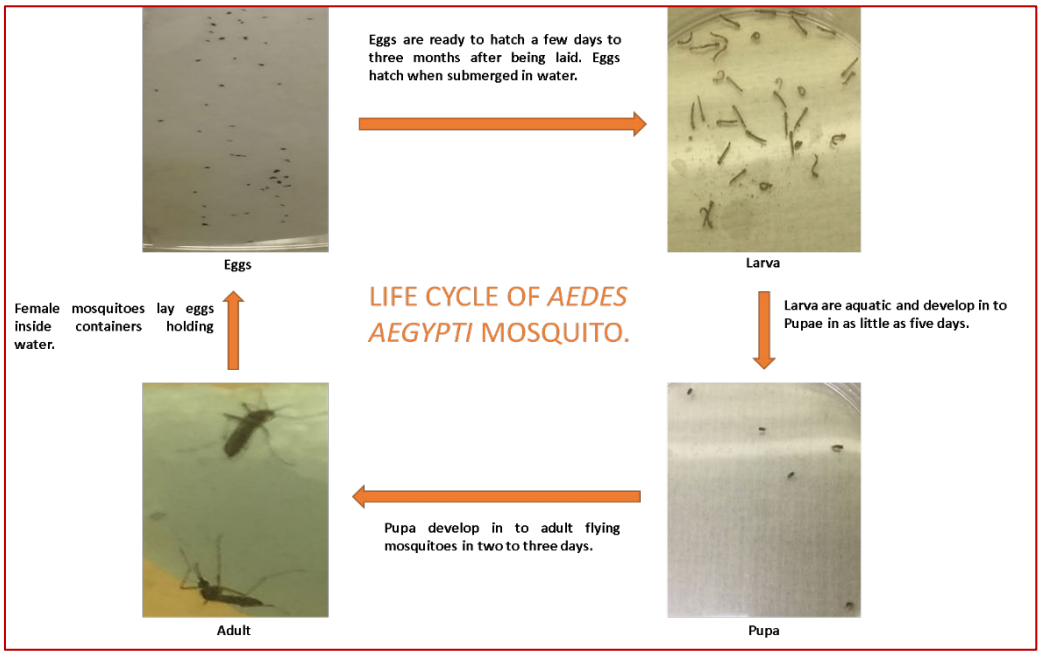



***Figure 1.5.1 Life cycle of Aedes aegypti mosquito. The orange arrows point towards the direction of metamorphosis.***

**Adults**: *Aedes aegypti* is a holometabolous insect, which means that it undergoes a complete metamorphosis; starting from egg, larva, pupa, and to the adult stage. The life span of an adult mosquito can range from two weeks to a month base on the environmental conditions (CDC, 2019). *Aedes aegypti* occur in three polytypic forms: domestic, peridomestic and sylvan. The domestic form usually breeds in urban locations, often around or inside houses. The peridomestic form grows well in environmentally

modified areas such as farms and coconut groves, whereas the sylvan form is a more rural form, which breeds in tree holes and forests (Tabachnick *et al*., 1979).

**Eggs**: The female *Aedes aegypti* mosquitoes produces an average of 100 to 200 eggs per batch after ingesting a blood-meal; however, this number is mainly dependent on the size of the blood meal ingested. During their life time, the females can produce up to five batches of eggs (Nelson, 1986). The eggs are usually laid on damp surfaces in areas that are likely to flood temporarily e.g. tree holes and manufactured containers. The eggs are not laid in mass but rather singly. The laying of eggs can be spread out over hours or days depending on substrates availability, and they are not all laid at once (Clements, 1999). The eggs are, most often times, placed at varying distances above the water line, and the female do not lay the entire clutch at a single site, but rather spread them out over two or more sites (Foster, 2002).

Eggs of *Aedes aegypti* are approximately one millimetre long, smooth, and ovoid shaped. The eggs usually appear white when first laid but they turn to shiny black within few minutes. They can develop within two days in warm climates, such as the one obtained in the tropics, whereas their development can take up to a week in cooler temperate climates (Foster, 2002). *Aedes aegypti* eggs have the ability to withstand desiccation for months and can hatch once submerged in water, making the control of *Aedes aegypti* difficult (Nelson, 1986).

**Larvae:** The mosquito larvae are usually referred to as "wrigglers" or "wigglers," because of their ability to wiggle intermittently in the water when disturbed. They use their posteriorly located siphon, which is usually held above the surface of the water, to breathe oxygen while the rest of the body hangs vertically. Most *Aedes* larvae are easily differentiated from the other genera through their short siphon even by the use of bare eyes (Nelson, 1986). Larvae feed on organic particulate matter such as algae and other microscopic organisms found in the water.

The larvae are often found at homes in puddles, tyres, or within any object that holds water. The development of the larvae is temperature dependent. They pass through four instar stages, spending a short period in the first three, undergoing three moulting

processes, and spending up to three days in the fourth instar stage. The fourth instar larvae are approximately eight millimetres long. The development of the male *Aedes* mosquitoes occur faster compare to the females, so the males generally pupate earlier. At cooler temperatures, *Aedes aegypti* can remain in the larval stage for provided there is sufficient water supply (Foster, 2002).

**Pupae:** This is the next stage of *Aedes aegypti* development after the fourth instar. The pupae of mosquitoes behave different from those of many holometabolous insects because they are mobile and can respond to stimuli. Pupae, are also known as “tumblers," they do not feed and develop in approximately two days. Adults emerge by ingesting air thus expanding the abdomen and splitting open the pupal case, and emerge head first.

### 1.5.2 Current approaches to *Aedes aegypti* population control

Vector-based control has been the global strategy for the control of mosquito-borne diseases for a very long period and the use of chemical insecticides such as DDT, Malathion, or pyrethroids have been the most important components in this effort. However, despite the initial promising results obtained through this method (Gomez-Dantes and Willoquet, 2009), it is being halted by the emergence of insecticide resistance and cross-resistance (Buttler, 2011). Therefore, this has resulted in the re-emergence of mosquito-borne diseases in many parts of the world. In addition, insecticides are usually toxic against non-target organisms and contribute to environmental pollution. Physical methods involving the elimination of mosquito-breeding sites has some limitations in its applications such as:

i. If the mosquito breeding sites in a particular location are large, they can hardly be covered.
ii. Some important species in the ecosystem, which share a habitat with mosquitoes are being destroyed as well, which may lead to their extinction.
iii. Important medicinal plants are also burnt because of bush burning during the control of mosquito breeding sites.

Therefore, biological methods including the introduction of parasites and predators, or use of pathogens to target mosquitoes are now an alternative. The current and most

promising of these methods for mosquito control has been the 'Release of Insects with Dominant Lethality' (RIDL) program (Alphey *et al*., 2013). Although effective in reducing populations by up to 90% (Carvalho *et al*., 2015), there are nonetheless some challenges associated with this method. For example, it can lead to the decline of species that rely primarily on mosquitoes/mosquito larvae as their source of food like the western mosquitofish, *Gambussia affinis* etc. There is also the possibility for development of resistance via assortative mating (Koyama *et al.,* 2003) or overcoming the zygotic killing mechanism (Alphey *et al*., 2011). Although rare, resistance genes could appear and spread rapidly. They are manageable by developing new strains or stacking traits, but this program is expensive to maintain, most especially in developing countries bedevilled by diseases spread by *Aedes aegypti* mosquitoes.

Therefore, the use of spray formulations developed from a crystal toxin produced by *Bacillus thuringiensis*, which are toxic to mosquitoes, very specific in their actions, environmentally friendly, and which have no reported cases of field resistance against *Aedes aegypti,* is considered the only alternative.

### 1.5.3 Factors that affect the susceptibility of *Aedes aegypti* to Cry toxins

The implementation of proper control programs against *Aedes aegypti* and other mosquito species requires that the susceptibility profiles of the target field populations to the intended control agent(s) be carried out. In some cases, laboratory colonies were employed as surrogates to establish susceptibility status. However, such colonies may still underestimate the presence of resistance alleles in the field due to founder and bottleneck effects in maintaining laboratory colonies (Robertson *et al.*, 1995). Therefore, some factors, which may affect the susceptibility to Cry toxins (Bti based insecticides) of *Aedes aegypti* that are the current agents used for the control of mosquito population and to which there is no any case of field resistance reported yet are discussed here.

Susceptibility is species-dependent and relates to the insecticidal proteins. The toxicity of Bti against mosquito larvae is linked to a crystal produced during sporulation, which contains mainly Cry11Aa, Cry4Aa, Cry4Ba and Cyt1Aa toxins. Therefore, for toxicity to occur there must be proper interaction between the Cry toxin and the mosquito larvae in question. As such, anything that could limit these interactions can affect toxicity.

Despite the efficacy of products based on *B*. *thuringiensis israelensis,* they still have some limitations as biopesticides leading to no/or reduced activity on the target organism. One of these is the fact that the crystals settle at the bottom of the water column away from the larval feeding range just within few days of application. This problem could be overcome by the development of live recombinant algae or bacteria that expresses toxin(s) and can remain within the feeding range of the mosquito larvae (Romero *et al*., 2001, Zhang *et al*., 2017). The disadvantage to this approach is the high risk of development of resistance by the target insects as resistance to individual Bti toxins has already been observed in laboratory colonies of *Culex quinquefasciatus* (Georghiou and Wirth, 1997).

Elleuch *et al*. (2015) observed two mechanisms that affect the susceptibility of *Aedes aegypti* larvae to Bti toxins. The first of which involves the change or removal of any of the Cry toxin genes, which form part of the Bti toxin by mutagenesis, or plasmid transfer between Bti and other closely related *Bacillus* isolates. This could lead to decreased crystal toxin production and loss or inactivation of such cry genes. This, consequently, results in reduced toxin to interact with the *Aedes* larvae, resulting in decreased effectiveness. The second mechanism involves the stage of the toxin processing and stability. Elleuch *et al*. (2015) also observed early degradation of mutated Bti toxin by *Aedes* mid gut protease compared to the wild type, suggesting that the amino acid changes in the mutated Bti toxin might have caused alteration in the number and or the accessibility of the protease cleavage sites as a result of tertiary structure modifications. Hence, accessibility of the *Aedes* protease to the Cry toxin affects its activation step by not allowing proper interaction between them leading to decreased activity. For instance, mutagenesis of loop I and loop II of Cry4Ba has been shown to abolish its activity against *Aedes aegypti* and Anopheles larvae (Abdullah *et al*., 2003), which may suggest early degradation of the mutated Cry4Ba by the insect's mid gut due to structural modifications caused by mutagenesis leading to early degradation and hence lack of activity due to decrease interaction between the toxin and the insect's mid gut protease.

Toxin sequestration is another factor that can affect the susceptibility of *Aedes aegypti* larvae to Cry toxin. This is because the toxin, after its processing, must remain stable in

the gut epithelium for it to exert its toxicity. But, the presence of some specific binding sites on the peritrophic membrane have been proposed to exert a trapping effect thereby reducing the ability of specific toxins to diffuse across the peritrophic membrane and interact with the gut epithelium (Hayakawa *et al*., 2004).

The receptor binding stage in the mechanism of action of Cry toxin has been shown to be one of the important stages (Bravo *et al*., 2007). Cry toxins when activated bind to specific mid gut proteins, and a number of these have been identified in *Aedes aegypti* larvae (Table 1.4-1). In addition, mutagenesis of the putative loop α-8 residues of Cry11Aa confirmed that this region is important for its interaction with *Aedes aegypti* Brush Border Membrane Vesicles (BBMV) and toxicity (Fernandez *et al*., 2006). Therefore, this showed that binding of the Cry proteins to their receptors on the insect's mid-gut is very essential for their interactions and hence susceptibility.

Cry proteins are applied as insoluble proteins because mosquitoes are filter feeders as such able to absorb them in that form. Therefore, due to the fact that the proteins are in an insoluble form, some settled at the bottom of the water and not accessible to the larvae, and only little are absorbed by the larvae resulting in low mortality (McNeil and Dean, 2011). In addition, the age of the *Aedes* mosquito larvae has a role to play in their susceptibility because sensitivity to Cry toxin decreases with decrease larval age. Volume of the water to larval number also has a critical effect on larval stress and sensitivity to toxin; thus affecting susceptibility (McNeil and Dean, 2011). If the volume of the water is high, the larvae die of stress rather than sensitivity to the toxin. Thus, 4ml of water to a larvae is generally recommended (WHO, 2005b).

## 1.6 Mechanism of *Aedes* resistance to insecticides

Following the first report of resistance to chlorinated hydrocarbon insecticides by mosquitoes (Gjullen and Peters, 1952), there have been many researches aimed at understanding the mechanism underlying the development of resistance in mosquitoes. The ability of *Aedes* mosquitoes to resist chemical insecticides such as pyrethroid is a major threat against the control of major arbovirus diseases, the world over. It was discovered that there is increase in mosquito resistance to all classes of insecticides in more than sixty countries with respect to all the major vector species (WHO, 2013).Therefore, until an alternative control strategies are widely introduced, the

monitoring of resistance levels of insecticides and understanding their mechanisms of resistance by mosquito populations in the field is crucial for implementing appropriate management plan.

Two basic mechanisms have now been widely accepted to be generally responsible for an insect's resistance to insecticides: increased metabolic detoxification of insecticides and decreased sensitivity of the target proteins on which an insecticide acts, so-called target sites insensitivity (Ranson *et al*., 2011, Li and Liu, 2010, Wang *et al*., 2015).

Target site insensitivity, arises from mutation of genes encoding proteins that interact with insecticides (Casida and Durkin, 2013). Insecticides such as dichlorodiphenyltrichloroethane (DDT) and pyrethroids appear to target sodium channels causing a repetitive discharge on the nervous system of the insect after binding to the sodium channels, resulting in depolarization of the its nerve membranes and ultimately death (Narahashi, 1988). Acetylcholinesterase (AChE) is key enzyme in the nervous system, which hydrolyses acetylcholine neurotransmitters and terminating nerve impulses, it appears to be the target for organophosphates and carbamate insecticides. The γ-aminobutyric acid (GABA) receptors appear to be the target for cyclodiene and fipronil insecticides (Cole, 1993. , Ffrench-Constant et al., 2000). Two mutations in the active site of AChE1 of mosquitoes has been shown to result in their insensitivity or reduced sensitivity to organophosphate and carbamate insecticides. A G119S substitution has been reported in many mosquito species, including *Anopheles albimanus*, *Culex pipiens*, *Anopheles gambiae, Culex vishnui*, and *Culex quinquefasciatus* (Alout and Weill, 2008).

The GABA receptor is a type A receptor for γ-aminobutyric acid, a neurotransmitter, and is the target site for many cyclodiene insecticides such as dieldrin, and phenyl pyrazoles such as fipronil. It is made up of five subunits, and each contains an extracellular Cys loop as well as four transmembrane domains (M1–M4). The second transmembrane domain designated M2 represents the main portion of the ion channel. A296G substitution has been observed in *Anopheles gambiae* (Du *et al*., 2005), whereas a A296S substitution is linked to dieldrin resistance in *Anopheles stephensi*, *Anopheles arabiensis, Anopheles funestus*, and *Aedes aegypti* (Ffrench-Constant *et al*., 2000, Du *et al*., 2005). Two novel mutations within the sodium channel gene (F1552C and F1554C) were also

linked to pyrethroid and DTT resistance in *Aedes aegypti* populations from Thailand (Yanola *et al*., 2011).

The second mechanism is via metabolic detoxification of insecticides. Three major detoxification gene families namely: cytochrome P450s (P450s), esterases, and glutathione S-transferases (GSTs) are mainly responsible for detoxification of insecticides in mosquitoes. One of the remarkable features of insect's P450s and GSTs is their ability to upregulate transcription process, which results in increased enzymatic activities and consequent increase in protein production levels. This in turn increases the metabolic detoxification of insecticides resulting in the development of resistance to insecticides (Ponlawat *et al*., 2005). Esterase is a group of heterogeneous enzymes, which are present in many organisms. The overproduction of this enzyme has been studied extensively. It has been shown that the amplification and/or random overexpression of esterase genes increases the level of production of detoxification proteins and hence resistance (Pasteur and Raymond, 1996).

The silencing of a GST gene in *Aedes aegypti* has been demonstrated to show its role in insecticide resistance (Lumjuan *et al.*, 2011). In addition, some Cytochrome P450 genes: CYP6BB2, CYP6M11, CYP6N12, CYP9J9, CYP9J10 and CCE3 were implicated in conferring resistance to *Aedes aegypti* populations as they were found to be upregulated in the resistant populations, and hence their involvement in resistance is highly suggested (Dusfour *et al*., 2015).

From the above it is obvious that there are many cases of insect resistance to chemical insecticides and hence the need to rely on biological insecticides for mosquito control. The biological insecticides mostly used for mosquito eradication are the Bti based larvicides. There is no established case of field resistance to Bti toxin. There is considerable difference between laboratory-selected resistance and field selected resistance as the former may have considerably lower genetic diversity. The most important one being considered mostly is the field resistance.

Several studies carried out test for resistance in *Aedes aegypti* populations against Bti based insecticides from various parts of the world and discovered that this mosquito species is still highly susceptible to the Bti based larvicides (Marcombe *et al*., 2012, Araujo *et al*., 2013, Suter *et al*., 2017). Some researchers have reported cases of laboratory resistance to Cry toxins (Cadavid-Restrepo *et al*., 2012, Stalinski *et al*., 2014).

Cadavid-Restrepo *et al*. (2012) reported a case of resistance in Cry11Aa, which forms part of the Bti toxin, by *Aedes aegypti*. He observed the development of resistance to Cry11Aa toxin by the 54$^{th}$ generation of *Aedes aegypti*. Stalinski *et al*. (2014) used Cry4Aa, Cry4Ba and Cry11Aa toxins from a strain that consists of 80% of susceptible Bora-Bora strain 20% of LiTOX strain that are resistant to Bt for the selection of three strains of *Aedes aegypti* LR4A, LR4B and LR11 from the 22$^{nd}$ generations. A mixture of Bti Cry toxins in a similar amount to those found in Bti (12.5% Cry4Aa, 12.5% Cry4Ba, 75% Cry11Aa) with unformulated Bti, or not selected, to select this composite strain for up to 5 generations in order to generate the LR3Tox, LR3Bti and LR3NS strains. The degree of resistance of these strains to each of the three Cry toxins, Bti, and the mixture of the Cry toxins was determined using bioassay. They observed an increased in the level of resistance in each of the three strains of *Aedes aegypti* to their selected toxins, and a cross resistance between each pair of toxins. Nevertheless, no case of field resistance to Bti larvicide, as a whole, by this insect is reported yet.

## 1.7 The Cry2A family of toxins

The Cry2A proteins constitute one of the largest classes among the Cry family of toxins, with a group of 11 toxins with molecular weight ranging between 61-72kDa, present in cuboidal crystals produced by Bt (Donovan *et al*., 1988, Nicholls *et al*., 1989, Dankocsik *et al*., 1990). Cry2A proteins include some toxins, which exhibit dual activity spectra to both the lepidopteran and dipteran orders of insects that pose threats to agriculture and public health. For instance, the Cry2Aa toxin is toxic to both moths and mosquitoes, which makes it an attractive platform to combat diseases caused these insects (McNeil and Dean, 2011).

The crystal structure has been solved for Cry2Aa at 2.2Å resolution. It consists of a 633-amino acid protoxin containing 49-amino acid peptides at the N-terminal region, which is speculated to be cleaved on activation, and three domains that form the mature toxin (Morse *et al*., 2001). There is remarkable structural similarity between the three domains of Cry2Aa to those of the activated toxins of Cry3Aa (Li *et al*., 1991) and Cry1Aa (Grochulski *et al*., 1995) despite the fact that they have only little sequence identity to Cry2Aa; 20% in the case of Cry3Aa and 17% for Cry1Aa.

The structure of Cry2Aa toxin consists of domain 1 (residues 1–272), which is a pore-forming seven-helical bundle (Schnepf *et al*., 1998). It is cleaved at around amino acid 144$^{th}$ position for the active Cry2Aa toxin to be formed, leading to the loss of the N-terminal region, which has 49 amino acid residues. The second domain comprises of (residues 273–473) and is a β prism with a three-fold symmetric arrangement of β sheets, each of which has a Greek key fold (Figure 1.7.1), this domain is known for receptor binding. The third domain comprises of (residues 474–633) is associated with both larval receptor binding (Lee *et al*., 1995, de Maagd *et al*., 1999) and pore function (Schwartz *et al*., 1997) and has a lectin-like β-sandwich (Fig 1.7.1). Morse *et al*. (2001) suggested based on modelling studies using Cry2Aa that proteolytic activation of the toxin might involve the cleavage of the 49 N-terminal amino acids which results in exposing a residue comprising a putative toxin-receptor binding surface.

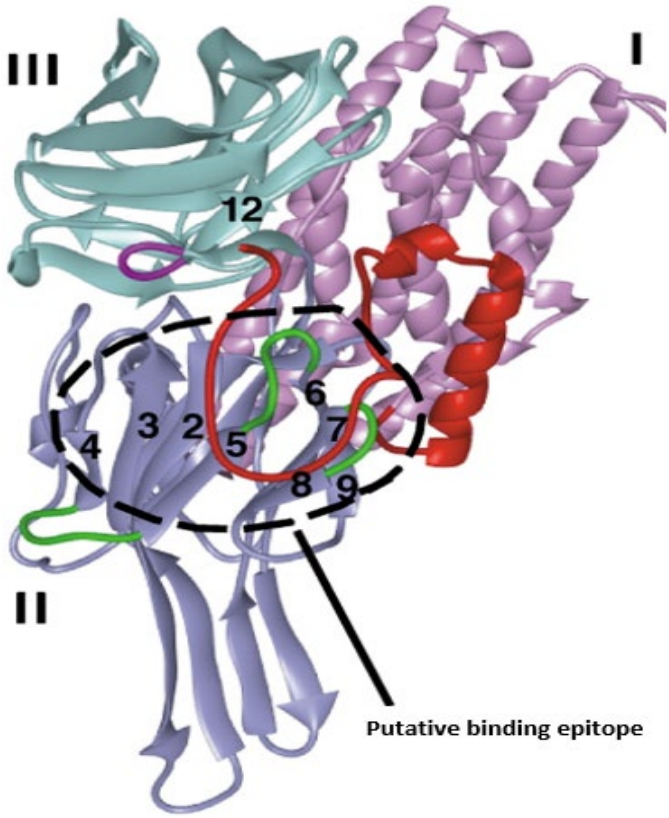



***Figure 1.7.1 The three-domain crystal structure of Cry2Aa.Domain I is highlighted in magenta colour, domain II highlighted in blue colour; domain III highlighted in cyan colour whereas the 49-N-terminus sequence is highlighted in red. The putative 800 Å$^2$ binding epitope is bordered by dashed black line, and the β-strands have been numbered by their order in the sequence. Taken from Morse et al. (2001).***

## 1.8 Determination of Cry toxins' specificity to insects

Specificity of a crystal protein is defined as the extent of species or taxa to which it is active on. In other words, it is referred to its activity spectrum. The corollary, however, referred to the susceptibility spectrum of a particular species tested with different toxins i.e. the range of toxins that are active against a particular species.

There has been an extensive review by van Frankenhuyzen (2009), who looked at the specificities of toxins grouped at that time in to 55 Cry and 2 Cyt families by Crickmore *et al*. (1998). van Frankenhuyzen (2009) gathered the information on biological specificity, which have been generated for over 25 years, and carried out a comprehensive review on this information. The review seems quite complicated because the bioassay results were confused by many factors apart from the toxin type.

van Frankenhuyzen (2009) made of the data base for toxin specificity limited to spore-free preparations of protoxins or crystals obtained by the expression of cloned genes or purified from single-gene strains, which were individually bioassayed (with binary toxins being exempted). Genetically altered crystal proteins were not included in his review except a few that were changed through single amino acid substitutions e.g. (Lambert *et al*., 1996).

Specificity within orders was examined by analysing the activity of the toxins at the species level (tertiary rank) depending on mortality using a binary response (active or not active). Activity was examined with no reference to the life stage of the organism under consideration, the method employed in the bioassay, or the nature of the toxin used (protoxin, crystal or activated toxin). Crystal proteins were considered 'not active' when they failed to cause mortality response at the highest concentration used and 'possibly active' when there are conflicting reports.

van Frankenhuyzen (2009) also assessed specificity across orders by rolling up qualitative data by secondary toxin rank and order of test organism and supplementing them with information that were published but failed to meet the requirements for inclusion in to the data base. A family of toxin is considered active against a given order if at least one toxin in the family has been reported to be active against at least a species belonging to that order. However, it is a toxin is considered not active when none of the toxins that caused a considerable mortality response in any of the tested species, and 'possibly active' when there are conflicting reports.

There are some limitations associated with specificity determination as highlighted by van Frankenhuyzen (2009). First of which is that our current understanding on specificity is limited by the number of toxins tested to date and the range of species in those tests as presented in Figure 1.8.1.

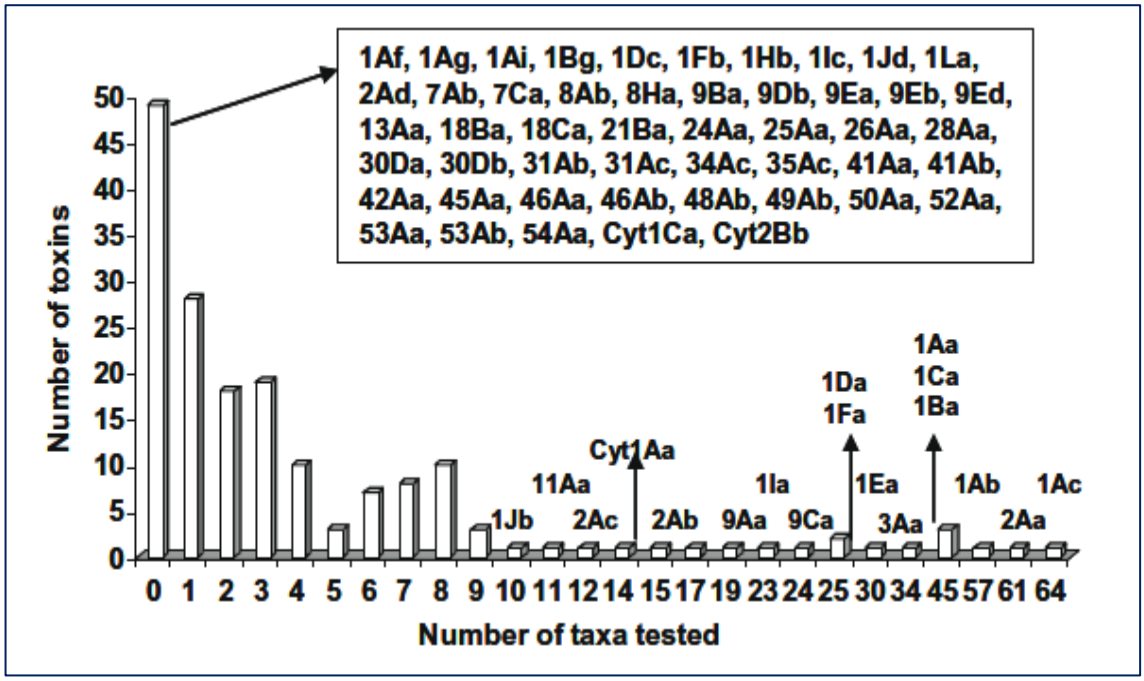



***Figure 1.8.1 The number of holotype toxins (tertiary rank) that have been bioassayed as a function of the number of taxa (mostly species) tested. The toxins listed in the box have no any published bioassay data. Taken from (van Frankenhuyzen, 2009).***

Figure 1.8.1 above, for specificity looks fragmentary as the majority of the toxins (91%) were tested against only small number of species (10 or less), 49 out of the 174 holotype toxins have not been tested at all (the box in Figure 1.8.1 above) and the species and toxin tested were not distributed equally across the families of protein and taxa.

Secondly, the difference between toxins considered to be active and non-active is undoubtedly subjective, because some toxins that were reported as non-active might evoke mortality response when tested at higher concentrations.

van Frankenhuyzen (2009) carried out the specificity determination of the Crystal proteins, as a qualitative assessment across, and within orders of insects as follows:

### 1.8.1 Specificity determination across orders

The specificity based on order was considered across toxin families at the secondary rank as depicted in Figure 1.8.2. It is obvious here, that the number of crystal proteins

that show activity across order has increased considerably after the first classification done by Hofte and Whiteley (1989), who identified four major pathotypes based on order specificity. These are: Lepidoptera-specific (CryI, now Cry1), Coleoptera- specific (CryIII, now Cry3), Diptera-specific (CryIV, now Cry4, Cry10, Cry11; CytA, now Cyt1A), and lastly CryII (now Cry2), which was the only family that was known to possess dual specificity at that time (Lepidoptera and Diptera) specificity but has been found now to have additional cross activity against Hemiptera. This additional cross activity is because of Cry2Aa being active against this order of insects (Sims, 1997). Cross-order activity displayed by 15 of the 87 pesticidal crystal protein families as shown in Figure 1.8.2.

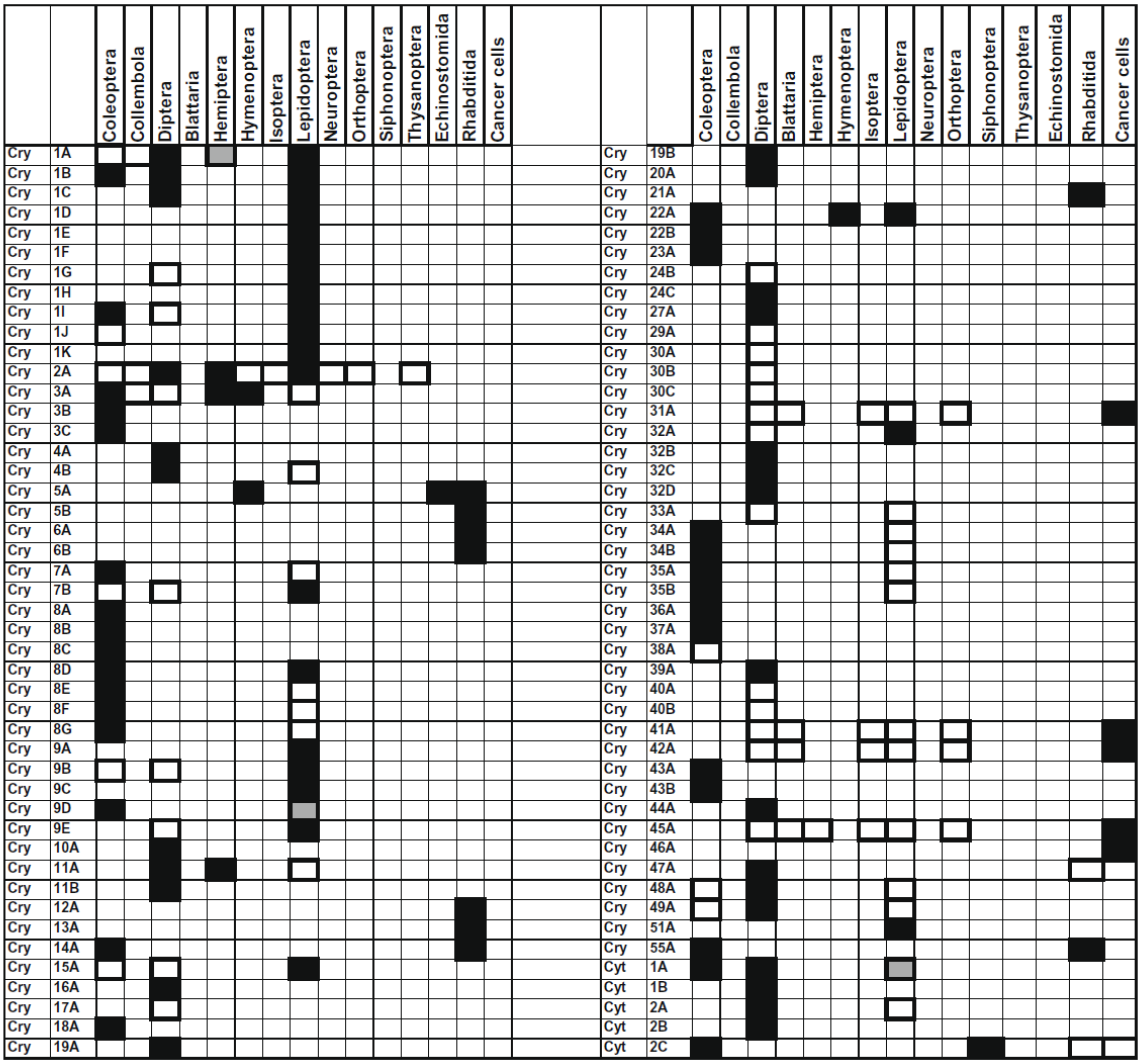



***Figure 1.8.2 Specificity of Cry and Cyt toxin families (secondary rank) across orders. Toxin families are indicated as being active ■, not active □, possibly active ▒, or not tested ▫. Toxin families for which no bioassay data are available are not shown (Cry1L, 7C, 8H, 18B, 18C, 21B, 24A, 25A, 26A, 28A, 30D, 42A, 50A, 52A, 53A, 54A, Cyt1C). Cry toxins are displayed horizontally while the insect orders are displayed vertically. Taken from van Frankenhuyzen (2009).***

### 1.8.2 Specificity determination within orders

The specificity was also considered within orders for three major orders of insects namely: Lepidoptera, Diptera, and Coleoptera. Other insects were then grouped together. For the purpose of this thesis, which is concerned with the Cry2A group of toxins, I will consider only those orders to which Cry2A toxins appeared to be active.

1. Lepidoptera

Considering all the bioassays previously carried out on Lepidoptera where 59 holotype toxins have been tested against 71 species in 1,182 bioassays; van Frankenhuyzen (2009) presented a figure for susceptibility spectra of these species in the rows as depicted in Figure 1.8.3. Four members of Cry2A toxins (Cry2Aa, Cry2Ab, Cry2Ae and Cry2Af) were shown to have specificity towards this order of insects as indicated in Figure 1.8.3. *P. xylostella* appeared to be the most frequently tested species (9.8% of total bioassays), followed by *Spodoptera exigua* (8.7%), *Heliothis virescens* (6.4%), *Manduca sexta* (5.9%), *Trichoplusia ni* (5.3%), *Ostrinia nubilalis* (5.1%), *Helicoverpa armigera* (4.8%), *Heliothis zea* (4.0%) and *Bombyx mori* (3.9%)"(van Frankenhuyzen, 2009) as shown in Figure 1.8.4.

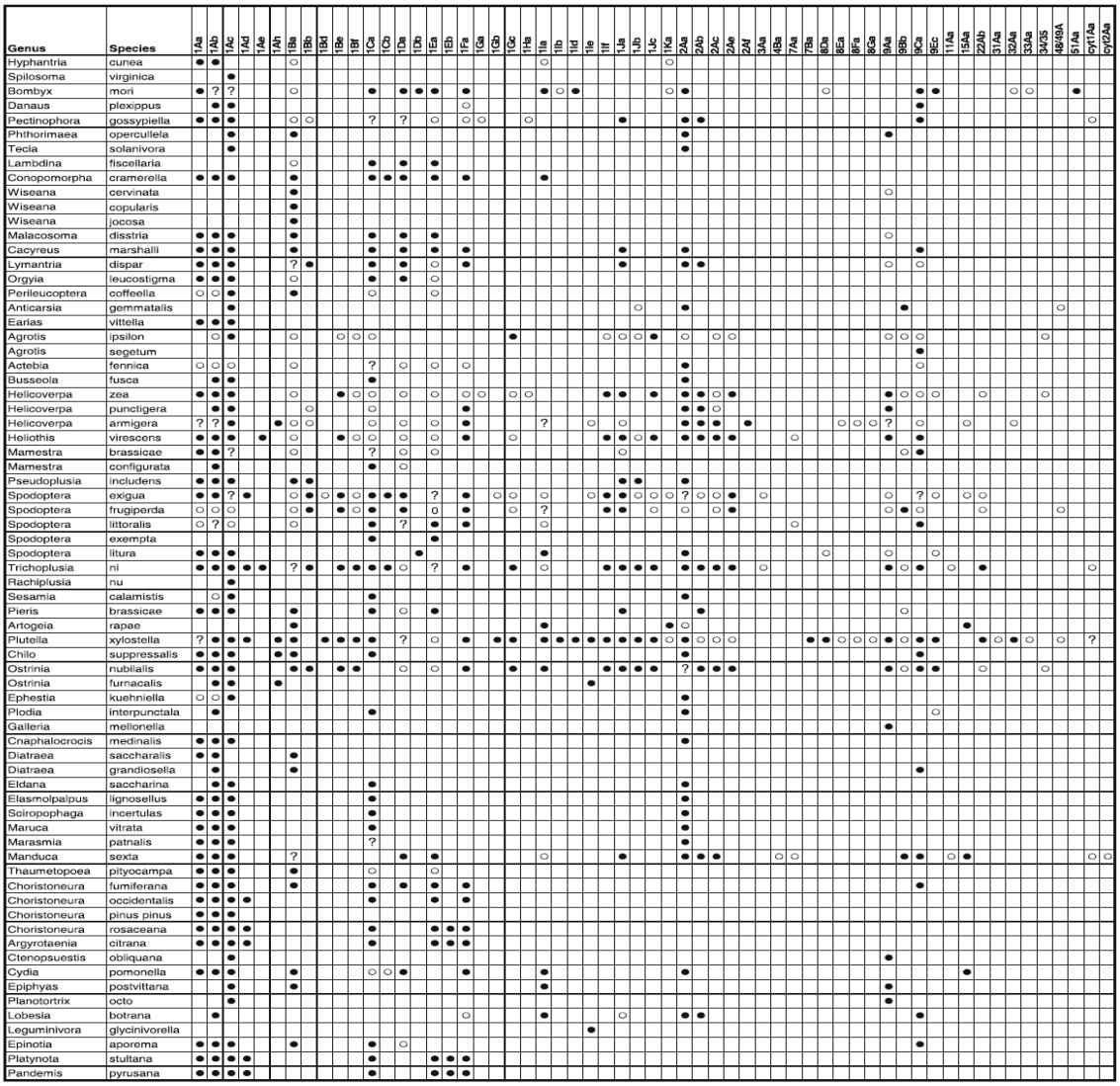



***Figure 1.8.3 Activity spectrum of Cry and Cyt holotype toxins that were tested against species of Lepidoptera. Toxins are indicated as active indicated by black dots inside a square, not active indicated by plain dots inside a square or possibly active indicated by a question mark. Taken from van Frankenhuyzen (2009).***

The activity spectra of the 59 holotype toxins that were tested are represented by columns in Figure 1.8.3. The 96.2% of total bioassays resulted from Cry1, Cry2 and Cry9 toxin families, with Cry1 and specifically Cry1A toxins responsible for 80% and 36.6% respectively. The widest range of toxins was tested against *Plutella xylostella* (43 toxin types) and it was one of only 12 species that were tested with 15 or more toxins as shown in Figure 1.8.4 below.

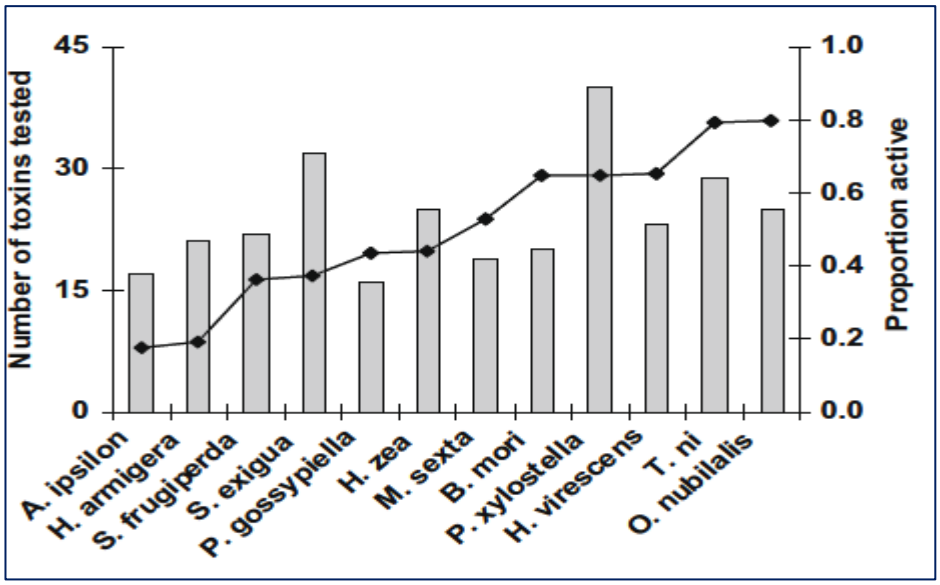



***Figure 1.8.4. Permissiveness of the most frequently tested species as indicated by the proportion of toxin types that displayed toxicity. Taken from van Frankenhuyzen (2009).***

The activity spectra of the holotype toxins that were tested against at least 15 species was presented as a proportion of the susceptible species (Figure 1.8.5). Cry2Aa and Cry2Ab, from the Cry2A group of toxins, were found to be among the active toxins in the activity spectrum of toxins presented as depicted in Figure 1.8.5.

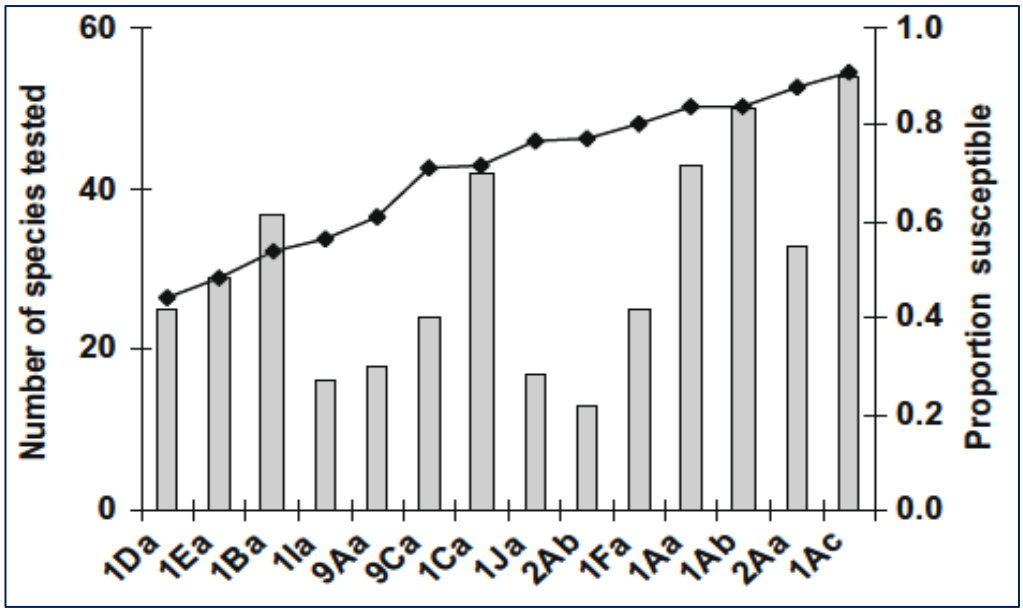



***Figure 1.8.5 Activity range of holotype toxins that were tested against at least 15 species as indicated by the proportion of species that were susceptible. Taken from van Frankenhuyzen (2009).***

2. Diptera

The dipteran species are very crucial here, as the species of insects investigated in this work fall within this order. van Frankenhuyzen (2009) presented the activity spectra of 53 toxins, which were tested against 23 dipteran species in 233 bioassays with their corresponding susceptibility spectra in rows and columns depicted in Figure 1.8.6.

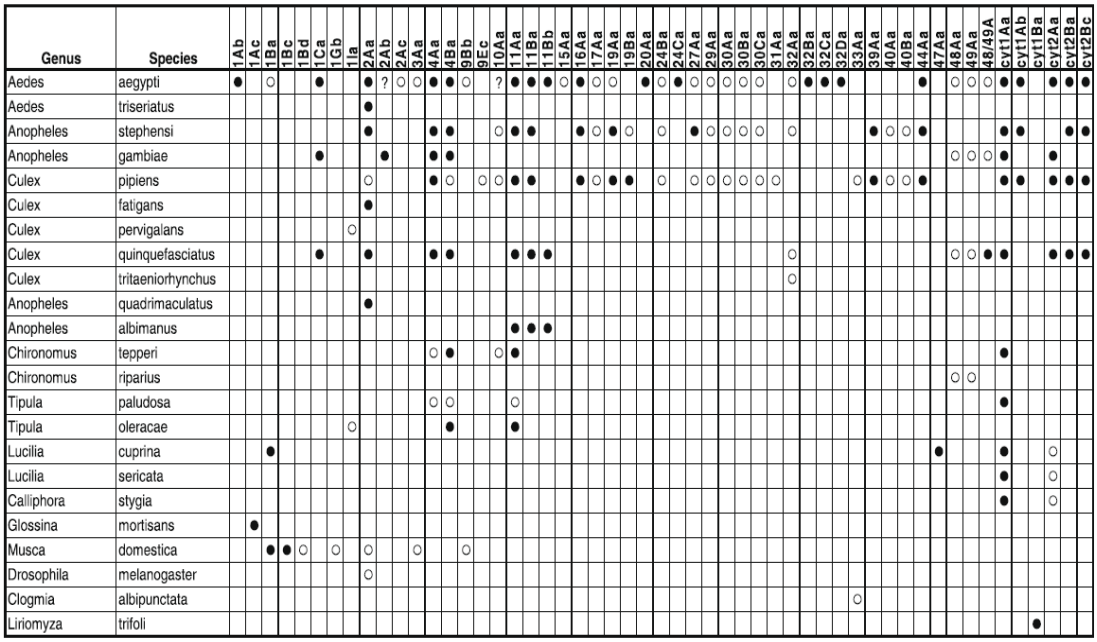



***Figure 1.8.6 Activity spectra of Cry and Cyt holotype toxins that were tested against species of Diptera. Toxins, which are active, are indicated by black dots inside a square, not active indicated by plain dots inside a square or possibly active indicated by a question mark. Taken from van Frankenhuyzen (2009).***

From the above (Figure 1.8.6), three of the toxins from the Cry2A group of toxins (Cry2Aa, Cry2Ab and Cry2Ac) were all tested against at least an insect in this order. Cry2Aa was found to be active against *Aedes aegypti*, *Aedes triseriatus*, *Anopheles stephensi*, *Culex fatigans*, *Culex quinquefasciatus*, and *Anopheles quadrimaculatus*, and non-active to *Culex pipiens*, *Musca domestica*, *and Drosophila melanogaster*. Cry2Ab was found to be active against *Anopheles gambiae*, and in terms of *Aedes aegypti* it is not very clear as there are conflicting reports hence it is indicated as being possibly active. Cry2Ac was reported in this review (van Frankenhuyzen, 2009) to be nontoxic to *Aedes aegypti*. Furthermore, it was shown that only four species were responsible for 77.7% of the total number of bioassays, with *Aedes aegypti* as the species that was tested most frequently

(37.2%), followed by *Culex pipiens* (15.8%), *Anopheles stephensi* (14.9%) and *Culex quinquefasciatus* (9.8%) as shown in Figure 1.8.7. The 84% of all the bioassays was against *Culicidae* alone, and it was the only family that was tested against more than 10 toxins, and having 60% of the species–toxin combinations being positive (van Frankenhuyzen, 2009).

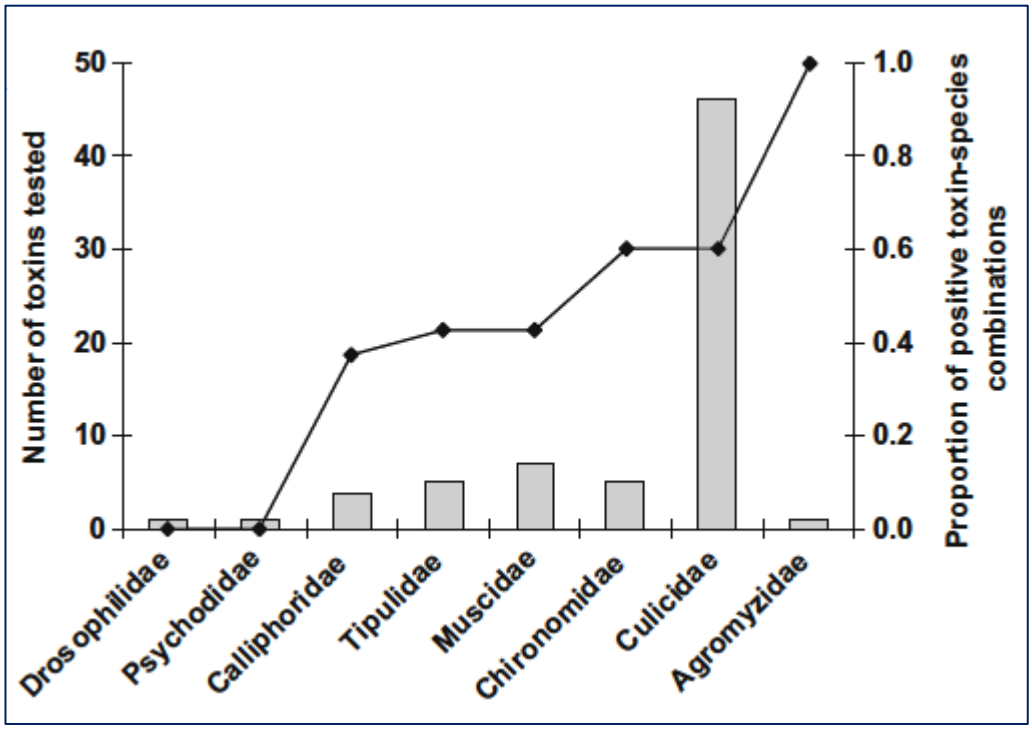



***Figure 1.8.7 Number of toxins that were tested for each family of diptera and the proportion of positive species–toxin combinations within those families. Taken from van Frankenhuyzen (2009).***

Considering the number of species which were susceptible against toxins tested on five or more dipteran species, as reviewed by van Frankenhuysen (2009), it was only Cry2Aa among the Cry2A group of toxins, which fall in this category of toxins as depicted in Figure 1.8.8 below.

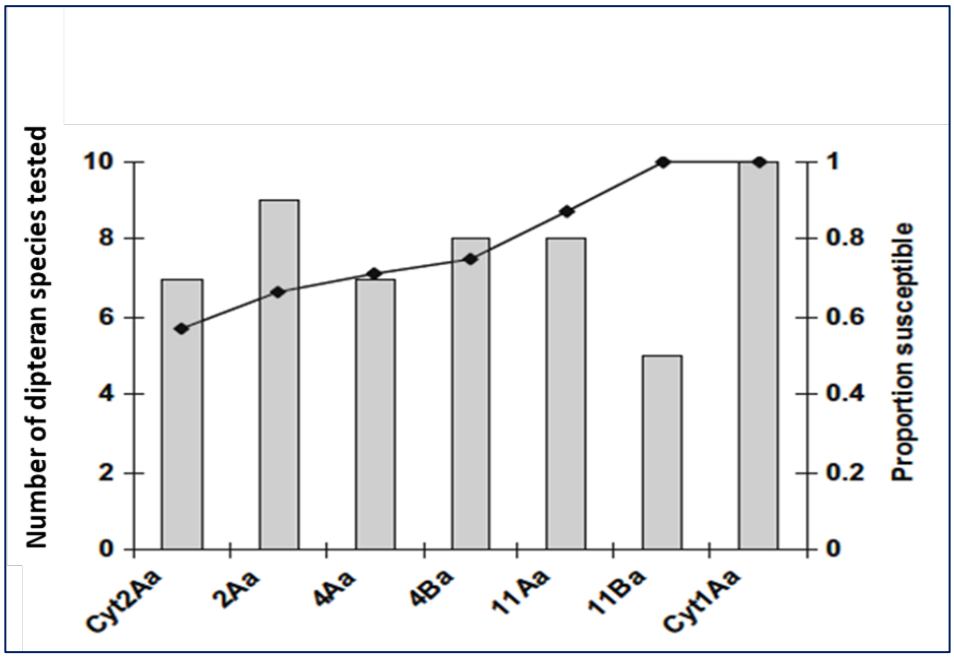



***Figure 1.8.8 Proportion of susceptible species for toxins that were tested against five or more dipteran species. Taken from van Frankenhuyzen (2009).***

## 1.9 Determination of regions/residues responsible for specificity in Cry2A toxins

Specificity region of a Cry toxin refers to amino acid residues/motifs within a particular region(s) of the Cry toxin structure that is or /are responsible for its activity against one or more insects. One of the major characteristics of Bt is the production of proteinaceous crystal toxins that possess specific activity against many insect pests including dipteran, lepidopteran and coleopteran. Therefore, determination of the region/ residues responsible for specificity of Cry2A toxins against *Aedes aegypti* involves studying the different associations between the amino acid sequences of Cry2A toxins and their toxicities against this insect. Understanding the specificity region of Cry2A toxins of *Bacillus thuringiensis* is essential in the risk assessment of novel insecticidal toxins from this bacterium many of which are being considered for commercialisation to ensure that they are not detrimental to non-target organisms within the environment. It will also provide a platform for the design of Cry toxin with broader species spectra of activities since a member of this group of toxins, Cry2Aa, has a broad spectrum of activity against both dipteran and lepidopteran insects.

Previous studies on Cry toxins specificity region determination focused on identifying the residues that define dipteran and lepidopteran specificities or either of these

through chimeric scanning mutagenesis (Widner and Whiteley, 1990, Liang and Dean, 1994), or elucidating the structure of Cry toxin by multiple isomorphs replacement using six heavy atoms derivatives and refined to 2.2Å resolution (Morse *et al*., 2001).

Widner and Whiteley (1990) constructed 16 hybrids by the combinations of Cry2Aa and Cry2Ab as shown in Figure 1.9.1.

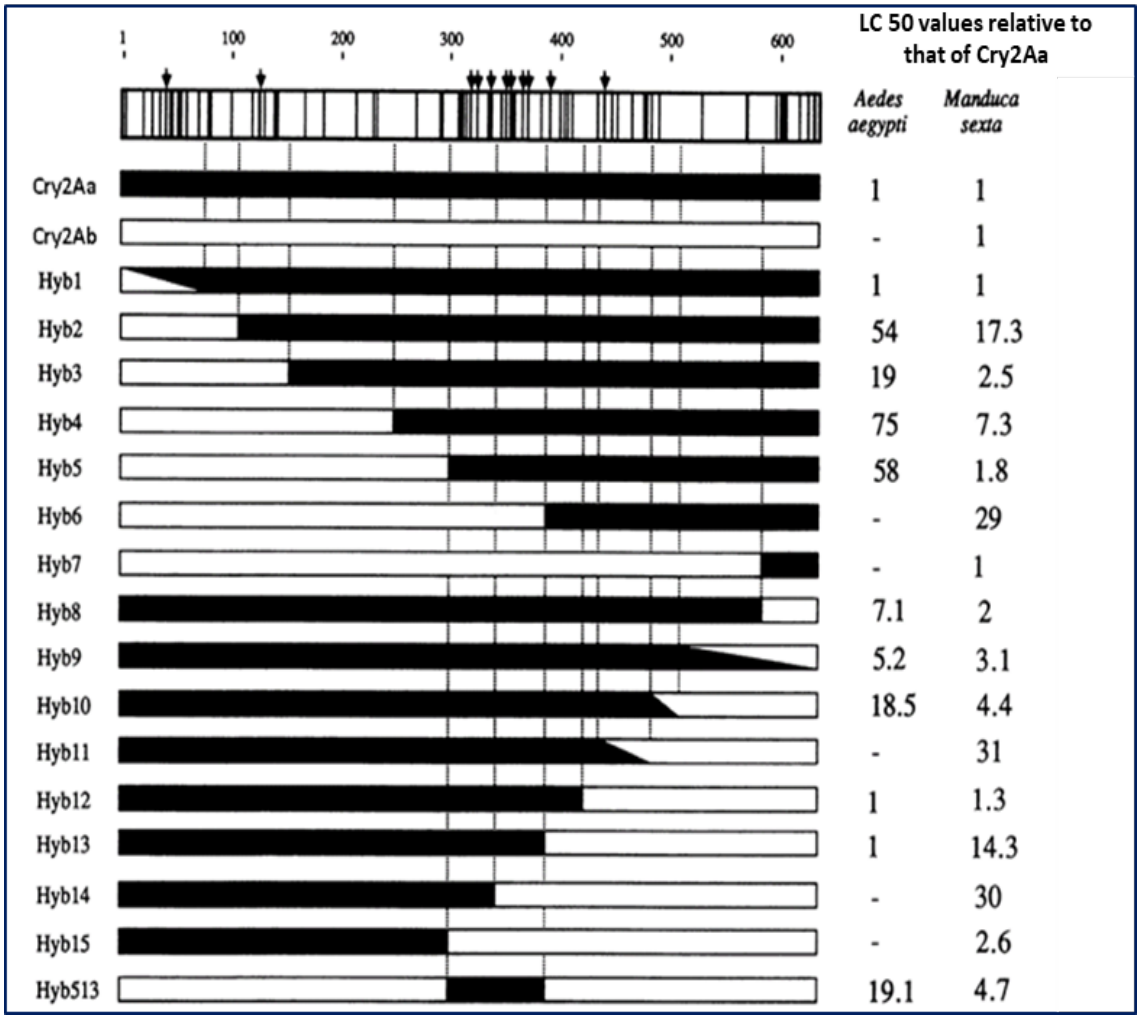



***Figure 1.9.1 Hybrids created between Cry2Aa and Cry2Ab. Cry2Aa (shaded bar), Cry2Ab (non-shaded bar), and hybrid gene products (combination of the two patterns) and their toxicities to A. aegypti and M. sexta. All of the toxicities are relative to that of Cry2Aa (value of 1); a fivefold difference in toxicity is considered significant, the bigger the number the lesser the toxicity of the hybrid. The bar at the top of the figure is a diagram depicting a FASTP alignment of the Cry2Aa and Cry2Ab polypeptides; vertical lines represent differences between the two and arrows above the bar denote the locations of non-conservative changes. Vertical broken lines show locations of the hybrid junctions determined by restriction mapping (hybrids 1, 9, 10, and 11) and DNA sequence analysis (hybrids 2 to 4, 6, 8, 12 to 14, and 513). The dotted lines extend upwards to the alignment diagram to show where the junctions are located with regard to the amino acid differences that exist between the two polypeptides (Widner and Whiteley, 1990).▬ Sign represents hybrid junctions determined through DNA sequence analysis, and ◣ sign represents hybrid junctions determined by restriction mapping. Taken from Widner and Whiteley (1990).***

From Figure 1.9.1 from Widner and Whiteley (1990), the amino-terminal end, hybrids 1 to 7 (Cry2Ab-Cry2Aa hybrids) contain decreasing amounts of Cry2Aa sequences and increasing amounts of Cry2Ab sequences; the amounts of Cry2Aa sequences in hybrids 8 to 15 (Cry2Aa-Cry2Ab hybrids)decrease from the carboxyl-terminal end.

Widner and Whiteley (1990) tested the activity of these hybrids against *A. aegypti* larvae and *Manduca sexta*, but I will focus on the results for *Aedes aegypti* being the species considered in this thesis. Hybrid 1 exhibited the same toxicity as Cry2Aa, and inclusions from the Cry2Ab containing strain were nontoxic. Inclusions from hybrids 2 to 5, which contained more of Cry2Ab in the N-terminal region, were found to be substantially less toxic than the Cry2Aa control. Inclusions from hybrids 6 and 7, with a difference of Cry2Ab sequence from position 382 to around 580 replacing Cry2Aa in hybrid 7, and lacking Cry2Aa sequence in the region 307-382, were nontoxic to *Aedes aegypti* mosquito larvae, even when tested at high concentrations (250 ng/ml).

When they tested the Cry2Aa-Cry2Ab inclusions (hybrids 8 to 15), for activity, they discovered that hybrids 12 and 13 were as toxic to *Aedes aegypti* larvae as was Cry2Aa. Hybrids 8 to 10 were progressively less toxic, and hybrid 11 as well as hybrids 14 and 15, were nontoxic. Except for the results they obtained with inclusions from hybrid 11, the absence of mosquitocidal activity in hybrids 6, 7, 14, and 15 suggests that the boundaries of hybrids 5 and 13 delineate the Cry2Aa sequences that are minimally required for toxicity to mosquito larvae. Therefore, to determine whether the short segment of Cry2Aa defined by these boundaries is, in fact, sufficient to influence specificity, they further constructed a Cry2Ab-Cry2Aa-Cry2Ab hybrid gene in vitro between hybrids 5 and 13 to generate hybrid 513 (Figure 1.9.1). The resulting gene product was toxic to both test insects, indicating that residues 307 through 382 of the Cry2Aa polypeptide influence mosquitocidal activity. However, the toxicity of hybrid 513 inclusions to *A. aegypti* was reduced 20-fold relative to that of Cry2Aa, which is still somehow negligible considering the fact that only 5-fold reduction in activity was considered significant in their research. This may suggest that sequences outside this putative mosquitocidal region may also be important for specificity determination and therefore they have not clearly defined the region and/or amino acid motifs responsible for the specificity of Cry2A toxins to *Aedes aegypti*.

The results of Widner and Whiteley (1990), were further studied by Liang and Dean (1994) who also located the regions responsible for specificity of Cry2A toxins to both dipteran and lepidopteran insects by creating hybrids between Cry2Aa which possessed activity against mosquito larvae and gypsy moth larvae, with Cry2Ab, which possessed only lepidopteran activity.

They located the specificity regions of Cry2Aa against lepidopteran and dipteran insects, by replacing the putative domain II of Cry2Aa by the putative domain II of Cry2Ab. They made used of the sequence alignment generated by Hodgman and Ellar (1990) for the different domains of Cry proteins. Domain II of Cry2Aa was aligned between amino acid 278 and amino acid 487, which is encoded by the naturally existing *NheI- NarI* fragment of the Cry2Aa gene. The DNA fragment for domain II of Cry2Ab (also aligned between amino acids 278 and 487) was amplified and they use the PCR technique to introduce *NheI* and *NarI* sites into the fragment ends. With this approach, they created a recombinant gene DL105 as shown in Figure 1.9.2 below.

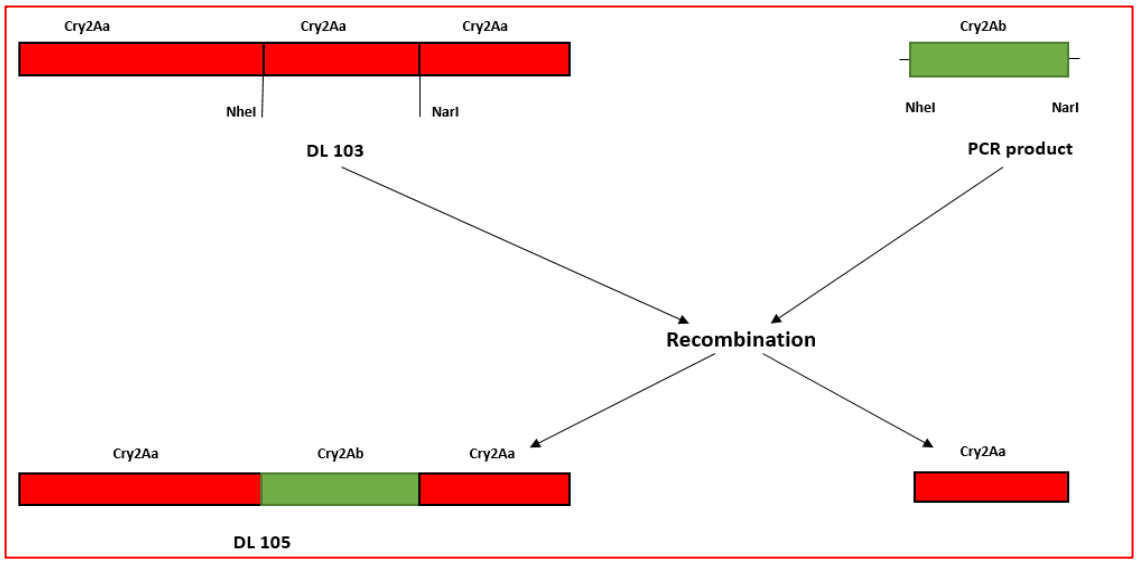



***Figure 1.9.2 Generation of Hybrid DL105 (BBB) containing Cry2Ab at domain II of wild type Cry2Aa (DL103) by recombination using the restriction enzymes NheI and NarI. The red colour denotes Cry2Aa and the green colour denotes Cry2Ab (Liang and Dean, 1994).***

They further introduced *MluI* and *XhoI* sites in to both the wild-type Cry2Aa gene DL103, and the recombinant gene DL105, at positions that appear to divide the domain II of both genes in to three regions as shown in Figure 1.9.3. They designated these regions as region 1, region 2 and region 3, encoding polypeptide fragments from amino acid 278 to amino acid 340, from 341 to 412 and from 413 to 487, respectively. Therefore, they

produced six chimeric genes (Table 1.9-1) by homologue-scanning mutagenesis by substituting one or two regions of domain II of Cry2Aa with the corresponding region of Cry2Ab.

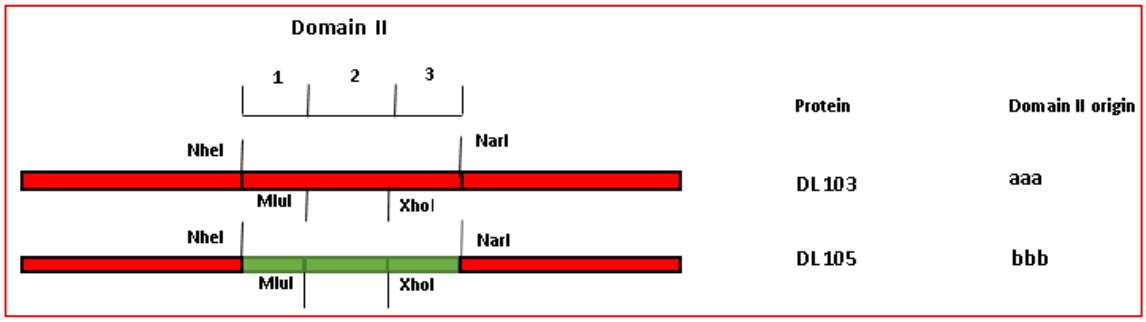



***Figure 1.9.3 MluI and XhoI sites introduced into DL103 and DL105. MluI and XhoI almost equally divide domain II into three regions, which have been named (from N-terminal to C-terminal) regions 1, 2, and 3, NheI and NarI are naturally occurring sites bordering domain II of Cry2Aa (Liang and Dean, 1994)***

Table 1.9-1 below shows the various hybrids created from chimeric scanning mutagenesis involving changes among the three fragments created between the domain II of Cry2Aa and that of Cry2Ab, and their associated toxicities against both *Aedes eagypti* and *Lymantria dispar*.

| **Protein** | **Domain II origin** | **LC 50 of *Aedes aegypti* (95% confidence interval) (ng/ml)** | **ID 50 of *Lymantria dispar* (95% confidence interval) (ng)** |
|---|---|---|---|
| DL103 | aaa | 65.5 (41.1-100) | 102(77-181) |
| DL105 | bbb | $>10^5$ | 304(226-418) |
| DL111 | abb | ND | ND |
| DL112 | baa | $1.23 \times 10^5$ ($2.82 \times 10^4$-$8.33 \times 10^5$) | 126(85.7-187) |
| DL113 | bab | $1.50 \times 10^5$ ($1.05 \times 10^5$-$1.02 \times 10^6$) | 88.7(58.0-129) |
| DL114 | aba | ND | ND |
| DL115 | bba | $>10^5$ | 3200(1340-51900) |
| DL116 | aab | 52.2 (25.7-107) | 90.6(57.7-136) |

***Table 1.9-1 Bioassays of homologue-scanning mutants against A. aegypti and L. dispar. Letters indicate the origin of each of the three regions in domain II of each mutant. a, stands for amino acids from Cry2Aa origin, b stands for amino acids from Cry2Ab origin. ND stands for not determined.***

They carried out the bioassay as shown in Table 1.9-1 above and discovered that the wild type Cry2Aa was toxic against *Aedes aegypti* and slightly toxic against *Lymantria*

*dispar* larvae as well, but Cry2Ab was about three times less toxic than Cry2Aa to *Lymantria dispar* larvae. Dankocsik *et al*. (1990) who used spores/crystal mixes also got similar toxicity ratios for these two Cry2A toxins. When domain II of Cry2Aa was replaced by domain II of Cry2Ab (Hybrid D105), it was three times less toxic to *L. dispar* than wild-type Cry2Aa and showed a similar toxicity to wild-type Cry2Ab. As with wild-type Cry2Ab, it did not show any mosquitocidal activity even at concentrations as high as 100µg/ml. Their results, therefore, demonstrated that the specificity regions of Cry2Aa against *L. dispar* larvae and *A. aegypti* larvae are both located in domain II. The mutants they created by substituting regions within domain II behaved differently. While DL116 (containing Cry2Ab at amino acids position 413 to 487) was as active as wild-type Cry2Aa against both *L. dispar* and *A. aegypti* larvae, DL115 (containing Cry2Ab at amino acid position 278 to 412) lost its activity against both insects. Its activity against *L.* dispar larvae reduced by 10 and 30 times when compared to the wild type toxins DL103 and DL105, respectively, and had no activity against *A. aegypti* larvae. DL112 (containing Cry2Ab at amino acid positions 278 to 340) and DL113 (containing Cry2Ab at amino acid positions 278 to 340 and 413 to 487 respectively) appeared to show similar activities against both insects. While their activities against *Lymantria dispar* larvae was similar to that of wild-type Cry2Aa, their activities against *Aedes aegypti* larvae were both reduced by approximately 2000-fold.

Therefore, from their findings it was clear that only when both region 1 and region 2 of the recombinant protein were of Cry2Aa origin (DL116) did the protein possessed mosquitocidal activity at an extent that could be comparable to wild-type Cry2Aa. This indicated that the specificity region against the mosquito larvae was located in regions 1 and 2 (amino acids 278-412) and not region 3 (amino acids 413 to 487). When they further analysed this region using their bioassay data (table 1.9-1, it showed that region 1 of Cry2Aa was required for activity against mosquitoes (DL112 and DL113). The findings that substituting region 1 of Cry2Ab with Cry2Aa had no effect on the stability of the protein, its ability to form crystals, or its activity against *L. dispar* (DL112 and DL113) but had dramatically affect its activity against A. *aegypti* indicated that the difference in region 1 (amino acids 278-340) between Cry2Aa and Cry2Ab is only associated with mosquitocidal activity. However, region 2 of Cry2Aa was also essential

for production of a functional toxin against mosquitoes but Liang and Dean (1994) were unable to exclude or demonstrate the role of region 2 in the toxicity due to the inability of their two hybrids (DL111 and DL114) to form crystals.

Research by Morse *et al*. (2001) was based on understanding the structural determinants of Cry toxin specificity. Therefore, to achieve this, they elucidated the structure of Cry2Aa from *Bacillus thuringiensis* subspecies *kurstaki* by multiple isomorphic replacement using six heavy atoms derivatives and refined to 2.2Å resolution. They chose Cry2Aa because it is among the unusual subset of Cry proteins possessing broad insect species specificity by exhibiting high specificity against both Dipteran and Lepidopteran insects (Yamamoto, 1981, Donovan *et al*., 1988). Therefore, it could serve as an important platform for the design of Cry toxin with broader species spectra of activities.

Morse *et al*. (2001) were able to identify a putative candidate toxin receptor-binding surface, which appeared to be consistent with the available chimeric-scanning mutagenesis data (Widner and Whiteley, 1990, Liang and Dean, 1994).

This defines a continuous 106 amino acid block (307-412), of specificity-distinguishing residues, within which there are 23 residues that differ between Cry2Aa and Cry2Ab (Morse *et al*., 2001).

Liang and Dean (1994) demonstrated that substitution of residues 278-340 resulted in loss of *Aedes* activity in Cry2Aa, DL115 in figure 1.9.4, while Widner and Whiteley (1990) demonstrated that substitution of residues 307-382 of Cry2Aa to Cry2Ab conferred dipteran specific activity to Cry2Ab Hyb513. Thus, the sequence responsible for the lack of dipteran activity in Cry2Ab, which falls in its amino acid residues 307-382 is now tracked with Cry2Aa (Hyb 513).

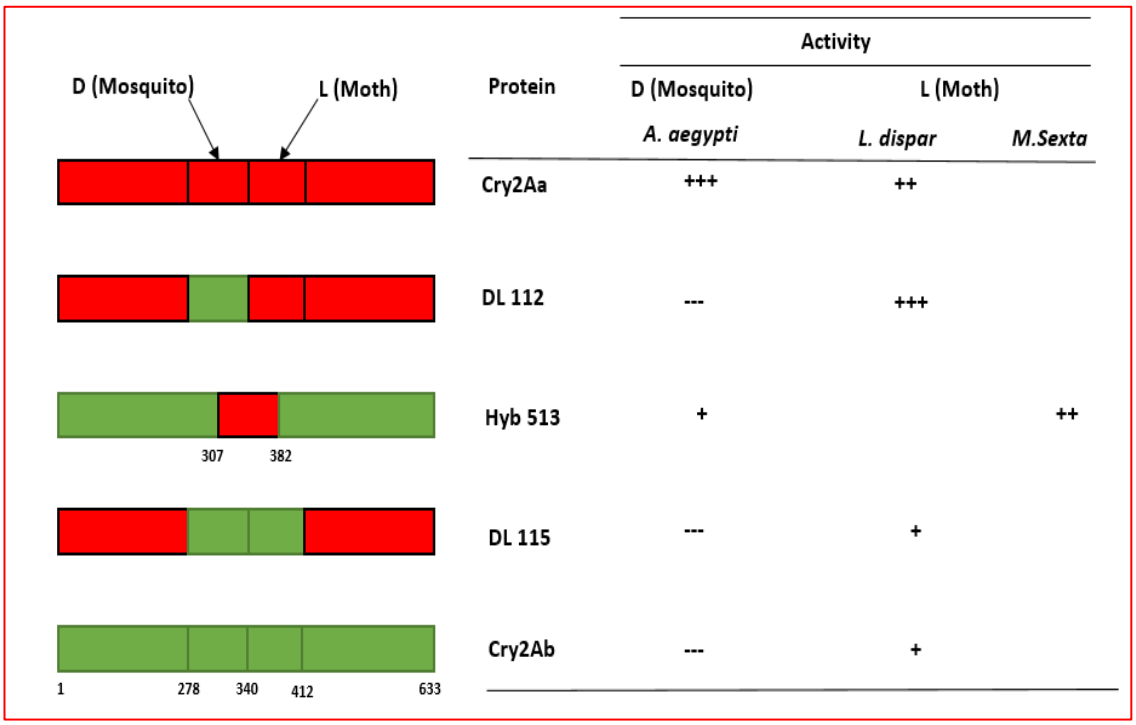



***Figure 1.9.4 Schematic presentation of Chimeric-Scanning mutagenesis data. Figure adapted from Morse et al. (2001).The top band and all other red rectangles indicate Cry2Aa sequence. The bottom band and all other green rectangles indicate Cry2Ab sequence. DL112 and DL115 are data from Liang and Dean (1994). Hyb513 is a data from Widner and Whiteley (1990). Activity representations indicate an approximate log scale. For reference, (+) indicates an ID50 (infectious dose) of 126 (85.7-187) ng, and (+++) indicates an ID50 of 3,200 (1,340-51,900) ng. (---) represents negligible toxicities.***

The chimeric data above was reported by Morse *et al*. (2001) to have enabled the determination of a candidate toxin-receptor binding surface on Cry2Aa, after the structure of Cry2Aa was determined by multiple isomorphic studies replacement using heavy atoms and comparing the structure to those of Cry1Aa and Cry3Aa. In addition, the amino sequence of Cry2Aa and Cry2Ab were aligned around these regions defined by the chimeric mutagenesis studies to identify the specificity distinguishing residues. This binding surface is composed of an arrangement of hydrophobic residues Val365, Leu369 from the β5-β6 loop, β4-β5 and Leu402 – Leu404 from the β7-β8 loop), across the solvent-exposed surface of the β- prism and β -sandwich domains as earlier depicted in Figure 1.7.1.

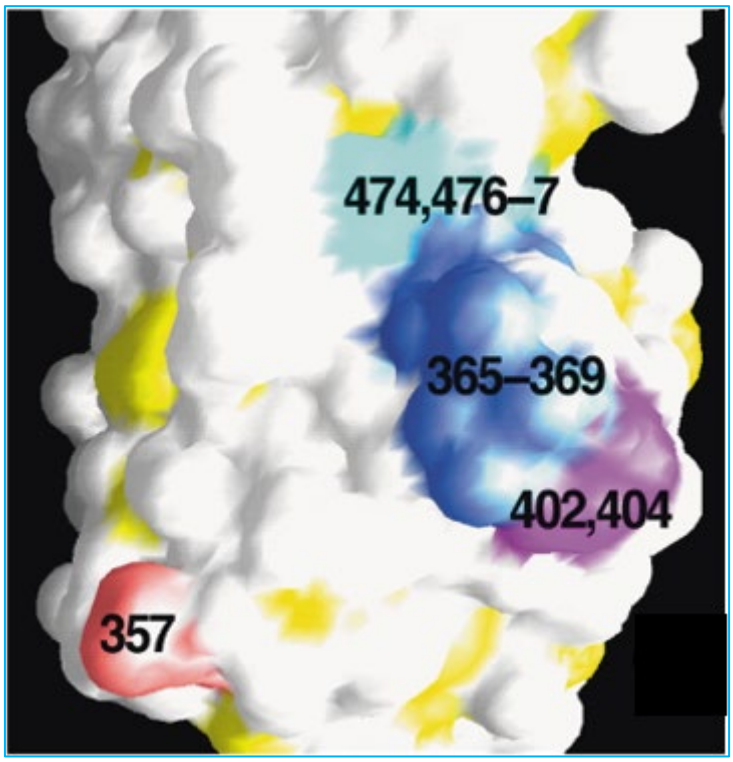



***Figure 1.9.5 The solvent accessible surface of domains II and III of Cry2Aa. The projection of residue hydrophobicity onto this surface is shown in colour. Portions of the hydrophobic surface contributed by residues 474, 476, and 477 are shown in cyan, those contributed by residues 365–369 are shown in blue, those contributed by residues 402 and 404 are shown in magenta, and the remainder of the surface contributed by hydrophobic residues is shown in yellow. The remaining surface that is identified as non-hydrophobic is coloured white. For orientation, the portion of the surface contributed by residue 357 of the β4-β5 loop is shown in red. Figure taken from Morse et al. (2001).***

Most of the amino acid differences between Cry2Aa and 2Ab spotted by Morse *et al*. (2001) are found within or about the domain II/III 800Å$^2$ hydrophobic patch depicted in figure 1.9.5 above and the surrounding residues from the β5- β6, β7- β8, and β4- β5 loops shown in Figure 1.7.1 but not domain I.

Proteolytic activation of the toxin was shown to involve the removal of the 49-N-terminal amino acid (Audtho *et al*., 1999), through cleavage around amino acid at the 144$^{th}$ position and this was reported by Morse *et al*. (2001) to expose the residues comprising the putative toxin-receptor binding surface shown in Figure 1.9.6 below. In addition, removal of the 49 N-terminal amino acids exposes these residues comprising the putative toxin-receptor binding surface. However, removal of the 49 amino terminal residues comprised of α0, α0a, and an N-terminal coil (Figure 1.9.6), they reported, would have no effect on the structure of the seven-helical membrane insertion domain, as examined through comparing the structures of the activated toxin from Cry1Aa and that of the protoxin from Cry2Aa.

The diagram showing the 49N-terminal residues of Cry2Aa, comprising α0, α0a, and the N-terminal coil. These components, based on the structure of Cry2Aa, may be suggested to sterically hinder access to the putative binding epitope, β5-β6 and β7-β8 loops, and the exposed parts of domain III closest to domain II as shown in the ribbon structure of Cry2Aa, depicted in Figure 1.9.7 below.

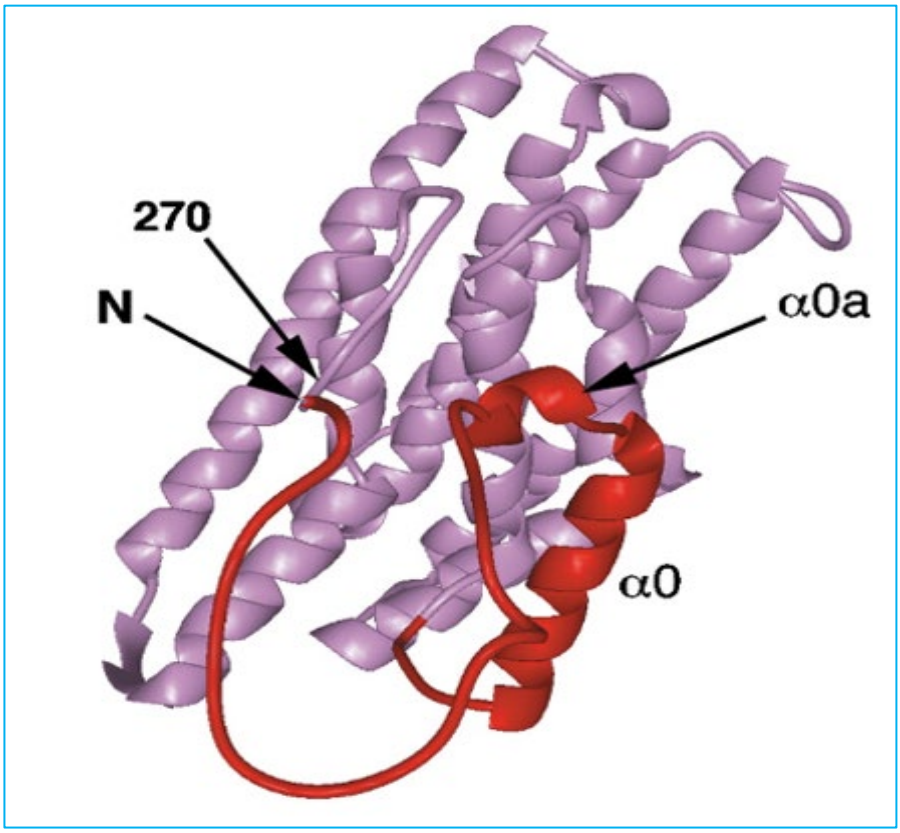



***Figure 1.9.6 Diagram of Domain I of Cry2Aa showing the 49N-terminal amino acid residues coloured red. Labels with amino acid numbers indicate the visible N and C termini of the domain. Taken from Morse et al. (2001).***

Morse *et al*. (2001) showed that projection of hydrophobicity onto the solvent accessible surface of domains II and III reveals an 800A°$^2$ hydrophobic patch (Figure 1.9.5) proximal to these loops. Nevertheless, they asserted that while the structure indicates that the 49 N-terminal residues (α0, α0a, and the N-terminal coil Figure 1.9.6 above) may sterically prevent access to the putative binding epitope, the biological logic for this function is unclear. That, it is very improbable that Bt has a receptor with affinity for the activated toxin. Hence, it does appear possible that the N-terminus acts to hinder premature activation of the toxin within Bt. The explanation they gave was that the blockage of the hydrophobic patch of the putative binding epitope prevents nonspecific aggregation of the toxin with itself or other host proteins, and that the N-terminal amino

acids might play a role in the formation of an environmentally stable crystalline inclusions.

### 1.9.1 Recent works toward finding the specificity determining regions (SDRs) in Cry2A toxins

Work carried out by a previous project student, Jake Evans, involved the analysis of the amino acid sequences of four Cry2A toxins namely: Cry2Aa, Cry2Ab, Cry2Ac and Cry2Ag based on previous bioassay data from the literature, which is presented in table 1.9-2 below. The bioassay data presented in table 1.9-2 below are unreliable because they are not comparable as different types of samples such as spore crystal mixes, purified proteins, single toxin producing strains and multi toxin producing strains were assayed, hence the need for me to carry out a comprehensive bioassay of all the available Cry2A toxins in our laboratory in order to produce a much more reliable bioassay data.

## Bioassay data for Cry2A toxins ●= toxic o= nontoxic

| Genus | Species | Qualitative | Quantitative (Toxicity Measure) | Mortality recorded (time) | Instar | Origin of gene used (Subspecies, Strain, Plasmid or isolated gene) | Reference |
|---|---|---|---|---|---|---|---|
| ***Aedes*** | ***Aegypti*** | ● | $LC_{50}$ = 0.5-1 µg/ml | | L1 | *kurstaki* HD-1 (not assayed) - WRW30 recombinant plasmid expressing Cry2Aa protein | Widner and Whiteley (1989) |
| | | ● | $LD_{50}$ = 0.1-1.0 µg of cells, wet weight/ml | 72hrs | L3 & L4 | *kurstaki* – expressed in *B.megaterium* cells harboring the plasmid pEG204 (cry2Aa) | Donovan *et al*., (1988) |
| | | ● | $LC_{50}$ = 6.25 µg/ml | 24hrs | 4 to 6 day old | *kurstaki* HD-1 (using strain information from the nomenclature database to identify which toxin is produced) | Nicholls *et al*. (1989) |
| | | ● | $LD_{50}$ = 1-5 µg/ml of deionized water | 72hrs | L4 | *kurstaki* HD-1 and HD-263 - not assayed - isolated gene | Dankocsik *et al*. (1990) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | ● | 100% mortality | Daily intervals up to 1 week | L3 | YBT-226 (whole crystal from this strain also toxic) | Hodgman *et al*., (1993) |
| | | ● | $LC_{50}$ = 65.5ng/ml | 72hrs | L3 | Plasmid pSB304.3 containing cry2Aa operon. Orf1 and orf2 deleted leaving cry2Aa gene in vector pTZ18R forming pDL103 | Liang and Dean (1994) |
| | | ● | $LC_{50}$ = 37.06 ug/ml | 48hrs | L3-4 | *kurstaki* - no assay with strain - purified cry2Aa protein | Sims (1997) |
| | | ● | 20µg/m l = ~38% 50µg/m l = ~95% 100µg/ml = 100% mortality | 48hrs | L3 | *kenyae* HD549 - shown to be toxic when bioassayed with all 6 species in previous papers (Amonkar *et al*., 1979; 1985; Donovan *et al*., 1988) | Misra *et al*. (2002) |
| | | o | No toxicity observed at different doses:30 µl | 24 and 48hrs | L2 | *kurstaki* Brazilian S477 strain - recombinant viruses | Lima *et al*. (2008) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | containing 30, 20, 15, 10, 5 and 1 µg/ml, respectively | | | (vAcCry2Aa and vSynCry2Ab) were amplified in *Trichoplusia ni* (BTI-Tn5B1-4) cells and used to infect *Spodoptera frugiperda* larvae - crystals were purified and used in bioassays - S477 not assayed | |
| | | ● | $LC_{50}$ >5000ng/ml - slightly toxic | 24hrs | L3 | Plasmid pSB304.3 containing cry2Aa operon. Orf1 and orf2 deleted leaving Cry2Aa gene in vector pTZ18R forming pDL103 (Liang and Dean, 1994) | McNeil and Dean (2011) |
| | | o | $LC_{50}$ >100 µg/ml | 24hrs | Early L4 | Rpp39 | Liang *et al.,* (2011) |
| ***Aedes*** | ***triseriatus*** | ● | $LC_{50}$ = 2.84 µg/ml | 48hrs | L3-4 | *kurstaki* - no assay with strain - purified cry2Aa protein | Sims, (1997) |
| ***Anopheles*** | ***stephensi*** | ● | Mortality measured at 20, 50 and 100µg/ml = 90, 100 and 100% respectively | 48hrs | L3 | *kenyae* HD549 - no assay with strain | Misra *et al*., (2002) |
| ***Anopheles*** | ***gambiae*** | ● | $LC_{50}$ = 0.13µg/ml | 24hrs | 4 to 6 day old | *kurstaki* HD-1 - native crystal toxic | Nicholls *et al*., (1989) |
| | | ● | $LC_{50}$ = 110 ng/ml | 24hrs | L3 | Plasmid pSB304.3 containing cry2Aa operon. Orf1 and orf2 deleted leaving Cry2Aa gene in vector pTZ18R forming pDL103 (Liang and Dean, 1994) | McNeil and Dean (2011) |
| ***Anopheles*** | ***quadrimaculatus*** | ● | LC50 = 0.37 ug/ml | 24hrs | L3-4 | *kurstaki* - no assay with strain - purified cry2Aa protein | Sims (1997) |
| | | ● | $LC_{50}$ = 38ng/µl | 24hrs | 2 day | Cry2Aa gene construct, pGEM103-9, made by subcloning cry2Aa | Audtho (2001) |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| | | | | | | gene from pDL103 (Liang and Dean., 1994) into pGEM-3Z(+) vector (promega) | |
| ***Culex*** | ***pipiens*** | o | $LC_{50}$ >200 µg/ml | 48hrs | L2-4 | *kurstaki* - no assay - purified Cry2Aa protein | Sims (1997) |
| | | ● | $LT_{50}$ at a concentration of 100 µg/ml of the spore/crystal mixture = 70hrs | 70hrs | L2 | *kurstaki* - no assay with strain | Zghal *et al*.(2006) |
| | | ● | $LC_{50}$ >5000 ng/ml (slightly toxic) | 24hrs | L3 | Plasmid pSB304.3 containing cry2Aa operon. Orf1 and orf2 deleted leaving Cry2Aa gene in vector pTZ18R forming pDL103 | McNeil and Dean (2011) |
| ***Culex*** | ***fatigans*** | ● | mortality measured at 20, 50 and 100µg/ml = 90, 100 and 100% respectively | 48hrs | L3 | *kenyae* HD549 - shown to be toxic when bioassayed with all 6 species in previous papers - isolated protein assayed in this paper | Misra *et al*., (2002) |
| ***Culex*** | ***quinquefasciatus*** | ● | $LC_{50}$ = 1.63 µg/ml | 48hrs | L2 | *Kurstaki* NRD-12 isolate | Moar *et al*. (1994) |
| | | o | No toxicity observed at different doses: 30 µl containing 30, 20, 15, 10, 5 and 1 µg/ml, respectively | 24 and 48hrs | L2 | *kurstaki* Brazilian S477 strain - recombinant viruses (vAcCry2Aa and vSynCry2Ab) were amplified in *Trichoplusia ni* (BTI-Tn5B1-4) cells and used to infect *Spodoptera frugiperda* larvae - crystals were purified and used in bioassays - S477 not assayed | Lima *et al*., (2008) |
| | | ● | $LC_{50}$ >200µg/ml | 48hrs | L4 | Recombinant plasmid with cry2Aa (from pCL-92 into pDBF69 plasmid: (Ge et al., 1998)) - no assay of galleriae | Bideshi *et al.,* 2013 |

**Bioassay data for Cry2Ab:** ● = toxic, o = non-toxic

| Genus | Species | Qualitative | Quantitative (Toxicity Measure) | Mortality recorded (time) | Instar Age | Origin of gene used (Subspecies, Strain, Plasmid or isolated gene) | Reference |
|---|---|---|---|---|---|---|---|
| ***Aedes*** | ***Aegypti*** | o | $LC_{50}$ >50µg/ml | | L1 | *kurstaki* HD-1 - WRW50 plasmid (Cry2Ab) | Widner and Whiteley (1989) |
| | | o | $LD_{50}$ >20µg/ml | 72hrs | L4 | EG7219 (pEG259 Cry3A/2Ab fusion) | Dankocsik *et al*., 1990 |
| | | o | $LC_{50}$ >100µg/ml | 72hrs | L3 | EG7219 (Dankocsik *et al*, 1990), | Liang and Dean (1994) |
| | | ● | $LC_{50}$ = 23.42µg/ml | 24hrs | early L4 | Ywc5-4 | Liang *et al*., 2011 |
| | | o | $LC_{50}$ >6000ng/ml ) | 24hrs | L3 | *kurstaki* HD-1- isolated Cry2Aa (Morse et al., 2001) | McNeil and Dean (2011) |
| ***Anopheles*** | ***gambiae*** | ● | $LC_{50}$ = 540ng/ml | 24hrs | L3 | *kurstaki* HD-1- isolated Cry2Aa (Morse et al., 2001) | McNeil and Dean (2011) |
| ***Culex*** | ***pipens*** | o | $LC_{50}$ > 6000ng/ml | 24hrs | L3 | *kurstaki* HD-1- isolated Cry2Aa (Morse et al., 2001) | McNeil and Dean (2011) |

**Bioassay data for Cry2Ac:** ● = toxic, o = non-toxic

| Genus | Species | Qualitative | Quantitative (Toxicity Measure) | Mortality recorded (time) | Instar Age | Origin of gene used (Subspecies, Strain, Plasmid or isolated gene) | Reference |
|---|---|---|---|---|---|---|---|
| ***Aedes*** | ***aegypti*** | o | $LD_{50}$ >50 µg/ml | | | *Bt* S1 - inclusions had mosquitocidal activity | Wu *et al*. (1991) |
| ***Aedes*** | ***albopictus*** | ● | 54·4% of corrected mortality | 72hrs | L3 | *Bt* LLB6 strain containing Cry2Ac toxin - LLB6 (75·6% of corrected mortality) | Zhang *et al*. (2007) |

**Bioassay data for Cry2Ag:** ● = toxic, o = non-toxic

| Genus | Species | Qualitative | Quantitative (Toxicity Measure) | Mortality recorded (time) | Instar Age | Origin of gene used (Subspecies, Strain, Plasmid or isolated gene) | Reference |
|---|---|---|---|---|---|---|---|
| ***Aedes*** | ***aegypti*** | ● | $LC_{50}$ =2.541 ug/ml | 24hrs | L1 & L4 | JF19-2 | Zheng *et al.* (2010) |

***Table 1.9-2 Bioassay data for some selected Cry2A toxins from the literature. Table taken from Evans (2014).***

He used amino acid sequence alignments to compare proteins with different specificities. He expected that the specificity determining residues would be conserved amongst homologues with the same specificity, which as he determined, was not the case. This is because when the sequences of the three active toxins against *Aedes aegypti* (Cry2Aa, Cry2Ac and Cry2Ag) were compared there was no sequence conservation along the regions identified by Widner and Whiteley (1990), Liang and Dean (1994) to be the SDRs (amino acid positions 307-412) depicted in Figure 1.9.7.

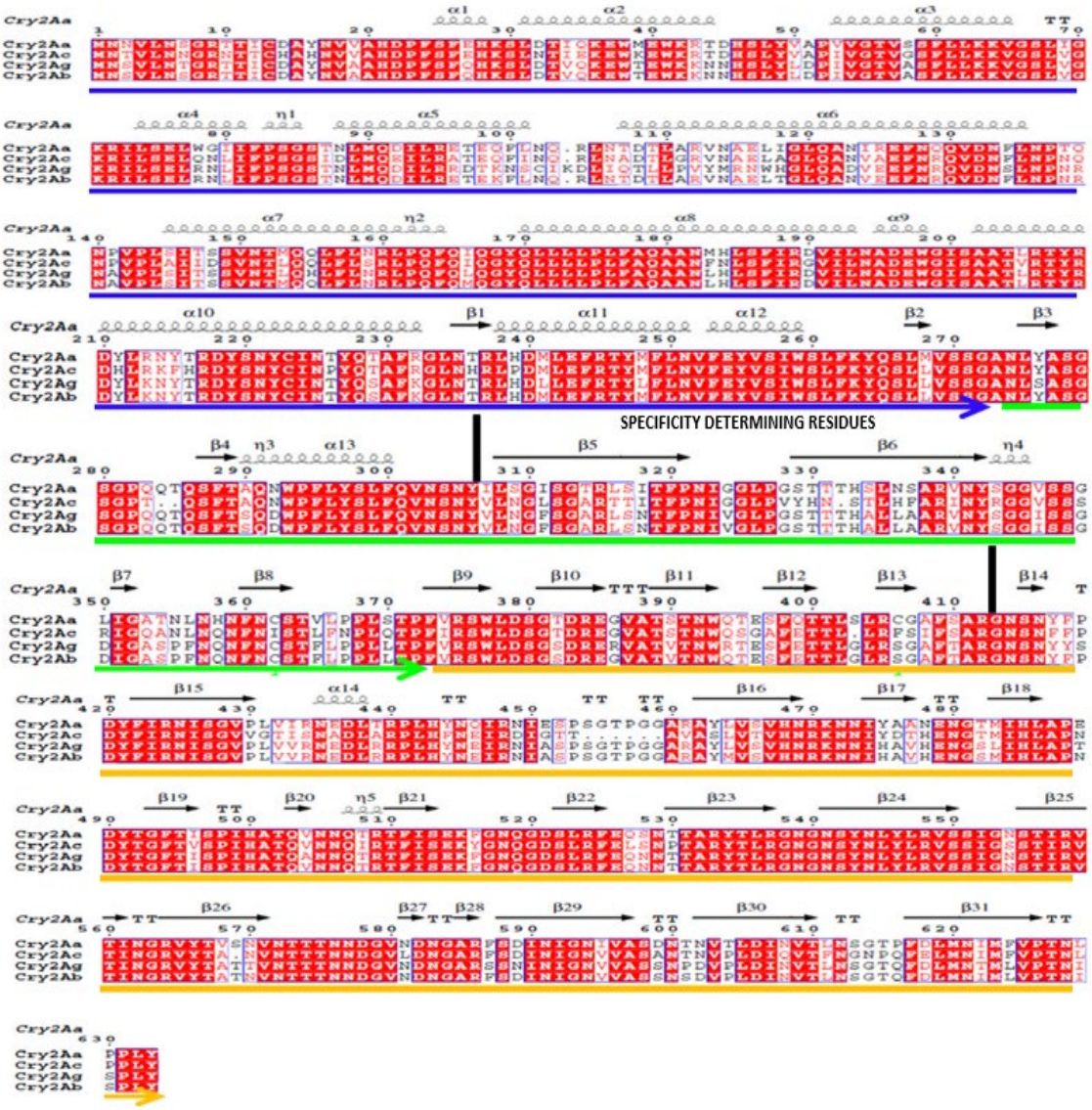



***Figure 1.9.7 Cry2A amino acid alignments (in Espript format). Amino acid sequences are aligned in for Cry2Aa, Cry2Ac, Cry2Ag and Cry2Ab. Cry2Aa alignment position has been changed to be at the top of the alignment in order to allow the programme to use the Cry2Aa secondary structure, which is mapped on the top of the alignment. The blue arrow spans domain I, the green arrow spans domain II and the orange arrow spans domain III. The position in the alignments are in reference to the position of amino acids in Cry2Aa. The region containing amino acid residues which comprise the specificity determining residues is indicated by black vertical lines. Figure taken from Evans (2014).***

He further used a method of grouping toxins by toxin specificity to genus *A. aegypti* where at a given amino acid position, no amino acid in the toxic group can be the same as the amino acid in the nontoxic group. With these, he identified some residues namely: Threonine 118, Methionine 464 and Serine 601 to be important for future mutagenesis studies in Cry2Ab. After further analysis of these identified amino acid using UCSF Chimera and Cry2Aa as a model structure, he discovered that Methionine 464 might be the most attractive target for future mutagenesis. His research work was complicated by the fact that the bioassay data in table 1.9-2 were having conflicting results as mentioned earlier. Therefore, he finally suggested that proper bioassay of all the Cry2A toxins be carried out, and at the same time, sequence analysis based on the results obtained be done in future research.

Recent research carried out to identify Cry2A toxin genes in a collection of 300 strains of Bt identified a novel toxin named Cry2Aa17 which showed sequence similarity to Cry2Ab in domain 1 whilst the domains 2 and 3 resembled Cry2Aa (Figure 1.9.8). When the toxicity profile of this novel toxin against three different insect orders was determined, it matched those of Cry2Ab, and hybrid creation through domain I swap between Cry2Aa and Cry2Aab gave similar findings thus implicating domain I in toxicity region determination (Shu *et al*., 2017). This led to the current project work.

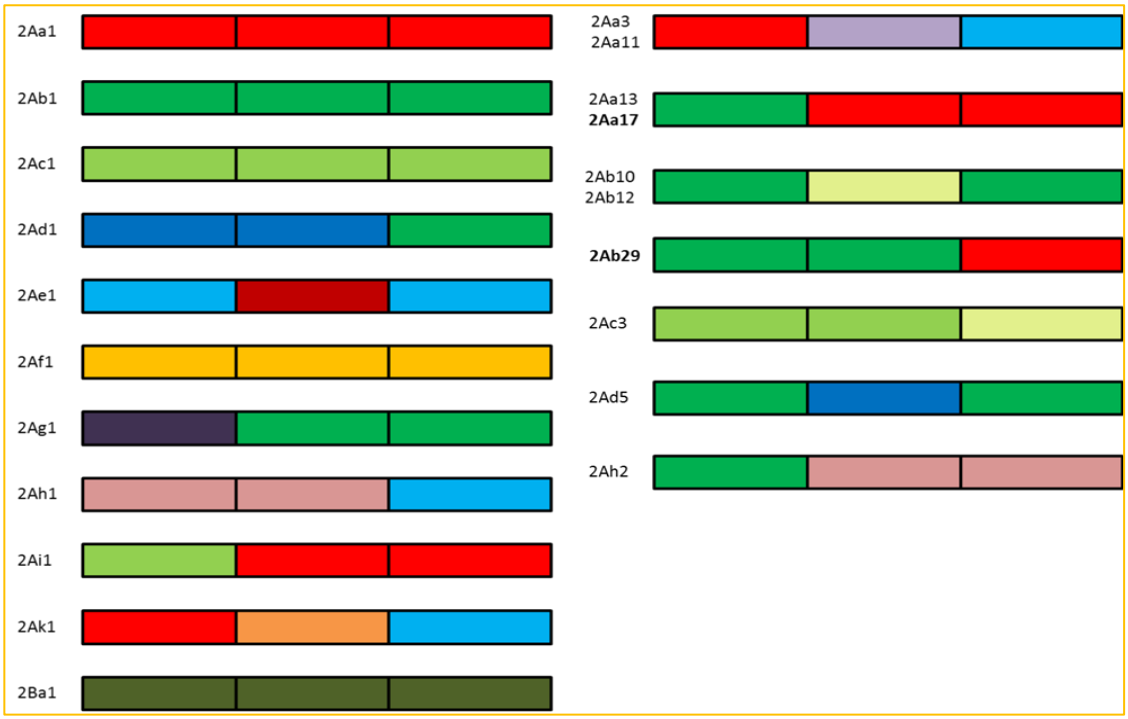



***Figure 1.9.8 Domain configurations of the Cry2A toxins. Each toxin is split into the three domains identified from crystallographic studies of Cry2Aa each of which is represented by a rectangle (Morse et al., 2001). Cry proteins with two or more different colours in their domains represent natural hybrids whereas those rectangles with two names of Cry proteins assigned to them represent toxins that have similar amino acids composition in their respective domains. Figure taken from Shu et al. (2017).***

## 1.10 Present work

### 1.10.1 Prelude

There is growing concern owing to the rapid increase in mosquito resistance to various chemical insecticides and their concomitant environmental pollution leading to the search for alternative means for mosquito control, such as the use of biological agents and insect growth regulators(Alphey *et al*., 2013). Therefore, Cry proteins from Bt strains having a wide toxicological spectrum and high specificity (Schnepf *et al*., 1998) became a common tool in mosquito control, Agriculture and Forestry and at the same time serving as a safer alternative to traditional pesticides. Importantly also, is the fact that it has been experimented and found that Cry2Aa toxin of Bt is toxic to mosquito vectors of human diseases including Zika fever, Chikungunya and yellow fever transmitted by *Aedes aegypti* making Cry2Aa an attractive mosquitocidal agent to control the spread of the disease (McNeil and Dean, 2011). Previous works were done to compare the activity of a closely related toxin, Cry2Ab that does not possess mosquitocidal activity against *Aedes aegypti* to Cry2Aa, which possessed mosquitocidal activity against *Aedes aegypti* (Hofte and Whiteley, 1989, Liang and Dean, 1994). However, with only two toxins it was difficult to find a good association between structure and function. That is why we want to use more Cry2A toxins- unfortunately; published data are confusing and often contradictory so we need to generate these here and use it to achieve the aim of this research, which is to understand the basis for the specificity of Cry2A toxins of *Bacillus thuringiensis* against the dipteran insect, *Aedes aegypti*.

### 1.10.2 Aims and objectives of the research

i. To carry out the characterisation, expression, harvesting and bioassay of the wild type Cry2A toxins against *Aedes aegypti* mosquito in order to generate a comprehensive and more reliable data for further work.

*ii.* To identify the domain(s) and/ or amino acid motifs that encode toxin specificity to *Aedes aegypti.*

iii. Last but not the least, to study the nature of the interactions between the mid gut juice of *Aedes aegypti* and Cry2A. This would be achieved by deciphering through in vitro studies if the cleaved fragments resulting from the activation of Cry2A toxins by protease within the *Aedes* mosquito mid gut

remain associated or dissociated from each other to exert their activity within the *Aedes* mid gut epithelium.

# 2. Materials and Methods

## 2.1 Materials

### 2.1.1 Bacterial strains and reagents

The Crickmore Laboratory of the School of Life Sciences, University of Sussex, provides all bacterial strains and reagents used in this study.

The Bacterial toxins used in this study comprise Cry2A toxins from *Bacillus thuringiensis*, which consist of the Bt toxin genes, and then two strains from *E. coli*, used for transformation and expression of the Bt gene. The various Cry2A used and their respective plasmids are shown in Table 2.1-1.

| Plasmid | Toxin |
|---|---|
| **pGEM** | Cry2Aa2 |
| **pEB** | Cry2Aa9 |
| **pEB** | Cry2Aa17 |
| **pEB** | Crym2Aa17 |
| **pGEM** | Cry2Ab (4D6-4) |
| **pGEM** | Cry2Ab (916-2) |
| **pEB** | Cry2Ab4 |
| **pEB** | Cry2Ab29 |
| **pGEM** | Cry2Ac |
| **pGEM** | Cry2AcAa |
| **pGEM** | Cry2Ad |
| **pEB** | Cry 2Ah1 |
| **pEB** | Cry 2Ax |
| **pGEM** | Cry2Ab (4D6-5 SP6) |
| **pGEM** | Cry2Ab (916-5 SP6) |

***Table 2.1-1 The Bacillus thuringiensis toxins used in this study***

The *E. coli* strains used in this study are shown in Table 2.1-2 below.

| Strain | Application |
|---|---|
| DH5α | Host for transformation |
| BL21(DE3)pLysS | Host for Expression |

***Table 2.1-2 The E. coli strains used in this study***

### 2.1.2 Buffers

10 x TBE buffer: 108 g of Tris base, 55 g of boric acid, 40 ml of0.5 M EDTA, 2 l of $dH_2O$, pH 8.0.

Resolving Gel Buffer (RGB): 18.18 g Tris-(Hydroxymethyl) amino methane, 0.4 g SDS, 100 ml of $dH_2O$, pH 8.8.

Stacking Gel Buffer (SGB): 6.06 g Tris-(Hydroxymethyl) amino methane, 0.4 g SDS, 100 ml of dH2O, pH 6.8.

Resolving Gel Buffer (RGB) for native gel: 18.18 g Tris-(Hydroxymethyl) amino methane, 100 ml of $dH_2O$, pH 8.8.

Stacking Gel Buffer (SGB) for native gel: 6.06 g Tris-(Hydroxymethyl) amino methane, 100 ml of $dH_2O$, pH 6.8.

10 ×SDS running buffer: 7.6g Tris-HCl, 36g glycine, 2.5g SDS, 250 ml of $dH_2O$, pH 8.3

10 x running buffer for native gel: 7.6g Tris-HCl, 36g Glycine, 250 ml of $dH_2O$, pH 8.3

2 × protein gel sample loading solution:2g SDS, 6 mg EDTA, 20 mg Bromophenol Blue, 5 ml of RGB, 50 ml glycerol, 100 ml of $dH_2O$.

2 x native protein gel sample loading solution: 6 mg EDTA, 20 mg Bromophenol Blue, 5 ml of RGB, 50 ml glycerol, 100 ml of $dH_2O$.

Coomassie blue stain: methanol, $dH_2O$, acetic acid (10:9:1 v/v/v), Brilliant Blue R-250 (0.25%, w/v).

De-staining solution: methanol, $dH_2O$, acetic acid (10:9:1, v/v/v).

10 ×PBS: 80 g of NaCl, 2g of KCl, 14.4g of $Na_2HPO_4$, 2.4g of $KH_2PO_4$,1 l of $dH_2O$, pH 7.4.

50mM $Na_2C0_3$ buffer (pH 10.5).

### 2.1.3 Reagents and Enzymes

Reagents obtained from Sigma-Aldrich: SDS, Tris-HCl, ammonium persulfate, Bromophenol Blue, β-mercaptoethanol, TEMED, acrylamide/bis-acrylamide 30%, sodium carbonate, Coomassie Brilliant Blue R-250, BSA, and IPTG. The following were purchased from AnalaR BDH: glucose, NaOH, EDTA, $CaCl_2$, methanol, 1-butanol, and sodium acetate. Chemicals obtained from Thermo Fisher Scientific: Sucrose, glycine, KCl, NaCl, HCl, glycerol, mono-potassium phosphate, di-potassium phosphate, glacial acetic acid, sodium hydrogen carbonate, $MgCl_2$. Pre-stained Protein Ladder, 1kb DNA ladder,

Gel red, DpnI, T4 DNA ligase, HaeIII and BSaAI restriction enzymes were obtained from New England Biolabs. The following reagents were obtained from Melford: Tris-base, ampicillin, LB Capsule (1kg), trypsin, and agarose. The protease inhibitor was obtained from Roche.

## 2.2 Methods

### 2.2.1 Expression and harvesting of protein from *E. coli* BL21 (DE3) pLysS

Colonies of *E. coli* BL21 (DE3) pLysS cells harbouring the relevant Cry2A clone were scraped from agar plate using a sterile loop into 500 ml of L Broth, prepared by dissolving an LB capsule (1 kg) in 500 ml of deionised water, to which 500 µl of 100 mg/ml ampicillin was added. Cells were grown for approximately 3 hours in a 37$^{o}$C shaking incubator checking the O.D at 30-minute intervals until an O.D of 0.4-0.6 was obtained. This was then followed by the addition of 250 µl of 1 molar IPTG.

The *E. coli* BL21 (DE3) pLysS cells in L-broth was then left overnight (14hours) at 25$^{o}$C in a shaking incubator. The cultures were then poured into a centrifuge bottle and centrifuged at 6,371 x g in JA 10 rotor for 10 minutes at 4$^{o}$C to form a pellet, and the supernatant was discarded. The pellet was re-suspended in 30 ml of distilled water, transferred to a 50 ml Falcon tube and sonicated at an amplitude of 20 microns for a total of 4 minutes with intermittent 1-minute rest times between each 1-minute sonication. The sonicated cells were then transferred to a 50 ml Oakridge tube, and centrifuge for 30 minutes at 27,216 x g. The supernatant containing the cell debris was discarded and the pellet containing the protein was transferred to a 50 ml Oakridge tube, and sonicated again for the second time, then centrifuged again at 27,216 x g for 20 minutes. It was then, finally, re-suspended in 1 ml of deionised water and viewed under the microscope to check for cell debris and protein inclusion. 5 µl of the re-suspended pellet was run on an SDS-PAGE gel.

### 2.2.2 SDS- PAGE gel

7.5% SDS-PAGE (Sodium Dodecyl Sulphate-Polyacrylamide Denaturing Gel Electrophoresis) gels were prepared for protein analysis. Glass plates were cleaned with ethanol and sealed with 200 µl of 1% SDS agarose, made up of 0.3g agarose in 30 ml 1xSDS gel running buffer. Resolving gels were made up in a small glass bottle using the

protocol below.

| Component | Volume (µl) |
|---|---|
| Distilled water | 2000 |
| Resolving Gel Buffer (RGB) | 1000 |
| Acrylamide/bisacrylamide | 1000 |
| Ammonium per sulphate (APS) | 8 |
| TEMED | 4 |

A 1 ml Gilson Pipette was used to introduce the resolving gel between the plates. 200 µl of water-saturated butanol was added to the top of the gel. When the gel was set after 20-30 minutes the water-saturated butanol was removed, washed with water, and blotted to remove excess liquid from between the plates. The stacking gel solution was

| Component | Volume (µl) |
|---|---|
| Distilled water | 1170 |
| Stacking Gel Buffer (SGB) | 500 |
| Acrylamide/bisacrylamide | 333 |
| Ammonium per sulphate (APS) | 4 |
| TEMED | 2 |

prepared using the protocol below.

The gel was allowed to set for 15-20 minutes, after which the plates were then set up in the electrophoresis apparatus. 1xSDS gel running buffer was added to the reservoirs and wells were washed out as well.

5 µl of the SDS-ME loading buffer, was added to 5 µl protein sample in a large Eppendorf tube. This was boiled for 4 minutes, and then centrifuged at 5,510 x g for 30 seconds.

The total 10 µl sample was then loaded into the gel alongside a protein marker. An SDS-PAGE gel was then run at 200V for 40 minutes. After the gel had run, it was then stained with Coomassie Brilliant Blue staining solution for 20 minutes on a shaker; this was followed by removal of the stain through the addition of different changes of de-staining solution for 20 minutes on a shaker until the bands became visible on the gels.

### 2.2.3 Native PAGE gel

The native gel used in this study was prepared using the following protocol. Glass plates were clean with ethanol before assembly and the bottom of the plates were sealed with 200 ul 1% agarose made up in 1 x running buffer (see Materials section). The resolving gel solution was made following the protocol below.

| **Component** | **7.5% Gel** | **12% gel** |
|---|---|---|
| Water | 2 ml | 2 x 700 µl |
| RGB | 1 ml | 1 ml |
| Acrylamide | 1 ml | 2 x 800 µl |
| 400mg/ml APS | 8 µl | 8 µl |
| TEMED | 4 µl | 4 µl |

The above components were carefully introduced between the plates using a 1 ml Gilson pipette. 200 ul of water or water-saturated butanol was carefully added to the top of the poured gel. After the gel has set, the top layer of water/butanol was removed, and washed with water and any traces of liquid remaining was blotted off.

The stacking gel solution was prepared using the protocol below.

| **Components** | **7.5% and 12% gel** |
|---|---|
| Water | 2 x 585 µl |
| SGB | 500 µl |
| Acrylamide | 333 µl |
| APS | 4 µl |
| TEMED | 2 µl |

The stacking gel was carefully added on top of the freshly poured resolving gel, and the comb was immediately inserted. When set, the comb was removed and the gel was transferred to the electrophoresis apparatus.

5 µl of the 2 x native protein gel sample loading buffer was added to 5 µl protein sample in a large Eppendorf tube then centrifuged at 5,510 x g for 30 seconds.

The total 10 µl sample was then loaded into the gel alongside a protein marker. The native gel electrophoresis was then run at 200V for 40 minutes. After the gel had run, it was then stained with Coomassie Brilliant Blue staining solution for 20 minutes on a shaker; this was followed by removal of the stain through the addition of different

changes of de-staining solution for 20 minutes on a shaker until the bands became visible on the gels.

### 2.2.4 Determination of protein concentration

Two methods were used to measure the protein concentration in this study. The first method was the Bradford method, which was used to measure the concentration of the *Aedes* mid gut juice protein and the densitometry method using Image J software to measure the Cry toxins concentration.

#### Bradford method for determination of protein concentration

Protein concentration was determined by the Bradford method (Bradford, 1976) using a Bio-Rad Protein Assay Kit (Bio-Rad) with BSA as the standard. All presented concentrations represent final concentrations used (unless stated otherwise). The mixture was incubated for 5 - 10 min at RT before measurement. Concentration of the unknown sample was determined by comparing its absorbance value against a plotted BSA standard curve. The standard curve showed near linear response (R2=0.9975) over 0 - 1 mg/ml BSA concentration range, which is the range absorbance values from the least to the highest by which the standard curve obeys Beer-Lambert's law.

#### Determination of protein concentration using image J

The concentrations of the toxins within the crude sample was determined using densitometric method by running the proteins along with three or four sets of BSA (Bovine Serum Albumin) standards at different concentrations. The gel obtained was then subjected to the Image J software, which quantified each toxin, as well as the BSA standards as area under the peak. The concentrations of the toxins were obtained by interpolation.

### Sucrose gradient protein purification

Toxin inclusions were partially purified for bioassay by sucrose gradient purification. In a 1.5 ml Eppendorf tube, 100 µl of undiluted sample was layered on top of 1 ml of 50 % (w/v) sucrose solution, and then centrifuged for 10 minutes at 14,549 x g. The supernatant and any floating debris were discarded and 100 µl of deionised water was used to re-suspend the pellet.

### 2.2.5 PCR Amplification of the DNA

PCR procedure was carried out in order to amplify the various domains which are to be ligated to form the various hybrids required using the wild type Cry toxins as the template strand. In addition, to be sure that the various domains amplified using PCR are free from interference by the template/parental DNA, each of the amplified products is further digested with DpnI.

Primers were designed based on the specific amplification products desired at each stage of the study, and were ordered from the MWG Eurofins after making sure that they met the optimum conditions for PCR reaction. The primers were all adjusted to a concentration of 100 pmol/µl with deionised water and then each diluted 1:10 again with deionised water.

The reaction mixture for the Cry2A toxins domains amplification is shown below.

| | |
|---|---|
| Master mix | 25 µl |
| Primer DI or DII F | 1 µl |
| Primer DI or DII R | 1 µl |
| DNA template (pGEM Cry2A) | 1 µl |
| Deionised water | 22 µl |
| Total volume | 50 µl |

The PCR Program used for this procedure was PFU ULTRA 6KB.

The reaction conditions for the reaction were as follows.

1. Initial denaturation $95^0$C for 2minutes
2. Denaturation $95^0$C for 20 seconds
3. Annealing $50^0$C for 20 seconds
4. Elongation $72^0$C for 1 minute and 20 seconds

Reactions 2, 3 and 4 were repeated for 30 consecutive cycles then finally

5. Final extension for 3 minutes

The product obtained above was run on a gel to see if the desired product had been amplified after which 45 µl of the PCR product was being mixed with 1 µl of DpnI enzyme, and incubated for 1 hour to digest the parental/ template strand.

### 2.2.6 Agarose Gel Electrophoresis

To prepare the gels, 0.3 g of agarose was mixed into 30 ml TBE (Tris/Borate/EDTA) inside a sterile 50 ml Erlenmeyer flask. The mixture was microwaved until visibly clear of agarose traces. Once cooled down, 1.5 µl of a 1:3 dilution of GelRed™ was introduced, and the liquid gel was poured into the receptacle of the electrophoresis device and a comb inserted. 5 µl of each amplified DNA sample was mixed with 1 µl of gel loading buffer prior to loading. 5 µl of 1 kb marker was loaded alongside for identification of the PCR products present in each sample. The gel was run at 120 V and imaged using a UV transilluminator.

### 2.2.7 Purification of DNA from Agarose gels

We used two different approaches to purify the DNA samples based on the observed purity of the DNA amplification products seen on the gel. These were:

#### Liquid Purification

Liquid purification was performed on DNA gel samples displaying a single band on an agarose gel following the QIAquick® PCR Purification Kit Protocol. 50 µl of the sample was placed inside a 1.5 ml Eppendorf tube and 250 µl of PB buffer was added to each tube. The samples were then placed in a spin column inside a collection tube, and centrifuged for 30 seconds at 8,609 x g. The flow-through was discarded. 750 µl of PE buffer was added to each spin column prior to two rounds of 30 seconds of centrifuging at 8,609 x g each time to get rid of residual buffer. The spin columns were then transferred to a 1.5 ml Eppendorf tubes prior to eluting by adding 30 µl EB buffer. Finally, the samples were given 1 minute to rest before being centrifuged for 1 minute at 14,549 x g. The flow-through now contained the purified PCR products for visualising in later DNA gels.

#### Gel Purification

Excised gel samples were placed in a 1.5 ml Eppendorf tubes. The protocol from QIAquick® Gel Extraction Kit was used in this situation. 600µl of QG buffer was added to each tube prior to placing them on a pre-heated warming block at 50°C for 5 minutes to

dissolve the gel. The contents of each tube were then poured into individual spin columns and centrifuged for 1 minute at 14,549 x g. The flow through was discarded and 500 µl of QG was added to the spin column. The samples were centrifuged again for 30 seconds at 14,549 x g, the flow-through discarded, and 750 µl of PE buffer was added to each column and centrifuged for another 30 second at 14,549 x g and the flow-through discarded. At this point, the spin columns were transferred to 1.5 ml Eppendorf centrifuge tubes and 30 µl of EB buffer was added to each of them before leaving them to stand for 1 minute. This was then centrifuged for 1 minute at 14,549 x g. The flow through now contained the eluted and purified DNA samples.

### 2.2.8 Ligation of the PCR products

The ligation reaction was set up following the recommended ligation ratio of 1:3 to 1:5 of vector to insert in the right estimated proportion based on the intensity of their respective bands on a gel, plus 1 µl of ligase buffer and 0.5 µl of DNA Ligase then adding deionised water to make a total of 10 µl mixture. This was left to stand overnight for ligation reaction to take place. A second ligation reaction that we later adopted in this work was the one using the Blunt/TA master mix reagent from New England BioLabs.

1. The master mix was transferred to ice prior to reaction set up. The tube was mixed by finger flicking before use
2. 20-100 ng of the Vector was combined with a 3- fold molar excess of insert and the volume was adjusted to 5 µl with deionised water.
3. 5 µl of the Blunt/TA master mix was added to the above mixture by pipetting up and down 7-10 times or by finger flicking
4. The above mixture was incubated at room temperature ($25^0$C) for 15 minutes and placed on ice

The above ligation mixture was then used for transformation or stored at $-20^0$C for future use.

### 2.2.9 *E. coli* transformation

We employed two methods (chemical and electroporation).

#### Electroporation method

*E. coli* strains were grown on 25 ml L-agar at 37°C overnight. The strains were then prepared for transformation by scraping a culture from the agar plate using a sterile toothpick into 100 ml L-broth and grown in a shaking incubator until it reached an OD of over 0.4-0.6. The cells were then harvested by centrifugation- the 100 ml of broth containing the *E. coli* was spun at 17,696 x g for ten minutes, the supernatant discarded and the pellet re-suspended in 100ml of deionised water at 4°C. The re-suspended cells were centrifuged again at 17,696 x g for a further ten minutes and the pellet re-suspended in approximately 1ml of 4°C water, which was then transferred to a 1.5 ml Eppendorf. The Eppendorf was centrifuged at 14,549 x g for 1 min and the pellet re-suspended in 200µl of 4°C sterile water.

50 µl of the *E. coli* cell suspension was transferred to a small Eppendorf tube, and stored on ice- 1 µl of DNA was added and mixed before being transferred into a sterile 2 mm electroporation cuvette and tapped to ensure the cells settled at the bottom. The gene pulser was set to 1.8 kV, 200 Ohms and 25 µF, the electroporation cuvette placed in it and a short electrical pulse was applied to the cells. The cells and 0.5 ml of L-broth were mixed with a sterile Pasteur pipette, and transferred into small glass bottles. Then left to rest for one hour at room temperature.

#### Heat shock method

The protocol outlined below is the one specified by the New England BioLab for transformation using this method. The protocol is as follows:

i. A tube containing 50 µl of NEB5-α competent *E. coli* cells was thawed on ice for 10 minutes
ii. 2 µl of the ligation mixture was added to the 50 µl NEB5-α competent *E. coli* cells above and carefully flick the tube 4-5 times to mix the cells and DNA.
iii. The mixture was placed on ice for 30 minutes without mixing
iv. The above mixture was then heat shocked at exactly $42^0$C for exactly 30 seconds without mixing.
v. It was then placed on ice for 5 minutes

vi. 950 µl of recovery media (SOC) maintained at room temperature was added to the mixture

vii. It was then placed on a shaker maintained at $37^0$C, 250 rpm and was allow to rotate for 1 hour

viii. 500 µl of the above was poured on an ampicillin plate that was warmed at $37^0$C and the plate was placed in an incubator maintained at $37^0$C and was allow to stay overnight for cell growth.

### 2.2.10 PCR and restriction digest

The cells obtained from the transformation procedure, which is expected to contain the hybrid comprising the two DNA fragments that were ligated together, needs to be checked for the presence of the insert (the DNA fragment that does not contain the vector), and to be sure that the insert is in the right orientation. This is because the insert could be ligated to the vector in two possible orientations; T7 (A) orientation, that is in the same direction with the promoter region and SP6 (B) orientation, meaning in opposite direction with the promoter region. In addition, the vector could self-ligate itself, and this could be picked by the NEB5-α competent *E.coli* cells during transformation process. Therefore, this confirmation was achieved using two approaches, each of which was employed at some points in this research work. The first approach involves the use of the plasmid DNA extracted from the colonies and then using enzyme digestion to confirm which of the colonies contain the insert whereas in the second approach the technique of colony PCR and HaeIII restriction enzyme digestion were used to confirm the presence of the insert (domain I in this case). The colony PCR procedure used in this research is outlined as follows:

The colonies produced from the NEB5-α transformation were selected using a toothpick and were suspend on a tube containing the mixtures below.

1. Master mix 12.5 µl
2. Forward primer 0.5 µl
3. Reverse primer 0.5 µl
4. D/water 12.5 µl

The above mixture in a tube was run on a PCR using the program RMMG with the following conditions:

Heated lid $105^0$C

Initial denaturation 2 minutes at $90^0$C

Seq 1 Denaturation for 30seconds at $95^0$C

Seq 2 Annealing for 30 seconds at $50^0$C

Seq 3 Elongation for 30 seconds at $72^0$C

The above three reactions are repeated for 25 cycles

Then finally the final extension for 5 minutes at $72^0$C

The restriction enzyme digestion procedure is same for both the two approaches and it is as shown below:

| | | |
|---|---|---|
| 1. | Deionised water | 6.5 µl |
| 2. | Plasmid DNA | 2 µl |
| 3. | Buffer | 1 µl |
| 4. | Restriction enzyme | 0.5 µl |
| | Total | 10 µl |

This was incubated in a water bath for 20-30 minutes at $37^0$C

After which 2 µl of DNA loading buffer was added and run on a gel. The gel was scanned and the restriction fragments produced, which appeared as bands in the gel was compared to those predicted by NEB cutter for both A and B orientations. This then enabled us to know which of the colonies has the insert in the right orientation.

### 2.2.11 Mosquito rearing.

#### Insect population

*Aedes aegypti* were obtained from Cardiff University, UK and subsequently cultured here.

#### Feeding the mosquitoes

The adult male mosquitoes were fed on 20% sucrose solution, which was prepared as soon as it is finishes and/or if any form of fungal or algal growth is noticed on the cotton. The sucrose solution was poured inside a bottle and a cotton wool was made in a thread-

like fashion, and dipped inside it. The sucrose moves through the cotton via a capillary action. The adult males drink the sucrose through the soaked cotton wool.

The female adult mosquitoes were fed on heparinised horse serum, freshly prepared after every two days when there was no high demand for eggs or on daily basis when there was high demand for eggs. The blood meal was prepared by pouring some blood in a bottle lid and the exterior of the lid was dried using tissue paper. After which, a para film was stretch and used to cover the lid containing the blood meal; the para film automatically sticks to the dry sides of the lid containing the blood meal. The extra para film extending from the lid was cut using scissors. Then the bottle lid containing the blood meal was carefully placed inside the cage in the position where a hole was already created in the cage, the parafilm facing the inside of the cage while the bottom of the bottle lid attached to the heating block maintained at $37^0$C.

The mosquito larvae were fed on ground tetramine fish food. This was ground to powder using pestle and mortar. Then a loop full of the feed was placed inside the water containing the larvae. This was repeated daily or after every two days depending on the demand for eggs.

### Changing the water containing the larvae or pupae

The water containing the larvae and pupae was changed regularly, especially; any moment I noticed that it was cloudy or to have contained some fungal or algal growth. This was done by pouring clean water on the container containing the larvae or pupae and discarding it by pouring it away making sure the pupae or larvae were not poured along with the water. This was done continuously with many changes of the water until it becomes clear.

### Hatching of the eggs

The eggs were hatched by putting the filter paper containing the eggs on a beaker that is 1/3 filled with a lukewarm water containing some feed and was allow staying at a temperature of $27^0$C. Usually, the larvae began to appear after a period of 24 hours. This procedure was enhanced by placing the beaker containing the eggs inside an incubator maintained at $27^0$C, this way the eggs began to hatch after 12 hours. The filter paper was removed after the eggs have hatched, but those still containing unhatched eggs were placed in a new beaker and returned in to the incubator until hatched.

### Harvesting and transfer of the pupae

Provided the larvae were fed regularly, they metamorphosed to pupae within a period of five days to a week. They were then transferred in to a cage to avoid their escape on metamorphosing to adult. Therefore, a pipette dropper was used to collect the pupae and transfer them to a new container, which was then placed inside the cage. The pupae metamorphosed to adults within a period of two to four days, hence the reason why they were quickly transferred in to the cage.

### 2.2.12 Bioassay

The standard bioassay method by WHO (2005c) was used in this study. This involved using 4 ml of deionised water per larvae. To 80 ml of deionised water containing five different concentrations of the toxins in a 100 ml beaker and the control containing only the deionised water with no toxin, 20 late 3rd instar larvae were carefully added using a pipette dropper. They were then allowed to stay for a period of 24 to 72 hours at $27^0$C and 18 light; 6-hour dark photoperiod. A little amount of larval food was added to each beaker since the exposure period is more than 24 hours. The number of dead larvae in each beaker was counted after a period of 24 hours, 48 hours and 72 hours. During counting of dead larvae, moribund larvae that are larvae incapable of rising to the surface of the water or not showing the characteristic diving reaction when the water is disturbed, were counted as dead. The result is expressed as percentage mortality, which was obtained by dividing the total number of dead larvae by the total number of larvae in each container for the number of replicates and multiplying the result by 100. During the bioassay experiment if more than 10% of the control larvae pupate or if more than 20% mortality was recorded in the control, the test was discarded and repeated. For mortalities between 5% and 20% in the control group, the result of the treatment groups was treated using the Abbot's formula designated as follows:

**Corrected percentage mortality**= X-Y/X multiply by 100.

Where X = Percentage survival in the untreated control, and Y= Percentage survival in the treatment group.

### 2.2.13 Preparation of the *Aedes* mid gut protease

The *Aedes* mid gut protease was prepared by extracting 50 mid guts from late third instar larvae of *Aedes aegypti* through dissection on ice cold. The motility of the larvae

was restricted by putting them on ice cold for five minutes before commencing the dissection. The 50 mid guts dissected were suspended in 100 µl of 1X PBS buffer and was homogenised by sonication for 8 minutes setting it at 20 sec on and off cycle or using a homogeniser. The above mixture, which contains both extracellular and intra cellular fluids as the mid gut of *Aedes aegypti* is too small and the mid gut juice comprising the intracellular fluid cannot be extracted alone, was centrifuged for 10 minutes at 6,268 x g and $4^0$C. The supernatant was decanted on a 1.5 ml Eppendorf tube as the *Aedes* mid gut juice (AMJ) and was stored at $4^0$C for future use while the pellet was discarded.

### 2.2.14 Protein solubilisation and activation

Pellet containing Cry2Aa crystals was solubilized in 50 mM sodium carbonate (pH 10.5), in the presence of 5 mM dithiothreitol (DTT) and incubated at 37°C for 1 hour (1:1 w/v ratio of pellet to buffer). Sample was then spun down at 14,549 x g for 5 - 10 min and supernatant containing solubilized protein was treated with a protease. 10 mg/ml of chymotrypsin solution in the buffer was used for activation at the ratio of 1:5 (v/v) enzyme to supernatant at 37°C for 2hour whereas 1:3 ratio of the *Aedes aegypti* mid gut juice (AMJ) to the solubilised protein was used for activation using same temperature and time interval. After digestion, Complete™ mini EDTA-free protease inhibitor was added to stop further proteolysis in a ratio of 1:6 of the inhibitor to the activated protein in both instances.

# 3. Characterisation, Expression, Harvesting and Bioassay of Wild type Cry2A toxins.

## 3.1 Introduction

This chapter involves the characterisation, expression, harvesting and bioassay of all the Cry2A toxins displayed in Table 2.1-1 of the Materials and Methods section of this project. The characterisation was achieved by sending the DNAs for sequencing and using the sequencing results to carry out sequence alignment, using blastn and Clustal Omega, with the sequences of already characterised Cry2A toxins from the Bt nomenclature data base (Crickmore *et al*., 1998). This is to enable us confirm their identity through the degree of their sequence similarity to those of the database. The Cry2A toxin genes were sequenced in both the T7, which is in the same direction with the promoter region, and SP6, which is in the opposite (reverse) direction with the promoter region.

Further confirmation was done by running a protein gel and confirming the molecular weight of the toxin bands and comparing them to those found in the Bt nomenclature data base (Crickmore *et al*., 1998),and the ones predicted from a program in Expasy to be sure that there is actual agreement. This was done in order to have a much more comprehensive data for all the Cry2A toxins, as previous researches mostly concentrate on comparing the activity of a closely related toxin, Cry2Ab that does not possess mosquitocidal activity against *Aedes aegypti* to Cry2Aa that possessed mosquitocidal activity against *Aedes aegypti* (Widner and Whiteley, 1990, Liang and Dean, 1994). However, with only two toxins it was difficult to find a good association between structure and function. That is why we want to use more Cry2A toxins- unfortunately; published data are confusing and often contradictory (Table 1.9-2), so we need to generate these here.

The host of expression for all the Cry2A proteins chosen in this research was *E.coli.* Therefore, we carried out an experiment to show that the proteins from *E.coli* are nontoxic to *Aedes aegypti*. This implies that any activity observed from the bioassay can thereafter, be attributed to the toxin alone but not the *E.coli* proteins, which may still be found in solution with the toxin.

Qualitative bioassay was also carried out to screen out those Cry2A that are toxic to *Aedes aegypti* larvae from those that are nontoxic to this insect. This was followed by quantitative bioassay for those Cry2A toxins that were toxic to *Aedes aegypti* larvae to be able to determine their LC 50 values.

## 3.2 Results

### 3.2.1 Confirmation of Cry2A toxin sequence

The seven of the Cry2A toxins presented in table 2.1-1 (Cry2Aa9, Cry2Aa17, mCry2Aa17, Cry2Ab4, Cry2Ab29, Cry2Ah1, and Cry2Ax), were provided and sequenced by the institute of plant protection, Chinese Academy of Agricultural Sciences and were transformed into *E.coli* BL 21 strain by a previous student (Nicholas Stevens, 2015). A previous student (Phipps, 2015, BSc Dissertation) confirmed the sequence of Cry2Ac toxin. Cry2AcAa hybrid toxin, which encodes domain I of Cry2Ac, and domain II and III of Cry2Aa, was also designed by a previous student (Evans, 2014, BSc Dissertation). The two Cry2Ab protein, 4D6-5(SP6) and 916-5(SP6), which were designed to be in SP6 (reverse orientation), that is in a direction that is opposite to the promoter region and as such could not be expressed, were also done by a previous project student (Evans, 2014). Cry2Aa2 sequence was taken from the Bt nomenclature data base (Crickmore *et al*., 1998). Therefore, we sequenced Cry2Aa2 toxin available in our lab using a pGEM T7 primer and compare it to the one in the Bt nomenclature database to confirm if it is truly Cry2Aa2, the result is shown in Figure 3.2.5.

The two Cry2Ab proteins i.e. Cry2Ab (4D6-4) and (916-2) which contained Cry2Ab gene with the pGEM plasmid DNA were extracted and sent off to 'MWG eurofins' lab to be sequenced so we could confirm their sequence as they were yet to be determined. Therefore, in order to sequence the entire gene, three primers were designed as one or two primers cannot be sufficient to sequence the entire gene. These primers are:

T7 with the sequence: 5'-TAATACGACTCACTATTAGGG-3'

Cry2F with the sequence: 5'-TATTACCTTTATTTGCACAGGCA-3'

SP6 with the sequence: 5'-ATTTAGGTGACACTATAG-3'

The sequencing results received were pieced together in each case to form the nucleotide sequence for the gene of the Cry2Ab being determined. The primer SP6, is a

reverse primer, therefore, the reverse complement of the sequence from this primer was obtained in order to carry out the analysis. Since there were areas of overlaps among the three sequences received from the sequencing results, we therefore, aligned them with the DNA sequence of Cry2Ab4 from the database as a template (Figure 3.2.1 and 3.2.2). This is to enable us understand where to stop the sequence from one primer and start the sequence of the next primer to avoid repetition of the overlapping regions. Positions where there were differences between our sequence and that of the database were noted, and carefully studied to see if they are real differences, or just a case of ambiguity in the sequencing process but fortunately enough there were only few differences, which fall within the overlapping regions and were all resolved. The diagram illustrating the procedure for mapping the sequences of the two Cry2Ab (4D6-2 and 916-2) received from the sequencing results to that of Cry2Ab4 from the database is shown in Figure 3.2.1 and 3.2.2 respectively. Thereafter, the DNA sequence generated was converted to protein using a program in EXpasy.

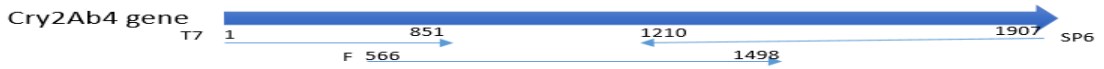



***Figure 3.2.2: The diagrammatic representation of gene mapping for Cry2Ab (4D6-4 gene). T7, F and SP6 denote the three results obtained from the sequencing results for the entire gene. Arrows shows the direction of sequencing.***


***Figure 3.2.1 The diagrammatic representation of gene mapping for Cry2Ab (916-2). T7, F and SP6 denote the three results obtained from the sequencing results for the entire gene. Arrows shows the direction of sequencing.***

The sequence of the two Cry2Ab (4D6-4 and 916-2) generated from the above analysis are shown in Figure 3.2.3 and 3.2.4 below.

**GCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAAGGAGGAATTTTATATGAATAATGTATTGAATAGCGGAAGAACTACTATTT**
**GTGATGCGTATAATGTAGCGGCTCATGATCCATTTAGTTTTCAACACAAATCATTAGATACCGTACAAAAGGAATGGACGGAGTGGA**
**AAAAAAATAATCATAGTTTATACCTAGATCCTATTGTTGGAACTGTGGCTAGTTTTCTGTTAAAGAAAGTGGGGAGTCTTGTTGGAAA**
**AAGGATACTAAGTGAGTTACGGAATTTAATATTTCCTAGTGGTAGTACAAATCTAATGCAAGATATTTTAAGAGAGACAGAAAAATT**
**CCTGAATCAAAGACTTAATACAGACACTCTTGCCCGTGTAAATGCGGAATTGACAGGGCTGCAAGCAAATGTAGAAGAGTTTAATCG**
**ACAAGTAGATAATTTTTTGAACCCTAACCGAAACGCTGTTCCTTTATCAATAACTTCTTCAGTTAATACAATGCAACAATTATTTCTAAA**
**TAGATTACCCCAGTTCCAGATGCAAGGATACCAACTGTTATTATTACCTTTATTTGCACAGGCAGCCAATTTACATCTTTCTTTTATTAG**
**AGATGTTATTCTAAATGCAGATGAATGGGGAATTTCAGCAGCATCATTACGTACGTATCGAGATTACTTGAAAAATTATACAAGAGA**
**TTACTCTAACTATTGTATAAATACGTATCAAAGTGCGTTTAAAGGTTTAAACACTCGTTTACACGATATGTTAGAATTTAGAACATATA**
**TGTTTTTAAATGGATTTGAGTATGTATCTATCTGGTCGTTGTTTAAATATCAAAGTCTTCTAGTATCTTCCGGTGCTAATTTATATGCAA**
**GTGGTAGTGGACCACAGCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTTTCCAAGTTAATTCAAATTATGTGT**
**TAAATGGATTTAGTGGTGCTAGGCTTTCTAATACCTTCCCTAATATAGTTGGTTTACCTGGTTCTACTACAACTCACGCATTGCTTGCTG**
**CAAGGGTTAATTACAGTGGAGGAATTTCGTCTGGTGATATAGGTGCATCTCCGTTTAATCAAAATTTTAATTGTAGCACATTTCTCCCC**
**CCATTGTTAACGCCATTTGTTAGGAGTTGGCTAGATTCAGGTTCAGATCGGGAGGGCGTTGCCACCGTTACAAATTGGCAAACAGAAT**
**CCTTTGAGACAACTTTAGGGTTAAGGAGTGGTGCTTTTACAGCTCGCGGTAATTCAAACTATTTCCCAGATTATTTTATTCGTAATATTT**
**CTGGAGTTCCTTTAGTTGTTAGAAATGAAGATTTAAGAAGACCGTTACACTATAATGAAATAAGAAATATAGCAAGTCCTTCAGGAA**
**CACCTGGTGGAGCACGAGCTTATATGGTATCTGTGCATAACAGAAAAAATAATATCCATGCTGTTCATGAAAATGGTTCTATGATTCA**
**TTTAGCGCCAAATGACTATACAGGATTTACTATTTCGCCGATACATGCAACTCAAGTGAATAATCAAACACGAACATTTATTTCTGAAA**
**AATTTGGAAATCAAGGTGATTCTTTAAGGTTTGAACAAAACAACACGACAGCTCGTTATACGCTTAGAGGGAATGGAAATAGTTACA**
**ATCTTTATTTAAGAGTTTCTTCAATAGGAAATTCCACTATTCGAGTTACTATAAACGGTAGGGTATATACTGCTACAAATGTTAATACT**
**ACTACAAATAACGATGGAGTTAATGATAATGGAGCTCGTTTTTCAGATATTAATATCGGTAATGTAGTAGCAAGTAGTAATTCTGATG**
**TACCATTAGATATAAATGTAACATTAAACTCCGGTACTCAATTTGATCTTATGAATATTATGCTTGTACCAACTAATATTTCACCACTTT**
**ATTAAGGTTTG**

***Figure 3.2.3 Sequence generated for Cry2Ab (4D6-4) after analysing the results obtained from sequencing the entire gene.***

**TGCATGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAAGGAGGAATTTTATATGAATAATGTATTGAATAGCGGAAGAACTAC**
**TATTTGTGATGCGTATAATGTAGCGGCTCATGATCCATTTAGTTTTCAACACAAATCATTAGATACCGTACAAAAGGAATGGACGGAG**
**TGGAAAAAAAATAATCATAGTTTATACCTAGATCCTATTGTTGGAACTGTGGCTAGTTTTCTGTTAAAGAAAGTGGGGAGTCTTGTTG**
**GAAAAAGGATACTAAGTGAGTTACGGAATTTAATATTTCCTAGTGGTAGTACAAATCTAATGCAAGATATTTTAAGAGAGACAGAAA**
**AATTCCTGAATCAAAGACTTAATACAGACACTGTTGCCCGTGTAAATGCGGAATTGACAGGGCTGCAAGCAAATGTAGAAGAGTTTA**
**ATCGACAAGTAGATAATTTTTTGAACCCTAACCGAAACGCTGTTCCTTTATCAATAACTTCTTCAGTTAATACAATGCAACAATTATTTC**
**TAAATAGATTACCCCAGTTCCAGATGCAAGGATACCAACTGTTATTATTACCTTTATTTGCACAGGCAGCCAATTTACATCTTTCTTTTA**
**TTAGAGATGTTATTCTAAATGCAGATGAATGGGGAATTTCAGCAGCAACATTACGTACGTATCGAGATTACTTGAAAAATTATACAA**
**GAGATTACTCTAACTATTGTATAAATACGTATCAAAGTGCGTTTAAAGGTTTAAACACTCGTTTACACGATATGTTAGAATTTAGAACA**
**TATATGTTTTTAAATGTATTTGAATATGTATCTATCTGGTCGTTGTTTAAATATCAAAGTCTTCTAGTATCTTCCGGTGCTAATTTATATG**
**CAAGTGGTAGTGGACCACAGCAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTTTCCAAGTTAATTCAAATTATG**
**TGTTAAATGGATTTAGTGGTGCTAGGCTTTCTAATACCTTCCCTAATATAGTTGGTTTACCTGGTTCTACTACAACTCACGCATTGCTTG**
**CTGCAAGGGTTAATTACAGTGGAGGAATTTCGTCTGGTGATATAGGTGCATCTCCGTTTAATCAAAATTTTAATTGTAGCACATTTCTC**
**CCCCCATTGTTAACGCCATTTGTTAGGAGTTGGCTAGATTCAGGTTCAGATCGGGAGGGCGTTGCCACCGTTACAAATTGGCAAACAG**
**AATCCTTTGAGACAACTTTAGGGTTAAGGAGTGGTGCTTTTACAGCTCGCGGTATTTCAAACTATTTCCCAGATTATTTTATTCGTAAT**
**ATTTCTGGAGTTCCTTTAGTTGTTAGAAATGAAGATTTAAGAAGACCGTTACACTATAATGAAATAAGAAATATAGCAAGTCCTTCAG**
**GAACACCTGGTGGAGCACGAGCTTATATGGTATCTGTGCATAACAGAAAAAATAATATCCATGCCGTTCATGAAAATGGTTCTATGAT**
**TCATTTAGCGCCAAATGACTATACAGGATTTACTATTTCGCCGATACATGCAACTCAAGTGAATAATCAAACACGAACATTTATTTCTG**
**AAAAATTTGGAAATCAAGGTGATTCCTTAAGGTTTGAACAAAATAACACGACAGCTCGTTATACGCTTAGAGGGAATGGAAATAGTT**
**ACAATCTTTATTTAAGAGTTTCTTCAATAGGAAATTCCACTATTCGAGTTACTATAAACGGTAGGGTATATACTGCTACAAATGTTAAT**
**ACTACTACAAATAACGATGGAGTTAATGATAACGGAGCTCGTTTTTCAGATATTAATATCGGTAATGTAGTAGCAAGTAGTAATTCTG**
**ATGTACCATTAGATATAAATGTAACATTAAACTCCGGTACTCAATTTGATCTTATGAATATTATGCTTGTACCAACTAATATTTCACCAC**
**TTTATTAAGGTT**

***Figure 3.2.4 Sequence generated for Cry2Ab (916-2) after analysing the results obtained from sequencing the entire gene.***

The sequence alignment result for Cry2Aa2 obtained from sequencing result is shown in figure 3.2.5.

```
Cry2Aa2-T7      TGCTCCGGCCGCCATGGCGGCCGCGGGAATTCGATTAAGGAGGAATTTTATATGAATAAT  60
Cry2Aa2         ----------------------------------------------------ATGAATAAT  9
                                                                     *********

Cry2Aa2-T7      GTATTGAATAGTGGAAGAACAACTATTTGTGATGCGTATAATGTAGTAGCCCATGATCCA 120
Cry2Aa2         GTATTGAATAGTGGAAGAACAACTATTTGTGATGCGTATAATGTAGTAGCCCATGATCCA  69
                ************************************************************

Cry2Aa2-T7      TTTAGTTTTGAACATAAATCATTAGATACCATCCAAAAAGAATGGATGGAGTGGAAAAGA 180
Cry2Aa2         TTTAGTTTTGAACATAAATCATTAGATACCATCCAAAAAGAATGGATGGAGTGGAAAAGA 129
                ************************************************************

Cry2Aa2-T7      ACAGATCATAGTTTATATGTAGCTCCTGTAGTCGGAACTGTGTCTAGTTTTTTGCTAAAG 240
Cry2Aa2         ACAGATCATAGTTTATATGTAGCTCCTGTAGTCGGAACTGTGTCTAGTTTTTTGCTAAAG 189
                ************************************************************

Cry2Aa2-T7      AAAGTGGGGAGTCTTATTGGAAAAAGGATATTGAGTGAATTATGGGGGATAATATTTCCT 300
Cry2Aa2         AAAGTGGGGAGTCTTATTGGAAAAAGGATATTGAGTGAATTATGGGGGATAATATTTCCT  249
                ************************************************************

Cry2Aa2-T7      AGTGGTAGTACAAATCTAATGCAAGATATTTTAAGGGAGACAGAACAATTCCTAAATCAA 360
Cry2Aa2         AGTGGTAGTACAAATCTAATGCAAGATATTTTAAGGGAGACAGAACAATTCCTAAATCAA  309
                ************************************************************

Cry2Aa2-T7      AGACTTAATACAGATACCCTTGCTCGTGTAAATGCAGAATTGATAGGGCTCCAAGCGAAT 420
Cry2Aa2         AGACTTAATACAGATACCCTTGCTCGTGTAAATGCAGAATTGATAGGGCTCCAAGCGAAT  369
                ************************************************************

Cry2Aa2-T7      ATAAGGGAGTTTAATCAACAAGTAGATAATTTTTTAAACCCTACTCAAAACCCTGTTCCT 480
Cry2Aa2         ATAAGGGAGTTTAATCAACAAGTAGATAATTTTTTAAACCCTACTCAAAACCCTGTTCCT  429
                ************************************************************

Cry2Aa2-T7      TTATCAATAACTTCTTCGGTTAATACAATGCAGCAATTATTTCTAAATAGATTACCCCAG 540
Cry2Aa2         TTATCAATAACTTCTTCGGTTAATACAATGCAGCAATTATTTCTAAATAGATTACCCCAG  489
                ************************************************************

Cry2Aa2-T7      TTCCAGATACAAGGATACCAGTTGTTATTATTACCTTTATTTGCACAGGCAGCCAATATG 600
Cry2Aa2         TTCCAGATACAAGGATACCAGTTGTTATTATTACCTTTATTTGCACAGGCAGCCAATATG  549
                ************************************************************

Cry2Aa2-T7      CATCTTTCTTTTATTAGAGATGTTATTCTTAATGCAGATGAATGGGGTATTTCAGCAGCA 660
Cry2Aa2         CATCTTTCTTTTATTAGAGATGTTATTCTTAATGCAGATGAATGGGGTATTTCAGCAGCA  609
                ************************************************************

Cry2Aa2-T7      ACATTACGTACGTATCGAGATTACCTGAGAAATTATACAAGAGATTATTCTAATTATTGT 720
Cry2Aa2         ACATTACGTACGTATCGAGATTACCTGAGAAATTATACAAGAGATTATTCTAATTATTGT  669
                ************************************************************

Cry2Aa2-T7      ATAAATACGTATCAAACTGCGTTTAGAGGGTTAAACACCCGTTTACACGATATGTTAGAA 780
Cry2Aa2         ATAAATACGTATCAAACTGCGTTTAGAGGGTTAAACACCCGTTTACACGATATGTTAGAA  729
                ************************************************************

Cry2Aa2-T7      TTTAGAACATATATGTTTTTAAATGTATTTGAATATGTATCCATTTGGTCATTGTTTAAA 840
Cry2Aa2         TTTAGAACATATATGTTTTTAAATGTATTTGAATATGTATCCATTTGGTCATTGTTTAAA  789
                ************************************************************

Cry2Aa2-T7      TATCAGAGTCTTATGGTATCTTCTGGCGCTAATTTATATGCTAGCGGTAGTGGACCACAG 900
Cry2Aa2         TATCAGAGTCTTATGGTATCTTCTGGCGCTAATTTATATGCTAGCGGTAGTGGACCACAG  849
                ************************************************************
```

***Figure 3.2.5 Sequence alignment result for Cry2Aa2.***

The sequencing results showed that the sequence is for Cry2Aa2 toxins due to the complete homology between the sequence of our Cry2Aa2 (T7) and that of the database as shown above.

### 3.2.2 Expression and harvesting of toxins.

The Cry2A toxins in this research were expressed using *E. coli* BL 21 (DE3) pLysS cells containing the T7 RNA polymerase gene under the control of the lac UV5 promoter in its chromosomal DNA by induction with IPTG.

The expressed toxin was grown, harvested (figure 3.2.6) and its concentration measured by running the toxin on a gel along with BSA standards as depicted in Figure 3.2.7(a,b,c,d and e) below, by densitometry method using Image J software.

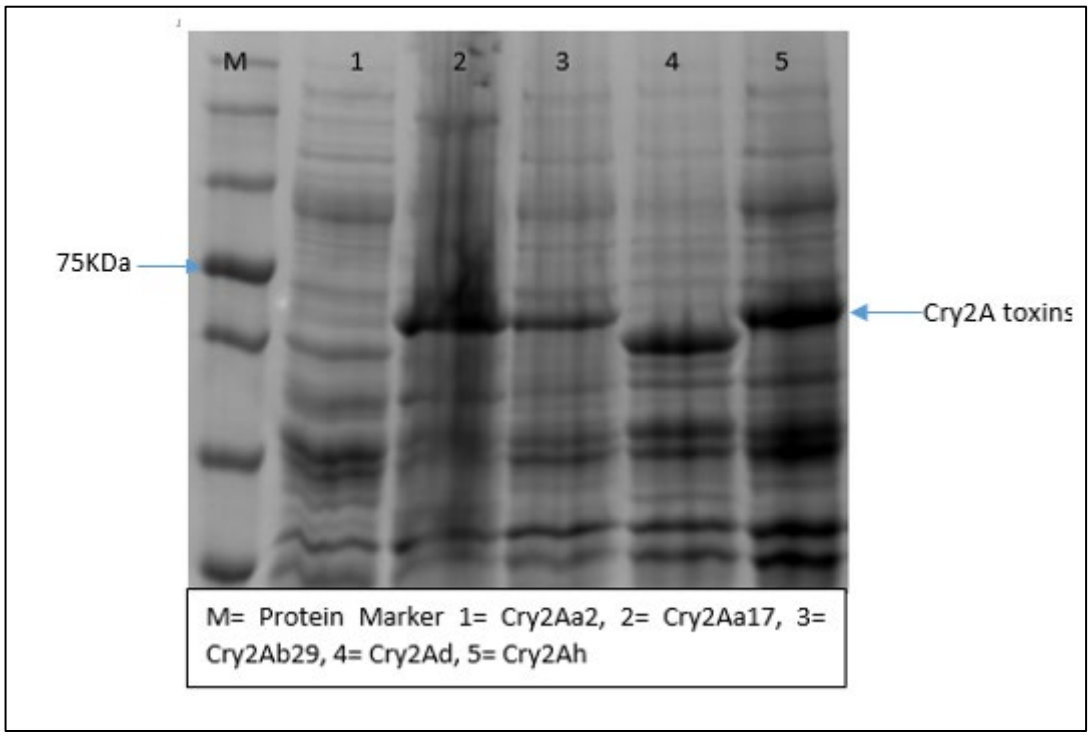



***Figure 3.2.6 Protein SDS-Page gel showing Cry2A toxins bands. The arrow denotes the position of the Cry2A toxin bands.***

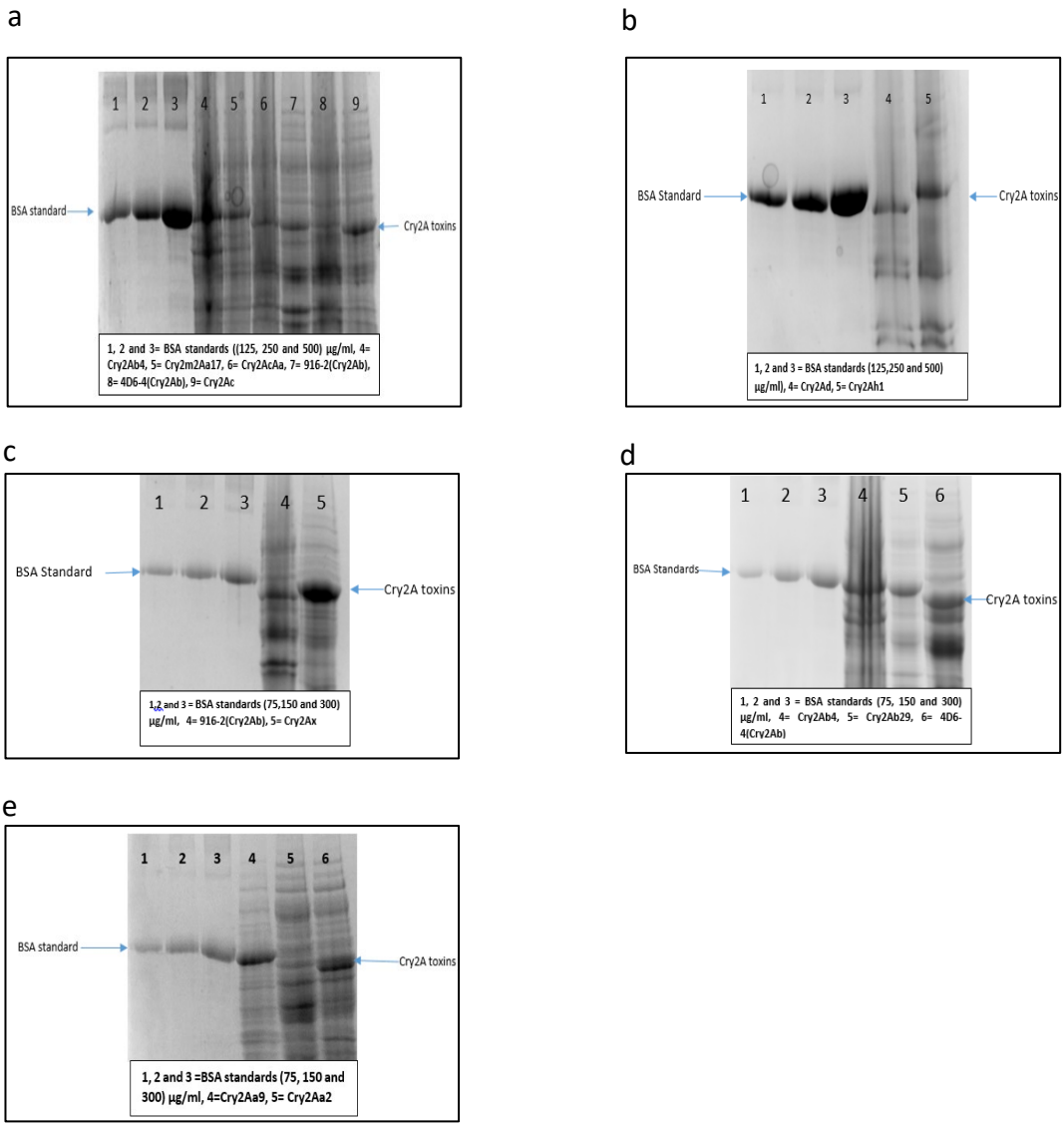



***Figure 3.2.7(a, b, c, d and e): Protein SDS-Page gel showing the various Cry2A toxins run along with BSA standards. The arrows on the right of the gel denote the position of the Cry2A toxin bands while those on the left denote the position of the bands for the BSA Standards.***

The bands observe at the position of the Cry2A toxins clearly show that these toxins were expressed and the concentration was measured.

| Plasmid | Protein | Concentration(µg /ml) | Predicted molecular weight (KDa) |
|---|---|---|---|
| **pGEM** | Cry2Aa2 | 600 | **70.85** |
| **pEB** | Cry2Aa9 | 600 | **70.85** |
| **pEB** | Cry2Aa17 | 700 | **70.64** |
| **pEB** | Crym2Aa17 | 300 | **70.71** |
| **pGEM** | Cry2Ab(4D6-4) | 200 | **70.75** |
| **pGEM** | Cry2Ab(916-2) | 600 | **70.71** |
| **pEB** | Cry2Ab4 | 600 | **70.73** |
| **pEB** | Cry2Ab29 | 300 | **70.82** |
| **pGEM** | Cry2Ac | 100 | **69.75** |
| **pGEM** | Cry2AcAa | 300 | **70.55** |
| **pGEM** | Cry2Ad | 60 | **70.63** |
| **pEB** | Cry 2Ah1 | 100 | **70.78** |
| **pEB** | Cry 2Ax | 800 | **70.81** |

***Table 3.2-1 Concentrations of the available Cry2A toxins used in this research as measured using Image J.***

Table 3.2-1 above showed the plasmid, name of protein, predicted molecular weight and the concentrations of the proteins as measured using densitometry. The results of the concentrations clearly showed that even though the same methods and procedures were used for the harvesting of the toxins, the amount of protein obtained from each toxin differed significantly, with Cry2Ax toxin giving the highest amount of Cry protein and Cry2Ad giving the least amount of Cry protein.

The next thing I did after measuring the concentrations of these toxins was to calculate the amount of each toxin that could give the required concentration for bioassay experiment in each case.

### 3.2.3 Control experiment to see if *E.coli* protein contributes to the activity of Cry2A grown using *E.coli* expression system.

Therefore, before we can start any proper bioassay for these toxins, as stated in the introductory section of this chapter there is need for an experiment, which could clearly show if *E. coli* proteins which may still remain in solution with our toxins, even after purification, is actually nontoxic to this insect. This is necessary to be established because all the Cry2A toxins to be used in this experiment will be expressed using *E. coli* expression system. Therefore, we used the cells from pGEM 4D6-5 and pGEM 916-5, which were cloned in *E.coli* DH5-α strain by a previous project student Evans (2014). Therefore, the cells were miniprepped and a pure DNA obtained from them, which were run on an agarose gel for confirmation as shown in Figure 3.2.8. These were then cloned in *E.coli* BL21 strain for expression of their proteins.

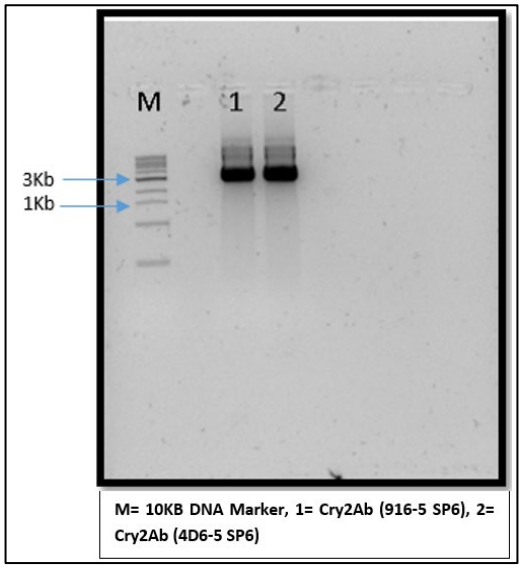



***Figure 3.2.8 DNA agarose gel showing the Plasmid DNAs for Cry2Ab toxins (4D6-5 and 916-5) which are to be used for the control experiment. The two plasmid DNAs above were cloned in E.coli DH5-α.***

The idea behind using either of these two toxins in this experiment was that since they have their respective genes in the opposite direction to the promoter region, as such, the *E.coli* expression system, which expresses only if the gene is in the same direction to the promoter, cannot express them. Therefore, when grown on a shaker we will have only the *E.coli* protein but no Cry toxin protein, which will enable us to test the effect of the *E.coli* protein alone on *Aedes aegypti* mosquito to be sure that it has no interference with the bioassay results.

The cells obtained from the *E.coli* BL21 strain cloning were then grown on a shaker and the protein harvested. The protein SDS- PAGE gel is shown in Figure 3.2.9 below.

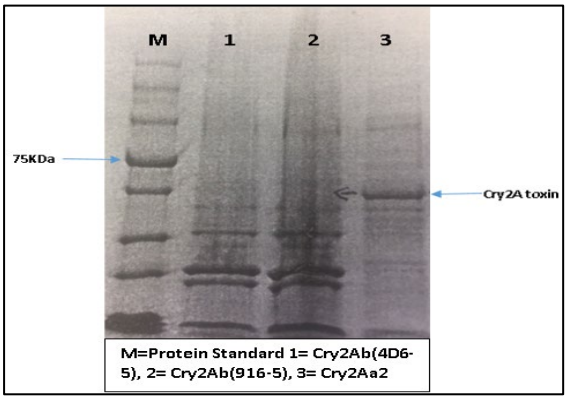



***Figure 3.2.9.SDS-PAGE gel for the two proteins harvested along with Cry2Aa2 as a positive control for expression. The arrow on the left indicates the position of the protein marker while the one on the right indicate the position of the Cry2A toxin band.***

From the above protein gel for the two Cry2Ab toxins (4D6-5 and 916-5) all expressed in the reverse orientation, and Cry2Aa, which is toxic to *Aedes aegypti* used as a positive control. It is very apparent that there is no Cry2A protein expressed for the two Cry toxin genes in the reverse direction; therefore, any protein resulting from these two after they are grown will just be only *E.coli* protein. The band indicated by an arrow is from Cry2Aa protein, which is the only one expressed. The concentration of the three proteins Cry2Aa, Cry2Ab(4D6-5) and Cry2Ab (916-5) were each measured as shown in table 3.2-2 below.

| Plasmid | Protein | Concentration(µg/ml) | Predicted m.weight (KDa) |
|---|---|---|---|
| **pGEM** | Cry2Aa2 | 600 | **70.85** |
| **pGEM** | Cry2Ab (4D6-5 SP6) | 3700 | **NA** |
| **pGEM** | Cry2Ab (916-5 SP6) | 1500 | **NA** |

***Table 3.2-2 Concentration of the E.coli proteins. NA stands for not-available.***

The concentrations of all the three proteins Cry2Ab (4D6-5), Cry2Ab (916-5), and Cry2Aa2 were measured using the Bradford method described in the materials and methods section of this thesis to ensure fair comparison among the three proteins. This is because the Cry toxin genes from the two Cry2Ab proteins (4D6-5 and 916-5) were

not expressed so they cannot be measured using the image J method as they do not form visible bands on the agarose gel rather just crude protein resulting from the *E. coli.*

The above proteins were bioassayed qualitatively by estimating 2 mg/l from individual protein and testing it against *Aedes aegypti* mosquito, the graph of the bioassay results is depicted Figure 3.2.10.

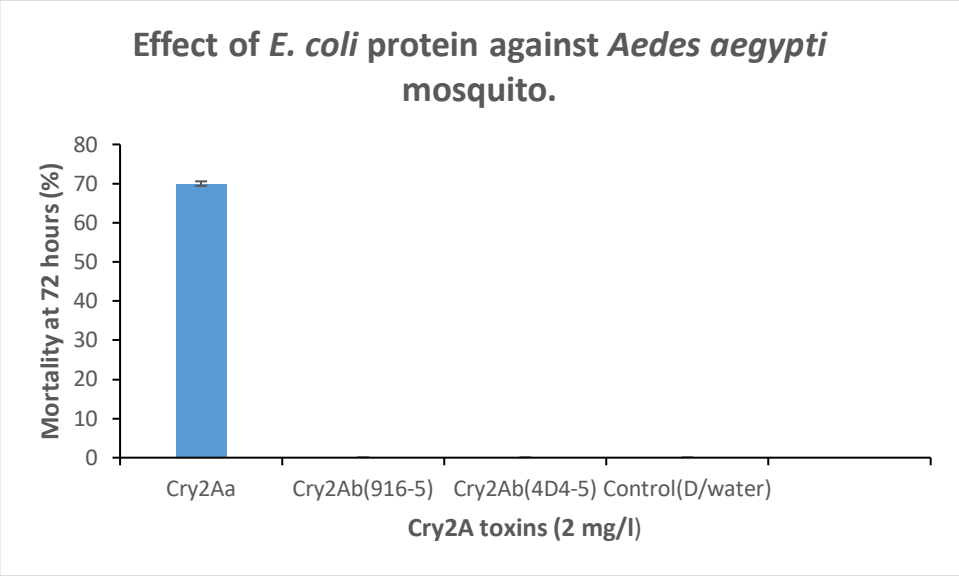



***Figure 3.2.10. Graph showing the effect of E.coli inclusion protein on Aedes aegypti. The results are shown for the two proteins resulting from Cry2Ab (916-5 and 4D6-5) respectively, Cry2Aa as a positive control and deionised water as negative control. Mortality is presented as mean percentage of three replicate bioassay. Cry2A toxins with mortality below 10% are considered nontoxic while those with mortality above 10% are considered toxic. The toxins were all expressed, grown and harvested together at the same time. Error bar represents Standard error of mean (SEM).***

From the above graph, it is obvious that the *E.coli* protein has no effect on the *Aedes aegypti* larvae. This is because all the three Cry toxins were cloned in *E.coli* BL21 strain for expression, though only Cry2Aa2 was expressed, and were all grown together at the same time, but it appears, from the results that the two Cry toxins that were not expressed (916-5 and 4D6-5), did not show activity against *Aedes aegypti.* They have 0% mortality rate, whereas the Cry toxin that was expressed (Cry2Aa2) and known to possess activity against *Aedes aegypti* showed activity with 70% mortality rate against *Aedes aegypti* based on our bioassay results.

### 3.2.4 Bioassay results for all available Cry2A toxins

The bioassay experiment was carried out for all the available Cry2A toxins following the method of WHO (2005c) described under materials and methods. Two bioassay experiments were done; first of which was a qualitative bioassay to screen out those Cry2A toxins that are toxic from the nontoxic ones whereas the second bioassay was the quantitative bioassay experiment which was carried out on only those Cry2A toxins that showed toxicity to *Aedes aegypti*. The second bioassay was carried out so that their respective LC50 values could be determined.

The qualitative bioassay was carried out using a discriminatory dose of 0.2mg/l of each of the Cry2A toxin harvested to be able to know those toxins that are toxic from those that are nontoxic to *Aedes aegypti*. These data are presented graphically as shown in Figure 3.2.11 below.

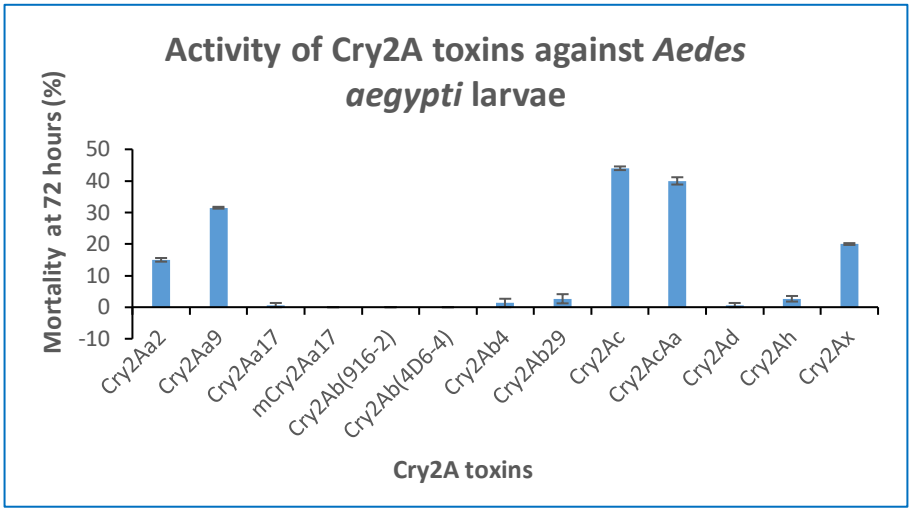



***Figure 3.2.11 Activity of Cry2A toxins against Aedes aegypti. Cry toxins with mortality below 10% are considered nontoxic while those with mortality above 10% are considered toxic. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three-repeated experiments, each with a new batch of the same Cry2A toxins. Error bars represent standard error of mean (SEM).***

From the above graph it is obvious that Cry2Aa2, Cry2Aa9, Cry2Ac, Cry2AcAa, and Cry2Ax all induced mortalities of more than 10% against *Aedes aegypti* larvae whereas Cry2A17, mCry2Aa17, Cry2Ab (916-2), Cry2Ab (4D6-4), Cry2Ab4, Cry2Ab29, Cry2Ad and Cry2Ah each with mortality rate below 5% are nontoxic.

Therefore, the above results showed that despite the 87.4% to 99.7% sequence identities, which is a measure of the empirical relationship between two or more

sequences with a common objective of establishing the likelihood for sequence homology; the chance that sequences have evolved from a common ancestor, among the Cry2A toxins (Table 3.2-4). They still seem to differ in their spectrum of activities and hence specificities (Liang and Dean, 1994). None of the Cry2Ab toxins appear to show activity against *Aedes aegypti* larvae whereas the other toxins except for Cry2Aa17 and mCry2Aa17 whose domain 1 resembled those of Cry2Ab (Shu *et al*., 2017), showed activity against *Aedes aegypti* mosquitoes.

The next aspect of the study was to carry out a quantitative bioassay for those Cry2A toxins, which were found to be toxic against *Aedes aegypti* namely: Cry2Aa2, Cry2Aa9, Cry2Ac, Cry2AcAa, and Cry2Ax respectively and use the results to determine their LC30 values. The quantitative bioassay was done using a range of concentrations for each Cry2A toxins from 0.0125 mg/l to 0.2 mg/l. The result for the qualitative bioassay is shown in Figure 3.2.12 below.

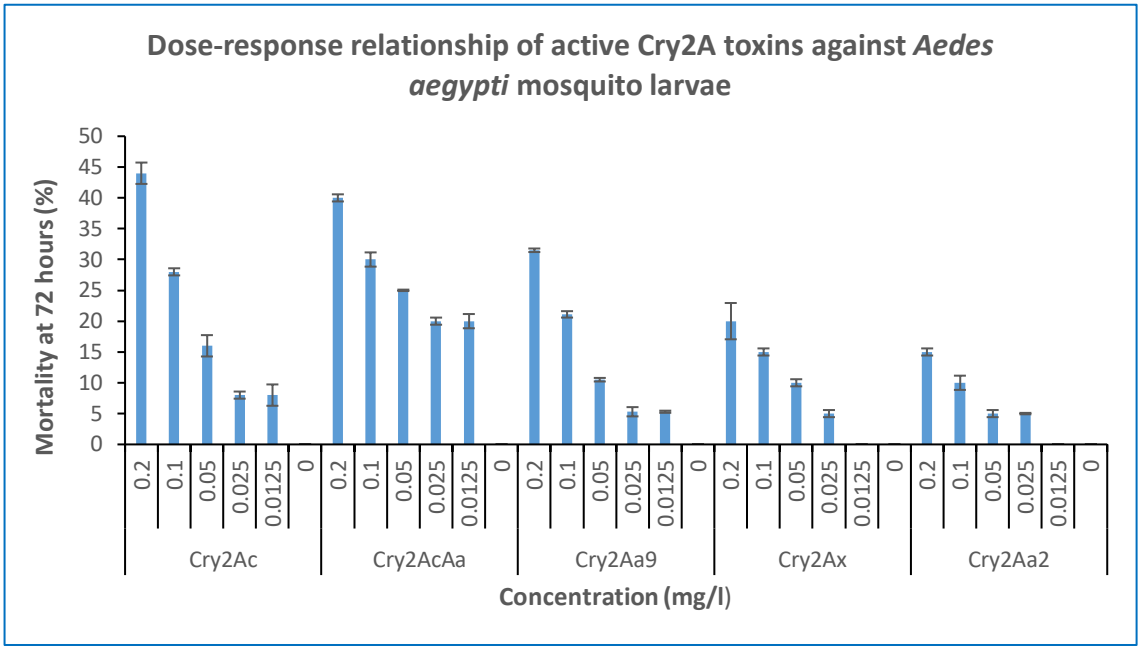



***Figure 3.2.12 Graph showing the dose-response relationship of Aedes aegypti larvae to all Cry2A toxins toxic against Aedes aegypti mosquito larvae at various concentrations of the toxins. The mortality rate on the y-axis is presented as percentage of the death larvae to those of the total number of larvae whereas the x-axis contained the various Cry2A toxins at varying concentrations. Error bars represent standard error of mean (SEM).***

The graph above for the quantitative bioassay showed that among the Cry2A toxins that are toxic against *Aedes aegypti* mosquito, Cry2Ac showed the highest degree of toxicity (44%) at a concentration of 0.2 mg/l whereas Cry2Aa2 toxin, at this instance, showed the least degree of toxicity (15%) at the same concentration. The results indicated that

though all the toxins in (Figure 3.2.12) are toxic against *Aedes aegypti* some appeared to exert more activity than others as could be seen by the differences in the percentage mortality recorded even though the same concentration ranges of toxin were used. The values for the concentrations and percentage mortalities were used to calculate the LC30 as the LC50 of the toxins, which is used in calculating the diagnostic concentration of the toxins for effective formulation of an active biopesticides against *Aedes aegypti* mosquitoes and other insects, is not realisable using the current bioassay data.

The LC30 values were calculated using Probit in analysis of variance by the SPSS software and is presented in the Table 3.2-3.

| Plasmid | Toxin | LC30 (mg/l) | 95% Confidence limits (mg/l) |
|---|---|---|---|
| pGEM | Cry2Aa2 | 0.60 | (0.30-4.00) |
| pEB | Cry2Aa9 | 0.20 | (0.10-0.40) |
| pGEM | Cry 2Ac | 0.10 | (0.10-0.20) |
| pGEM | Cry2AcAa | 0.10 | (0.10-0.20) |
| pEB | Cry 2Ax | 0.30 | (0.20-0.80) |

***Table 3.2-3 LC30 values of Cry2A toxins toxic against Aedes aegypti***

The table above represents the LC30 values of the Cry2A toxins that are toxic against *Aedes aegypti* mosquito larvae. The LC50 normally represents the value for concentration of the toxin that killed 50% of the mosquito larvae. Here, we obtained the LC30 values by extrapolation because the LC50 values could not be obtained from our current data due to not enough toxin being used in the assays. Therefore, the current data could be use only qualitatively as they are vague and not very reliable quantitatively.

For better understanding of the relationship between toxicity and sequence identity amongst Cry2A toxins, I carried out a pairwise sequence alignment amongst all the Cry2A toxins using Clustal omega and presented the result as percentage identity. This will help us decipher if overall percentage sequence identity among Cry2A toxins infers how close their toxicity spectrum can be. This is presented in Table 3.2-4 below.

| Percentage sequence identities amongst Cry2A toxins | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 2Aa9 | 2Aa17 | m2Aa17 | 2Ab4 | 2Ab29 | 2Ah1 | 2Ax | 2Ad | 2Ac | 2Aa2 | 4D6-4 | 916-2 | 2AcAa |
| 2Aa9 | | 98.1 | 98.4 | 94.5 | 95.4 | 94.2 | 96.1 | 93.5 | 89.3 | 100 | 94.5 | 94.5 | 97.5 |
| 2Aa17 | 98.1 | | 99.7 | 96.4 | 97.3 | 94.8 | 94.5 | 95.1 | 88.9 | 98.1 | 96.4 | 96.4 | 97 |
| m2Aa17 | 98.4 | 99.7 | | 96.1 | 97 | 95.1 | 94.5 | 95.1 | 89.1 | 98.4 | 96.1 | 96.1 | 97.3 |
| 2Ab4 | 94.5 | 96.4 | 96.1 | | 99.1 | 96.8 | 97.2 | 96.2 | 88.3 | 94.5 | 99.7 | 100 | 93.4 |
| 2Ab29 | 95.4 | 97.3 | 97 | 99.1 | | 96.8 | 96.2 | 95.6 | 88.2 | 95.4 | 99.1 | 99.1 | 94.3 |
| 2Ah1 | 94.2 | 94.8 | 95.1 | 96.8 | 96.8 | | 95.3 | 94.8 | 87.5 | 94.2 | 96.8 | 96.8 | 93.2 |
| 2Ax | 96.1 | 94.5 | 94.5 | 97.2 | 96.2 | 95.3 | | 94 | 87.8 | 96.1 | 96.8 | 97.2 | 93.5 |
| 2Ad | 93.5 | 95.1 | 95.1 | 96.2 | 95.6 | 94.8 | 94 | | 87.4 | 93.5 | 96.2 | 96.2 | 92.4 |
| 2Ac | 89.3 | 88.9 | 88.3 | 88.2 | 87.5 | 87.8 | 87.5 | 87.4 | | 89.3 | 88.3 | 88.3 | 91.8 |
| 2Aa2 | 100 | 98.1 | 98.4 | 93.5 | 95.4 | 94.2 | 96.1 | 93.5 | 89.3 | | 94.5 | 94.5 | 97.5 |
| 4D6-4 | 94.5 | 96.4 | 96.1 | 99.7 | 99.1 | 96.8 | 96.8 | 96.2 | 88.3 | 94.5 | | 99.7 | 93.4 |
| 916-2 | 94.5 | 96.4 | 96.1 | 100 | 99.1 | 96.8 | 97.2 | 96.2 | 88.3 | 94.5 | 99.7 | | 93.4 |
| 2AcAa | 97.5 | 97 | 97.3 | 93.4 | 94.3 | 93.2 | 93.5 | 92.4 | 91.8 | 97.5 | 93.4 | 93.4 | |

***Table 3.2-4 Percentage sequence identities amongst Cry2A toxins.***

The results of the pairwise sequence identity above clearly showed that toxicity in Cry2A toxins is not dependent on the overall sequence identity signifying there may be some residues amongst the Cry2A toxins that may determine their specificity to *Aedes aegypti* but not the overall percentage sequence identity. For example, Cry2Aa2 has 98.4 percentage sequence similarity to mCry2Aa17 (Table 3.2-4) but the latter being nontoxic while the former is toxic against *Aedes aegypti*. In the same vein, Cry2Aa2 and Cry2Ac have only 89.3 percentage sequence identity but all of them appear to be active against *Aedes aegypti* larvae further buttressing this point.

## 3.3 Discussion

This study seeks to understand the basis for the mosquitocidal specificity of Cry2A group of toxins against *Aedes aegypti*. The sequences of some of the available Cry2A toxins used in this study which were not initially confirmed were done so by sequencing the gene and comparing the results of the gene sequencing obtained to those of the sequences of known Cry2A toxins from the Bt nomenclature database (Crickmore *et al.*, 1998). In addition, clustal Omega was used to determine the degree of sequence identity among the Cry2A toxins (Table 3.2-4). For those toxins whose sequence were not available in the database the sequence from the sequencing results were pieced together using a closely related Cry2A toxins as a template to take care of overlapping regions (Figure 3.2.1 and 3.2.2). These were used to determine the gene of these two Cry2Ab toxins (Cry4D6-5 and 916-5) in Figure 3.2.3 and 3.2.4 respectively.

Therefore, to further, confirm the identity of these toxins, they were grown, the respective proteins harvested, and the protein SDS-PAGE was ran comparing their molecular weights to those of standard protein marker and were checked using the predicted values obtained from Expasy (Table 3.2-1).

The concentration of a toxin should be accounted for to carry out a standard bioassay procedure that enables proper comparison amongst different groups of toxins. Therefore, unlike previous research data published that use Bradford method, which measures all the crude proteins, in quantifying the protein (Wu *et al*., 1991, Wu and Aronson, 1992, Liang and Dean, 1994), we decided to use a different densitometry method (Image J) that measures the concentration of only the toxin band and leaving behind all other proteins. This, we believe will improve the quality of bioassay results (Figure 3.2.7 a to e).

The control experiment which histogram was presented in Figure 3.2.10 clearly showed that there was no activity in Cry2Ab (both 916-5 and the 4D6-5) which contained only the *E.coli* proteins but no toxins because the Cry toxin genes were not expressed. This, therefore, means that proteins that may arise from the *E.coli* cannot interfere with our bioassay results. Several researchers have used *E.coli* expression systems to express their Cry toxin genes, but they did not report any case of toxicity arising from *E. coli* protein (Mandal *et al*., 2007, Audtho *et al*., 1999, Pang *et al*., 1992, Xu *et al*., 2016, Wu *et al*., 1991).Hence this experiment has explained why there are no such reports. The Cry2Aa toxin, which was used as positive control showed toxicity against *Aedes aegypti*. Different researchers have already shown this toxin to possess activity against *Aedes aegypti* (Widner and Whiteley, 1990, Liang and Dean, 1994, Morse *et al*., 2001, Shu *et al*., 2017).

The bioassay results for all the available Cry2A toxins shown in table 3.2.11 indicated that some of the Cry2A toxins are toxic against *Aedes aegypti* while others are nontoxic despite having close percentage sequence identity to each other, as this does not determine the nature of their activity and /or specificity against *Aedes aegypti*. For example, Cry2Aa2 has 98.4 percent sequence identity to mCry2Aa17 (Table 3.2-4) but the latter appeared to be nontoxic while the former is toxic against *Aedes aegypti*. In contrast, Cry2Aa2 and Cry2Ac have only 89.3-percentage sequence identity, which is

slightly higher compare to the first example, but these two Cry toxins appear to be toxic against *Aedes aegypti* larvae. This therefore, clearly showed that though there is 87.4 to 99.7% sequence identity amongst Cry2A toxins (Table 3.2-4), their toxicity is not dependent on the overall sequence identity. This signifies that, there may be some few residues amongst the Cry2A toxins that might determine their specificity to *Aedes aegypti* but not the overall percentage sequence identity, which makes Cry2A toxins an interesting group of Cry proteins. Interestingly, almost all the Cry2Aa toxins appeared to be toxic against *Aedes aegypti* mosquitoes while all the Cry2Ab toxins appear not to be toxic against this species of mosquitoes, a result that agreed with previous findings where these two toxins were compared (Widner and Whiteley, 1990, Liang and Dean, 1994). Among the Cry2Aa toxins, it was only Cry2Aa17 and mCry2Aa17, whose domain 1 resemble that of Cry2Ab that appear not to be toxic against *Aedes aegypti.* This is not surprising because the toxicity spectrum of Cry2Aa17 against three order of insects was reported to be like those of Cry2Ab toxins thus implicating domain 1 as a specificity-determining region (Shu *et al*., 2017).

The bioassay results for the five Cry2A toxins that are toxic against *Aedes aegypti* mosquitoes shown in (Figure 3.2.12), indicated the fact that though all of them have activity against this species of insects, some appear to exert more toxic effects than others did. This is apparent in the different values recorded for percentage mortality, which showed Cry2Ac, having the highest mortality rate (44%) and Cry2Aa2 having the least (15%) at this instance. These results, clearly, buttressed the fact that even among Cry toxins that are specific against an order of insect there are still differences in their level of specificities. This fact, which has already been stressed by a previous researcher (Liang and Dean, 1994), who stated that despite the high sequence identities among the Cry2A group of toxins (Table 3.2-4), they seem to differ in their spectrum of activities and hence toxicity, with some being more active than others while some were not active at all.

Final bioassay results; are preferably presented as LC50 values but is presented as LC30 in (Table 3.2-3). This is because the current bioassay data we have cannot give us up to the LC50 values, and remain within a good range of confidence limit. For instance, the 95% confidence limits for some of the Cry toxins (Cry2Aa2 and Cry2Ah1) are wide and

so making the data for those toxins not very reliable (Table 3.2-3), hence the need for us to present these values as LC30.

From available literatures, there are many differences in the LC50 values obtained for a given toxin even when tested on a given order of insects. For instance, different results for LC50 values were obtained for Cry2Aa toxin, which is toxic against *Aedes aegypti,* by different researchers (Widner and Whiteley, 1990, Liang and Dean, 1994, McNeil and Dean, 2011) but surprisingly Liang *et al*. (2011) reported this same Cry2Aa toxin to be nontoxic against *Aedes aegypti*. Similarly, Cry2Ac was reported by Wu *et al*. (1991) to be nontoxic against *Aedes aegypti* at an LC50 value greater than 50 µg/ml but, contrarily, the same Cry2Ac was reported to be toxic against *Aedes aegypti* with an LC50 value of 70 ng/ml by Liang and Dean (1994). A result, which agrees with our current findings on Cry2Ac in terms of toxicity, even though we only obtained the LC30 value of 0.11 mg/l, instead of LC50, which would have made comparison of results much better.

These variations in bioassay results, most especially in the case of *Aedes aegypti* mosquitoes could be attributed to the following factors.

i. Differences in exposure periods, which affects the extent to which the larvae interact with toxin to bring about the desirable effects.
ii. Difficulty in quantification; due to the fact that the toxin must be applied as crystals not as soluble proteins since mosquitoes are filter feeders and some of the toxins settled at the bottom of the water in the container and could not be accessible to the larvae(McNeil and Dean, 2011).
iii. Age of the larvae; this is because sensitivity to Cry toxin decreases with decrease larval age, hence the need to use same instar stage of larvae for all bioassay in order to eliminate these discrepancies.
iv. Volume of the water to larval number; which has a critical effect on larval stress and sensitivity to toxin (McNeil and Dean, 2011).

These differences in results made comparison of bioassay results very difficult even within a given order of insect. This difficulty, can also arise due to complications on the bioassay methods used (McNeil and Dean, 2011), differences in the host of expression which affects toxicity (Lima, 2008), and lastly, the fact that intra species variation in toxin susceptibility may occur between test colonies obtained from different parts of the

world. A variation of 1-2 orders of magnitude has been observed, therefore, even insects from the same geographical region or colony may vary by 1 order of magnitude between cohorts or successive generations (van Frankenhuyzen, 2009).

Therefore, to address the bioassay problems outlined above the method of WHO (2005c) described under Materials and Methods section of this report was adopted because it proffered solutions to most of the problems highlighted. This involves setting up a bioassay protocol involving the use of larvae at the same growth stage, same larvae to water ratio, same protocol for toxin preparation and the usage of the same unit of measurement for all bioassays.

# *4.* Identification of the domain(s) and/ or amino acid motifs that encode toxin specificity to *Aedes aegypti.*

## 4.1 Introduction

This chapter seeks to use the results obtained from the bioassay of the available Cry2A toxins carried out previously, to find the domain/domains that is/are responsible for the activity of this group of toxins against *Aedes aegypti*. This was achieved by using the data obtained from the crystallographic studies of Cry2Aa by Morse *et al*. (2001), and by comparing the amino acid sequences of the Cry2A toxins through multiple sequence alignments using Clustal Omega software. These gave us information on the relative sequence identities of the three domains in all the Cry2A toxins, which enabled us to carry out domain analysis, and subsequently create some hybrids through making informed domain swaps. The creation of hybrids through informed domain swaps enabled us to determine which domain(s) is/are responsible for the activity of this group of toxins against *Aedes aegypti*. The functional domain(s) discovered were used in determining the amino acid(s) of importance through mutagenesis of the conserved amino acids either within the group found to be active or inactive against this insect. Previous research had shown that comparisons of the amino acid sequences of Cry toxins and their structures have led to the identification of conserved regions, which are important in basic toxin function and insect specificities (Hofte and Whiteley, 1989). In addition, small sequence differences among Cry toxins can strongly affect specificity and therefore the role of sequence dissimilarities is important in specificity determination (Bravo *et al*., 2007, de Maagd *et al*., 2001). Therefore, our current research on Cry2A toxins have focused on identifying the region that defines *Aedes* specificity. Moreover, it has been established that one of the most common and well-studied means of altering the toxicity spectrum and hence improving insect susceptibility to Bt toxins is through hybrid creation. This is because some of the hybrids created have appeared to exert more toxic effects on insects, compared to the wild type when bioassay experiments were conducted (Hu *et al*., 2014, Liang and Dean, 1994, Mandal *et al*., 2007, Widner and Whiteley, 1990).

Previous studies on Cry2A specificity region determination focused on identifying the regions that define dipteran and/or lepidopteran specificities through chimeric scanning

mutagenesis (Widner and Whiteley, 1990, Liang and Dean, 1994, Morse *et al*., 2001). They utilised the fact that Cry2Aa has both dipteran and lepidopteran activity whereas Cry2Ab is lepidopteran specific. Therefore, they created hybrids between these two toxins through random swapping then testing these hybrids for activity on these insect types (Liang and Dean, 1994, Widner and Whiteley, 1990). Therefore, with this approach all the authors cited were able to speculate on the amino acid regions that confer both dipteran and lepidopteran specificity in Cry2Aa, and lepidopteran specificity in Cry2Ab. We used a similar approach, after analysing the results of Cry2A toxins and knowing those that are toxic to *Aedes* and those that are not, we then created hybrid toxins through domain swaps between those that were toxic and those that were not.

Recent research carried out to identify Cry2A toxin genes in a collection of 300 strains of Bt identified a novel toxin named Cry2Aa17 which showed sequence similarity to Cry2Ab in domain I, whilst domains II and III resembled Cry2Aa. When the toxicity profile of this novel toxin against *Aedes aegypti* was determined, it matched that of Cry2Ab more than Cry2Aa, thus implicating domain I as a toxicity determining region in Cry2A toxins (Shu *et al*., 2017).

## 4.2 Results

### 4.2.1 Domain I implicated as the specificity-determining region of Cry2A toxins against *Aedes aegypti.*

The results of the bioassay of the wild type Cry2A toxins depicted in Figure 3.2.11 was analysed. The domains of those Cry2A toxins that were toxic were compared to those that were non-toxic. This was achieved by carefully taking some representatives from the group of toxins that were toxic to *Aedes* (Cry2Aa, Cry2Aa9, Cry2Ac, Cry2AcAaAa, and Cry2Ax), and from those that were non-toxic (Cry2A17, mCry2Aa17, Cry2Ab (916-2), Cry2Ab (4D6-4), Cry2Ab4, Cry2Ab29, Cry2Ad and Cry2Ah) then examined the composition of their domains. We decided to use six representatives of Cry2A toxins for the domain analysis (Cry2Aa, Cry2Ac, Cry2AcAa, Cry2Aa17, Cry2Ab29, and Cry2Ab) respectively. This is because they represent all the possible domain combinations and activities required for comparison as Cry2Aa and Cry2Ac are active against *Aedes aegypti*, and Cry2AcAaAa was a hybrid between the two of them. In addition, Cry2Ab was nontoxic to *Aedes aegypti* and Cry2Ab29 and Cry2Aa17 are both natural hybrids containing both Cry2Ab and Cry2Aa in their domains and were non-active against *Aedes aegypti*.

Cry2AcAaAa which was one of the most toxic ones based on our bioassay has the following domain composition; **Domain I:** (Cry2Ac), **Domain II:** (Cry2Aa) and **Domain III:** (Cry2Aa).

Cry2Ab29, a native hybrid toxin that was **non- toxic,** has the following domain composition. **Domain I:** (Cry2Ab), **Domain II** (Cry2Ab), and **Domain III** (Cry2Aa).

Cry2Aa17, which was also **non-toxic** has the following domain composition: **Domain I** (Cry2Ab), **Domain II** (Cry2Aa), **and Domain III** (Cry2Aa).

Also, from the bioassay results in Figure 3.2.11, the native Cry2Ac, which has all its three domains comprising Cry2Ac and native Cry2Aa2, which has all its three domains comprising Cry2Aa are all toxic against *Aedes aegypti*. These descriptions are presented diagrammatically in Figure 4.2.1 below:

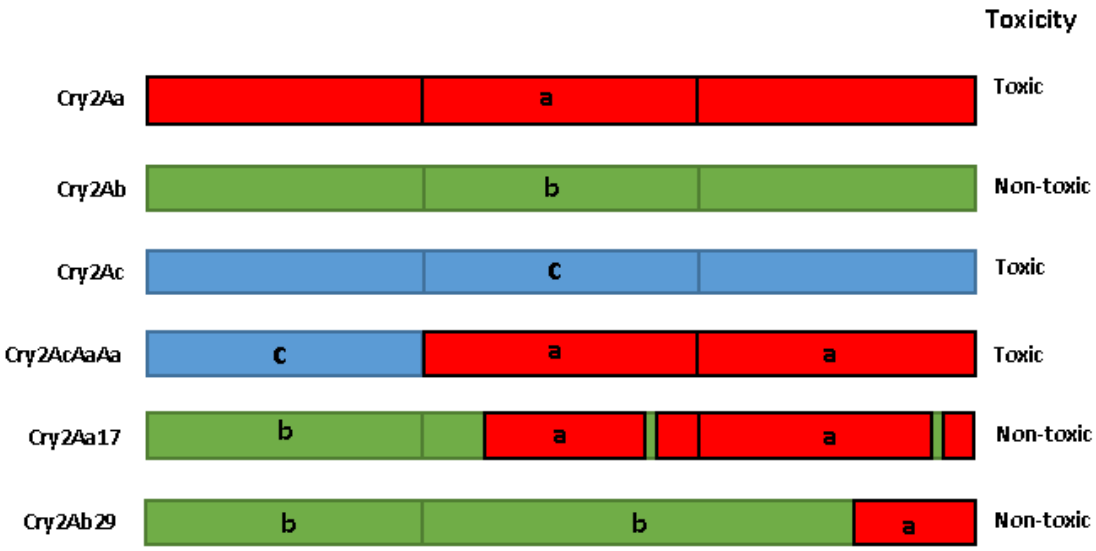



***Figure 4.2.1.Domains matching of some representatives of Cry2A toxins that are toxic and some that are non-toxic against Aedes aegypti. The colours with letters represent each of the wild type Cry2A toxin and the hybrids; (red) stands for wild type Cry2Aa, b (green) stands for wild type Cry2Ab and c (blue) stands for wild type Cry2Ac.***

From the analysis of the different domain compositions of the native Cry2A toxins and natural hybrids above, it is clear that all those that were toxic against *Aedes aegypti* mosquito, have their domains I from either Cry2Aa or Cry2Ac whose wild type are toxic against this insect. In contrast, all those that were nontoxic against this insect contain a Cry2Ab in domain I, whose wild type was nontoxic against *Aedes aegypti*. This, therefore, implicated domain I as a toxicity-determining region of Cry2A toxins against *Aedes aegypti* but this remained to be proven. With these findings, we therefore formulated a hypothesis that domain I influences the specificity of Cry2A toxins against *Aedes aegypti* mosquito. This implies that if we could swap the domain I of Cry2Ac or Cry2Aa2 into a native Cry2Ab toxin which was non-toxic, we could obtain a hybrid toxin that would be toxic against *Aedes aegypt*i.

We decided to start domain I hybrid creation by swapping the domain I of Cry2Ac and Cry2Aa into the Cry2Ab (4D6-4) and swap the domain I of Cry2Ab (4D6-4) into Cry2Ac and Cry2Aa following the plan designed in the diagram depicted in Figure 4.2.2 below.

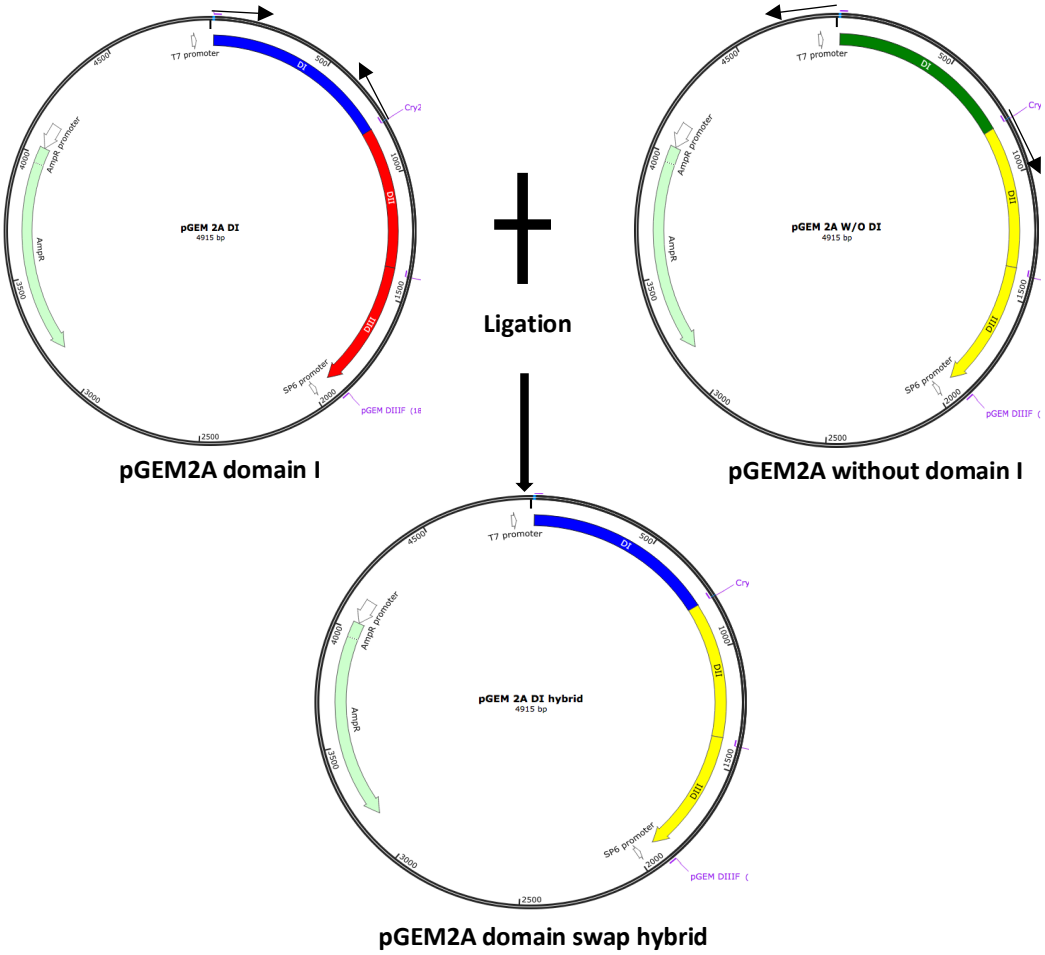


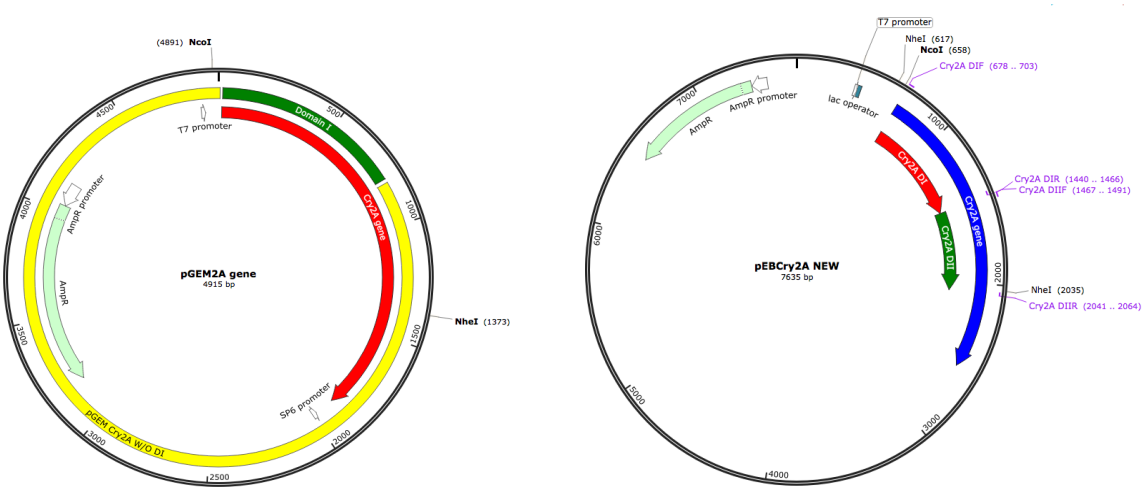

***Figure 4.2.2 Plasmid diagram showing the plan for Cry2A hybrids created through domain I swap. The part coloured blue denotes the domain I portion of the pGEM2A gene, whereas the part coloured yellow represents pGEM2A without domain I.***

Domain I was amplified using two primers named Cry2A domain I forward and Cry2A domain I reverse respectively as shown in Figure 4.2.2. The second part, which is pGEMCry2A without domain I contained the plasmid together with domain II and domain III but without domain I, and this portion was also amplified using two primers namely, Cry2ADIIF and pGEM reverse respectively as shown in Figure 4.2.2.

### Primer design for domain I swap among Cry2A toxins

We checked Cry2A gene for possible restriction enzyme sites, which we could use for creating hybrids for the above design (Figure 4.2.2). Unfortunately, we could not get a clear restriction enzyme sites in Cry2A genes that could cut out the domain I region from the rest of the Cry2A gene. However, there was NcoI enzyme site upstream of the start codon of the Cry2A gene in both plasmids (pGEM and pEB) but additionally pEB plasmid

also had NheI site upstream of the NcoI site. Moreover, both the two plasmids have NheI site at the boundary of domain II with domain III of the Cry2A gene as shown in Figure 4.2.3, but we were concerned with excising domain I, hence these two restriction enzymes could not be used in this instance.




**pGEMCry2A gene** **pEBCry2A gene**

***Figure 4.2.3. Plasmid diagram showing the NcoI and NheI restriction enzyme sites in both pGEMCry2A gene on the left and pEBCry2A gene on the right respectively. Diagrams, which explained why these enzymes could not be used to amplify domain I of Cry2A gene in these plasmids.***

Therefore, since we could not use the restriction enzyme sites for the amplification of domain I, we decided to design universal primers for the amplification of domain I of all the Cry2A toxins using Cry2Aa toxin sequence as a template. In addition, all necessary adjustments were made to make the sequences of other Cry2A toxins conform to those of Cry2Aa along the sequences that were used for the universal primer design. The area for the domain I forward primer designed for the Cry2A toxins is shown in Figure 4.2.4 below.

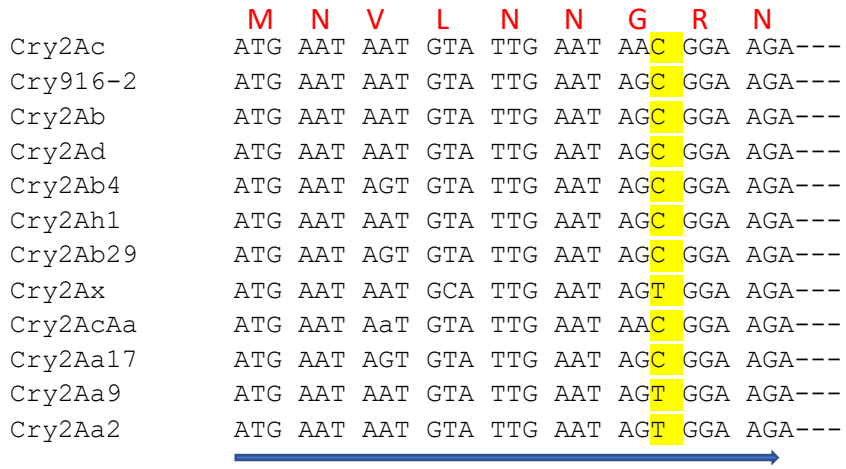



***Figure 4.2.4 Sequence alignment for domain I forward universal primer designed. Areas shaded yellow represent those with sequence dissimilarities within this portion among the Cry2A toxin genes, ATG stands for the start codon.***

The only difference spotted in the sequence alignment above is where T substitutes C in some of the Cry2A toxins. Therefore, this had to be resolved since it was around the 3'end and as such, it can affect the binding of the primers to the template strand. This was resolved by making this primer degenerate. In this case, this primer was made degenerate through replacing this position by Y as it can now bind efficiently to either T or C in the nucleotide sequence of all the Cry2A toxins. Therefore, the final upper primer designed was as shown below.

**5'P -ATGAATAATGTATTGAATAAYGGAAG- 3'**

The reverse primer for the amplification of domain I, and that of the forward primer for the amplification of pGEMCry2A without domain I were designed using the sequences in Figure 4.2.5 below.

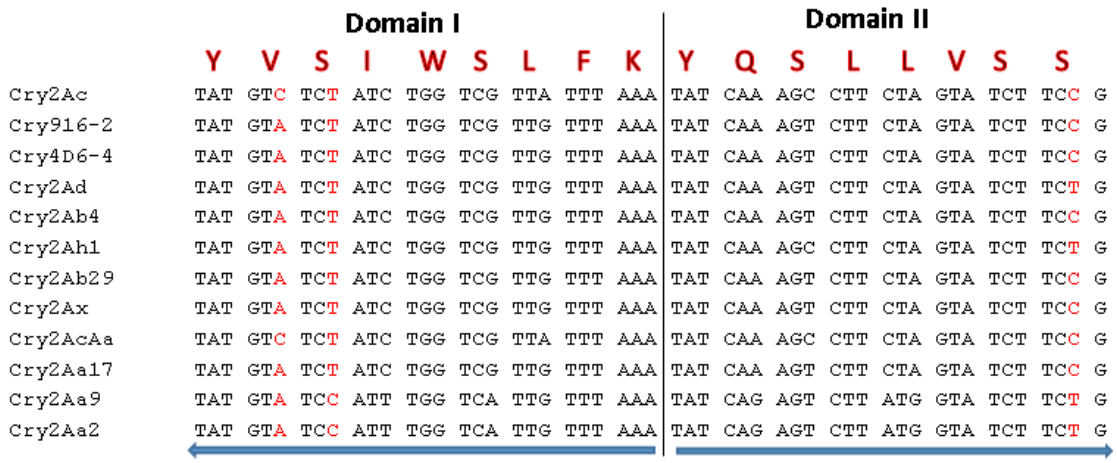



***Figure 4.2.5 Sequence alignment for domain I reverse and domain II forward universal primer designed. The sequences coloured red represent those with sequence dissimilarities within these portions among the Cry2A toxin genes. The vertical line represents the boundary between domain I and domain II of Cry2A toxin sequences.***

The reverse primer for domain I amplification was as shown below.

**5'P- TTTAAATAACGACCAGATRGAKACATA- 3'**

The areas replaced by R and K in the domain I reverse primer are areas where there were differences based on the sequence alignment, as such changing them to R and K enables the primer to bind to each of these nucleotides if found in any of the Cry2A toxin genes. The primers for domain I amplification were both phosphorylated at 5' position to allow ligation to the pGEMCry2A and/or pEBCry2A plasmid without domain I, as depicted in Figure 4.2.2.

The two vectors harbouring our Cry2A genes are pEB and pGEM, therefore we designed primers that can amplify both domain II and the rest of the plasmid in each case. The forward primer designed for amplification of pGEMCry2A without domain I or pEBCry2A without domain I of Cry2A toxins, which is taken from Figure 4.2.5 is shown below.

**5'- TATCAAAGCCTTCTAGTATCTTCYG-3'**

The sequence represented by "Y" in the primer for pGEMCry2A without domain I or pEBCry2A without domain I above represent the area where there is sequence difference in some of the Cry2A toxins sequence, which coloured red in Figure 4.2.5.

The reverse primer for pEBCry2A without domain I and/or pGEMCry2A without domain I amplification, which were designed upstream of domain I, at the beginning of the sequence from both plasmids respectively are shown below.
Reverse primer for pEBCry2A without domain I amplification is shown below.

**5' -CTCCCGGGATATCGCCATG- 3'**

The reverse primer for pGEMCry2A without domain I amplification is shown below.

**5' -ATAAAATTCCTCCTTAATCGAATTC -3'**

5'Phosphate group was not added to the 5' end in designing these primers in order to prevent self-ligation.

The two components of the Cry2A domain I hybrid designed as depicted in Figure 4.2.2, were therefore ligated by the process of blunt end ligation using DNA ligase enzyme, since it is clear that restriction enzyme method could not be employed in this instance as demonstrated in Figure 4.2.3. The product of the ligation reaction is a hybrid containing domain I from one of the Cry2A toxin and domain II and III with the pGEM plasmid from the other Cry2A toxin. The ligation product, which is the hybrid, was transformed in to *E.coli* DH5-α strain, and the right colony selected and further transformed using *E. coli* BL21 (DE3) pLysS strain in order to express the protein as described in the material and methods section.

### Hybrid toxins created among Cry2A toxins through domain I swap

The domain I swap hybrids based on our hypothesis were created using two wild type Cry2A toxins (Cry2Aa and Cry2Ac) as representatives of the active Cry2A toxins against *Aedes aegypti* whereas Cry2Ab(4D6-4) was used as a representative of the nontoxic ones. Therefore, giving rise to three native Cry2A toxins used for the creation of four hybrids through domain I swap among Cry2A toxins as shown diagrammatically in Figure 4.2.6.

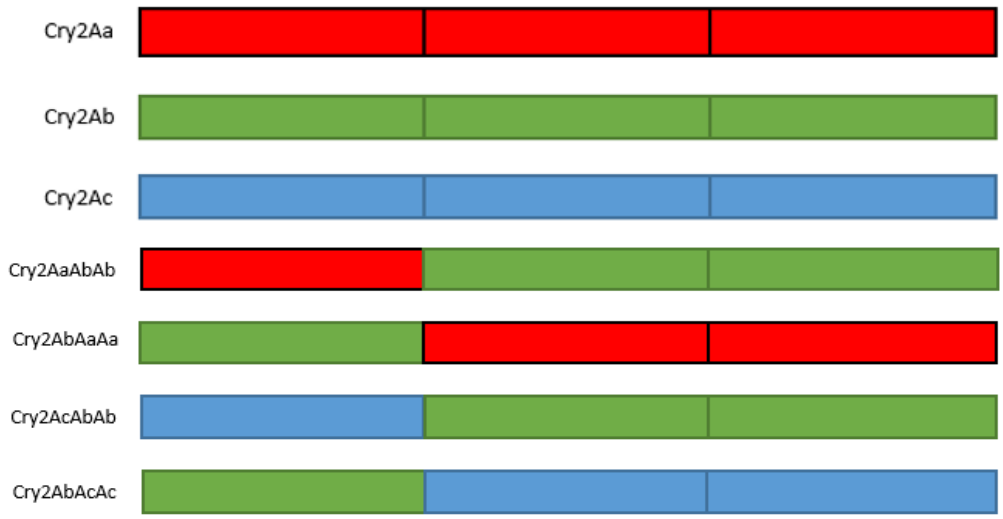



***Figure 4.2.6 Native Cry2A toxins and the domain I swap hybrids created from them. The colours represent each of the wild type Cry2A toxin and the hybrids: Red colour stands for wild type Cry2Aa, green colour stands for wild type Cry2Ab and blue colour stands for wild type Cry2Ac respectively. Each of the three rectangles in the figure that combined to form a Cry toxin represents a domain, I, II, and III moving from left to right. Each of the wild type toxins and the hybrids presented above was expressed, grown, and the protein harvested and tested against Aedes aegypti larvae.***

### PCR amplification, purification, and ligation of the respective domains from different Cry2A toxins were swapped.

The primers designed above were ordered from the MWG after making sure that they met the optimum conditions for PCR reaction. The primers were diluted 1:10 with deionised water to give a final concentration of 10 pmol/µl. PCR reaction was set up following the conditions outlined in the Materials and Methods chapter section in chapter two.

The product obtained was run on a gel to see if the desired product has been amplified, after which 45 µl of the PCR product was mixed with 1 µl of DpnI enzyme and incubated

for 1 hour to digest the parental/template strand. This was run on a DNA agarose gel following the procedure outlined in the materials and methods section in chapter two.

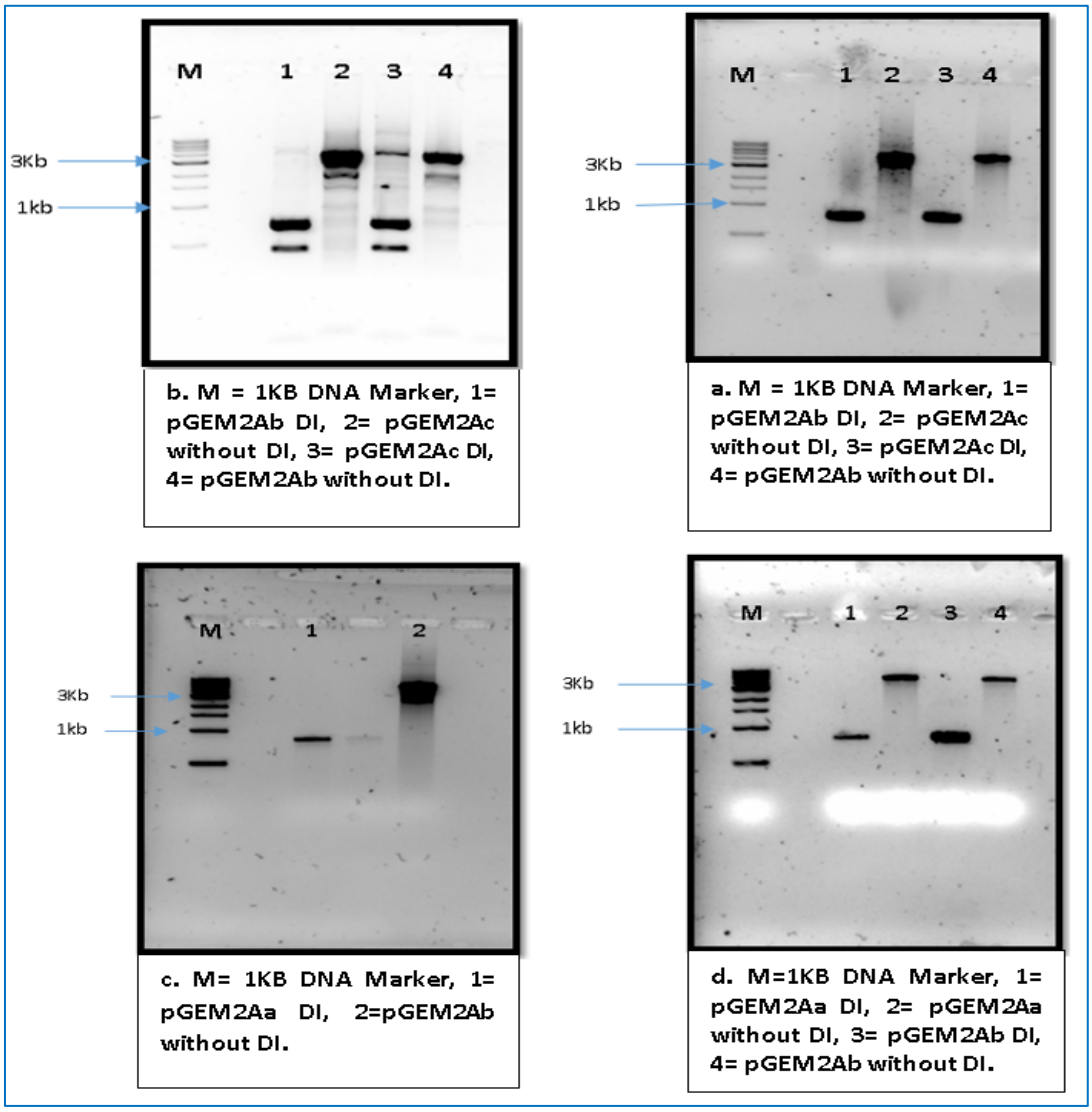



***Figure 4.2.7: DNA Agarose gels showing the purified PCR amplification products for the various domains from Cry2A toxins. The arrows indicate the positions of the DNA 3Kb and 1Kb Markers.***

The resulting gels (Figure 4.2.7) showed that all the desired domains for amplification were successfully amplified as can be seen by the presence and the positions of the respective DNA bands on the gel.

The ligation reactions to obtain the four hybrids outlined in figure 4.2.7 were set up following the recommended ligation ratio of 1:3 to 1:5 of vector to insert in an approximate proportion based on the intensity of their respective bands on the gel.

The products of the ligation reaction were introduced in to *E.coli* DH5-α strains following the procedures described in the Materials and Methods in chapter two.

The successfully transformed *E.coli* cells were identified by colonies from the L-agar plate, in that it was a blunt ligation, only the cells that contained the ampicillin resistant gene from the plasmid will grow on an ampicillin containing L. agar plate used in this transformation protocol. In addition, not all the colonies (transformants) contained the desired hybrid as others may pick only the plasmid, or the plasmid containing the domain in the wrong orientation, as such further screening was also performed to confirm the orientation of the domain. Colonies were selected, eight of which were then scraped up with a sterile toothpick and streaked on an ampicillin impregnated (100µg/ml) L-agar plate.

### Testing/confirming the transformants for domain orientation.

Two approaches for testing and confirming the transformants for gene orientation described in the Materials and Methods section of this project were both employed at some points in this research work. The first approach involved the use of the plasmid DNA extracted from the colonies and then followed by enzyme digestion to confirm the colonies that contained the insert; this method was employed for the two of the hybrids, Cry2AcAbAb and Cry2AaAbAb. The second approach involved the use of colony PCR to confirm the presence of the insert (domain I in this case) then followed by HaeIII restriction enzyme digestion. This method was employed for the remaining two hybrids created namely Cry2AbAcAc and Cry2AbAaAa.The HaeIII digest fragments from the gel was compared to those generated by the NEB cutter to see if they were correct, if they were, then the next thing done was to confirm the orientation of the gene in those colonies selected.

The plasmid DNA from the selected colony (ies) was tested for its orientation, since DNA can be ligated into the plasmid in two possible directions.

The restriction digestion reaction was done using HaeIII as mentioned above, as this confirmed the presence of the insert, after which BsaAI restriction enzyme was employed to know the orientation of the insert because there was no HaeIII site in domain I.

HaeIII enzyme digest of Cry2A domain I swap hybrids appeared to give the same fragments for both the A and B orientations (Table 4.2-1 a), the only exception is pGEM2AbAaAa which produces a slightly different HaeIII digest fragments as shown in table 4.2-1b. As such, another enzyme must be employed in addition to HaeIII, to be able to confirm the orientation of the insert. For hybrids creation involving domain I swap among different Cry2A toxins, BsaAI enzyme appeared to solve this problem as it has a restriction site within the domain I with different restriction enzyme fragments for A and B orientations, which enabled us to confirm if the insert was in the right orientation.

This was achieved by running the BsaAI digested DNA sample on a gel. The gel was scanned and the restriction fragments produced which appeared as bands on the gel (shown in Figure 4.2.8a to c), was compared to those predicted by NEB cutter for both A and B orientations (shown in Table 4.2-1a and b). This then enabled us to know which of the colonies had their respective inserts in the right orientation.

The gels for the HaeIII restriction digest of domain I swap hybrids are displayed in Figure 4.2.8 below.

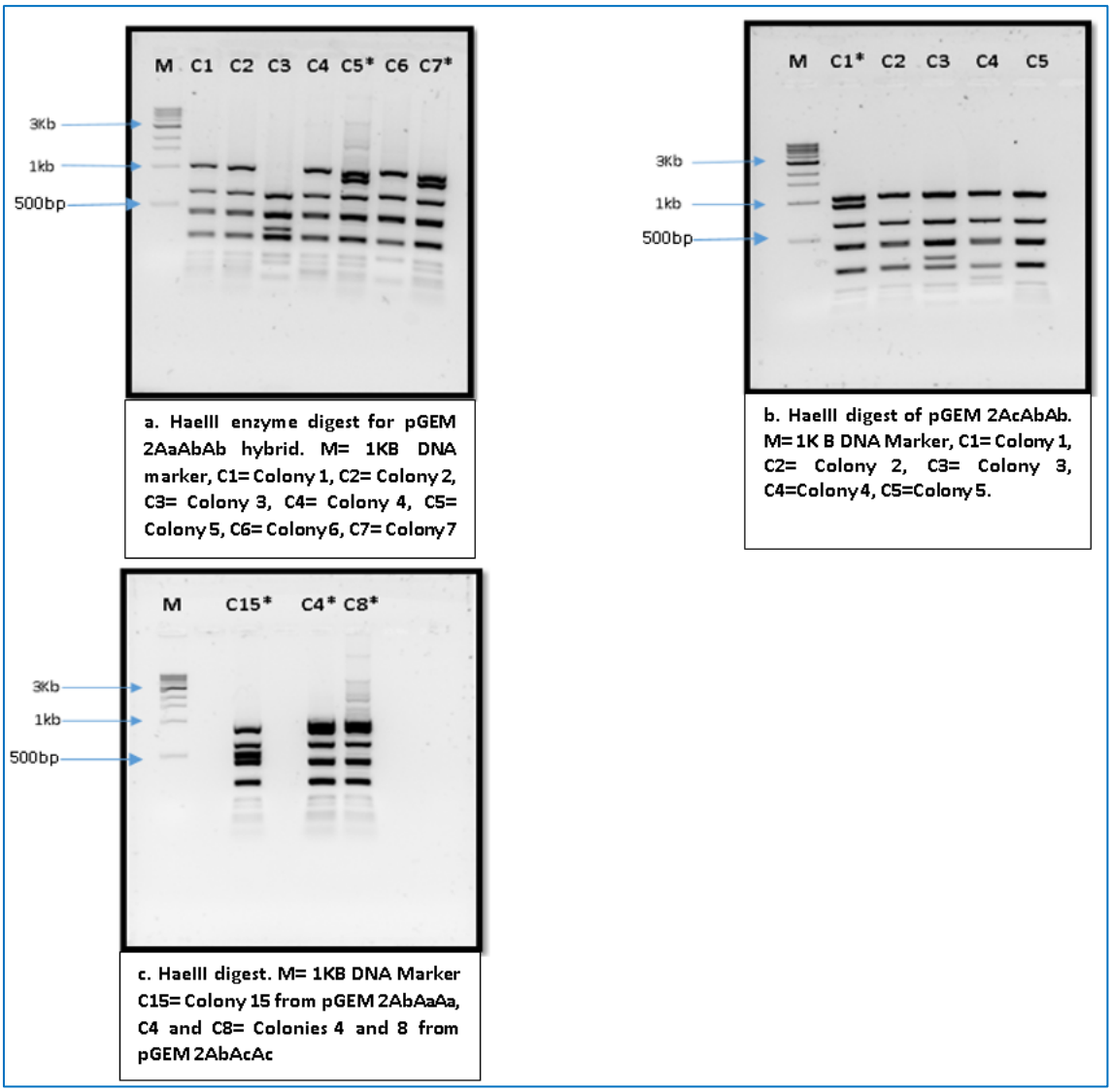



***Figure 4.2.8(a-c): DNA Agarose gels showing HaeIII digest fragments for the domain I swap hybrid toxins created. The arrows showed the positions of those bands on the marker, which enabled the detection of the positions and hence the length in kilo base of the unknown fragments on the gel; this applies to all the gels above. Asterisks indicate the colonies with correct HaeIII restriction fragments.***

The restriction digest fragments in Figure 4.2.8 above were compared to those fragments generated by the NEB cutter, which is shown Table 4.2-1 below

**a. pGEM 2AaAbAb DNA digested with HaeIII.**

| # | Ends | Coordinates | Length (bp) |
|---|---|---|---|
| 1 | HaeIII-HaeIII | 895-1953 | 1059 |
| 2 | HaeIII-HaeIII | 4935-894 | 910 |
| 3 | HaeIII-HaeIII | 3675-4328 | 654 |
| 4 | HaeIII-HaeIII | 2870-3327 | 458 |
| 5 | HaeIII-HaeIII | 2436-2869 | 434 |
| 6 | HaeIII-HaeIII | 4471-4759 | 289 |
| 7 | HaeIII-HaeIII | 1954-2232 | 279 |
| 8 | HaeIII-HaeIII | 3408-3674 | 267 |
| 9 | HaeIII-HaeIII | 2233-2406 | 174 |
| 10 | HaeIII-HaeIII | 4329-4470 | 142 |
| 11 | HaeIII-HaeIII | 4760-4861 | 102 |
| 12 | HaeIII-HaeIII | 3328-3407 | 80 |
| 13 | HaeIII-HaeIII | 4862-4901 | 40 |
| 14 | HaeIII-HaeIII | 4902-4922 | 21 |
| 15 | HaeIII-HaeIII | 2418-2435 | 18 |
| 16 | HaeIII-HaeIII | 4923-4934 | 12 |
| 17 | HaeIII-HaeIII | 2407-2417 | 11 |

**b. pGEM 2AbAaAa DNA digested with HaeIII**

| # | Ends | Coordinates | Length (bp) |
|---|---|---|---|
| 1 | HaeIII-HaeIII | 4929-894 | 910 |
| 2 | HaeIII-HaeIII | 3669-4322 | 654 |
| 3 | HaeIII-HaeIII | 1400-1947 | 548 |
| 4 | HaeIII-HaeIII | 895-1399 | 505 |
| 5 | HaeIII-HaeIII | 2864-3321 | 458 |
| 6 | HaeIII-HaeIII | 2430-2863 | 434 |
| 7 | HaeIII-HaeIII | 4465-4753 | 289 |
| 8 | HaeIII-HaeIII | 1948-2226 | 279 |
| 9 | HaeIII-HaeIII | 3402-3668 | 267 |
| 10 | HaeIII-HaeIII | 2227-2400 | 174 |
| 11 | HaeIII-HaeIII | 4323-4464 | 142 |
| 12 | HaeIII-HaeIII | 4754-4855 | 102 |
| 13 | HaeIII-HaeIII | 3322-3401 | 80 |
| 14 | HaeIII-HaeIII | 4856-4895 | 40 |
| 15 | HaeIII-HaeIII | 4896-4916 | 21 |
| 16 | HaeIII-HaeIII | 2412-2429 | 18 |

***Table 4.2-1(a and b). HaeIII restriction enzyme digest fragments generated from NEB cutter prediction for PGEM 2AaAbAb and 2AbAaAa respectively. The fragments were generated using the DNA sequence of the constructs of the domain I hybrids created.***

The above restriction digest fragments in Table 4.2-1a, was generated for all the Cry2A hybrid toxins that were created above using NEB cutter to confirm the right colony (ies) in that they all gave similar fragments to the one shown above. The only exception from all the other Cry2A hybrids created was pGEM2AbAaAa, which has a slightly different HaeIII restriction digest fragments as shown on the gel in Figure 4.2.8c, and the restriction fragments generated from the NEB cutter shown in Table 4.2-1b.

The results obtained through comparing the bands from the HaeIII restriction enzyme digest (Figure 4.2.8 a to c), and the fragments generated from the NEB cutter predictions (Table 4.2-1 a and b), showed that colonies 5 and 7 for pGEM2AbAaAa in Figure 4.2.8a, colony 1 for pGEM2AcAbAb in Figure 4.2.8b, colonies 15 for pGEM2AbAaAa, and colonies 4 and 8 for pGEM2AcAbAb in Figure 4.2.8c, all contained the right HaeIII enzyme digest fragments. Asterisks indicate these colonies. Nevertheless, we could not know if the inserts are in A or B orientation unless the colonies with the right fragments are digested with BsaAI enzyme. The DNA agarose gels for the BsaAI digest of the colonies selected from the HaeIII digest fragments in order to be screened for gene orientation are shown in Figure 4.2.9a to c.

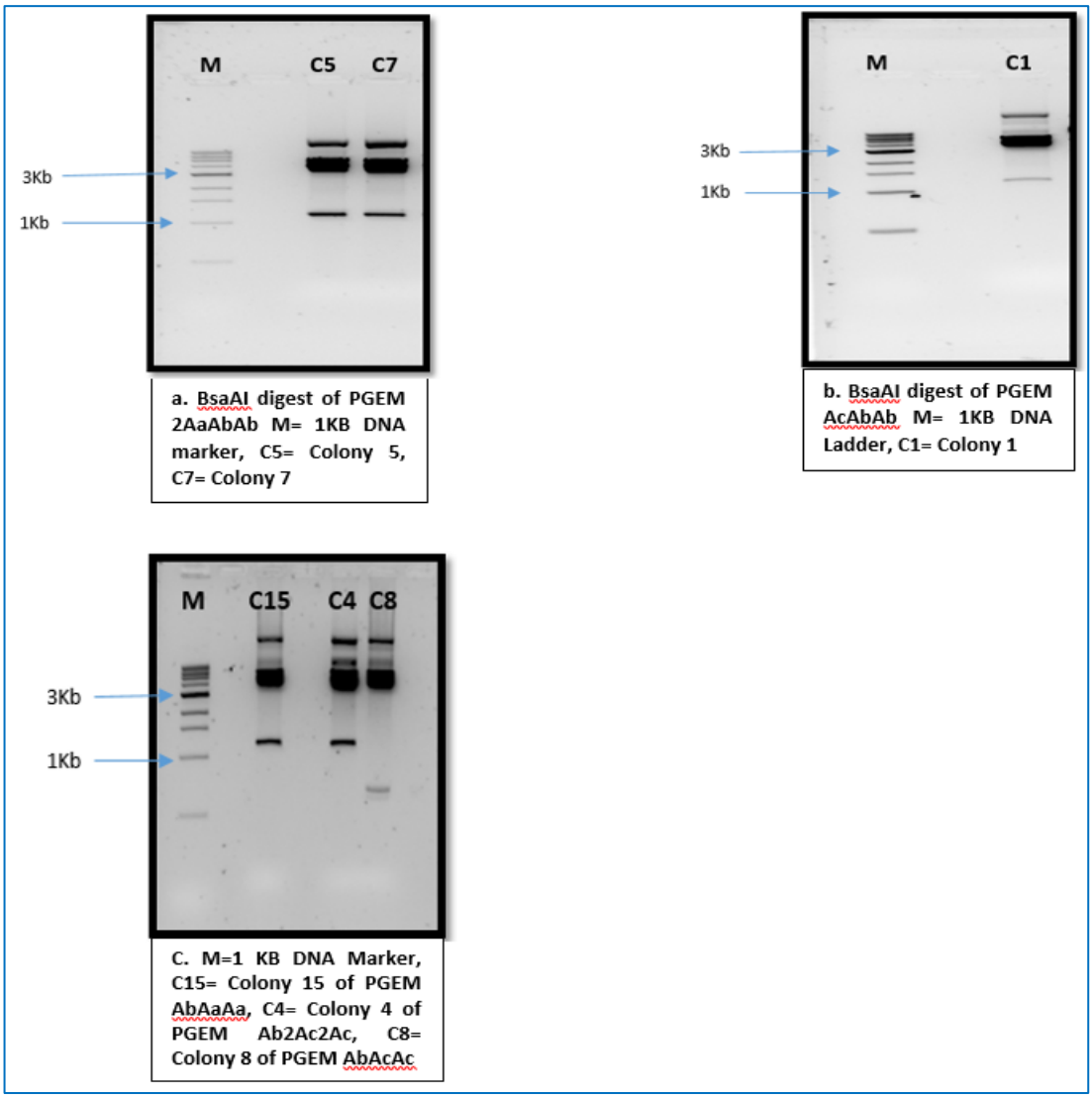



***Figure 4.2.9(a-c). DNA agarose gels containing the BSaAI digest of the colonies selected from HaeIII digests. Colonies selected from HaeIII digest, which appeared to contain the insert, are digested with BsaAI enzyme to confirm the orientation of the inserts. All the colonies in the gels displayed in this figure contained the inserts in the right orientation with the exception of colony 8 from figure 4.2.9c whose insert is not in the right orientation.***

BsaAI restriction digest fragments were generated from the constructs of the above hybrids to check if they are the same as they appeared on the gel and if they are truly in the right orientations. The BsaAI restriction digest fragments generated from the NEB cutter for both A and B orientations are shown in Table 4.2-2 below.

**a. pGEM2AaAbAb (A) digested with BsaAI**

| # | Ends | Coordinates | Length (bp) |
|---|---|---|---|
| 1 | BsaAI-BsaAI | 693-4478 | 3786 |
| 2 | BsaAI-BsaAI | 4479-631 | 1103 |
| 3 | BsaAI-BsaAI | 636-692 | 57 |
| 4 | BsaAI-BsaAI | 632-635 | 4 |

**b. pGEM2AaAbAb (B) digested with BsaAI**

| # | Ends | Coordinates | Length (bp) |
|---|---|---|---|
| 1 | BsaAI-BsaAI | 189-4472 | 4284 |
| 2 | BsaAI-BsaAI | 4473-127 | 599 |
| 3 | BsaAI-BsaAI | 128-184 | 57 |
| 4 | BsaAI-BsaAI | 185-188 | 4 |

***Table 4.2-2(a and b): BSaAI digest of PGEM 2AaAbAb fragments generated using NEB cutter predictions. The first one (a) is in the right(A) orientation whereas the second one (b) is when the gene is in the wrong(B) orientation, as can also be seen in colony 8 (C8) of the gel in figure 4.11c. For the B orientation, in order to generate the fragments from the NEB cutter, the reverse compliment of the DNA sequence of the construct was used.***

The colonies with the right restriction fragments when compared to the ones generated from the NEB cutter in the case of both HaeIII and BsaAI restriction fragments, they looked similar to the bands on the gel. In addition, when all the hybrids created were checked using NEB cutter they gave very similar fragments to those in Table 4.2-2. Hence, the two tables above (Table 4.2-2a and b) are representative of the NEB cutter predictions for the entire Cry2A domain I swap hybrids created.

Therefore, when the digestion fragments in (Figure 4.2.9a to c) were compared to the ones generated using NEB cutter, (Table 4.2-2 a and b). It was discovered that they do not really match the sizes in Figure 4.2-2; this is because we used an old BsaAI enzyme thus resulting in partial digest. Therefore, we only considered 1103 and 599 bands during confirmation. With this, we confirmed that both colonies 5 and 7 for pGEM2AaAbAb from Figure 4.2.9a, C1 for pGEM2AcAbAb from 4.9b, colony 15 for pGEM2AaAbAb, and Colony 4 for pGEM2AbAcAc, from Figure 4.2.9c, have the BsaAI fragments that matched those predicted by the NEB cutter. Colony 8 for pGEM2AbAcAc from Figure 4.2.9c was in the wrong orientation.

The DNAs from the various colonies identified to be in the right orientation, were sent for sequencing. The sequences received from the sequencing results were compared to the sequence of the construct we created through sequence alignment using either BLASTN or CLUSTAL omega, after which the ones confirmed to be correct were introduced in to *E. coli* BL21 (DE3) pLysS strain for the expression of the hybrid protein.

Confirmation of sequencing results for domain I swap hybrids created.

The sequence received from the sequencing results were confirmed by aligning them to the sequence of the wild type toxins whose domains were swapped together to form the hybrid. The idea was to check the alignment results along the boundary where they two wild type toxins were joined to form the hybrids. Before this boundary the wild type Cry toxin, whose domain I was used in the hybrid creation aligned with the hybrid sequence received from the sequencing results. Whereas, after the boundary it will be the sequence from the other wild type Cry toxin, whose domains II and III was used to form the other parts of the hybrids that aligned perfectly with the hybrid sequence. This procedure for hybrid confirmation was used along with the other procedure, which involved aligning the sequence received from the sequencing results with the constructs generated from the hybrid sequence. These two procedures were employed due to the high level of sequence similarity among the Cry2A group of toxins in order to be able to make sure that hybrids have been successfully formed between the two wild-type Cry2A toxins in question.

The various sequences for the confirmation of the domain I hybrids created are shown in Figure 4.2.10 below.

```
Cry2Ab                  ACATTACGTACGTATCGAGATTACTTGAAAAATTATACAAGAGATTACTCTAACTATTGT   597
Cry2Aa2                 ACATTACGTACGTATCGAGATTACCTGAGAAATTATACAAGAGATTATTCTAATTATTGT   589
Cry2AaAbAb-C7-T7        ACATTACGTACGTATCGAGATTACCTGAGAAATTATACAAGAGATTATTCTAATTATTGT   660
                        ************************* *** ****************** ***** ******

Cry2Ab                  ATAAATACGTATCAAAGTGCGTTTAAAGGTTTAAACACTCGTTTACACGATATGTTAGAA   657
Cry2Aa2                 ATAAATACGTATCAAACTGCGTTTAGAGGGTTAAACACCCGTTTACACGATATGTTAGAA   649
Cry2AaAbAb-C7-T7        ATAAATACGTATCAAACTGCGTTTAGAGGGTTAAACACCCGTTTACACGATATGTTAGAA   720
                        ***************** ******** *** ******** **********************

                                                                          DI
Cry2Ab                  TTTAGAACATATATGTTTTTAAATGTATTTGAGTATGTATCTATCTGGTCGTTGTTTAAA   717
Cry2Aa2                 TTTAGAACATATATGTTTTTAAATGTATTTGAATATGTATCCATTTGGTCATTGTTTAAA   709
Cry2AaAbAb-C7-T7        TTTAGAACATATATGTTTTTAAATGTATTTGAATATGTATCCATCTGGTCGTTATTTAAA   780
                        ********************************** ******** ** ***** ** ******

                                DII
Cry2Ab                  TATCAAAGTCTTCTAGTATCTTCCGGTGCTAATTTATATGCAAGTGGTAGTGGACCACAG   777
Cry2Aa2                 TATCAGAGTCTTATGGTATCTTCTGGCGCTAATTTATATGCTAGCGGTAGTGGACCACAG   769
Cry2AaAbAb-C7-T7        TATCAAAGCCTTCTAGTATCTTCCGGTGCTAATTTATATGCAAGTGGTAGTGGACCACAG   840
                        ***** ** *** * ******** ** ************** ** ****************

Cry2Ab                  CAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTTTCCAAGTTAAT   837
Cry2Aa2                 CAGACACAATCATTTACAGCACAAAACTGGCCATTTTTATATTCTCTTTTCCAAGTTAAT   829
Cry2AaAbAb-C7-T7        CAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTATATTCTCTTTTCCAAGTTAAT   900
                        ***** ***********  ***** ************************************
```

***Figure 4.2.10 Alignment results for the confirmation of the hybrid toxin PGEM2AaAbAb. The area shaded yellow indicate the sequences around the boundary where the two wild type Cry2A toxins forming the hybrid joined, the boundary between domain I of Cry2Aa and domain II and III of Cry2Ab is indicated by the vertical line. Sequences with red colour before the boundary indicates where there is difference between the hybrid sequence (Cry2aAbAb-C7-T7) and Cry2Ab within domain I whereas sequences shaded red after the junction indicates sequence differences between Cry2Aa and the hybrid toxin (Cry2aAbAb-C7-T7).***

From the above, it is apparent that there was successful hybrid formation between the two wild type Cry toxins (Cry2Aa comprising domain I and Cry2Ab comprising domain II and III of the hybrid toxin). This showed that the hybrid PGEM2AaAbAb was successfully formed by swapping the domain I of Cry2Aa to that of Cry2Ab.

The next hybrid formed is PGEM2AbAaAa, and the sequence alignment for its confirmation is shown in Figure 4.2.11 below.

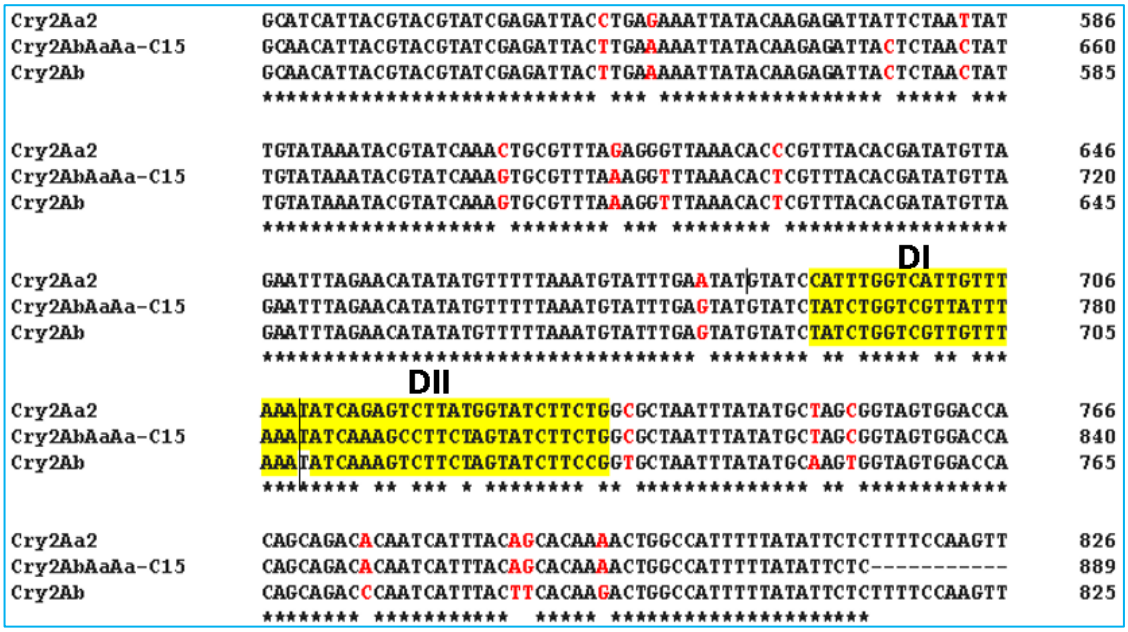



***Figure 4.2.11 Alignment results for the confirmation of the hybrid toxin PGEM2AbAaAa. The area shaded yellow shows the sequences around the boundary where the two wild type Cry2A toxins forming the hybrid joined, the boundary is indicated by the vertical line. Sequence with red colour before the boundary indicates where there is difference between the hybrid toxin sequence (Cry2AbAaAa-C15) and Cry2Aa within domain I, whereas sequences shaded red after the junction indicates sequence differences between Cry2Ab and the hybrid toxin (Cry2AbAaAa-C15).***

From the above, it is apparent that there was successful hybrid formation between the two wild-type Cry2A toxins (Cry2Ab comprising the domain I and Cry2Aa comprising the domain II and III of the hybrid toxin). This showed the hybrid PGEM2AbAaAa was successfully formed by swapping the domain I of Cry2Ab to that of Cry2Aa.

The third domain I swap hybrid formed was PGEM2AcAbAb, and the sequence alignment for its confirmation is shown in Figure 4.2.12 below.

```
Cry2Ab          ACATTACGTACGTATCGAGATTACTTGAAAAATTATACAAGAGATTACTCTAACTATTGT  597
Cry2Ac          ACAGTACGCACATATAGAGATCACCTGAGAAATTTCACAAGAGATTACTCTAATTATTGT  589
Cry2AcAbAb-C1   ACAGTACGCACATATAGAGATCACCTGAGAAATTTCACAAGAGATTACTCTAATTATTGT  660
                *** **** ** *** ***** ** *** *****  ********************** ******

Cry2Ab          ATAAATACGTATCAAAGTGCGTTTAAAGGTTTAAACACTCGTTTACACGATATGTTAGAA  657
Cry2Ac          ATAAATACGTATCAAACTGCATTTAGAGGTTTAAACACTCGTTTACACGATATGTTAGAA  649
Cry2AcAbAb-C1   ATAAATACGTATCAAACTGCATTTAGAGGTTTAAACACTCGTTTACACGATATGTTAGAA  720
                ********************************************************

                                                                      DI
Cry2Ab          TTTAGAACATATATGTTTTTAAATGTATTTGAGTATGTATCTATCTGGTCGTTGTTTAAA  717
Cry2Ac          TTTAGAACATATATGTTTTTAAATGTATTTGAATATGTaTCcATCTGGTCGTTATTTAAA  709
Cry2AcAbAb-C1   TTTAGAACATATATGTTTTTAAATGTATTTGAATATGTATCCATCTGGTCGTTATTTAAA  780
                ********************************** ******** *********** ******

                     DII
Cry2Ab          TATCAAAGTCTTCTAGTATCTTCCGGTGCTAATTTATATGCAAGTGGTAGTGGACCACAG  777
Cry2Ac          TATCAAAGCCTTCTAGTATCTTCCGGCGCTAATTTATATGCGAGTGGTAGTGGTCCAACA  769
Cry2AcAbAb-C1   TATCAAAGCCTTCTAGTATCTTCCGGTGCTAATTTATATGCAAGTGGTAGTGGACCACAG  840
                ******** ***************** ************** *********** ***

Cry2Ab          CAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTAAATTCCCTTTTCCAAGTTAAT  837
Cry2Ac          CAAT------CATTTACAGCACAAAACTGGCCATTTTTATATTCTCTTTTCCAAGTTAAT  823
Cry2AcAbAb-C1   CAGACCCAATCATTTACTTCACAAGACTGGCCATTTTTAAATTCCC--------------  886
                **        *******  ***** ************** **** *
```

***Figure 4.2.12 Alignment results for the confirmation of the hybrid toxin PGEM2AcAbAb. The area shaded yellow indicates sequences around the boundary where the two wild type Cry2A toxins forming the hybrid joined, the junction is indicated by the vertical line. Sequence with red colour before the junction indicates where there is difference between the hybrid (Cry2AcAbAb-C1) sequence and Cry2Ab within domain I (DI), whereas sequences shaded red after the junction indicate sequence differences between Cry2Ac and the hybrid toxin (Cry2AcAbAb-C1) starting from domain II (DII).***

The last domain I swap hybrid formed was pGEM 2AbAcAc, which has Cry2Ab comprising its domain I and Cry2Ac comprising its domain II and III. The sequence alignment for its confirmation is shown in Figure 4.2.13.

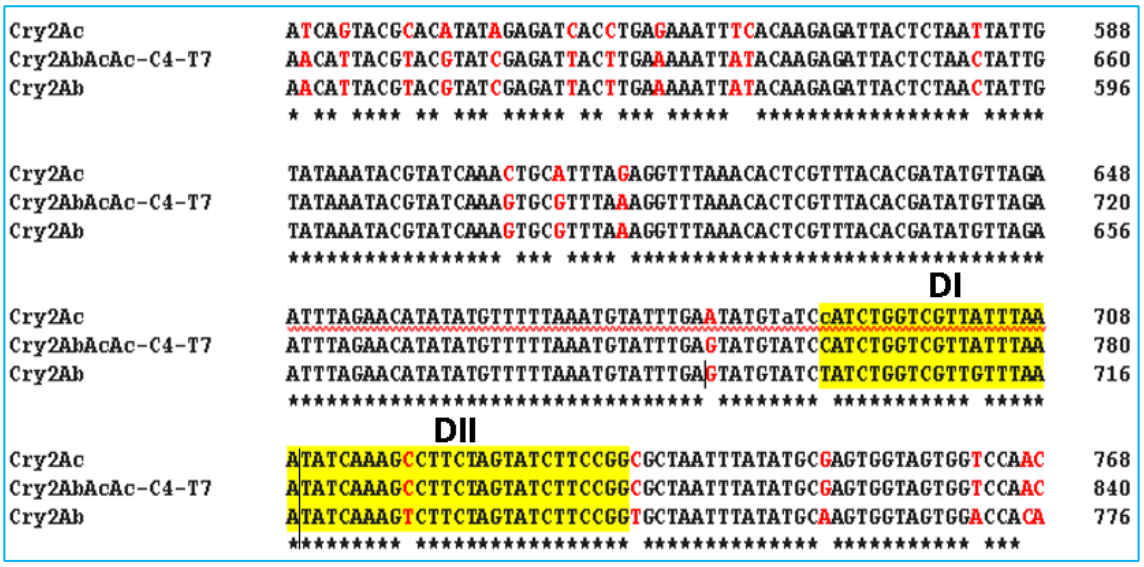



***Figure 4.2.13 Alignment results for the confirmation of the hybrid toxin PGEM2AbAcAc. The area shaded yellow showed the sequences around the boundary where the two wild type Cry2A toxins forming the hybrid joined, the junction is indicated by the vertical line. Sequence with red colour before the junction indicates where there is difference between the hybrid (Cry2AbAcAc-C4-T7) sequence and Cry2Ac within domain I, whereas sequences with red colour after the junction indicates sequence differences between Cry2Ab and the hybrid(Cry2AbAcAc-C4-T7) toxin starting from domain II.***

### Expression and harvesting of hybrid Cry2A toxin proteins from domain I swap.

The colonies from the hybrids that were confirmed to harbour the gene of interest in the right orientation were further introduced in to *E. coli* BL21 (DE3) pLysS strain following the protocol outlined in materials and methods. After the transformation, colonies from the *E. coli* BL21 (DE3) pLysS cells harbouring each plasmid were expressed and subsequently harvested, following the procedure described in the materials and methods in chapter two. They were run on an SDS-PAGE gel (7.5%), to confirm if they were successfully expressed, or not. The gel showing the Cry2A hybrid toxins is depicted in Figure 4.2.14.

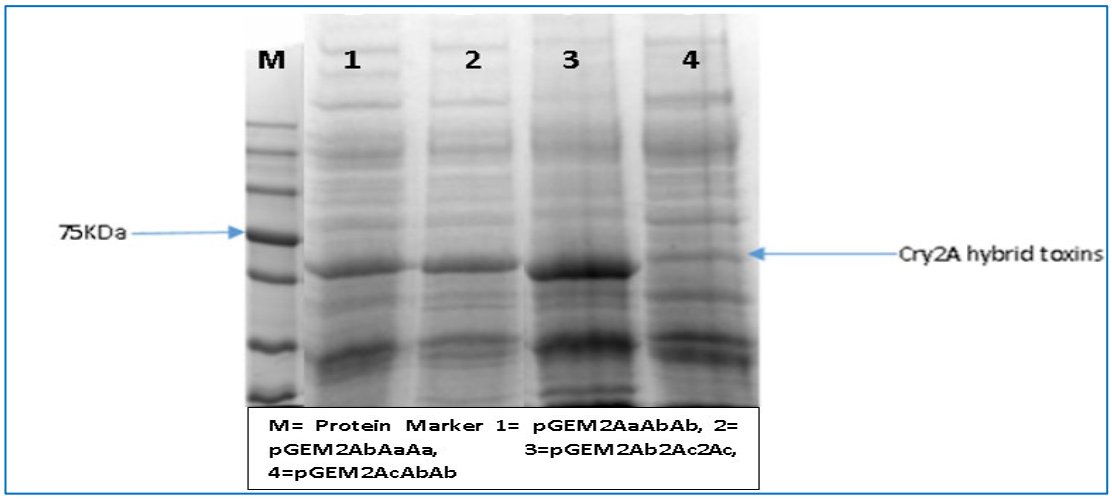



***Figure 4.2.14 Protein SDS-PAGE gel showing domain I swap hybrid Cry2A toxin proteins expressed. The arrow pointing towards the right showed the molecular weight protein marker used to estimate the weight of the Cry toxins, while the one pointing towards the left shows the position of the hybrid toxin bands on the gel.***

The toxins, were successfully expressed as shown on the gel in Figure 4.2.14 above, they were then run along with BSA standards on a gel and their concentrations was measured using Image J. The gel used to measure the concentration of the Cry toxins is shown in Figure 4.2.15 below.

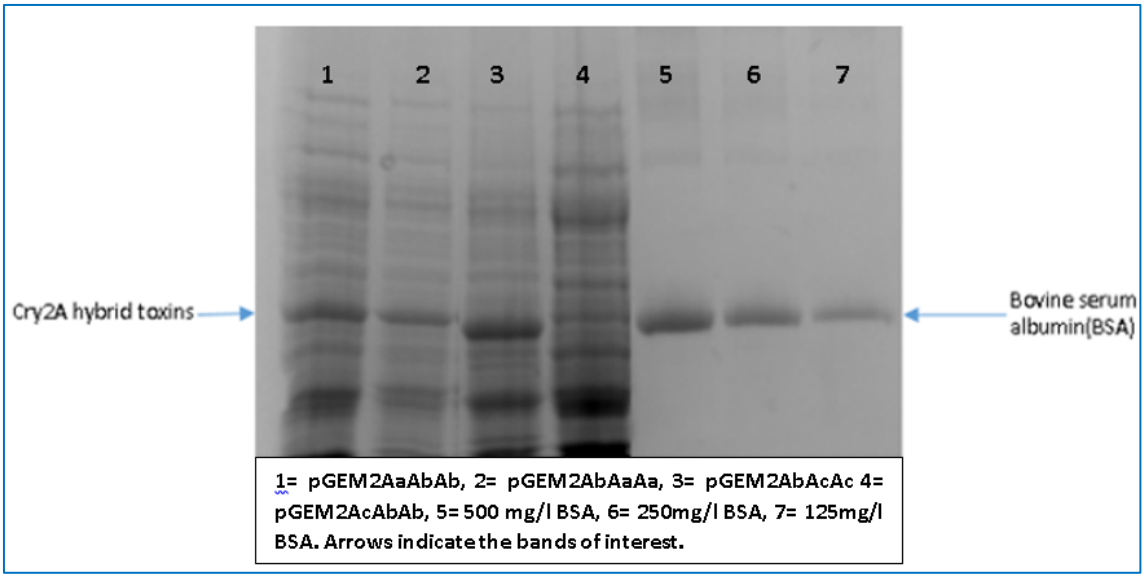



***Figure 4.2.15 Gel to measure the concentration of the hybrid proteins. Densitometry method using Image J was used to measure the concentration of each of the Cry toxins represented by the bands above and those of the BSA. Since the concentrations of the BSA standards are known already, they were used to determine the concentrations of the various hybrid Cry2A toxins using excel.***

The concentrations of the above hybrid toxins as measured using image J software is shown in Table 4.2-3.

| Hybrid Toxin | Concentration(µg/ml) | Predicted molecular weight (KDa) |
|---|---|---|
| Cry2AaAbAb | 1000 | 70.89 |
| Cry2AbAaAa | 500 | 70.73 |
| Cry2AbAcAc | 2100 | 69.92 |
| Cry2AcAbAb | 400 | 70.60 |

***Table 4.2-3 Concentrations of the Cry2A domain I swap hybrid toxins created as measured using Image J. The hybrids toxins were all expressed, and their relative molecular weight predicted using a program in Expasy.***

Qualitative bioassay was carried out for the above hybrids at a concentration of 2mg/l to be able to find out which ones among them were active against *Aedes aegypti*. The results of the bioassay are shown in Figure 4.2.16 below.

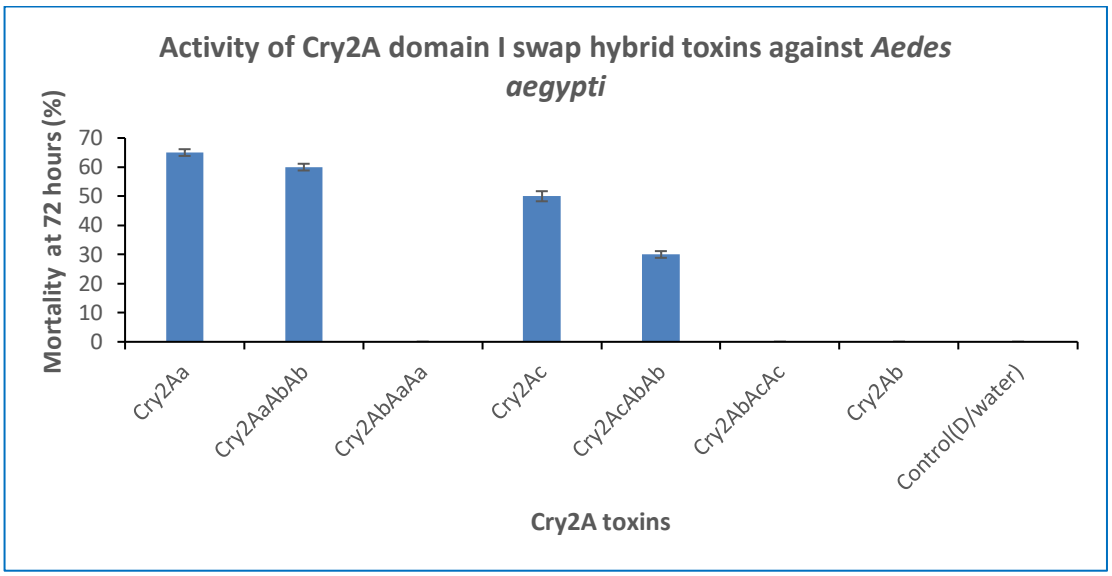



***Figure 4.2.16 Activity of Cry2A domain I hybrid toxins against Aedes aegypti mosquitoes. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three-repeated experiments, each with a new batch toxins. The wild type Cry2Aa and Cry2Ac were used as positive controls, whereas Cry2Ab and deionised water were used as the negative controls for the experiment. Error bars represent Standard error of mean (SEM).***

The results of the bioassay in Figure 4.2.16 clearly showed that domain I of Cry2A toxins might be responsible for their specificity and hence toxicity to *Aedes aegypti*. This is because all the hybrids toxins created from domain swaps involving domain I of toxic Cry2A proteins (Cry2Ac and Cry2Aa), with domain II and III from the non-toxic Cry2A protein Cry2Ab (4D6-4), namely Cry2AcAbAb and Cry2Aa2AbAb, showed toxicity against

*Aedes aegypti* mosquito larvae. Whereas those hybrids created by domain swaps involving domain I from a non-toxic Cry2A toxin Cry2Ab (4D6-4) with domain II and III from a toxic Cry2A toxins (Cry2Ac and Cry2Aa), namely Cry2AbAcAc and Cry2AbAaAa to showed no activity against this insect. Cry2Aa toxin appears to have higher mortality in the present bioassay compared to Cry2Ac and Cry2Aa that was earlier shown in Figure 3.2.11, this could be as a result of problems which may arise from the bioassay procedure that have been discussed earlier in chapter 3. These results upheld our earlier hypothesis that domain I of Cry2A group of toxins might be responsible for their specificity.

### 4.2.2 N-terminal region of Cry2A family of toxins as a determinant of specificity in *Aedes aegypti*.

Previous reports implicated domain II as responsible for specificity among Cry2A toxins (Liang and Dean, 1994, Morse *et al*., 2001, Widner and Whiteley, 1990). Therefore, we thought that there might be a link between these two domains, which could be responsible for specificity. We proposed a hypothesis that since the N-terminal folds back from domain I onto domain II as shown in the structure elucidated by Morse *et al*. (2001), depicted in Figure 1.9.6. It may, therefore, be the N-terminal loop folding back onto domain II that maybe responsible for the specificity determinant role previously apportioned to domain II by previous researchers (Liang and Dean, 1994, Morse *et a*l., 2001, Widner and Whiteley, 1990). Our hypothesis was that it is this N-terminal region comprising the first 49 amino acids, depicted in Figure 4.2.17, that folds back and becomes a functional part of domain II and thus influencing toxin binding and specificity.

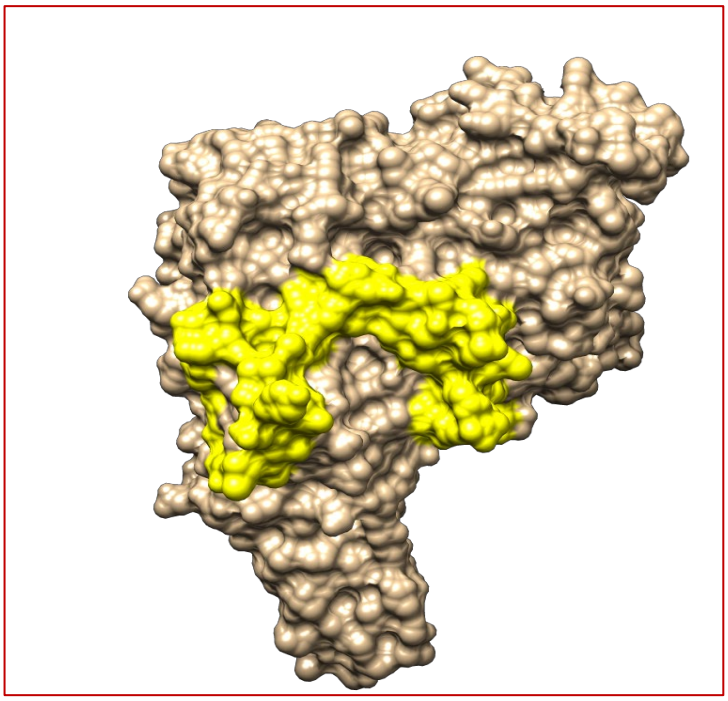


***Figure 4.2.17 Cry2Aa binding epitope formed by the N-Terminus loop (shaded yellow) which folds back onto the second domain influencing binding.***

Based on this hypothesis, we then decided to create the hybrids represented in Figure 4.2.18 below, this time swapping the N-terminal sequence of toxic Cry2A toxins (Cry2Ac and 2Aa) in to that of a representative of a nontoxic Cry2A toxin (Cry2Ab) and vice versa. This was to see the effect of the N-terminal 49 amino acids on the specificity/ toxicity of the Cry2A toxins.

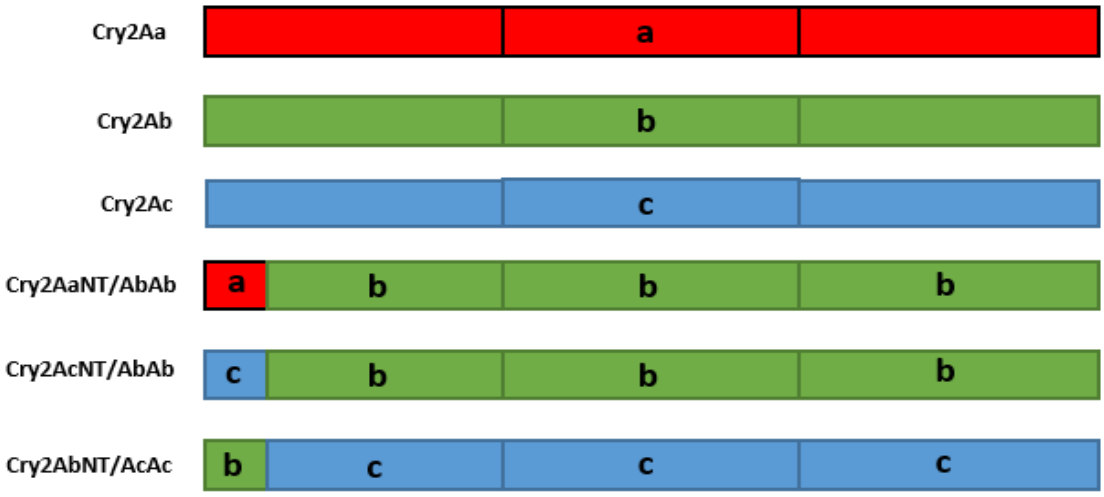



***Figure 4.2.18 N-terminal domain swaps hybrid created from Cry2A toxins. The colours represent each of the wild type Cry2A toxin and the hybrids; Red stands for wild type Cry2Aa, green stands for wild type Cry2Ab and blue stands for wild type Cry2Ac respectively.***

The amino acids as well as the nucleotide sequence showing the boundary between the N-terminus and the remaining portion of the Cry2Aa toxin is depicted in Figure 4.2.19 below.

```
D  P  F  S  F  E  H  K  S  L  D  T  I  Q  K  E  W  M  E  W  K
GAT CCA TTT AGT TTT GAA CAT AAA TCA TTA GAT ACC ATC CAA AAA GAA TGG ATG GAG TGG AAA
R  T  D  H  S  L  Y | V  A  P  V  V  G  T  V  S  S  F  L  L  K  K  V
AGA ACA GAT CAT AGT TTA TAC | CTA GAT CCT ATT GTT GGA ACT GTG GCT AGT TTT CTG TTA AAG AAA GTG
```

***Figure 4.2.19 Amino acids with their nucleotide sequence showing the boundary between the N-terminus and the remaining part of Cry2Aa toxin. The vertical line represents the junction between the two, while the sequence shaded yellow were those used for the design the Cry2AaNT reverse primer. ATG (M) at the beginning of the sequence represents the start codon.***

The N-terminal swap in the case of Cry2Ab to toxic Cry2A toxins i.e. Cry2Aa or Cry2Ac was done only for Cry2Ab N-terminus swapped into Cry2Ac domain as we presumed that it can provide the same information required even for hybrid that might be created using Cry2Ab N-terminus swapped into Cry2Aa.

To create the hybrids outlined in Figure 4.2.18, primers were designed for amplifying the N-terminus region and the pGEMCry2A without the N-terminus region for all the Cry2A toxins, which were used to create these hybrids. The general plan followed for the creation of the N-terminal swap hybrids is shown in Figure 4.2.20 below.

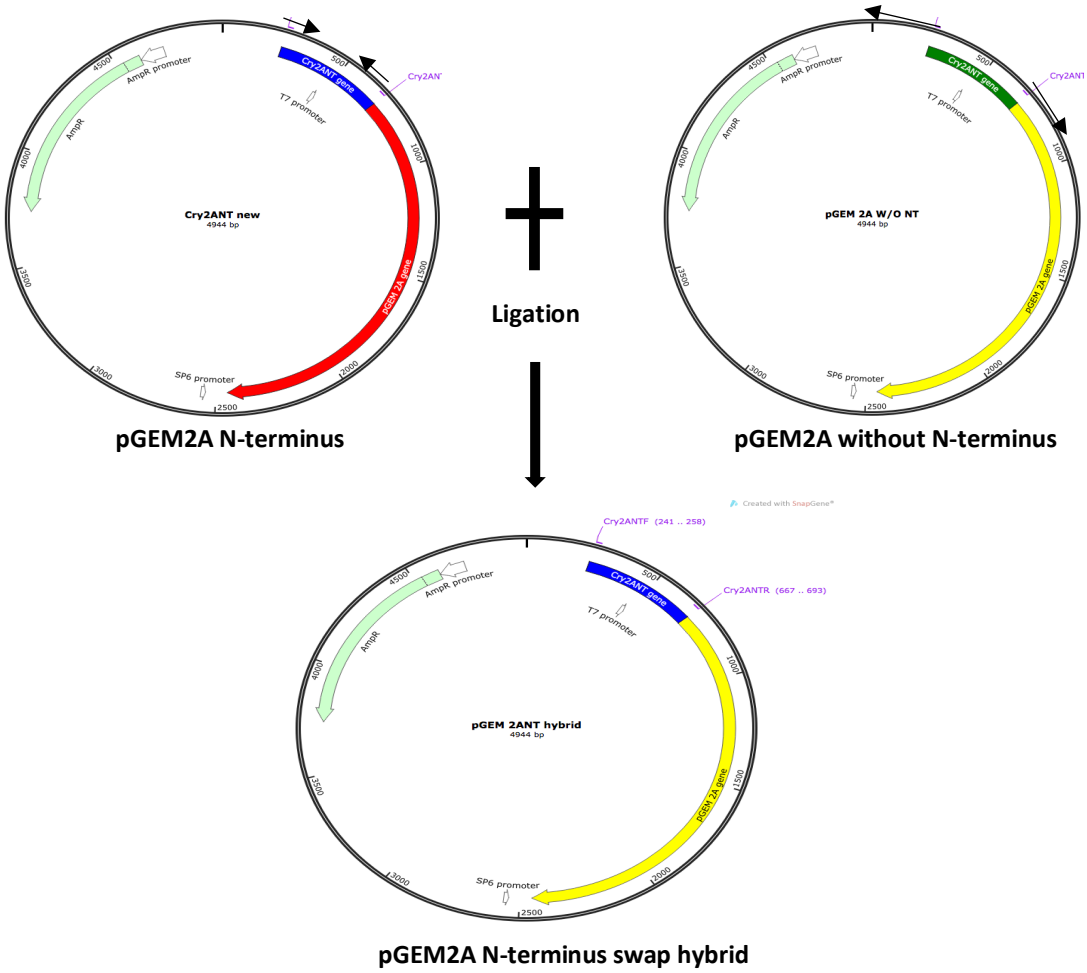



***Figure 4.2.20 Plasmid diagram showing the plan for pGEM2A hybrids created through N-terminus region swap. The part coloured blue denotes the N-terminus region of the pGEM2A gene, whereas the part coloured yellow represents the pGEM2A gene without the N-terminus region. The combination of the two regions through ligation gave rise to the pGEM2A N-terminus swap hybrid represented above.***

From the above diagram showing the plan for hybrids creation involving N-terminus swapping, the N-terminus region of the Cry2A toxin was amplified using two primers named Cry2ANT-F and Cry2ANT-R respectively as shown in Figure 4.2.20. The second part, which is pGEMCry2A without the N-terminal region contained the plasmid together with all the domains but lacking the N-terminal region and this portion was amplified using two primers namely, Cry2A W/O NT-F and Cry2A W/O NT-R respectively as shown in Figure 4.2.20 above. These two portions were ligated using DNA ligase

enzyme, the product of which is a hybrid containing an N-terminal region from one of the Cry2A toxin and the remaining portion including the pGEM plasmid from the other Cry2A toxin. The ligation product was introduced in to *E.coli* DH5-α strain, and the right colony was selected. This was transformed using *E.coli* BL21 strain for the expression of the protein as described in the Material and Methods section. The various primers designed are shown in Table 4.2-4 below.

| Share | Primer name | 5' to 3' | Modification | GC content |
|---|---|---|---|---|
| Yes | Cry2ANT-F | GCTGCGCGTAACCACCAC | 5'-PHO | 66.7% |
| No | Cry2AcNT-R | TAAACTATGATCAGTTCTTTTCCATTC | 5'-PHO | 29.6% |
| No | Cry2AbNT-R | TAAACTATGATATTTTTTTTCCACTCC | 5'-PHO | 25% |
| No | Cry2Aa2NT-R | TAAACTATGATCTGTTCTTTTCCACTC | 5'-PHO | 33.3% |
| No | Cry2Abplasmid-F | TACCTAGATCCTATTGTTGGAACTGTG | None | 40.7% |
| No | Cry2Acplasmid–F | TATGTAGCCCCTATTGTGGGAAC | None | 47.8% |
| Yes | Cry2A plasmid-R | GTGACCGCTACACTTGCCAG | None | 61.4% |

***Table 4.2-4 Primers used for the amplification of the fragments used for N-terminus swap hybrids of Cry2A toxins. Those primers indicated by 'Yes' on the column for share, are those primers that were used for all Cry2A toxins used in the creation of the hybrids.***

The PCR amplification products of the various fragments, which were used for creating the N-Terminal swaps hybrids, are shown in Figure 4.2.21(a-b) below.

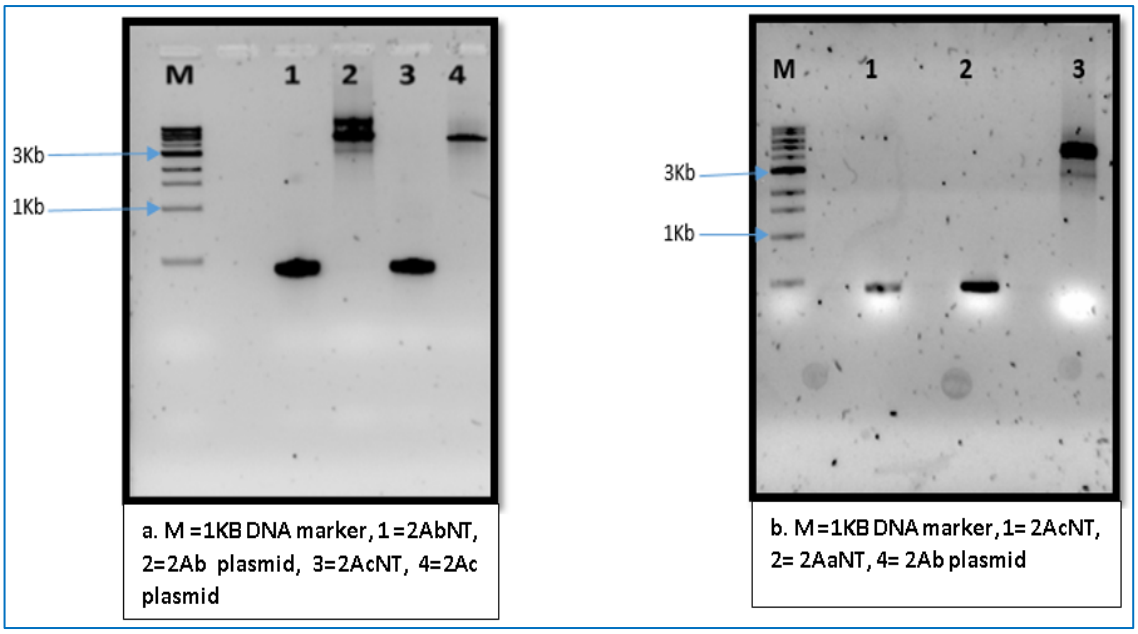



***Figure 4.2.21(a-b). PCR products for the components of Cry2A N-terminal swap hybrids created. The arrows pointing towards the right indicate the positions of the DNA Markers, which were used to understand if the amplified PCR products resolved on the gel, were of the required base pairs.***

The ligation reaction for the creation of the Cry2A N-terminal swap hybrids was set up by ligating 2AcNT+2Ab plasmid, 2AbNT+2Ac plasmid and 2AaNT+2Ab plasmid following the procedure for Blunt TA master mixed ligation described in the materials and methods section. Then the ligation products were introduced in to NEB-5α competent *E. coli* cells as outlined in the materials and methods. HaeIII enzyme restriction digests was used to confirm successful transformants. The HaeIII restriction enzyme digest for the three Cry2A N-terminal swap hybrids mentioned above are shown in Figure 4.2.22 below.

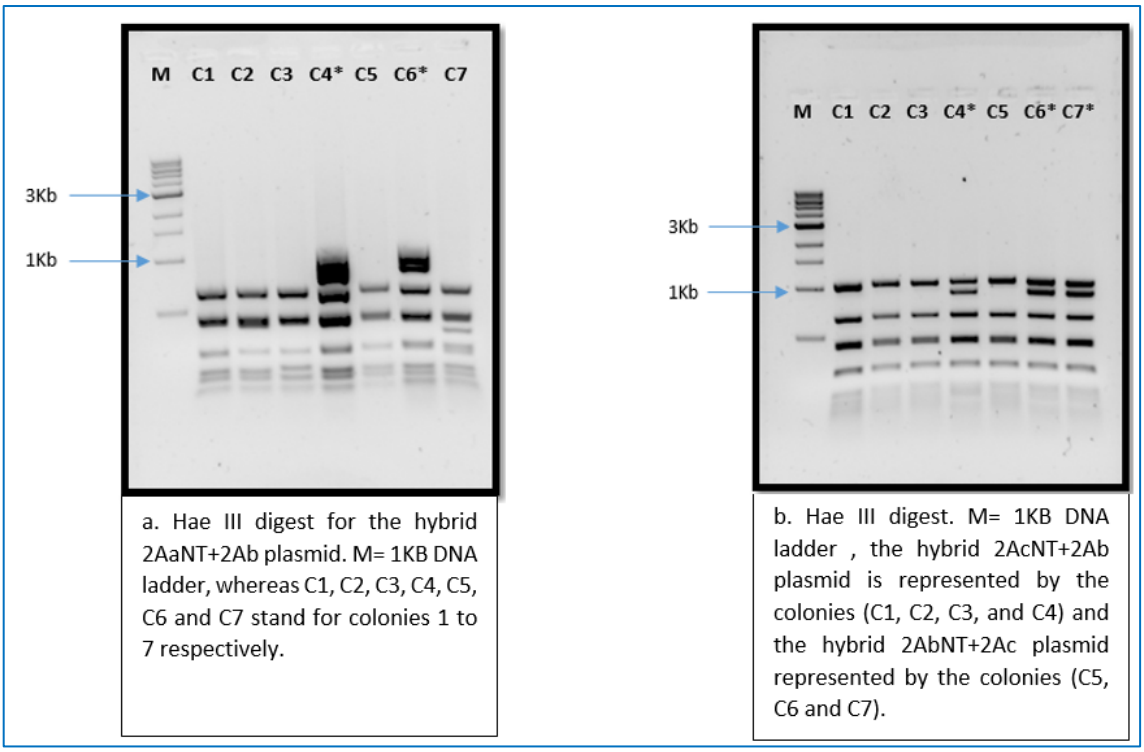



***Figure 4.2.22 (a-b). HaeIII digest for the colonies obtained from N-terminal swaps hybrids of Cry2A toxins. The arrows showed the positions of those bands on the marker to enable the detection of the positions, and hence the length in kilo base of the unknown fragments on the gel; this applies to the two gels above. Asterisks indicate colonies with the correct HaeIII restriction fragments.***

From the gels depicted in Figure 4.2.22, colony 4 and 6 for 2AaNT+2Ab, colony 4 for 2AcNT+2Ab, colony 6 and 7 for 2AbNT+2Ac, were picked and sent for sequencing. This was because the N-terminal sequence of Cry2A toxins, unlike domain I, did not contain any restriction site that could be used to confirm the orientation of the inserts. Therefore, this confirmation was done using the DNA sequencing results received after HaeIII digest by aligning them to the sequence of the construct generated from the hybrids created.

### Confirmation of sequencing results for the N-terminal swap hybrids created.

The sequencing results for the N-terminal swap hybrids created were confirmed following the same procedure done for the domain I swap hybrids. The sequences for the hybrid toxins received from the DNA sequencing results was aligned with the DNA sequences of the two wild type toxins, in which the N-terminal sequence of one was joined with the sequence of the other toxin excluding its N-terminal sequence portion,

through swapping. The various hybrids created and confirmed through alignment using Clustal Omega are shown in Figure 4.2.23 below.

The sequence alignment for the confirmation of the N-terminal swap hybrid, Cry2AaNT/AbAb formed by swapping the N-terminal sequence of Cry2Aa to the sequence of the rest of Cry2Ab without the N-terminal sequence portion is shown in Figure 4.2.23 below.

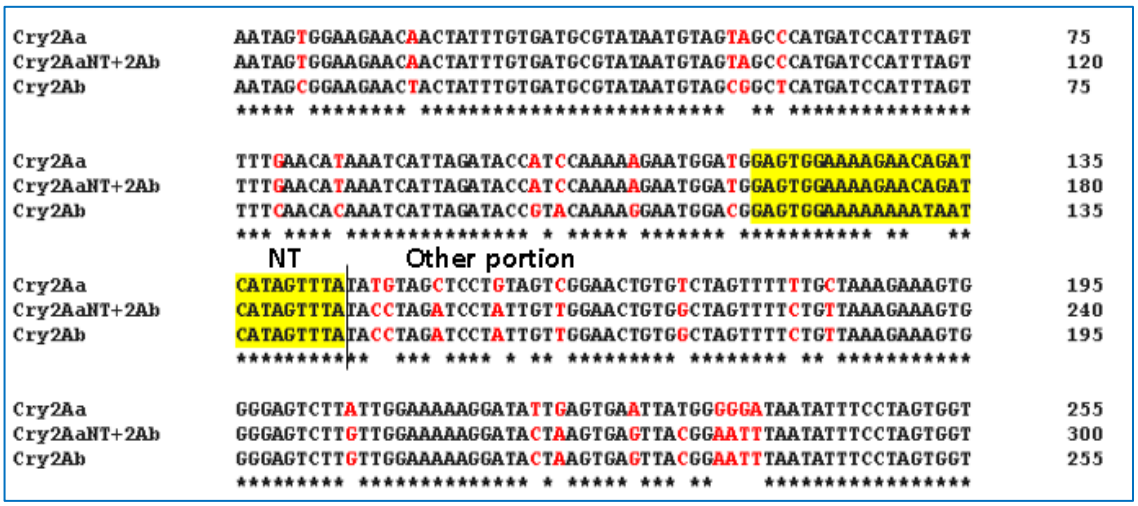



***Figure 4.2.23 Alignment results for the confirmation of the hybrid toxin PGEM2AaNT/AbAb. The area shaded yellow is the boundary where the two wild type Cry2A toxins forming the hybrid joined. The vertical line indicates the junction between them. Sequence with red colour before the boundary indicates where there is difference between the hybrid sequence and Cry2Ab within the N-terminal sequence whereas sequences shaded red after the junction indicates sequence differences between Cry2Aa and the hybrid toxin starting from the end of the N-terminal sequence. NT stands for N-terminal sequence.***

The sequence confirmation for Cry2AcNT/AbAb formed by swapping the N-terminal sequence of Cry2Ac to the sequence of the rest of Cry2Ab without the N-terminal sequence portion is shown in Figure 4.2.24 below.

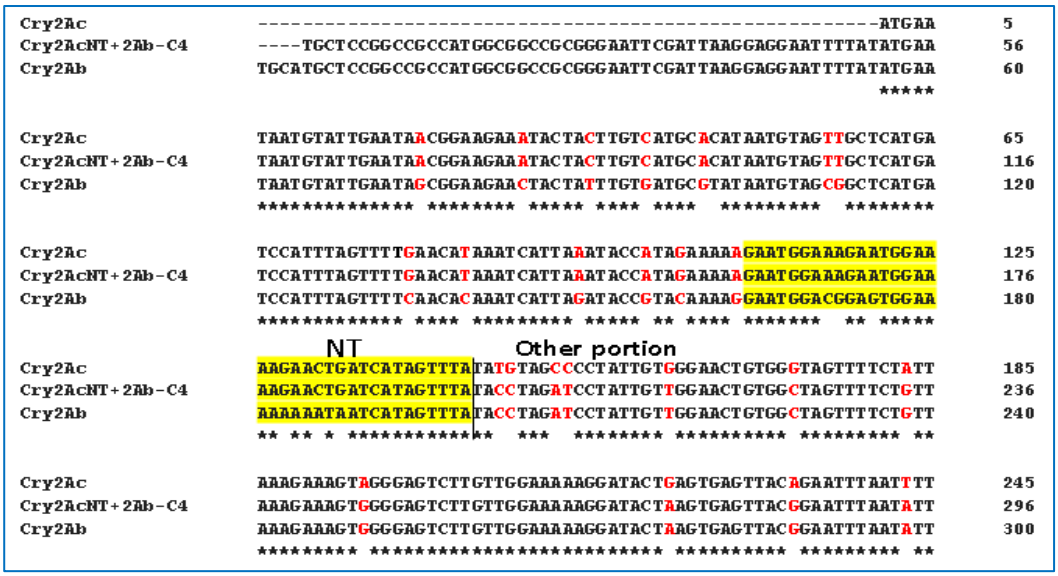



***Figure 4.2.24 Alignment results for the confirmation of the hybrid toxin PGEM2AcNT/AbAb. The area shaded yellow is the boundary where the two wild type Cry2A toxins forming the hybrid joined. The vertical line indicates the junction between them. Sequence with red colour before the junction indicates where there is difference between the hybrid sequence and Cry2Ab within the N-terminal sequence whereas sequences shaded red after the junction indicates sequence differences between Cry2Ac and the hybrid toxin starting from the end of the N-terminal sequence. NT stands for N-terminal sequence.***

The sequence alignment for the confirmation of the N-terminal swap hybrid, Cry2AbNT/AcAc formed by swapping the N-terminal sequence of Cry2Ab to the sequence of the rest of Cry2Ac without the N-terminal sequence portion is shown in Figure 4.2.25 below.

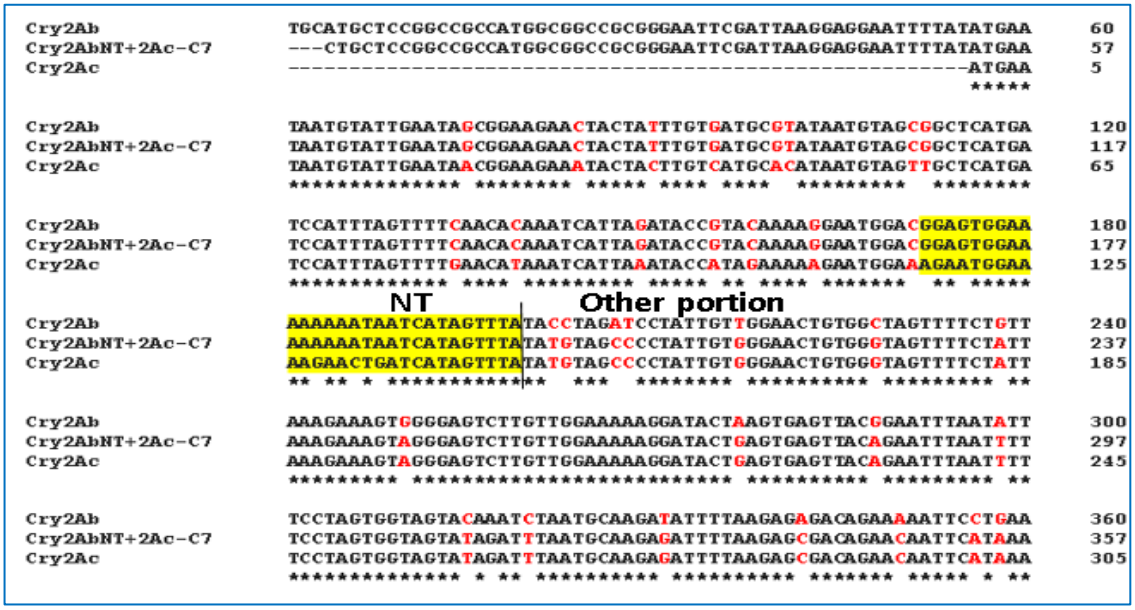



***Figure 4.2.25: Alignment results for the confirmation of the hybrid toxin PGEM2AbNT/AcAc. The area shaded yellow is the boundary where the two wild type Cry2A toxins forming the hybrid joined. The vertical line indicates the junction between the two. Sequence with red colour before the boundary indicates where there is difference between the hybrid sequence and Cry2Ac within the N-terminal sequence whereas sequences shaded red after the junction indicates sequence differences between Cry2Ab and the hybrid toxin starting from the end of the N-terminal sequence. NT stands for N-terminal sequence.***

The N-terminal sequence of the hybrid 2AbNT+2Ac, obtained from the sequencing results had aligned properly to Cry2Ab, which formed the N-terminal region for the hybrid as shown in the Figure 4.2.25 above. More also, the remaining sequence after the boundary aligned perfectly to Cry2Ac, which formed the other part of the two wild type Cry toxins that were ligated together to form the hybrid. This was found to be true from the alignment results in the entire N-terminal swap hybrids created, thus confirming that all the N-terminal swap hybrids were correct.

The SDS-PAGE gel showing all the N-terminal swap hybrid proteins created, expressed, grown and harvested were run on as SDS-PAGE gel along with their domain I swap counterparts as shown in Figure 4.2.26.

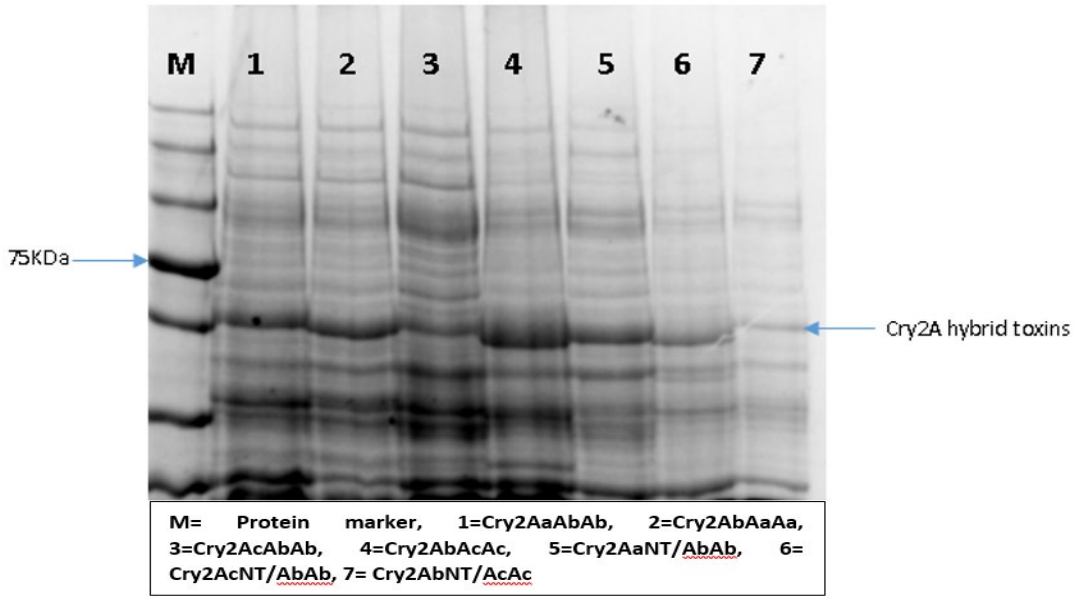



***Figure 4.2.26 Protein SDS-PAGE gel showing the N-terminal swap hybrid Cry2A toxin proteins expressed. The arrow pointing towards the right showed the molecular weight protein marker used to estimate the weight of the Cry toxins, while the one pointing towards the left shows the position of the hybrid toxin bands on the gel.***

The N-terminal swap hybrid proteins that were expressed were run along with BSA standards and their concentration measured by densitometry using image J software. The gel is shown Figure 4.2.27 below.

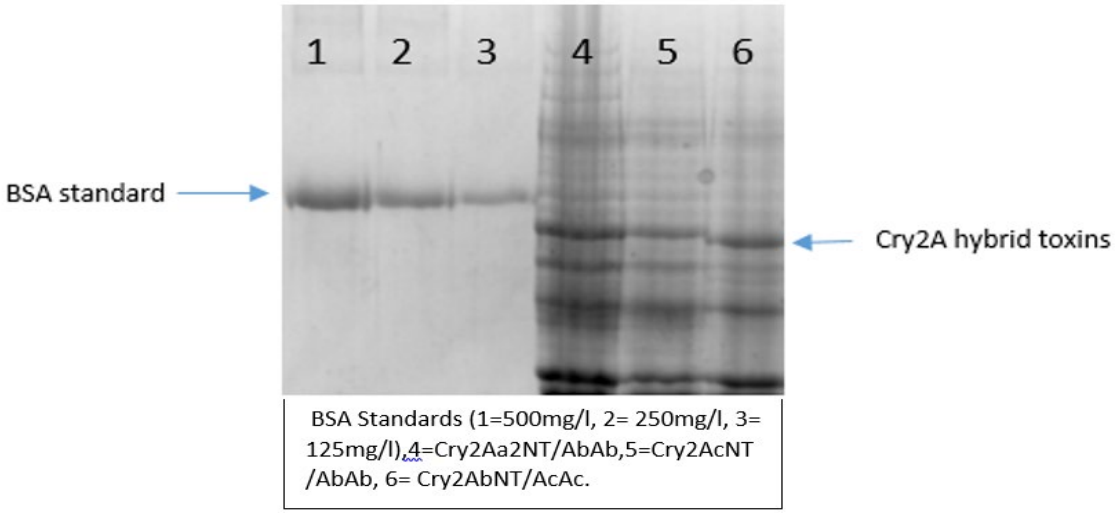



***Figure 4.2.27 Gel used to measure the concentration of the N-terminal swap hybrid proteins. The arrow pointing towards the right showed the Bovine Serum Albumin (BSA) standard, while the one pointing towards the left shows the position of the hybrid toxin bands on the gel.***

The table showing the concentration of the above hybrid toxins as measured from image J is shown in Table 4.2-5 below.

| Hybrid Toxin | Concentration(µg/ml) | Predicted molecular weight(KDa) |
|---|---|---|
| Cry2AaNT/AbAb | 500 | 70.81 |
| Cry2AcNT/AbAb | 200 | 70.83 |
| Cry2AbNT/AcAc | 300 | 69.65 |

***Table 4.2-5 Concentrations of the Cry2A N-terminal swap hybrid toxins created as measured using Image J. The hybrids toxins were all expressed, and their relative molecular weight predicted using a program in Expasy.***

A qualitative bioassay was performed using the above hybrid toxins, each at a concentration of 2mg/l following the procedures outlined by(WHO, 2005c). This was to know which among them was active against *Aedes aegypti*. The result of the bioassay is summarised in Figure 4.2.28 below.

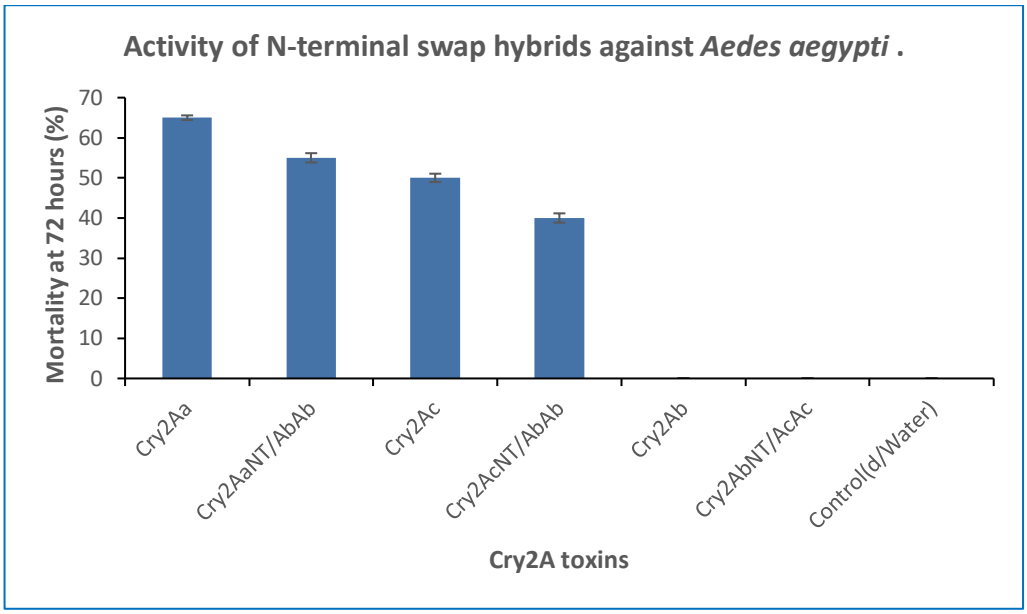



***Figure 4.2.28 Activity of Cry2A N-terminal swap hybrid toxins against Aedes aegypti mosquito. Cry2A toxins with mortality below 10% are considered nontoxic while those with mortality above 10% are considered toxic. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three-repeated experiments, each with a new batch of the toxins. The wild type Cry2Aa and Cry2Ac were used as positive controls, whereas Cry2Ab and deionised water were used as the negative controls for the experiment. Error bars represent Standard error of mean (SEM).***

From Figure 4.2.28, we could establish that the N-terminal sequence of Cry2A plays a role in their specificity against *Aedes aegypti* mosquito. This could be seen in that; Cry2Ab wild type toxin was not active against *Aedes aegypti* while the wild type Cry2Aa and Cry2Ac toxins were active against this insect. The swapping of the N-terminal sequence of Cry2Aa to Cry2Ab and that of Cry2Ac to Cry2Ab to form the hybrid toxins Cry2AaNT/AbAb and Cry2AcNT/AbAb respectively brought activity to the non-active Cry2Ab wild type toxin. Contrarily, the swapping of the N-terminal sequence of Cry2Ab to Cry2Ac to form the hybrid Cry2AbNT/AcAc abolished the activity seen in the active wild type Cry2Ac. These suggest that the basis for the activity/specificity of the Cry2A toxins resides in the N-terminal amino acid sequence. Hence, these findings supported our hypothesis that it is the N-terminal loop folding back onto domain II (Figure 4.2.20), which may be responsible for the specificity determinant role of this family of toxins against *Aedes aegypti.*

### 4.2.3 Deletion of 45 amino acids from the N-terminus of Cry2A toxins abolished activity against Aedes aegypti.

The previous results for the creation of hybrids through N-terminal swaps between the sequence of toxic Cry2A toxins (Cry2Ac and 2Aa) in to that of a representative of a nontoxic Cry2A toxin (Cry2Ab) and vice versa suggested that the N-terminal is responsible for the activity of Cry2A toxins against *Aedes aegypti*. Therefore, I decided to further confirm this by deleting the first 45 amino acids within the N-terminus of Cry2Aa2, to see if that will abolish the activity of Cry2A against *Aedes aegypti*. Two primers were designed for the creation of the truncated Cry2Aa toxin with 45- amino acid deleted from the N-terminus sequence, which we referred to as D45. Therefore, the forward primer starts from the 46$^{th}$ amino acid position whereas the reverse primer starts from the start codon ATG coding for methionine to allow the initiation of transcription after the deletion. The template strand used for this deletion mutant is Cry2Aa2 toxin.

The primers designed are as follows:

Forward primer

**F- 5'P- CATAGTTTATATGTAGCTCCTGTAG-3' (25)**

Reverse primer

**R-5'- CATATAAAATTCCTCCTTAATCG (23)**

The PCR conditions for the amplification of these products are as outlined in the material and methods section.

The amplified product was run on a DNA agarose gel to see if it had been amplified successfully. The gel is as shown in Figure 4.2.59 below.

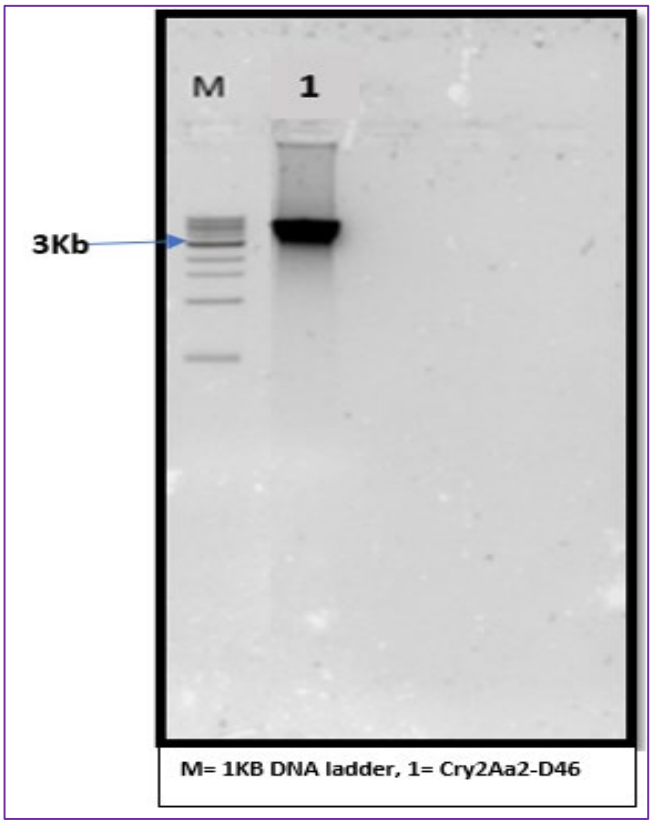



***Figure 4.2.29 Mutagenic PCR product for D-45 mutant from Cry2Aa N-terminus sequence.***

The purified D-45 PCR product in Figure 4.2.29 above was ligated using T4 DNA ligase and introduced in to *E.coli* DH5-α competent cells following the procedure described in the materials and methods section of this thesis. Colonies obtained after transformation were picked using toothpick and streaked in an ampicillin plate and was placed in an incubator maintained at $37^0$C and left to stay overnight. DNA miniprep was carried out using the cells harvested, and the purified DNA sample obtained from each of the

mutants was digested using HaeIII restriction enzyme. The gel of which is displayed in Figure 4.2.30 below.

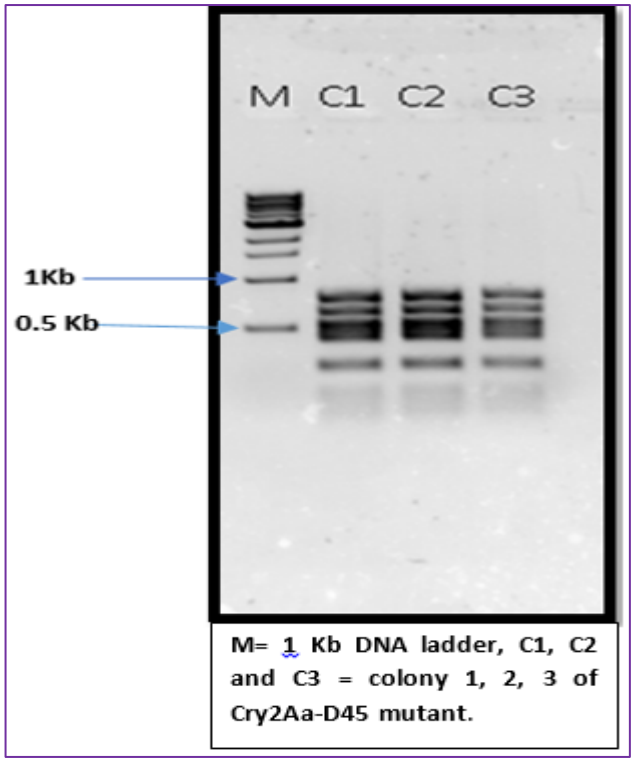



***Figure 4.2.30 DNA Agarose gel of the HaeIII restriction digest of colonies selected from Cry2Aa-D45 mutant.***

The HaeIII restriction digest fragments for Cry2Aa-D45 mutant in Figure 4.2.30 above was compared to the fragments generated from the NEB-Cutter using the sequence of the construct designed from the mutant, depicted in Table 4.2-13 below.

| # | Ends | Coordinates | Length (bp) |
|---|---|---|---|
| 1 | HaeIII-HaeIII | 4797-762 | 778 |
| 2 | HaeIII-HaeIII | 3537-4190 | 654 |
| 3 | HaeIII-HaeIII | 1268-1815 | 548 |
| 4 | HaeIII-HaeIII | 763-1267 | 505 |
| 5 | HaeIII-HaeIII | 2732-3189 | 458 |
| 6 | HaeIII-HaeIII | 2298-2731 | 434 |
| 7 | HaeIII-HaeIII | 4333-4621 | 289 |
| 8 | HaeIII-HaeIII | 1816-2094 | 279 |
| 9 | HaeIII-HaeIII | 3270-3536 | 267 |
| 10 | HaeIII-HaeIII | 2095-2268 | 174 |
| 11 | HaeIII-HaeIII | 4191-4332 | 142 |
| 12 | HaeIII-HaeIII | 4622-4723 | 102 |
| 13 | HaeIII-HaeIII | 3190-3269 | 80 |
| 14 | HaeIII-HaeIII | 4724-4763 | 40 |
| 15 | HaeIII-HaeIII | 4764-4784 | 21 |
| 16 | HaeIII-HaeIII | 2280-2297 | 18 |
| 17 | HaeIII-HaeIII | 4785-4796 | 12 |
| 18 | HaeIII-HaeIII | 2269-2279 | 11 |

***Table 4.2-6 HaeIII restriction digest fragments of Cry2Aa-D45 mutant generated from NEB-Cutter.***

Therefore, all the three colonies from Figure 4.2.30 above for Cry2Aa-D45 mutant appear to have same fragments to the one predicted from the NEB-cutter, hence all three colonies could contain the right transformant. Colony 1 and 2 were then sent for sequencing, and after the sequence from the sequencing results, were aligned with the two primers using CLUSTAL Omega they aligned perfectly well as shown in Figure 4.2.31 below. Hence, all the two colonies contained the right mutation.

```
Cry2Aa-D45-C2      CGATTAAGGAGGAATTTTATATGCATAGTTTATATGTAGCTCCTGTAG
Cry2Aa-D45-C1      CGATTAAGGAGGAATTTTATATGCATAGTTTATATGTAGCTCCTGTAG
Del-45-Primer      CGATTAAGGAGGAATTTTATATGCATAGTTTATATGTAGCTCCTGTAG
                   ||||||||||||||||||||||||||||||||||||||||||||||||
```

***Figure 4.2.31 Sequence alignment to confirm the creation of Cry2Aa-D45 mutant. Sequences shaded yellow showed the position of the forward primer and those shaded light green showed the position of the reverse primer.***

Colony 1 from Cry2Aa-D45 mutant was expressed in *E.coli* BL21 strain and the protein grown and harvested. The proteins were run on an SDS-PAGE along with a BSA standard and their concentrations measured using densitometry. The protein gel used to measure the concentrations of these toxins is shown in Figure 4.2.32 below.

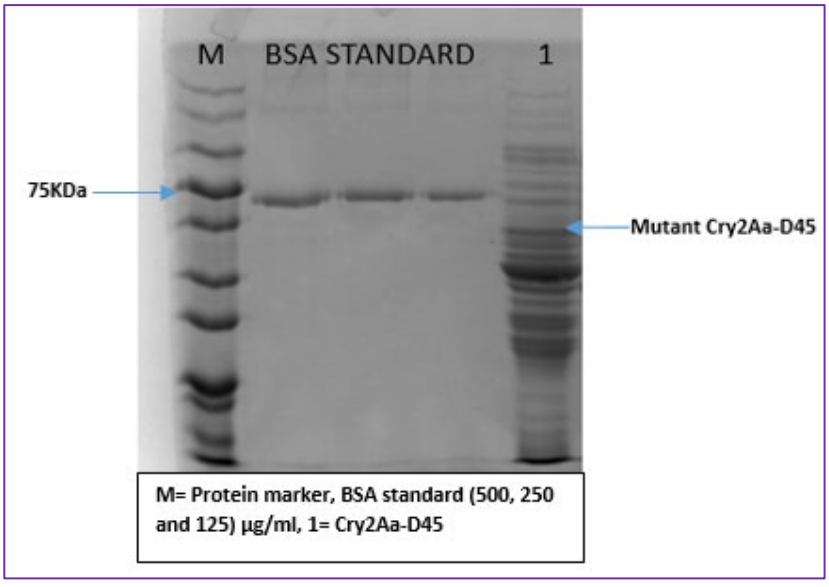



***Figure 4.2.32 Protein SDS-Page gel for measuring the concentrations of Cry2Aa-D45 mutant. The arrow pointing towards right indicate the protein marker while the one pointing towards the left indicate the mutant Cry2A toxins.***

A qualitative bioassay was performed for the mutant Cry2Aa-D45 in Figure 4.2.32 above using Cry2Aa as a positive control; each at a concentration of 2mg/l following the procedure outlined by WHO (2005c). Deionised water was used as a negative control. The graph is shown in Figure 4.2.33 below.

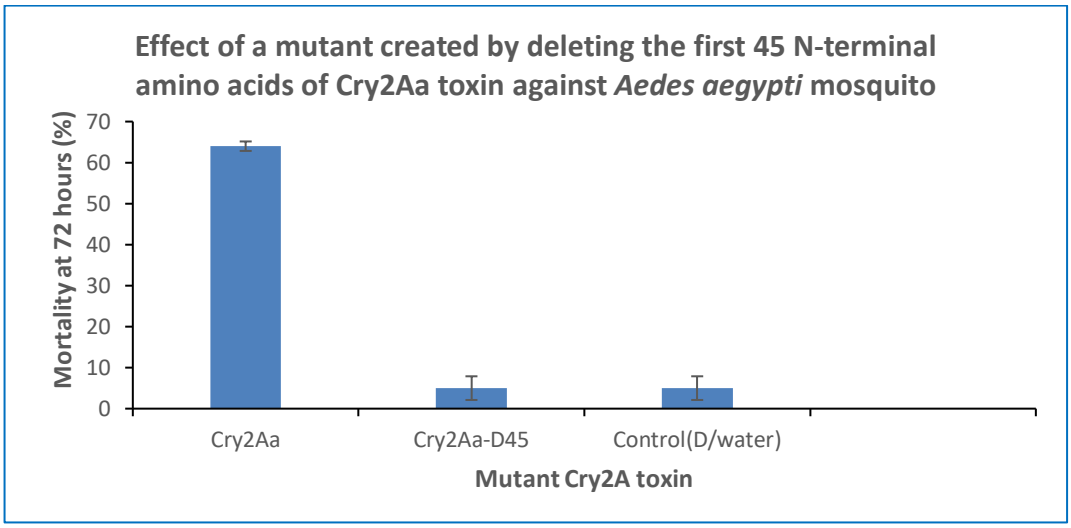



***Figure 4.2.33 Activity of mutant Cry2Aa-D45 toxin against Aedes aegypti. Cry2A toxins with mortality rate below 10% are considered non-active while those with mortality rate above 10% are considered active. The percentage mortality values on the graph represent the mean value of three-repeated experiments. The wild type Cry2Aa was used as positive control, whereas deionised water was used as the negative control for the experiment. Error bars represent Standard error of mean (SEM).***

From the results of bioassay depicted in the above graph (Figure 4.2.33), it was obvious that deletion of the first 45 amino acids from the N-terminus of Cry2Aa toxin abolished its activity against *Aedes aegypti* mosquito larvae. This is because the wild type Cry2Aa toxin from the bioassay was active against *Aedes aegypti* giving a percentage mortality of 65% whereas both the mutant Cry2Aa-D45 toxin and the control (deionised water) gave a percentage mortality of 5% each, less than the percentage mortality required (10%) to designate them as being active.

### 4.2.4 Finding the amino acid(s) residue responsible for specificity within the N-terminus region of Cry2A toxins.

Since we have established that the N-terminus region of Cry2A class of toxins is likely to be their specificity determinant region against *Aedes aegypti*. The next hurdle was to try to find the amino acid(s) residue, within the 49-amino acid N-terminal region, which is/are responsible for this activity among the Cry2A toxins. To achieve this, we aligned the 49 amino acids sequence comprising the N-terminus, for the toxic and nontoxic Cry2A toxins tested in this work, and at the same time of all the Cry2A available in the database using Clustal Omega.

MView v1.61 (Brown, 1998) was also used to render a multiple sequence alignment (MSA) of the N-termini of toxins used so far, alongside non-redundant N-terminus sequences of toxins, which we had in the lab and presented them in a colour format. A careful comparison between the sequences of the toxic ones and the non-toxic ones (Figure 4.2.34) was done to be able to see those amino acids that are conserved among the active ones and those conserved among the non-active ones.

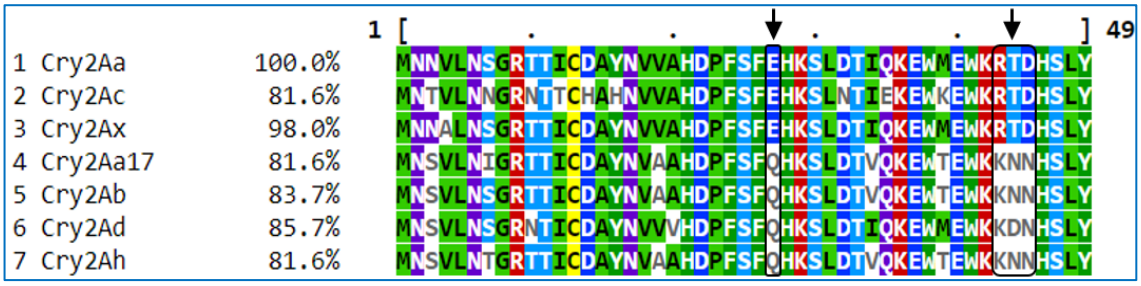



***Figure 4.2.34 Graphical representation of a multiple sequence alignment of the N-termini of selected Cry2A toxins using MView (http://www.ebi.ac.uk/Tools/msa/mview/). Percentages indicate the amount of sequence identity of each 49-mer sequence relative to the first sequence (Cry2Aa). Amino acids are coloured by their properties first, while uncoloured amino acids indicate that a residue is not identical in that position to the residue in the first sequence. Arrows above the encapsulating black boxes highlight putative specificity-determining residues. Order represents toxicity profiles with sequences found to be toxic at the top (Cry2Aa, Cry2Ac, and Cry2Ax) and sequences of toxins found to be inactive against Aedes at the bottom (Cry2Aa17, Cry2Ab, Cry2Ad, and Cry2Ah) respectively.***

A pattern emerged from the Multiple Sequence Alignment (MSA) results in Figure 4.2.34, which matched the toxicity profiles. Cry toxins found to be active against *A. aegypti* larvae (Cry2Aa, Cry2Ac and Cry2Ax), contained Glutamic acid (E) 27, and the triad of Arginine (R) 43, Threonine (T) 44, and Aspartic acid (D) 45, which I will refer to as RTD. In contrast, Cry toxins found to be inactive against *A. aegypti* larvae (Cry2Aa17,

Cry2Ab, Cry2Ad and Cry2Ah contained Glutamine (Q) 27 as well as the triad of Lysine (K) 43, Asparagine (N) 44, and Asparagine (N) 45, which I will refer to as KNN. Other differences did not correlate with activity.

To ensure this pattern was consistent, I carried out a far more intensive MSA of all currently known Cry2A toxins using a list created by another project student. In that work, the student had retrieved all sequences with >75% identity to Cry2Aa1 by running its sequence through Blastp, and removing all synthetic, hypothetical, misidentified or partial toxin sequences from the final list. The student found 99 sequences in this way, to which I have added new data for Cry2Aa18, Cry2Ac12, and Cry2Ax and removed data for U17(2Ab) since it displays a truncated N-terminus which bears no resemblance to all other Cry2A toxin N-termini (see appendix). This alignment showed that E / RTD and Q / KNN, are always associated as pairs and are both highly conserved among Cry2A toxins (See appendix).

However, we were yet to establish, which amino acid(s) among the four identified in Figure 4.2.34 is/are responsible for the specificity of this class of toxins. Therefore, we used mutagenesis to see which ones were important. The sequence representing the main areas of importance between the toxic and non-toxic Cry2A toxins is depicted in Figure 4.2.35 below.

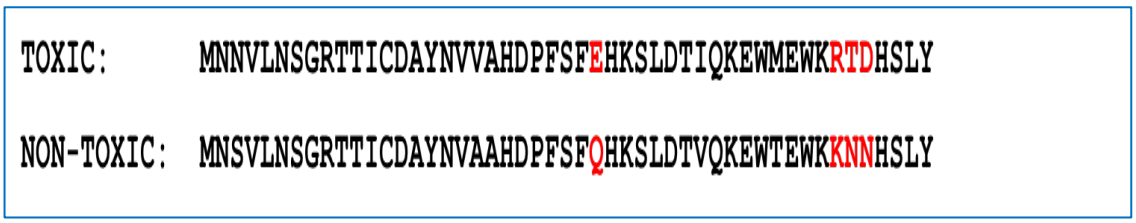



***Figure 4.2.35 A figure representing amino acids of importance for mutagenesis between the N-terminal sequence of toxic and Non-toxic Cry2A toxins against Aedes aegypti.***

I carried out site-directed-mutagenesis by PCR on Cry2Ab and Cry2Aa in an attempt to reverse their toxicity profiles. Single mutants for E/Q and RTD/KNN were constructed and bioassays performed to investigate whether either of the amino acid E/Q or the triad RTD/KNN was enough on its own to influence specificity, or if both are needed to be co-expressed in order to achieve specificity towards *A. aegypti* larvae.

I created three mutants from Cry2Ab. Firstly, I created a mutant 2Ab that possessed the amino acid E, at position 27 instead of Q i.e. Cry2Ab-E; secondly, I created another mutant that possessed the amino acids RTD instead of KNN at positions 43, 44 and 45 respectively i.e. Cry2Ab-RTD. Then lastly, I created a mutant that possessed all the amino acids substitutions mentioned for the two hybrids above (Q to E) at position 27 and (KNN to RTD) at positions (43, 44 and 45 respectively) i.e. Cry2Ab-ERTD.

### Mutagenic primers for the creation of Cry2Ab mutants

Mutagenic primers for the above-mentioned amino acid substitutions were created following two important properties:

I. The least substitution(s) that could give rise to the desired amino acid(s)
II. *E. coli* codon bias was also taken in to consideration, as some of the codons might not be preferable for expression by *E. coli* bacterium.

The general plan followed for the creation of the Cry2Ab-E and Cry2Ab-RTD mutants using Cry2Ab as the template strand and showing all the amino acids changes done on Cry2Ab are indicated in Figure 4.2.36a and b below.

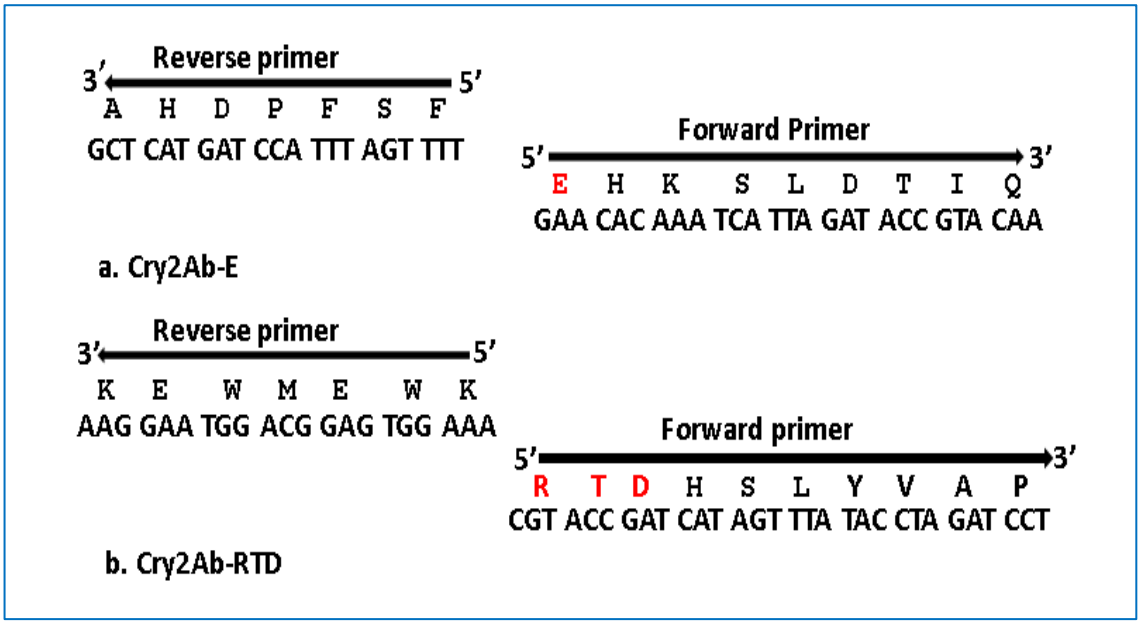



***Figure 4.2.36 Primers for the creation of Cry2Ab-E and Cry2Ab-RTD mutants showing the areas where amino acids changes were done on Cry2Ab toxin sequence, these are indicated by red colour.***

The mutagenic primers designed are shown in the Table 4.2-7 below.

| Oligo name | Sequence (5'-3') | Modification | GC content |
|---|---|---|---|
| Cry2Ab-RTD-F | CGTACCGATCATAGTTTATACCTAGATCC (29) | 5'-PHO | 41.4% |
| Cry2Ab-RTD-R | TTTCCACTCCGTCCATTCC (19) | 5'-PHO | 52.6% |
| Cry2Ab-E-F | GAACACAAATCATTAGATACCGTAC (25) | 5'-PHO | 36% |
| Cry2Ab-E-R | AAAACTAAATGGATCATGAGC (21) | 5'-PHO | 33.3% |

***Table 4.2-7 Mutagenic primers for the creation of Cry2Ab mutant toxins***

The PCR reaction was set following the procedure described in the material and methods section.

### PCR mutagenesis products for Cry2Ab mutants

The mutagenesis products obtained from the PCR reaction above were confirmed by running 5 µl of the DNA on a gel and the remaining 45µl were digested with 1µl of DpnI enzyme for 60 minutes to get rid of the parental DNA. This was then run on a gel, excised, and purified using the procedure outlined in the methods section for DNA gel purification. The gels for the PCR mutagenesis products are shown in Figure 4.2.37(a-b) below.

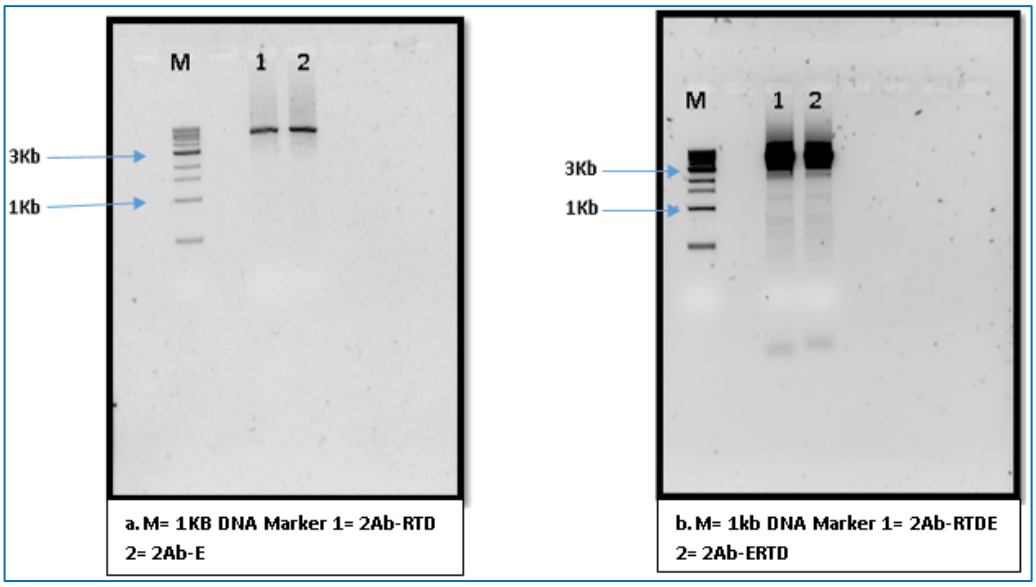



***Figure 4.2.37(a-b): Mutagenic PCR products for Cry2Ab mutant toxins.***

The mutagenic products above were ligated using blunt TA DNA ligase master mix after which they were introduced in to NEB-5α *E. coli* competent cells, and few colonies were selected. The selected colonies were digested with HaeIII restriction enzyme (Figure 4.2.38a and b), and the bands were compared to the fragments generated from NEB cutter (Table 4.2-1a); the correct colonies were selected, and sent for sequencing for further confirmation.

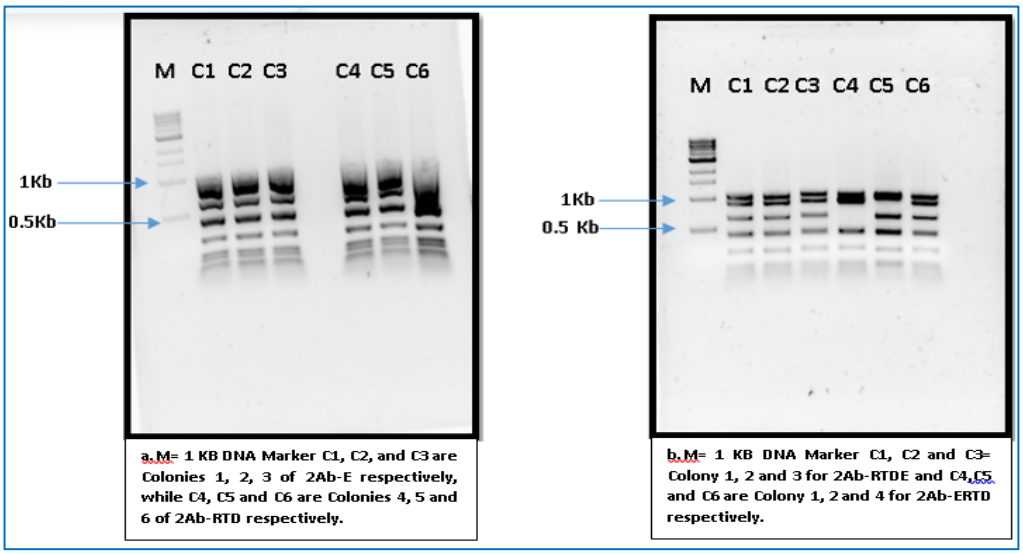



***Figure 4.2.38(a-b): DNA Agarose gel of the HaeIII restriction digest of colonies selected from Cry2Ab mutants***

The sequence received from the sequencing results for the creation of Cry2Ab-E by mutating glutamine (Q) at position 27 to Glutamic acid (E) was confirmed by aligning the sequence of the mutants received from the sequencing results (Cry2Ab-E-C4 and Cry2Ab-E-C5) with that of wild type Cry2Ab and checking at that position to see if the mutation has taken place. This is shown Figure 4.2.39 below.

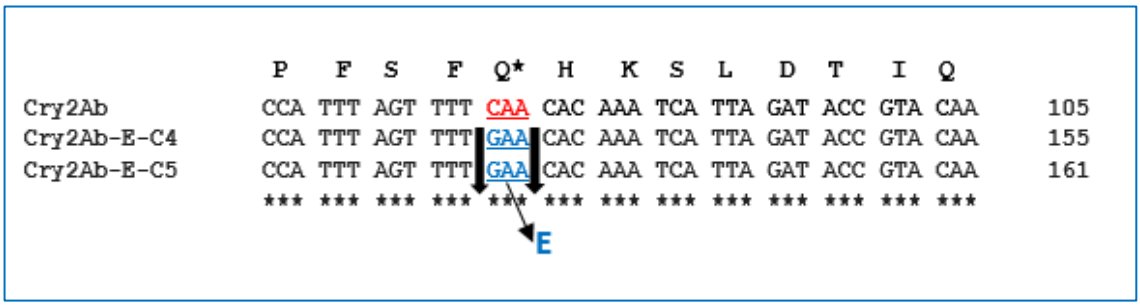



***Figure 4.2.39 Alignment results for the confirmation of Cry2Ab-E mutant. The alignment results is for mutating glutamine (Q) at position 27 of Cry2Ab to glutamic acid (E) which were underlined. The mutated nucleotides along with the corresponding amino acid are indicated by a blue colour; whereas an asterisk indicates the amino acid that was changed from Cry2Ab, whereas the non-mutated nucleotides in Cry2Ab are indicated by red colour.***

The mutant Cry2Ab-RTD made by mutating lysine, asparagine and asparagine (K, N, N) at positions 43, 44 and 45, to arginine, threonine, and aspartic acid (R, T, D) respectively, was confirmed by aligning the sequences of the mutants (Cry2Ab-RTD-C1 and Cry2Ab-RTD-C2) received from the sequencing results with that of wild type Cry2Ab as shown in Figure 4.2.40 below.

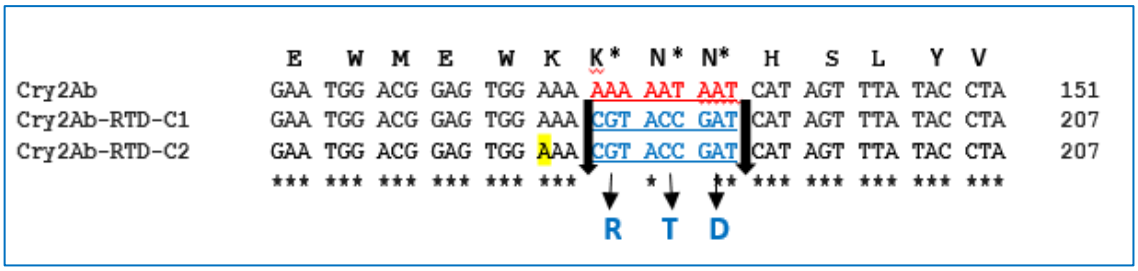



***Figure 4.2.40 Alignment results for the confirmation of Cry2Ab-RTD mutant. The alignment results is for mutating lysine, asparagine, asparagine (KNN) at position 43, 44 and 45 of Cry2Ab to arginine, threonine and aspartic acid (RTD) which are underlined. The mutated nucleotides along with the corresponding amino acids are indicated by a blue colour; whereas an asterisk indicates the amino acids that were changed from Cry2Ab, whereas the non-mutated nucleotides in Cry2Ab are indicated by red colour.***

The mutant Cry2Ab-ERTD was created by mutating glutamine, lysine, asparagine and asparagine (E, K, N, N) at positions 27,43, 44 and 45, to glutamic acid, arginine, threonine, and aspartic acid (E, R, T, D) respectively. This mutant was confirmed by aligning the sequences of the mutants (Cry2Ab-ERTD-C1 and Cry2Ab-ERTD-C4) received from the sequencing results with that of the wild type Cry2Ab as shown in Figure 4.2.41 below.

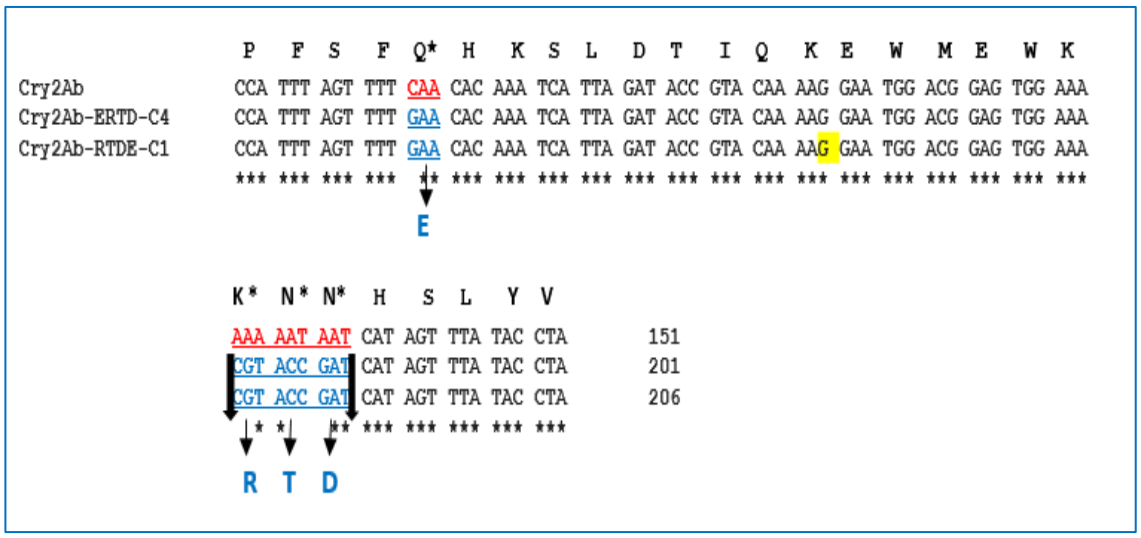



***Figure 4.2.41 Alignment results for the confirmation of Cry2Ab-ERTD mutant. The alignment results is for mutating glutamine, lysine, asparagine, asparagine (QKNN) at position27, 43,44 and 45 of Cry2Ab to glutamic acid, arginine, threonine and aspartic acid (ERTD) which are underlined. The mutated nucleotides along with the corresponding amino acids are indicated by a blue colour; whereas an asterisk indicates the amino acids that were changed from Cry2Ab, whereas the non-mutated nucleotides in Cry2Ab are indicated by red colour.***

From each of the three mutants represented in Figures 4.2.39, 4.2.40 and 4.2.41, a colony confirmed to have the right mutation was picked. Then introduced in to *E. coli* BL 21 strain for the expression of the mutant proteins. The gel for the expressed mutant Cry2Ab proteins is shown in Figure 4.2.42 below.

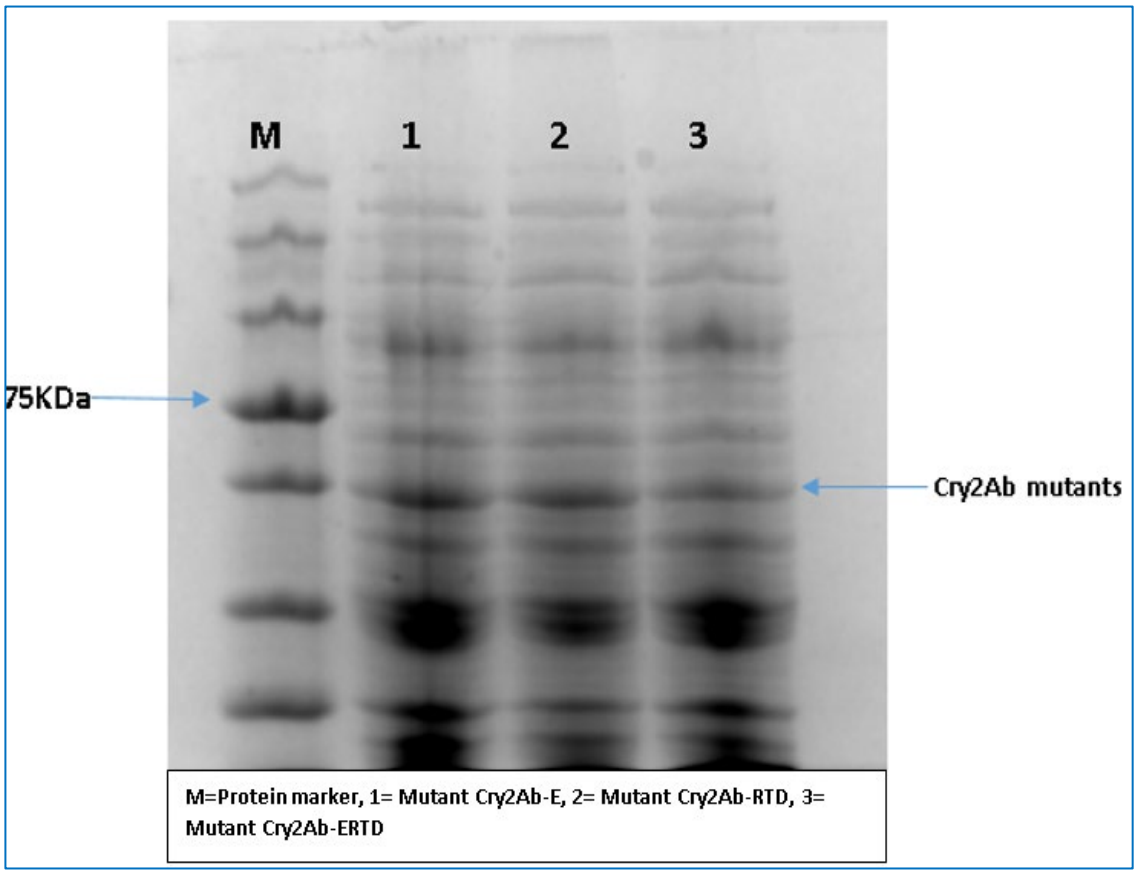



***Figure 4.2.42 Protein SDS PAGE showing the expression of the mutant Cry2Ab toxins. The arrow by the left side is pointing towards the protein marker, whereas the arrow towards the right is pointing towards the mutant Cry2Ab protein expressed.***

The concentration of the above expressed mutant proteins were measured by densitometry using Image J as described earlier in the Material and Methods section. The gel used in measuring the concentration of the above protein is shown in Figure 4.2.43 below.

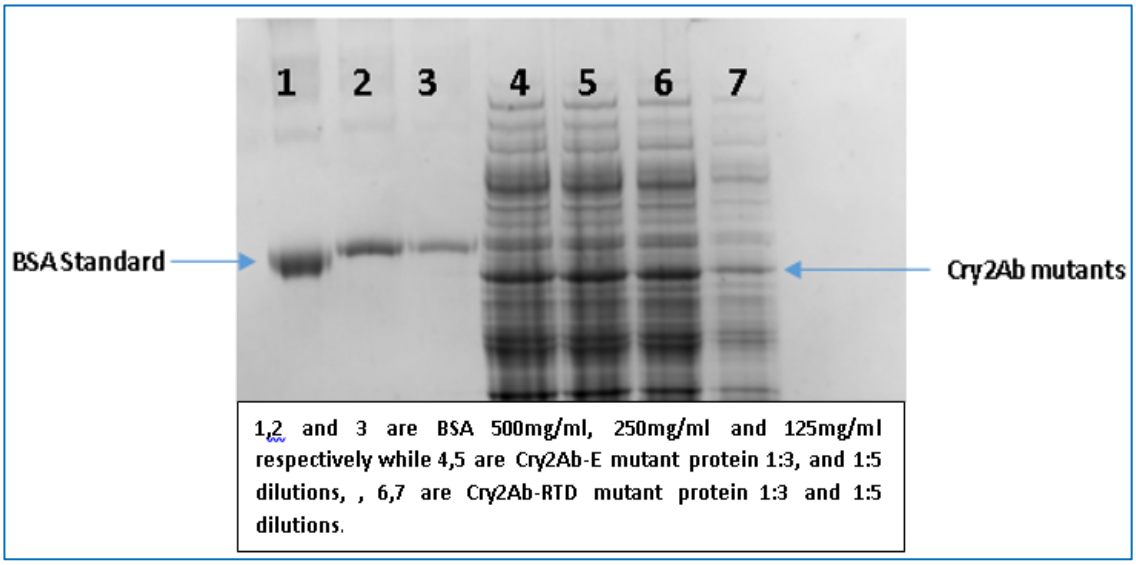



***Figure 4.2.43 Protein SDS PAGE for measuring the concentration of the mutant Cry2Ab toxins. The arrow pointing towards right indicate the BSA standard while the one pointing towards the left indicate the Cry2Ab mutant toxins.***

The concentrations of the above mutant toxins are depicted in Table 4.2-8 below.

| Mutant Cry2AbToxin | Concentration(µg/ml) | Predicted Weight (KDa) |
|---|---|---|
| Cry2Ab-E | 1,700 | 70.71 |
| Cry2Ab-RTD | 1,500 | 70.73 |
| Cry2Ab-ERTD | 600 | 70.72 |

***Table 4.2-8 Concentrations of the Cry2Ab mutant toxins created as measured using Image J. The hybrids toxins were all expressed, and their relative molecular weight predicted using a program in Expasy.***

A qualitative bioassay which discriminates between a toxic and non-toxic Cry2A proteins was performed using the above Cry2Ab mutants, each at a concentration of 2mg/l following the procedures outlined by (WHO, 2005b). This was done to enable us know which among the proteins was active against *Aedes aegypti*. The result of the bioassay is presented in Figure 4.2.44 below.

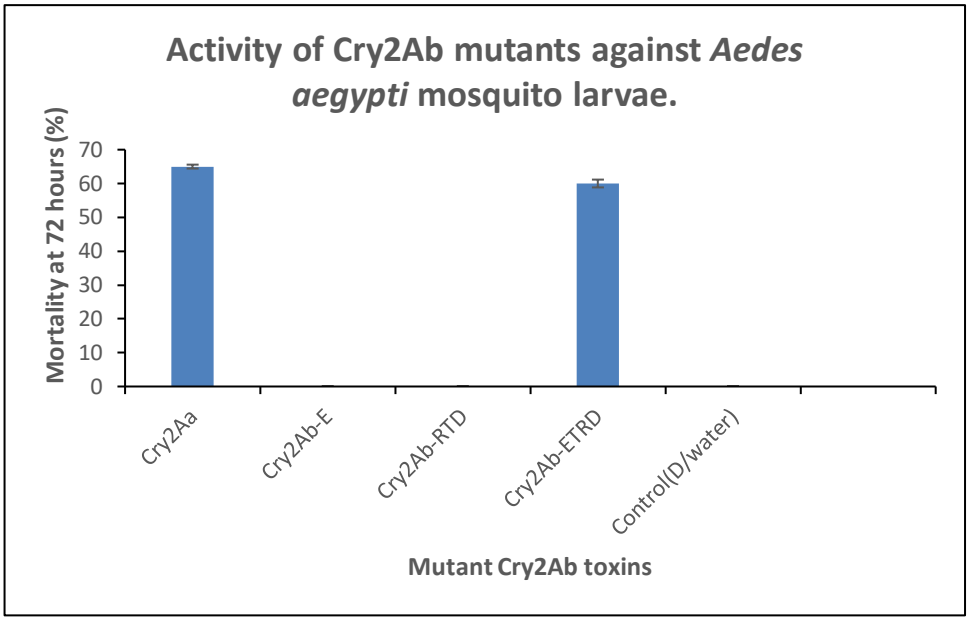



***Figure 4.2.44 Activity of Cry2Ab mutant toxins against Aedes aegypti mosquito. Cry2A toxins with mortality rate below 10% are considered non-active while those with mortality rate above 10% are considered active. The percentage mortality values on the graph represent the mean value of three-repeated experiments, each with a new batch of the toxins. The wild type Cry2Aa was used as positive control, whereas deionised water was used as the negative control for the experiment. Error bars represent standard error of mean (SEM).***

The graph in Figure 4.2.44 above showed that Cry2Ab-E and Cry2Ab-RTD mutants had no activity against *Aedes aegypti* larvae whereas Cry2Ab-ERTD was active against *Aedes*. These results showed that creation of single mutations, Q27E or KNN-43, 44, 45-RTD each in Cry2Ab (Cry2Ab-E and Cry2Ab-RTD) had no any effect on the activity of this inactive Cry toxin against *Aedes aegypti*. However, the combinatorial effect of the two mutations forming QKNN-27, 43, 44, 45- ERTD in Cry2Ab (Cry2Ab-ERTD) converted the non-active wild type Cry2Ab in to an active mutant toxin against *Aedes aegypti*.

### Creation of Cry2Aa mutant toxins

The effect of creating the same kinds of mutants as created in Cry2Ab toxin was also investigated in Cry2Aa toxin. This was to enable us to further understand if the two positions identified in Cry2A toxins i.e. Q27E and the triad KNN-43, 44, 45-RTD are important in specificity. To achieve this, we created two mutants Cry2Aa-Q and Cry2Aa-KNN to see if any of these two mutants can abolish the activity of Cry2Aa against *Aedes aegypti* mosquito hence asserting their roles in specificity determination.

The plan outlined in Figure 4.2.45a and b, showing the nucleotides used as primers as well as their corresponding amino acid sequences, was followed for the creation of the two mutants from Cry2Aa namely Cry2Aa-Q and Cry2Aa-KNN.

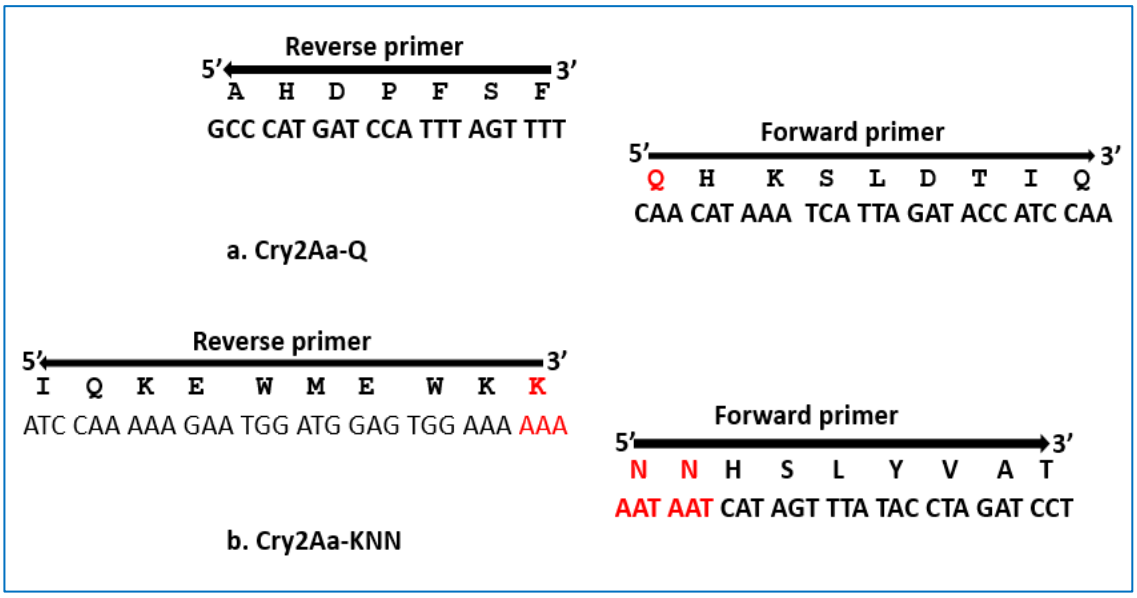



***Figure 4.2.45 (a-b). Plan for the primers designed for creation of Cry2Aa-Q and Cry2Aa-KNN mutants showing the areas where nucleotides as well as amino acids changes were done on Cry2Aa toxin sequence indicated by red colour.***

## Mutagenic primers for the creation of Cry2Aa mutants

The primers used in amplifying the components involved in creating Cry2Aa-Q and Cry2Aa-KNN mutant proteins are displayed in Table 4.2-9 below.

| Oligo name | Sequence (5'-3') | Modification | GC content |
|---|---|---|---|
| 2Aa2 E to Q-F | CAACATAAATCATTAGATACCATCC (25) | 5'-PHO | 44.1 |
| 2Aa2 E to Q-R | AAAACTAAATGGATCATGGG (20) | 5'-PHO | 44.1 |
| 2Aa2 RTD to KNN-F | AATAATCATAGTTTATATGTAGCTCC (26) | 5'-PHO | 44.1 |
| 2Aa2 RTD to KNN-R | TTTTTTCCACTCCATCCATTCTTTTTGG (28) | 5'-PHO | 44.1 |

***Table 4.2-9 Mutagenic primers for the creation of Cry2Aa mutant toxins.***

Cry2Aa toxin was used as the template strand for the PCR mutagenesis reaction for the mutants; Cry2Aa-Q and Cry2Aa-KNN. The PCR reaction was set following the procedure described in the material and methods section.

PCR mutagenesis products for Cry2Aa mutants

The PCR mutagenesis products for the two Cry2Aa mutants above were run on a gel to confirm if they have been amplified or not, this is shown in figure 4.2.46 below.

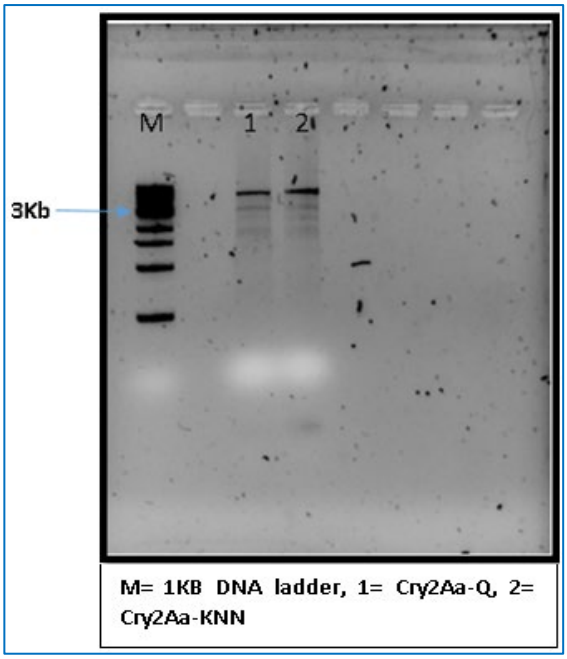



***Figure 4.2.46 Purified mutagenic PCR products for Cry2Aa mutant toxins. The arrow is pointing towards the 1Kb DNA marker used in estimating the size of the PCR products obtained on the agarose gel.***

The above gel showed the purified PCR products for the mutant toxins Cry2Aa-Q and Cry2Aa-KNN respectively. The DNA samples were then ligated, and introduced in to *E.coli* DH5-α strain. Successfully transformed colonies were picked using toothpick and further streaked on an ampicillin plate and was allowed to stay overnight in an incubator maintained at $37^0$C and mini prepped to obtain a pure DNA sample. The DNA obtained, which is circular was then digested using HaeIII restriction enzyme in order to get the colony containing the correct transformant. The SDS-page gel showing the HaeIII digest is depicted in Figure 4.2.47 below.

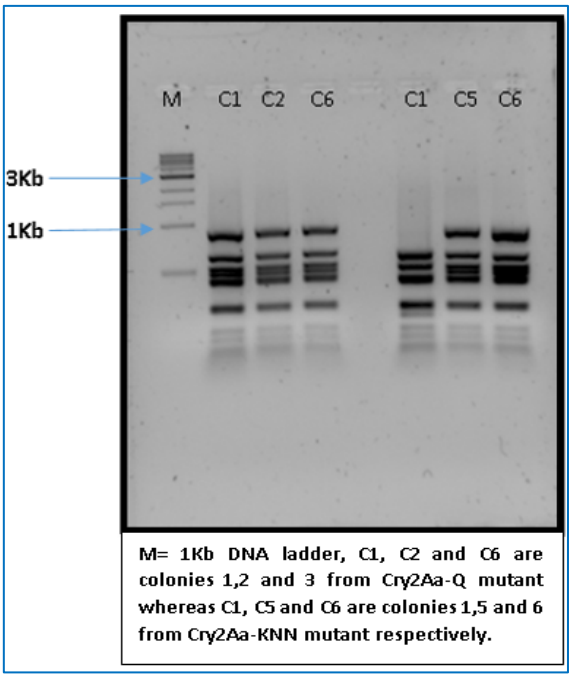



***Figure 4.2.47 DNA Agarose gel of the HaeIII restriction digest of colonies selected from Cry2Aa mutants.***

From the above gel. Colonies C1, C2 and C3 from the mutant Cry2Aa-Q all seemed to contain the correct HaeIII digest fragments when compared to those predicted by NEB-Cutter. Colonies C5 and C6 of the mutant Cry2Aa-KNN but not C1 also contained the right fragments from the HaeIII restriction digest.

The above colonies from each mutant were sent for sequencing after which colony 2(C2) from Cry2Aa-Q and colony 5(C5) from Cry2Aa-KNN were found to contain the desired mutation. The confirmation was done by aligning the sequence of the mutant toxin (Cry2Aa-Q-C2) received from the sequencing results to that of the wild type to see if the required mutations have been obtained, see Figure 4.2.48 below for the sequence alignment.

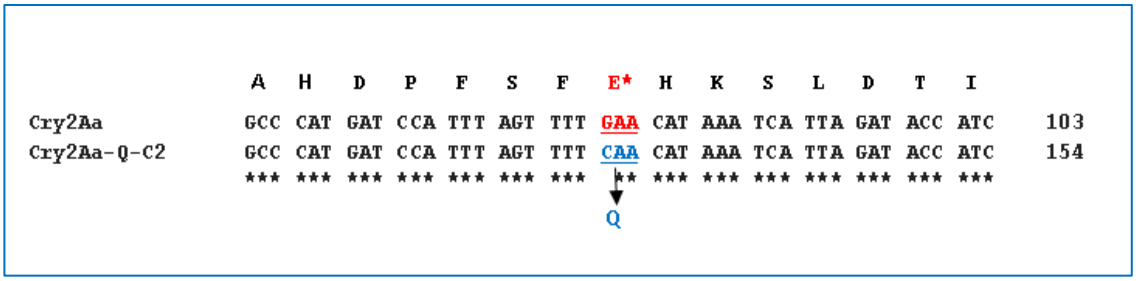



***Figure 4.2.48 Alignment results for the confirmation of Cry2Aa-Q mutant. The alignment results is for mutating glutamic acid (E) at position 27 of Cry2Ab to glutamine (Q) which is underlined. The mutated nucleotides along with the corresponding amino acid are indicated by a blue colour; whereas an asterisk indicates the amino acid that was changed from Cry2Aa, whereas the non-mutated nucleotides in Cry2Aa are indicated by the red colour.***

The alignment results for the confirmation of the Cry2Aa-KNN mutant is shown in Figure 4.2.49 below.

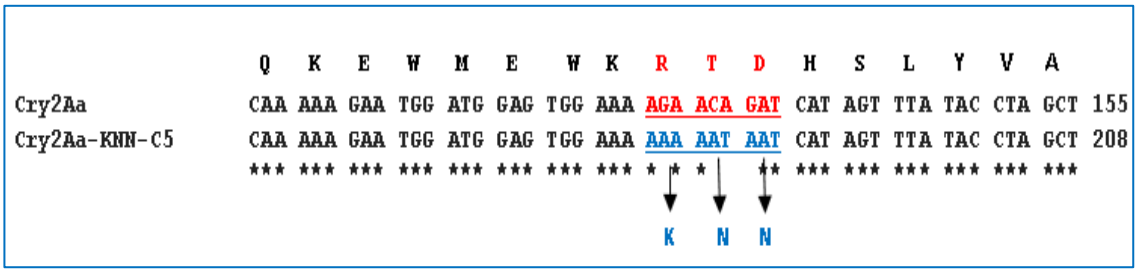



***Figure 4.2.49 Alignment results for the confirmation of Cry2Aa-KNN mutant. The alignment results is for mutating arginine, threonine and aspartic acid (RTD) at position 43, 44 and 45 of Cry2Aa to lysine, asparagine, and asparagine (KNN), which are underlined. The mutated nucleotides along with their corresponding amino acids are indicated by a blue colour; whereas red colour indicates the amino acids that were changed from Cry2Aa and the non-mutated nucleotides.***

The two colonies confirmed above for Cry2Aa-Q and Cry2Aa-KNN were introduced in to *E.coli* BL 21 for the expression of the protein and the protein was grown and harvested. The gel for which is displayed in Figure 4.2.50 below.

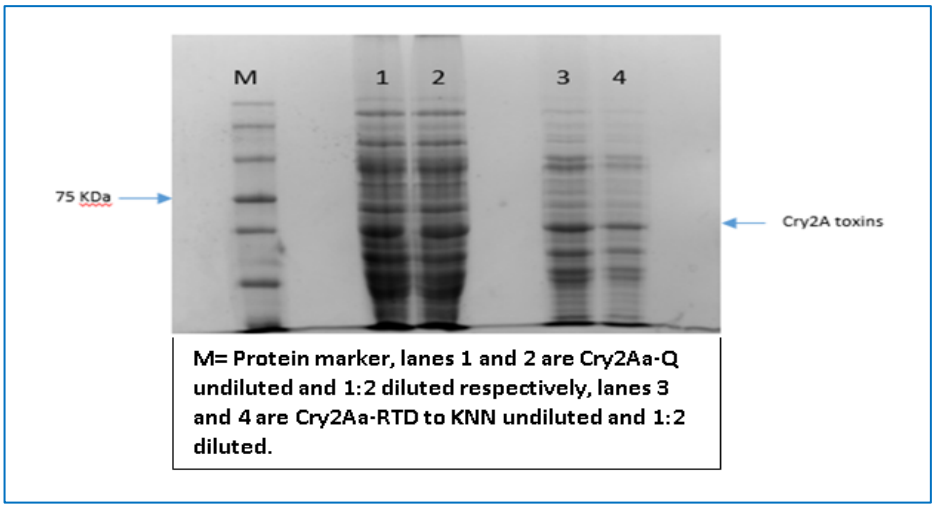



***Figure 4.2.50 Protein SDS PAGE showing the expression of the mutant Cry2Ab toxins. The arrow pointing towards right indicate the protein marker while the one pointing towards the left indicate the Cry toxins.***

The concentration of the above mutants was measured by densitometry using Image J after running the SDS-gel along with BSA standards as shown in the gel in Figure 4.2.51 below.

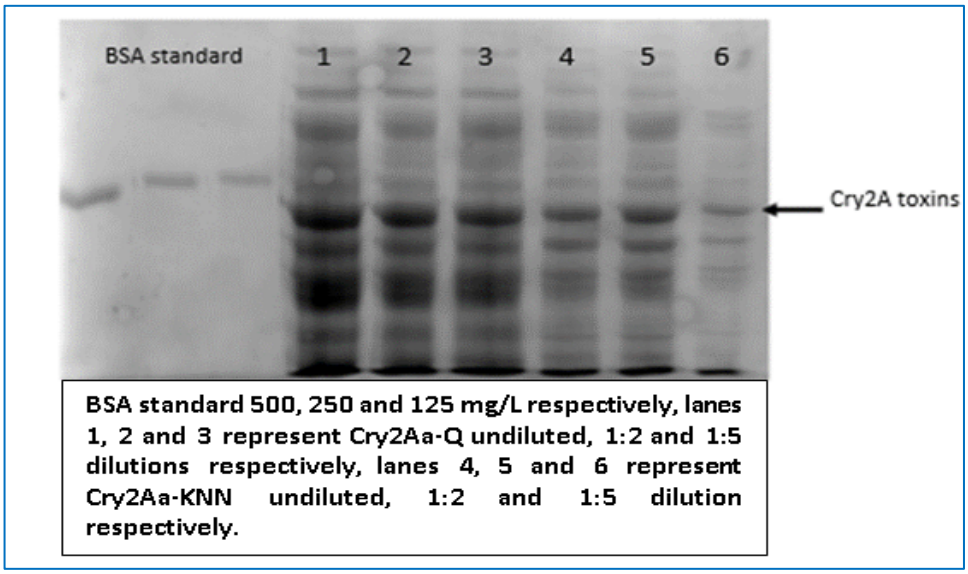



***Figure 4.2.51 SDS-Page gel for measuring the concentration of Cry2Aa-KNN mutant toxins. The arrow indicates the position of the Cry2A toxin bands.***

The concentration of the above toxins (Figure 4.2.51) as measured from image J is shown in Table 4.2-10 below.

| Plasmid | Cry2Aa Mutant | Concentration(µg/ml) | Molecular weight (KDa) |
|---|---|---|---|
| pGEM | Cry2Aa2 E to Q Mutant | 1300 | 70.85 |
| pGEM | Cry2Aa2 RTD to KNN mutant | 1500 | 70.85 |
| pGEM | Cry2Aa (positive control) | 1900 | 70.85 |

***Table 4.2-10 Concentrations of the Cry2Aa mutant toxins created as measured using Image J. The hybrids toxins were all expressed, and their relative molecular weight predicted using a program in Expasy.***

A qualitative bioassay which discriminates between a toxic and non-toxic Cry2A proteins was performed using the above Cry2Aa mutants each at a concentration of 2mg/l following the procedures outlined by WHO (2005b). This was to enable us know which amongst the Cry2Aa mutants created is active against *Aedes aegypti*. The result of the bioassay is summarised in the Figure 4.2.52 below.

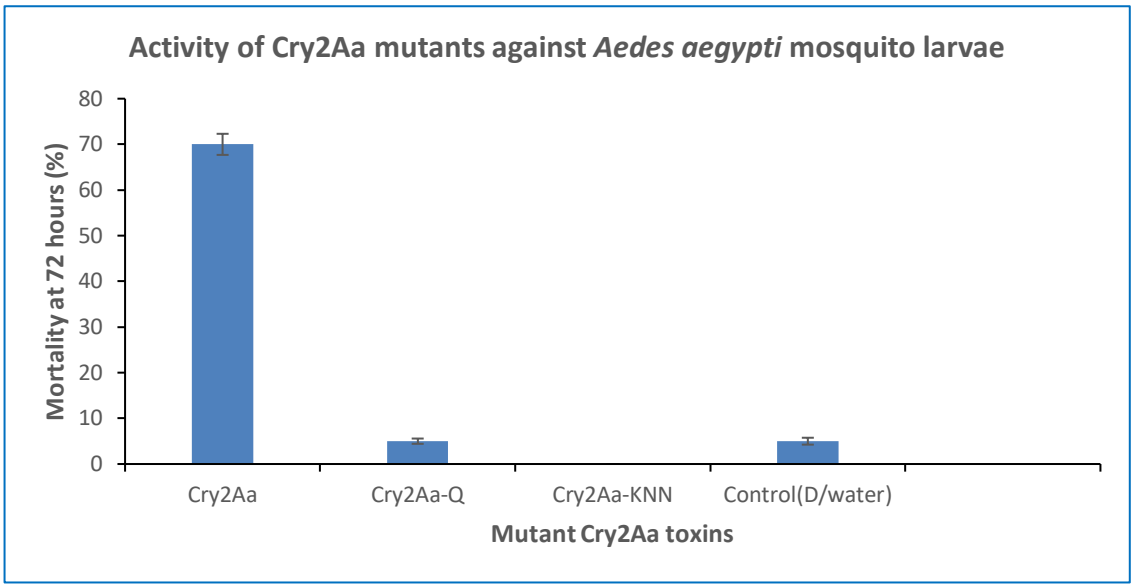



***Figure 4.2.52 Activity of Cry2Aa mutant toxins against Aedes aegypti mosquito. Cry2A toxins with mortality below 10% are considered non-active while those with mortality above 10% are considered active. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three-repeated experiments. The wild type Cry2Aa was used as positive control, whereas deionised water was used as the negative control for the experiment. Error bars represent Standard error of mean (SEM).***

The graph above (Figure 4.2.52) showed that both the two mutants Cry2Aa-Q and Cry2Aa-KNN have no activity against *Aedes aegypti* since they all possessed a percentage mortality of less than 10, which is non-significant. This, therefore, signified that all the four amino acids E/RTD found within the N-terminus of Cry2Aa or a few possible combinations from these four could be very significant for activity against *Aedes aegypti* mosquito larvae.

### Effect of single point mutagenesis of Cry2Ab-E/ KNN

The effect of creating a single point mutation within the triad KNN was investigated in order to see if single point mutation of any of this triad along with glutamic acid (E) on Cry2Ab i.e. the mutant Cry2Ab-E could lead to activity in Cry2Ab or all the four amino acids E/RTD must be present for activity in Cry2A toxins. Therefore, to achieve this we designed primers for the creation of the following three mutants:

Mutant Cry2Ab-ERNN

Mutant Cry2Ab-EKTN

Mutant Cry2Ab-EKND

The primers are as shown in Table 4.2-11 below.

| Oligo name | Sequence (5'-3') | Modification | GC content |
|---|---|---|---|
| Cry2Ab-ERNN-F | P-AATAATCATAGTTTATATATGTAGCTCC(26) | 5'-PHO | 44.1 |
| Cry2Ab-ERNN-R | p-TCTTTTCCACTCCATCCATTCTTTTTGG (28) | 5'-PHO | 39.3 |
| Cry2Ab-EKTN-F | P-ACTAATCATAGTTTATATGTAGCTCC(26) | 5'-PHO | 30.8 |
| Cry2Ab-EKTN-R | P-TTTTTTCCACTCCATCCATTCTTTTTGG (28) | 5'-PHO | 44.1 |
| Cry2Ab-EKND-F | P-AATGATCATAGTTTATATGTAGCTCC(26) | 5'-PHO | 30.8 |
| Cry2Ab-EKND-R | P-TTTTTTCCACTCCATCCATTCTTTTTGG (28) | 5'-PHO | 44.1 |

***Table 4.2-11 Mutagenic primers for the creation of single point mutants within the triad 'KNN'.***

Cry2Ab-E toxin was used as the template strand for the PCR mutagenesis reaction in the creation of the mutants in Table 4.2-11 above. The PCR reaction was set following the procedure described in the material and methods section. The amplified products were run on a DNA agarose gel to see if they have been amplified successfully. These are shown in Figure 4.2.53 below.

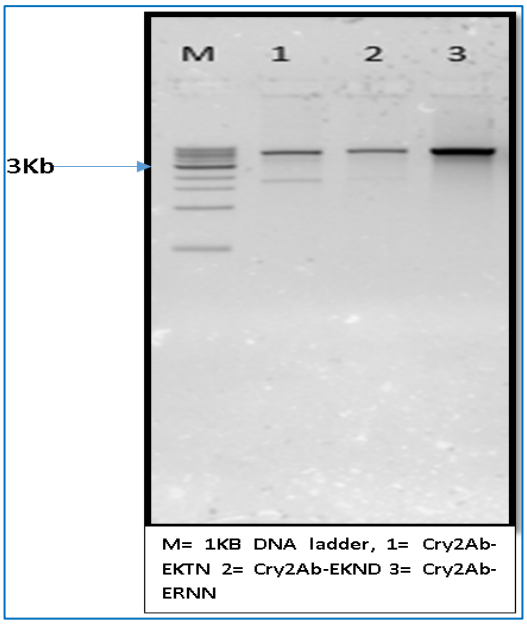



***Figure 4.2.53 Mutagenic PCR products for single point mutants within Cry2Ab-E 'KNN' triad.***

The above mutants (Figure 4.2.53) were ligated using T4 DNA ligase and introduced in to *E.coli* DH5-α competent cell, following the procedure described in the materials and methods sections. Transformed colonies were picked using toothpick, streaked in an ampicillin plate, placed in an incubator maintained at $37^0$C and was left to stay overnight. DNA miniprep was carried out using the cells harvested, and the pure DNA sample obtained from each of the mutants was digested using HaeIII restriction enzyme. The gels of which is shown in Figure 4.2.54 below.

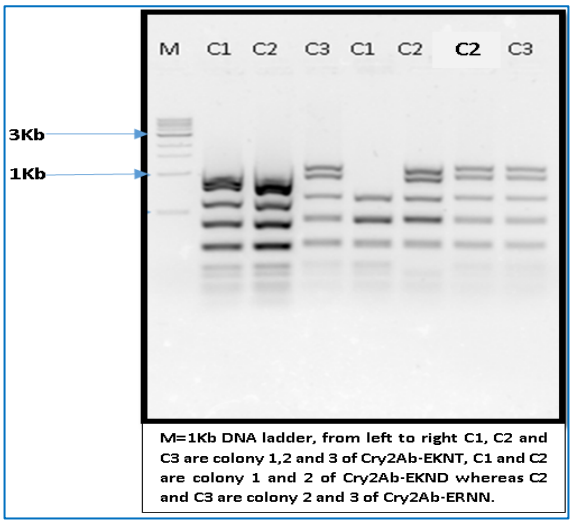



***Figure 4.2.54 DNA Agarose gel of the HaeIII restriction digest of colonies selected from the single point mutants created within Cry2Ab-E 'KNN' triad.***

From the gel in Figure 4.2.54 above, after comparison was made with the fragments generated from the NEB- cutter depicted in table 4.2-1a it showed that colony 1,2 and 3 from Cry2Ab-EKTN, colony C2 of Cry2Ab-EKND, and both colony 2 and 3 from Cry2Ab-ERNN may have the right transformants since they gave similar fragments to those generated by the NEB-cutter. Therefore, colony 1 and 2 of Cry2Ab-EKTN, Colony 2 from Cry2Ab-EKND and colony 2 and 3 from Cry2Ab-ERNN were sent for sequencing to confirm if they all contained the right mutations. This confirmation was done by aligning the sequence received from the sequencing results for each of these colonies from the mutants to that of Cry2Ab around the area where the mutation was expected to see if the right mutation has been successfully created. This is depicted in Figure 4.2.55.

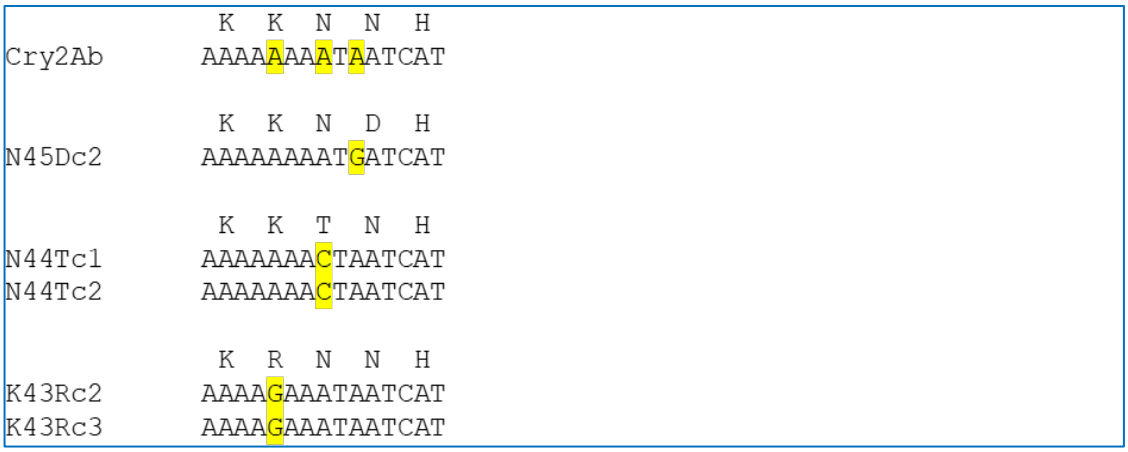



***Figure 4.2.55 Sequence alignment to confirm the creation of single point mutants within Cry2Ab-E 'KNN' triad.***

Therefore, from the sequence alignment in Figure 4.2.55 above, it was apparent that colony 2 from Cry2Ab-EKND, colony 1 and 2 from Cry2Ab-EKTN and colony 2 and 3 from Cry2Ab-ERNN all have the desired mutations created successfully. Furthermore, colony 2 from Cry2Ab-EKND, colony 1 from Cry2Ab-EKTN, and colony 2 from Cry2Ab-ERNN were selected and all put in *E. coli* BL21 strain for the expression of their respective proteins. The cells recovered after transforming each of these colonies with *E. coli* BL21 were grown and the proteins harvested and run on an SDS-Page gel. This is depicted in Figure 4.2.56.

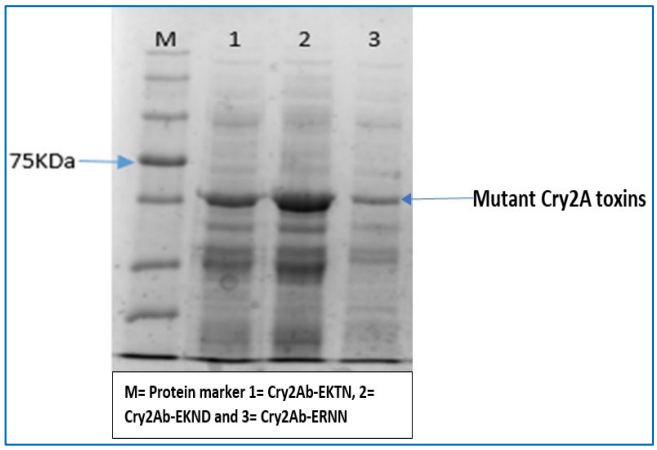



***Figure 4.2.56 protein SDS-Page gel for single point mutants created within Cry2Ab-E 'KNN' triad. The arrow pointing towards right indicate the protein marker while the one pointing towards the left indicate the mutant Cry2A toxins.***

The concentration of the mutants Cry2Ab-E toxins created within the 'KNN' triad were measured by densitometry using image J from the gel depicted in Figure 4.2.57 below.

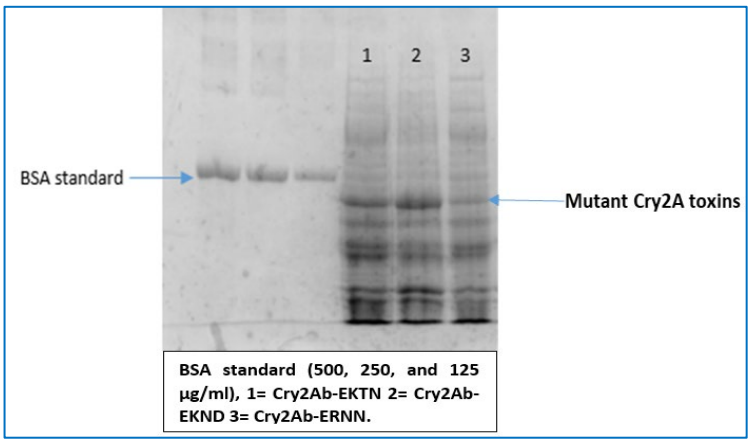



***Figure 4.2.57 Protein SDS-Page gel for measuring the concentrations of the single point mutants created within Cry2Ab-E 'KNN' triad. The arrow pointing towards right indicate the BSA standard while the one pointing towards the left indicate the mutant Cry2A toxins.***

The concentration of the above toxins (Figure 4.2.57) as measured from image J is shown in Table 4.2-12 below.

| Plasmid | Toxin | Concentration(µg/ml) | Predicted molecular weight (KDa) |
|---|---|---|---|
| pGEM | Cry2Ab-EKTN mutant | 900 | 70.71 |
| pGEM | Cry2Ab-EKND mutant | 1000 | 70.71 |
| pGEM | Cry2Ab-ERNN mutant | 500 | 70.71 |

***Table 4.2-12 Concentrations of the single point mutants created within Cry2Ab-E toxin 'KNN' triad as measured using Image J. The hybrids toxins were all expressed, and their relative molecular weight predicted using a program in Expasy.***

A qualitative bioassay was performed using the three mutants in Table 4.2-12 above along with Cry2Aa as a positive control; each at a concentration of 2 mg/l following the procedure outlined by WHO (2005c). Deionised water was used as a negative control. This was to determine which amongst the three mutants is active against *Aedes aegypti*. The result of the bioassay is summarised in Figure 4.2.58 below.

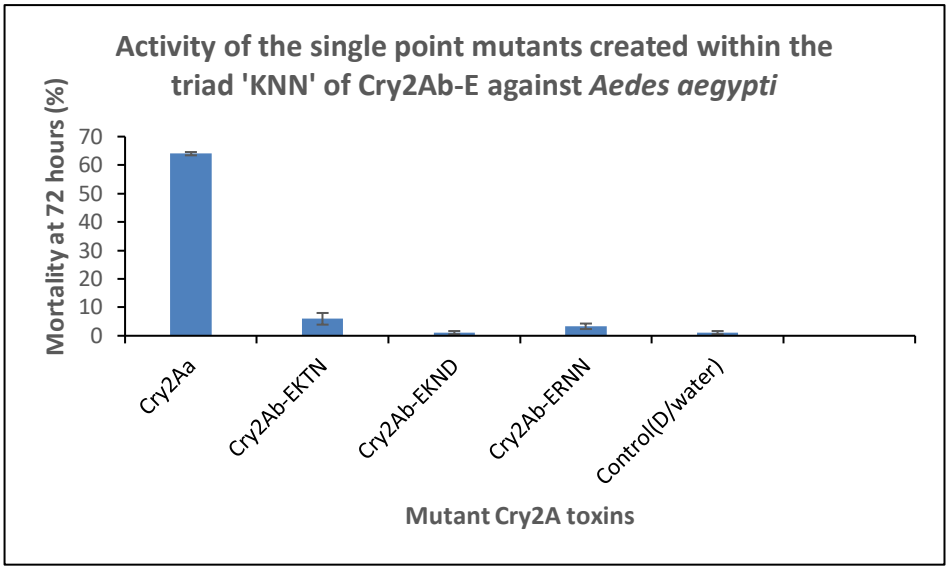



***Figure 4.2.58 Effect of the single point mutants created within the triad 'KNN' of Cry2Ab-E against Aedes aegypti. Cry2A toxins with mortality below 10% are considered non-active while those with mortality above 10% are considered active. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three-repeated experiments. The wild type Cry2Aa was used as positive control, whereas deionised water was used as the negative control for the experiment. Error bars represent Standard error of mean (SEM).***

The graph above showed clearly that none of the triad 'RTD' along with 'E27' was solely responsible for the activity of Cry2A toxins against *Aedes aegypti* since all the three mutants: Cry2Ab-EKTN, Cry2Ab-EKND and Cry2Ab-ERNN possessed a percentage mortality of less than 10, which is non-significant compared to the positive control (Cry2Aa) having a mortality rate of 65%. This, therefore, signified that no single amino acid in the triad (RTD) in combination with E could give activity.

### Effect of double point mutagenesis of Cry2Ab-E/KNN

Since it was obvious that none of the single point mutations on the triad KNN confers activity against *Aedes aegypti* mosquito larvae, I decided to create double mutants from the triad KNN to see the effect of double mutation on this position in Cry2A toxins. We used Cry2Ab-E mutant as the template strand; the three double mutants created are as follows:

Cry2Ab-ERTN

Cry2Ab-ERND

Cry2Ab-EKTD

The primers used to create the three double mutants above are depicted in Table 4.2-13 below.

| Oligo name | Sequence (5'-3') | Modification | GC content |
|---|---|---|---|
| Cry2Ab-ERTN-F | P-ACTAATCATAGTTTATATGTAGCTCC(26) | 5'-PHO | 30.8 |
| Cry2Ab-ERTN-R | p-TCTTTTCCACTCCATCCATTCTTTTTGG(28) | 5'-PHO | 39.3 |
| Cry2Ab-ERND-F | P-AATGATCATAGTTTATATGTAGCTCC(26) | 5'-PHO | 30.8 |
| Cry2Ab-ERND-R | p-TCTTTTCCACTCCATCCATTCTTTTTGG(28) | 5'-PHO | 39.3 |
| Cry2Ab-EKTD-F | p-AAAACTGATCATAGTTTATACCTAGATC(28) | 5'-PHO | 28.6 |
| Cry2Ab-EKTD-R | P-TTTTTTCCACTCCATCCATTCTTTTTGG(28) | 5'-PHO | 44.1 |

***Table 4.2-13 Mutagenic primers for the creation of double mutants in Cry2Ab-E within the triad 'KNN'***

The PCR reaction was set following the procedure described in the Material and Methods section. The amplified products were run on a DNA agarose gel to see if they have been amplified successfully. These are shown in Figure 4.2.59 below.

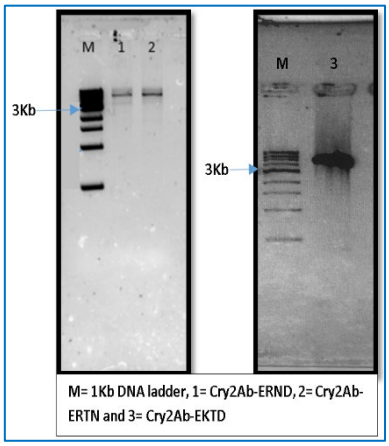



***Figure 4.2.59 Mutagenic PCR products for double point mutants within Cry2Ab-E 'KNN' triad.***

The mutants in Figure 4.2.59 were ligated using T4 DNA ligase and introduced in to *E.coli* DH5-α competent cells following the procedure described in the Materials and Methods section of this thesis. Colonies present after the transformation were picked using toothpick and streaked in an ampicillin plate and was placed in an incubator maintained at $37^0$C and was left to stay overnight. DNA miniprep was carried out using the cells harvested, and the purified DNA sample obtained from each of the mutants was digested using HaeIII restriction enzyme. The gels of which is shown in Figure 4.2.60 below.

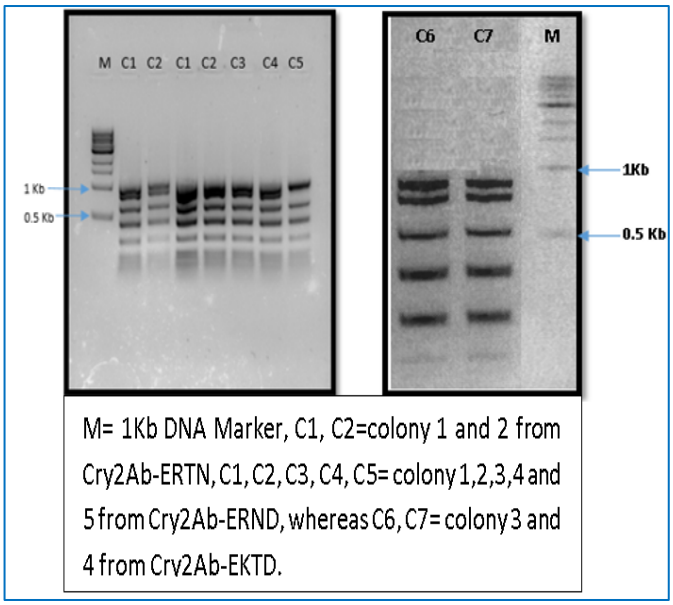



***Figure 4.2.60 DNA Agarose gel of the HaeIII restriction digest of colonies selected from the double point mutants created within Cry2Ab-E 'KNN' triad.***

From the above gel (Figure 4.2.60), colony 1 and 2 from Cry2Ab-ERTN, colony 1, 2, 3, and 4 from Cry2Ab-ERND, and colony 4 and 5 from Cry2Ab-EKTD appeared to have the right transformants when the bands from the HaeIII digestion were compared to the fragments generated from the NEB- cutter (Table 4.2-1a). Therefore, colony 1 and 2 from Cry2Ab-ERTN, colony 2 and 3 from Cry2Ab-ERND and colony 3 and 4 from Cry2Ab-EKTD were sent for sequencing, the results of which was aligned to the sequences of the constructs created in each case using Clustal omega. The result showed that all the colonies contained the desired mutations. This confirmation was done by aligning the sequence received from the sequencing results for each of these colonies from the mutants to that of Cry2Ab around the area where the mutation was expected, to see if the right mutation has been successfully created. This is depicted in Figure 4.2.61.

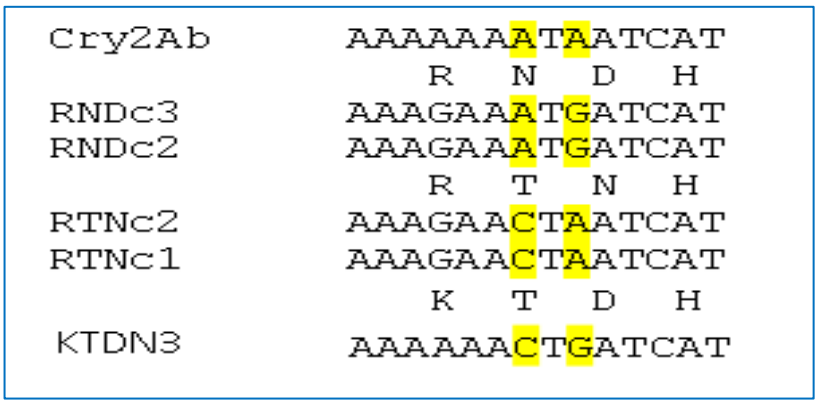



***Figure 4.2.61 Sequence alignment to confirm the creation of double point mutants within Cry2Ab-E 'KNN' triad.***

Therefore, colony 1 from Cry2Ab-ERND, colony 2 from Cry2Ab-ERTN and colony 3 from Cry2Ab-EKTD were expressed in *E.coli* BL21 strain and the protein grown and harvested. The proteins were run on an SDS-PAGE along with a BSA standard and their concentrations measured using densitometry. The protein gel used to measure the concentrations of these toxins is shown in Figure 4.2.62 below.

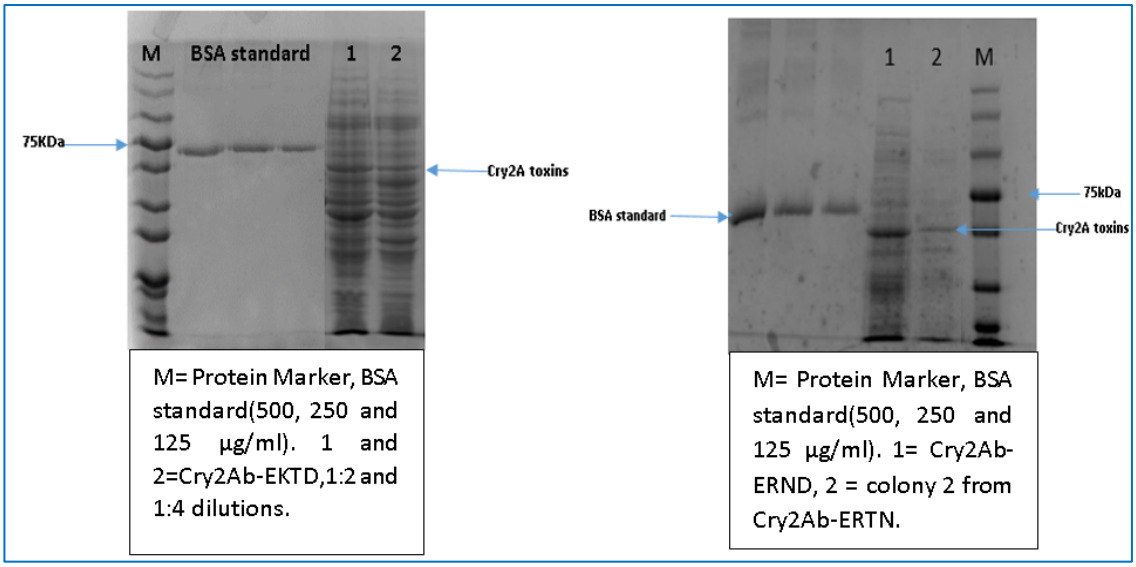



***Figure 4.2.62 Protein SDS-Page gel for measuring the concentrations of the double point mutants created within Cry2Ab-E 'KNN' triad. The arrow pointing towards right indicate the BSA standard while the one pointing towards the left indicate the mutant Cry2A toxins.***

The concentration of the above toxins (Figure 4.2.62) as measured from image J is shown in Table 4.2-14 below.

| Plasmid | Toxin | Concentration(µg/ml) | Predicted Molecular weight (KDa) |
|---|---|---|---|
| pGEM | Cry2Aab-ERND Mutant | 1500 | 70.71 |
| pGEM | Cry2Aab-ERND mutant | 800 | 70.71 |
| pGEM | Cry2Aab-EKTD Mutant | 500 | 70.71 |
| pGEM | Cry2Aa (positive control) | 1900 | 70.78 |

***Table 4.2-14 Concentrations of the double point mutants created within Cry2Ab-E toxin 'KNN' triad as measured using Image J. The hybrids toxins were all expressed, and their relative molecular weight predicted using a program in Expasy.***

A qualitative bioassay was performed using the three mutants in Table 4.2-14 above and Cry2Aa as a positive control; each at a concentration of 2 mg/l following the procedure outlined by WHO (2005c). Deionised water was used as a negative control. This was to determine if any of the three double point mutants created within Cry2Ab-E 'KNN' triad mutants is active against *Aedes aegypti*. The result of the bioassay is summarised in Figure 4.2.63 below.

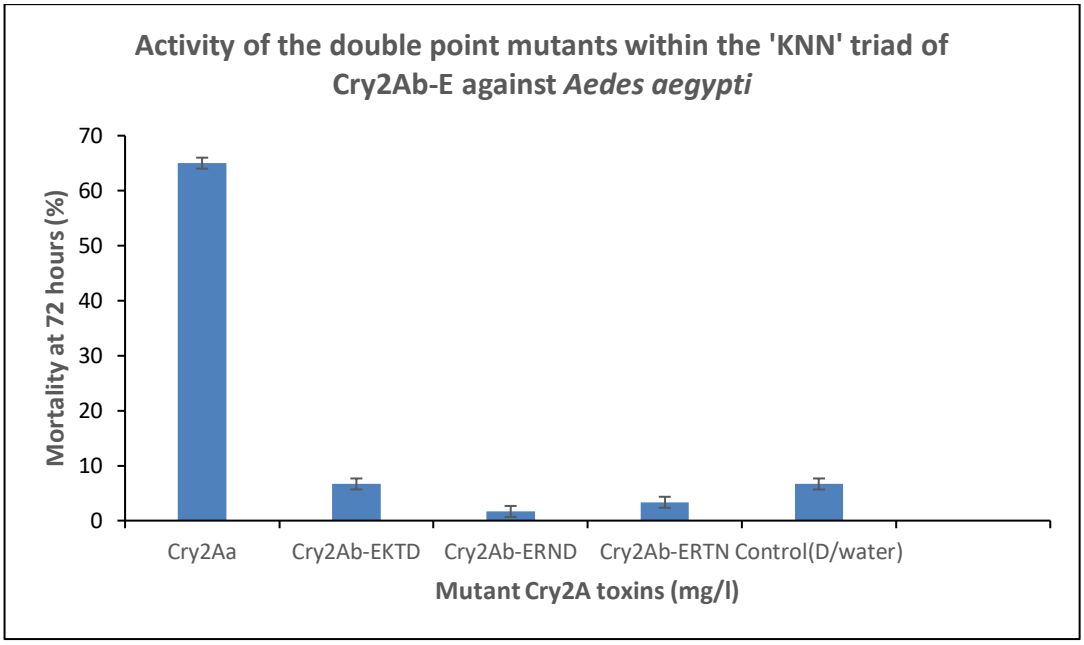



***Figure 4.2.63 Activity of the double point mutants created within the triad 'KNN' of Cry2Ab-E against Aedes aegypti. Cry2A toxins with mortality rate below 10% are considered non-active while those with mortality rate above 10% are considered active. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three repeated experiments. The wild type Cry2Aa was used as positive control, whereas deionised water was used as the negative control for the experiment. Error bars represent Standard error of mean (SEM).***

The above graph (Figure 4.2.63), showed that none of the double point mutants created within the triad 'KNN' of Cry2Ab-E has activity against *Aedes aegypti* since all the three mutants: Cry2Ab-EKTN, Cry2Ab-EKND and Cry2Ab-ERNN possessed a percentage mortality of less than 10, which is non-significant compared to the positive control (Cry2Aa) having a mortality rate of 65%. This, therefore, signified that no any combination of three among the four amino acids 'E/RTD' found within the N-terminus of Cry2A toxins influences their specificity against *Aedes aegypti*.

### Quantitative bioassay results for all Cry2A toxins active against *Aedes aegypti* mosquitoes.

This was to confirm the qualitative bioassay results for all the hybrid/mutant toxins created in the various stages of this study for more clarity and to carry out a quantitative bioassay for all the mutant/hybrid Cry2A toxins that were toxic against *Aedes aegypti*. For all the Cry2A hybrids created via domain I swapping between the wild type Cry2A toxins (Cry2Aa2, Cry2Ac) which were toxic against *Aedes aegypti* and the non-toxic Cry2A (Cry2Ab), the following hybrids were found to be active against *Aedes aegypti:* Cry2AaAbAb, Cry2AcAbAb whereas Cry2AbAaAa and Cry2AbAcAc were non-toxic (Figure 4.2.16). For those hybrids involving N-terminus swap of Cry2Aa and Cry2Ac and Cry2Ab, the following results were obtained: Cry2AaNT+2Ab and Cry2AcNT+2Ab were toxic whereas Cry2AbNT+2Ac was non-toxic (Figure 4.2.28). In addition, mutant created by the deletion of the first 45 amino acids within the N-terminus of Cry2Aa toxin was non-toxic against *Aedes* (Figure 4.2.33). The results for mutants created within the 49-amino acids comprising the N-terminal sequence of Cry2Ab toxin the following results were obtained: Mutants Cry2Ab-E and Cry2Ab-RTD were non-toxic whereas Mutant Cy2Ab-ERTD was toxic (Figure 4.2.44). The results for mutants created within the 49-amino acids comprising the N-terminal sequence of Cry2Aa toxin yielded the following: Mutants Cry2Aa-Q and Cry2Aa-KNN, which were each non-toxic to *Aedes* (Figure 4.2.52). Mutant toxins involving single point and double points mutagenesis of Cry2Ab within the 'KNN' triad were all non-active against *Aedes aegypti* mosquito larvae (Figure 4.2.58 and 4.2.63) respectively. Therefore, the quantitative bioassay results for those Cry2A wild type and hybrid/mutant toxins found to be active against *Aedes aegypti* mosquito (Cry2Ac, Cry2Aa, Cry2AaAbAb, Cry2AcAbAb, Cry2AaNT+2Ab, Cry2AcNT+2Ab, and Mutant Cry2Ab-ERTD) using a range of concentration is presented graphically in

Figure 4.2.64 for better comparison while those found to be non-active were excluded as their LC50 values could not be established.

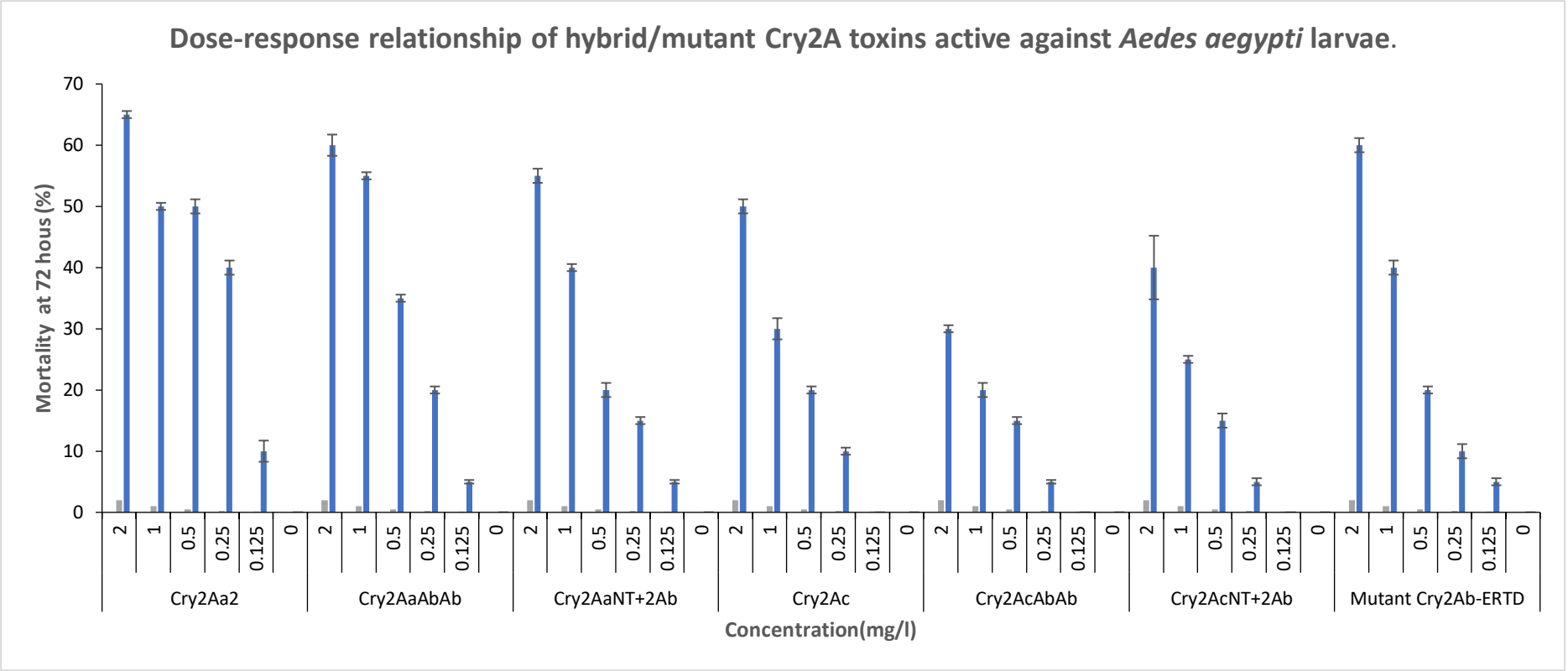



***Figure 4.2.64 Quantitative bioassay for hybrid/mutant Cry2A toxins active against Aedes aegypti. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three-repeated experiments. Deionised water represented by zero (0) in the concentration range was used as the negative control for the experiment. Error bars represent Standard error of mean (SEM).***

The values for the concentrations and percentage mortalities in Figure 4.2.64 above were used to calculate the LC50 values for all the hybrid/mutant Cry2A toxins as shown in Table 4.2-15 below.

| Toxin | LC50(mg/l) | 95% Confidence limits (mg/l) |
|---|---|---|
| Cry2Aa2 | 0.800 | (0.300-16.000) |
| Cry2AaAbAb | 1.000 | (0.700-2.100) |
| Cry2AaNT+2Ab | 1.600 | (1.300-2.300) |
| Cry2Ac | 1.900 | (1.500-2.700) |
| Cry2AcAbAb | 4.300 | (2.700-9.200) |
| Cry2AcNT+2Ab | 2.600 | (2.000-4.100) |
| Mutant 2Ab-ERTD | 1.500 | (1.200-1.900) |

***Table 4.2-15 LC50 values of Cry2A Hybrids and mutant toxins active against Aedes aegypti.***

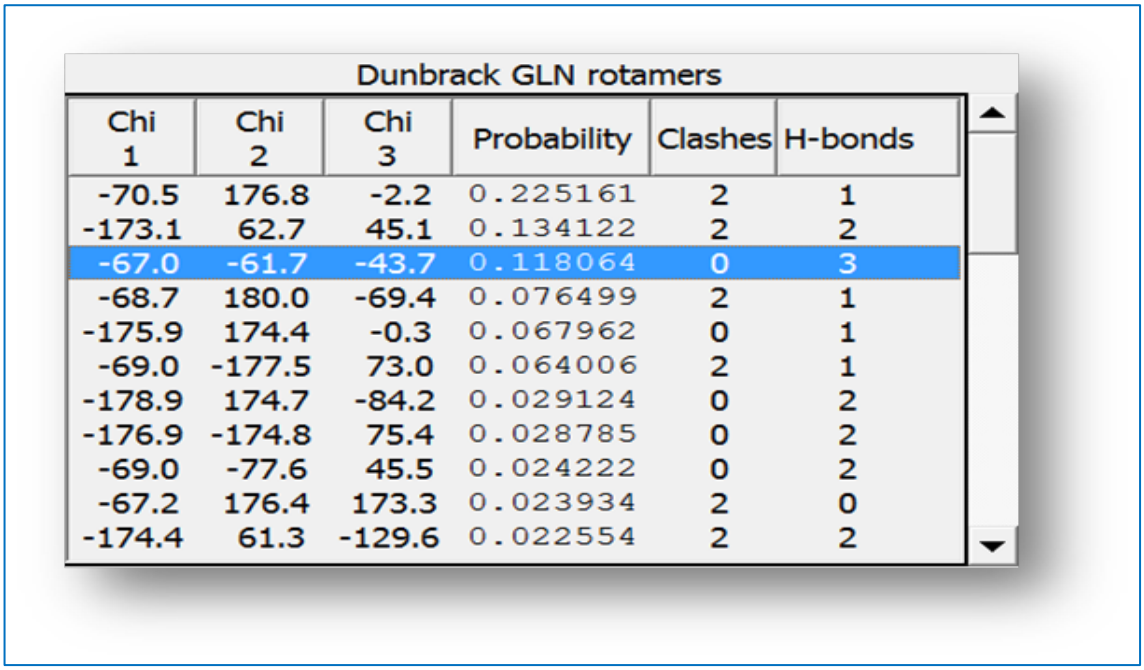
Table 4.2-15 above for the LC50 values of all the hybrid/ mutant toxins active against *Aedes aegypti* showed that generally all the confidence limits overlaps suggesting that there is no significant difference among the mutants as all of them appeared to be toxic with Cry2AcAbAb perhaps a little less toxic. There is a wide difference between the lower and upper confidence limits for Cry2Aa; this is perhaps due the fact that there is no difference in the percentage mortality values at concentrations of 1mg/l and 0.5 mg/l of the toxin (Figure 4.2.64).

### Structural analysis of mutant Cry2Aa and Cry2Ab toxins

Structural models for the mutated toxins were produced using Phyre2 v2.0 (Kelley *et al.*, 2015) using intensive modelling mode. Phyre2 uses the powerful Dunbrack rotamer library (Shapovalov and Dunbrack, 2011) to model mutations as best it can. The mutated and wild type toxins structural models produced were structurally compared in light of the results of bioassay obtained for both toxins, in an attempt to unravel the structural mechanism of action of Cry2A group of toxins.

Rotamers can be defined as isomers of a molecule (e.g. an amino acid) that differ in the rotations of its internal bonds. To gain more assurance that mutations were modelled properly in Phyre2, we used the rotamer function in Chimera v1.11.2 (Pettersen *et al.*, 2004) on the original crystal structure of Cry2Aa (Morse *et al.*, 2001) and followed the criteria for picking the most likely rotamer from the UCSF Chimera tutorial page. In the

absence of the density map of the protein, this consists in choosing the rotamers with the lowest clash score (least amount of overlaps with surrounding Van der Waals radii), then from that list, picking the rotamers with the highest number of hydrogen bonds. If there is still more than one choice left, the rotamer with the highest probability according to the literature was chosen. As an example, I have shown in Figure 4.2.65 the rotamer chosen for Cry2Aa-E27Q and its resultant structure. We did not find any differences with the models provided by Phyre2 in this way.




| Chi 1 | Chi 2 | Chi 3 | Probability | Clashes | H-bonds |
|---|---|---|---|---|---|
| -70.5 | 176.8 | -2.2 | 0.225161 | 2 | 1 |
| -173.1 | 62.7 | 45.1 | 0.134122 | 2 | 2 |
| -67.0 | -61.7 | -43.7 | 0.118064 | 0 | 3 |
| -68.7 | 180.0 | -69.4 | 0.076499 | 2 | 1 |
| -175.9 | 174.4 | -0.3 | 0.067962 | 0 | 1 |
| -69.0 | -177.5 | 73.0 | 0.064006 | 2 | 1 |
| -178.9 | 174.7 | -84.2 | 0.029124 | 0 | 2 |
| -176.9 | -174.8 | 75.4 | 0.028785 | 0 | 2 |
| -69.0 | -77.6 | 45.5 | 0.024222 | 0 | 2 |
| -67.2 | 176.4 | 173.3 | 0.023934 | 2 | 0 |
| -174.4 | 61.3 | -129.6 | 0.022554 | 2 | 2 |

***Figure 4.2.65. Image of Dunbrack rotamer library choice in Chimera for replacing Glu27 by Gln27 in Cry2Aa. Highlighted in blue is the optimal rotamer choice in this case.***

### Glu27 and Gln27

Both the Phyre2 model and the original crystal structure modified using the Dunbrack rotamer library revealed a clear change in structural conformation when Glu27 was substituted with a Gln27 (Figure 4.2.66). Figure 4.2.67 demonstrates how in Cry2Aa, Glu27 grants an opening to the cavity that is present behind the N-terminus, whereas the substitution to Gln27 sterically hinders the opening to the cavity.

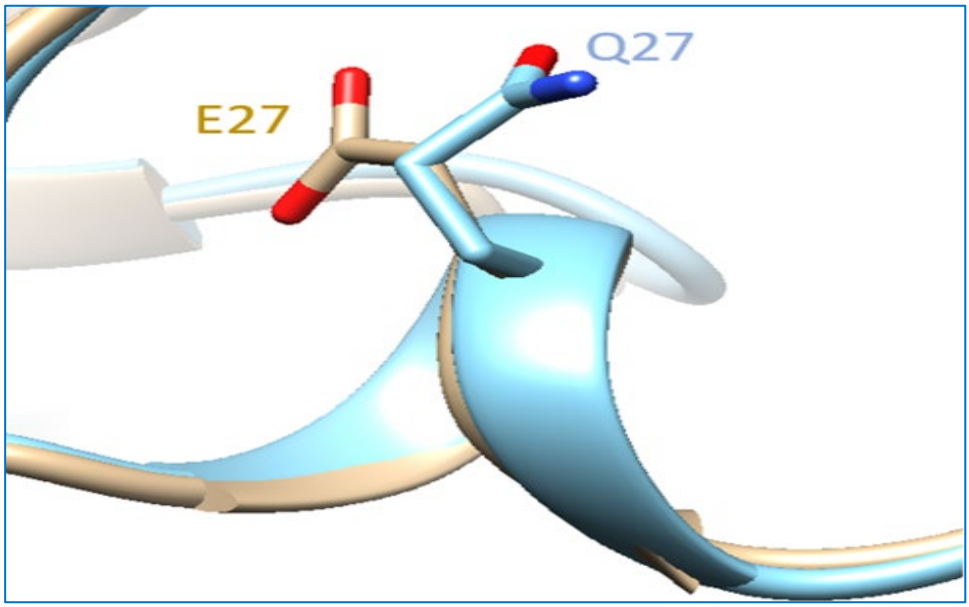



*Figure 4.2.66 Visual representation of superimposed Cry2Aa with Glu27 (tan) and Cry2Ab with Gln27 (cyan) using Chimera. PDB ID of Cry2Aa: 1i5p. Model for Cry2Ab produced using Phyre2. Red tips at the end of the residues represent oxygen atoms. The blue tip at the end of Gln27 represents a nitrogen atom.*

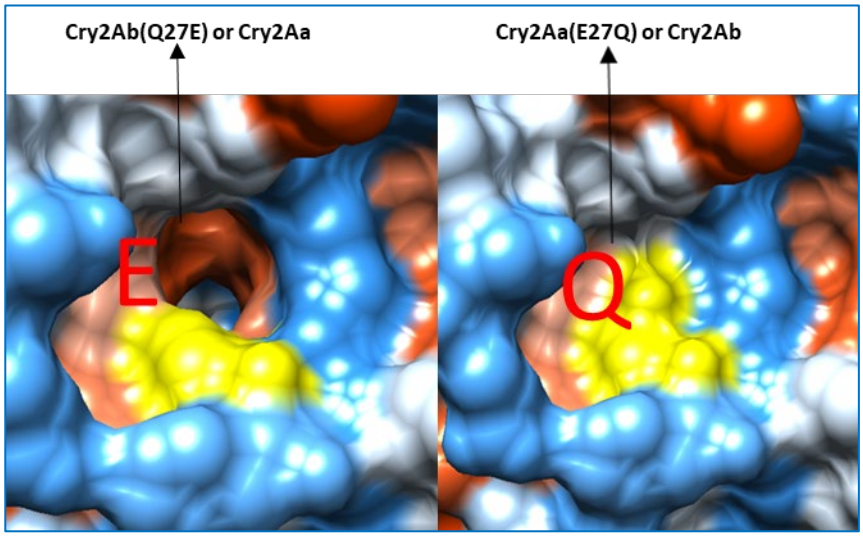



*Figure 4.2.67 Visual representation of the hydrophobic surfaces of Cry2Aa with Glu27 (left) and Cry2Ab with Gln27 (right) using Chimera. PDB ID of Cry2Aa: 1i5p. Both Glu27 and Gln27 are shown in yellow. Red surfaces indicate the Van der Waals (VdW) radii of hydrophobic residues. Blue surfaces represent the VdW radii of hydrophilic residues. White surfaces represent neither hydrophobic nor hydrophilic residue VdW radii.*

Therefore, the results of the structural analysis (Figure 4.2.66 and 4.2.67) of creating mutants by changing the amino acid glutamine (Q) at position 27 from a non-toxic Cry2Ab to glutamic acid (E), led to the formation of a hydrophobic pore (opening). Likewise, changing glutamic acid (E) at the same position from the toxic Cry2Aa to glutamine (Q) led to the closure of the opening and a consequent loss of activity. Therefore, a model to explain these results is that a cavity around the E/Q amino acid within the N-terminal region needs to be opened to allow docking with a receptor and hence activity against *Aedes aegypti*.

### RTD and KNN

Although the residues in both RTD and KNN have similar-to-identical biochemical properties only differing in that Asparagine (N) 45 is neutral and Aspartic acid (D) 45 is acidic- their conformations are different. As is shown in Figure 4.2.68, the 44$^{th}$ and 45$^{th}$ residues share roughly the same shape and conformation. Despite this similarity, the 43$^{rd}$ residues, Arginine (R) 43 in Cry2Aa and Lysine (K) 43 in Cry2Ab appeared to be almost facing away from each other, which leads to a substantial conformational shift. Nonetheless, both single and double mutants created within the 'KNN' triad of Cry2Ab-E (Figure 4.2.58 and 4.2.63) do not have activity against *Aedes aegypti* showing the absolute requirement for these three amino acids (RTD) along with the pore opening glutamic acid(E) depicted in Figure 4.2.67 for activity against this insect as shown with the mutant Cry2Ab-ERTD in Figure 4.2.44.

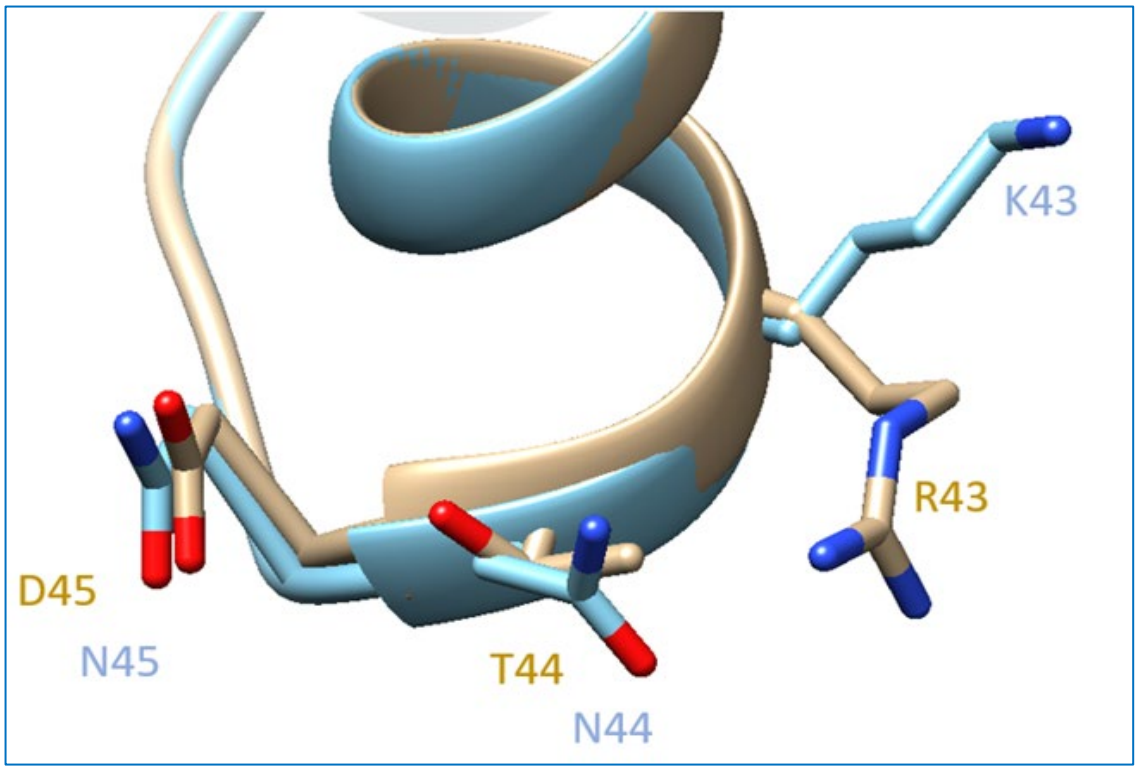



***Figure 4.2.68 Visual representation of superimposed Cry2Aa with RTD (tan) and Cry2Ab with KNN (cyan). PDB ID of Cry2Aa: 1i5p. Red tips at the end of the residues represent oxygen atoms. The blue tip at the end of residues represent a nitrogen atom.***

Therefore, it was obvious that all the four amino acids are required for activity against *Aedes aegypti* larvae as can be seen summarised structurally in Figure 4.2.69 below.

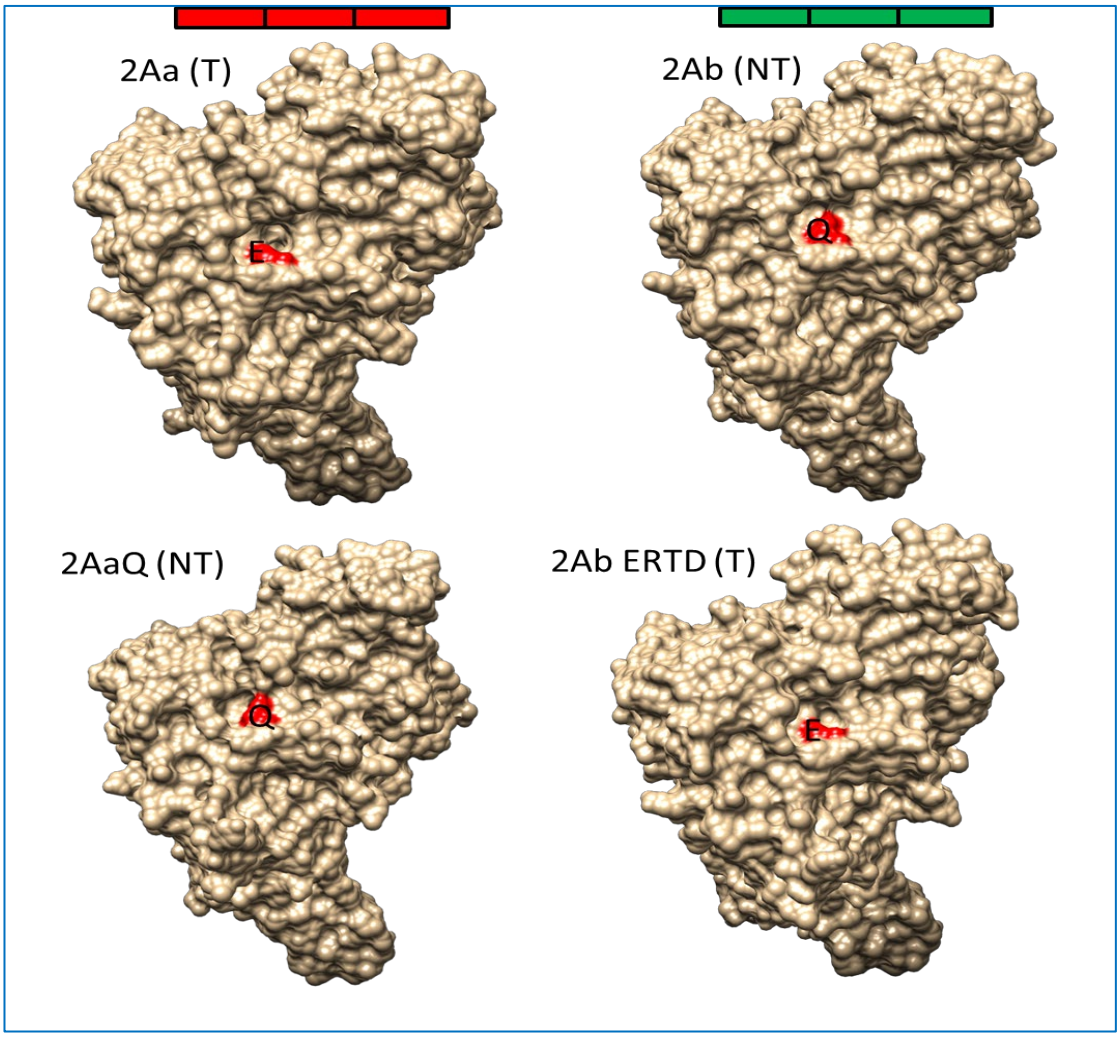



***Figure 4.2.69 Structural analysis of ERTD tetrad for activity against Aedes aegypti. The rectangles represent Cry protein domains. The red colour represents Cry2Aa while green represents Cry2Ab. The letters 'T' stands for toxic against Aedes aegypti while 'NT' stands for non-toxic against Aedes aegypti.***

From Figure 4.2.69 above, in Cry2Ab, which was non-toxic to *Aedes aegypti* and which has Q at position 27; the hole is closed, whereas in Cry2Aa, which was toxic against *Aedes* mosquito and has the amino acid E, at position 27 the cavity is open. However, changing the amino acid at position 27 to Q in the active Cry2Aa led to the closure of the cavity and a resultant loss in activity. In the same vein, changing Q to E in Cry2Ab-RTD led to the opening of the hole and a consequent gain of activity in Cry2Ab. This, therefore, led us to propose some roles for these four amino acids, that the cavity formed by the amino acid E is the cavity required for receptor docking whereas the other three amino acids (RTD) are thought to be directly involved in binding to the receptor, as is depicted in Figure 4.2.70 below.

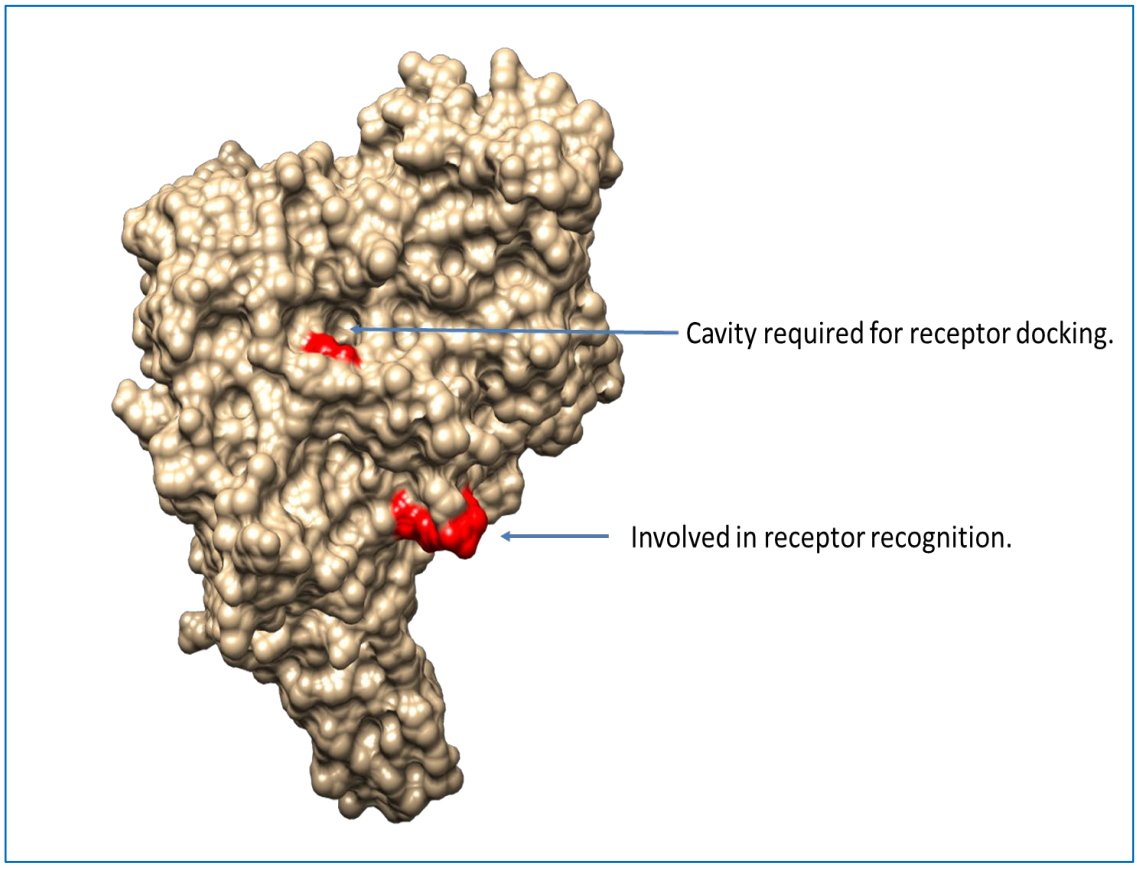



***Figure 4.2.70 Structure showing the positions of the tetrad 'ERTD' in Cry2A toxins and their proposed roles. The arrows point at their locations on Cry2Aa toxin structure. The areas shaded indicate the positions of the amino acids 'E' and 'RTD' respectively in Cry2Aa structure.***

Early hybrid created by Widner and Whiteley (1990) in which part of domain II of Cry2Ab was exchanged with Cry2Aa showed some little activity despite having Cry2Ab in domain I. Modelling showed that the cavity had opened in this hybrid as shown in Figure 4.2.71. This might indicate that domain II also played some significant role in specificity determination perhaps through its interaction with the N-terminal loop, which folds back in to this domain.

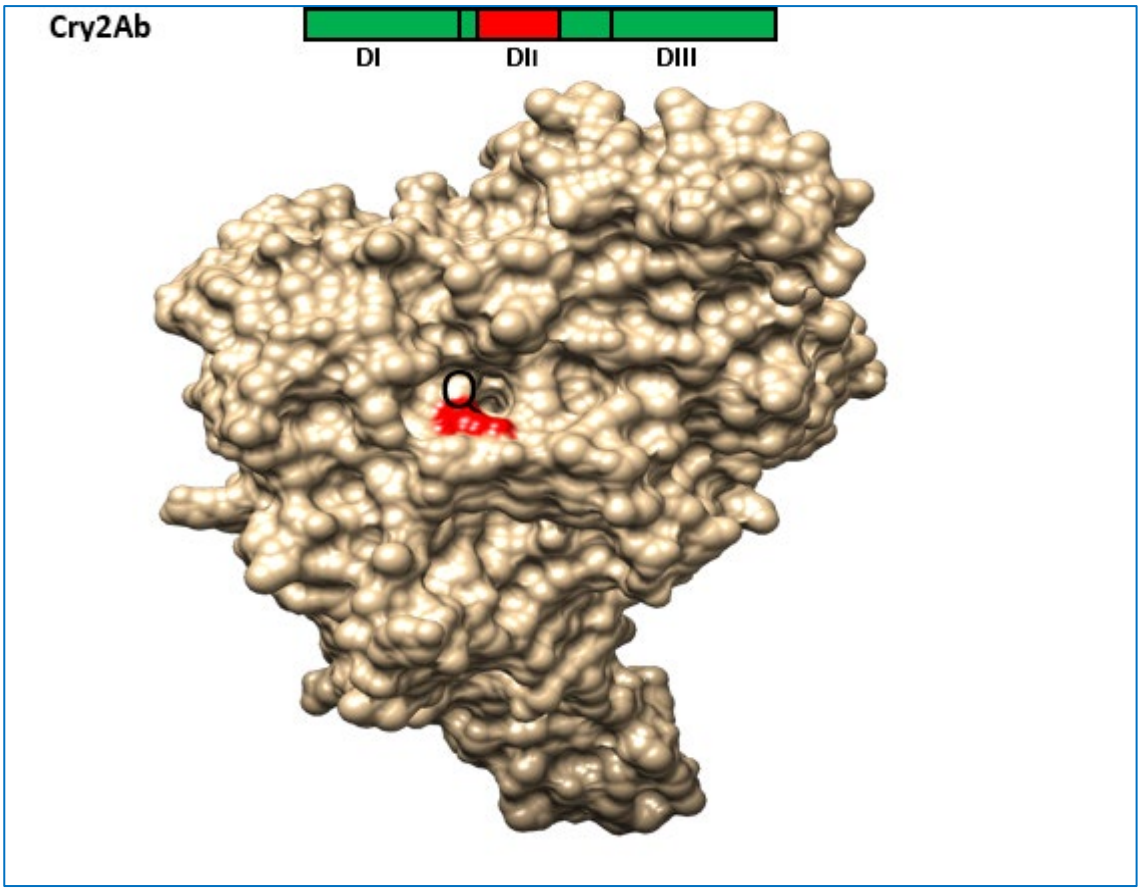



***Figure 4.2.71 Model structure for hybrid 513 created by Widner and Whiteley (1990). The rectangle indicates the different domains, with part of domain II of Cry2Ab exchanged with Cry2Aa coloured red while the remaining part of Cry2Ab is coloured green. Q is placed directly at the position of the opening on the structure, which indicated by a red colour.***

## 4.3 Discussion

The results obtained from the bioassay of the available Cry2A toxins carried out previously (Figure 3.2.11) to find the functional domain/domains among the Cry2A toxins were used to create hybrids/mutants towards the mosquito *Aedes aegypti,* combined with bioinformatic analyses to define regions that determine or influence activity towards this insect. I analysed the domains of the various Cry2A toxins, which were previously tested against *Aedes aegypti* and found to be toxic and / or non-toxic (Widner and Whiteley, 1990, Liang and Dean, 1994). Furthermore, it was discovered after the analyses that some naturally occurring hybrid Cry2A toxins, which we bioassayed and found to be active against this insect, have their domains I sequence similar to wild type Cry2Aa toxins, which were toxic against *Aedes aegypti*. Whereas, those found to be non-toxic have their domains I sequences similar to those of wild type Cry2Ab toxins, which were non-toxic against *Aedes aegypti* (Figure 4.2.1).

Therefore, based on the above findings, we created hybrid toxins containing domain I from a toxic wild type Cry2A toxins, and domains II and III from a non-toxic wild type Cry2A toxins namely: Cry2AaAbAb and Cry2AcAbAb, which were found to be toxic. In the contrary, those hybrids containing sequences in their domain I from those of non-toxic Cry2A toxins, while their domains II and III from toxic ones (Cry2AbAaAa and Cry2AbAcAc) appeared to be non-toxic against *Aedes aegypti,* results which further confirmed the involvement of domain I in specificity determination (Figure 4.2.16 and graphically in Figure 4.2.64). However, previous findings have implicated some amino residues in the middle of domain II (D-block) to be responsible for the specificity of Cry2A toxins (Widner and Whiteley, 1990, Liang and Dean, 1994). We, therefore, tried to find a connection between domain I and II, which based on the hypothesis we postulated that 'it is the N-terminal region comprising of the first 49 amino acids, depicted in Figure 1.7.1, that folds back and becomes a functional part of domain II and thus influencing toxin's binding and specificity'. Based on this, and results from some previous researches (Hu *et al*., 2014, Mandal *et al*., 2007, Morse *et al*., 2001), we strongly believe that the N-terminal loop comprising the first 49-amino acids presented in Figure 1.7.1 of this thesis might play a role in the specificity and hence toxicity of this group of toxins.

Therefore, we created three hybrids involving the N-terminal sequence from toxic Cry2A toxins (Cry2Ac and Cry2Aa2) and the other one having an N-terminal sequence from a non-toxic wild type Cry2A toxin (Cry2Ab) as depicted in Figure 4.2.20. The bioassay results obtained from these hybrids was shown in (Figure 4.2.28), which suggested that the N-terminal loop which folds back in to domain II is involved in specificity and hence toxicity of this important group of toxins against *Aedes aegypti*. This agreed with other findings, though carried out on different insects, which also showed that domain swapping between a toxic Cry toxin known to possess pore forming activity and a non-toxic Cry toxin not known with such property, brought pore forming activity and hence toxicity to the non-toxic Cry toxin (Hu *et al*., 2014). In addition, that some amino acids deletions and substitutions along the N-terminal sequence of the domain I of Cry2A sequence led to the formation of mutants with 4.1 to 6.6--fold increase in toxicity relative to the wild type tested (Mandal *et al*., 2007).

It is worthy of note to mention the findings of Widner and Whiteley (1990) who located the dipteran specificity region in a lepidopteran-dipteran crystal protein from Bt (Cry2Aa) by creating hybrids between this toxin and a toxin that is only lepidopteran specific (Cry2Ab) depicted in Figure 1.9.1. They discovered a short segment of Cry2Aa corresponding to residues 307-382, differing only by 18 amino acid residues from Cry2Ab, to be responsible for dipteran specificity. This is because when this region of Cry2Aa was swapped to Cry2Ab (Hybrid 513) the latter gained toxicity against dipteran insect (*Aedes aegypti* mosquito) and it remained toxic to lepidopteran insect (*Manduca sexta*) as well.

Their findings did contradict ours somehow, as our findings showed that the N-terminal region is responsible for specificity in Cry2A toxins, whereas in their case specificity is because of some few residues within domain II as mentioned above. This discrepancy could be explained by the fact that most of the hybrids we created showed lower activities compared to their respective wild type counterparts (Cry2Aa and Cry2Ac), which means that in the absence of structural stability data on these hybrid toxins, the lowered toxicities could relate to the absence of another specificity determining factor. This factor, may be the region in domain II of Cry2Aa that appears to confer *A. aegypti* specificity to Cry2Ab when swapped around(Widner and Whiteley, 1990).

Another area of discordance between our findings and theirs was the involvement of the C-terminus in specificity determination against *Aedes aegypti* even though with lower activity (Figure 1.9.1), which they did mention and which appeared not to be very relevant in our findings. Hence it could be explained that the interactions between domain I and domain II caused by the N-terminal loop triggered some structural alterations that affect domain III and hence the likely involvement of the C-terminus reported by them, but the C-terminus has no direct involvement in specificity determination based on our findings.

The findings of Liang and Dean (1994) was that the specificity region of Cry2Aa against the mosquito larvae was located in regions 1 and 2 (amino acids 278-412), and was not related to region 3 (amino acids 413 to 487). This is very similar to the findings of Widner and Whiteley (1990) in that the specified dipteran activity conferring residues(D-block) falls in domain II of Cry2Aa, hence same explanation. Since the domain I and N-terminus hybrids, as well as the mutant Cry2Ab-ERTD, created in this study are much closer to their wild type counterparts than Hyb513, which was 20-fold less toxic than Cry2Aa, and DL116. It suggests that the importance of the D-block has been overestimated relative to the role of the N-terminus in determining specificity to *Aedes aegypti*.

Also, it was speculated by Morse *et al*. (2001) that some residues, nine in total, within the putative receptor binding epitope (which constitutes residues 307-382) of Cry2Aa, could play a crucial role for the specificity of the toxin to dipteran insects. Thus, hypothesising that the cleavage of the 49 N-terminal amino acids of Cry2Aa exposes this binding motif (Figure 1.7.1). The findings by Morse *et al*. (2001) compared to our recent findings, which showed that:

i. Deletion of the first 45 amino acids from the N-terminal of Cry2Aa toxins abolished its activity against *Aedes aegypti* (Figure 4.2.33).
ii. Four amino acids substitutions within the N-terminal loop of a non-toxic Cry2Ab, at positions Q27E, KNN (43,44, and 45) to RTD; as in Cry2Aa, transformed the non-toxic Cry2Ab to a mutant Cry2Ab toxin, with activity against *Aedes aegypti* mosquito (Figure 4.2.44).

iii. That none of the triad (RTD) in combination with Cry2Ab-E mutant could result in activity against *Aedes aegypti* (Figure 4.2.58 and 4.2.63).

iv. Substitution of the amino acid Q to E led to opening of a cavity within the N-terminal region of Cry2Ab (Figure 4.2.67), which we earlier hypothesised that it has to be opened to allow docking with the receptor. In addition, that the other three amino acids, RTD, could be directly involved in binding to the receptor.

Based on the above results, unlike what was speculated by Morse *et al*. (2001) that the cleavage of the N-terminal residue exposed the binding motif. We propose that the N-terminal is actually the binding motif, since all the four amino acids, E/RTD, within the N-terminal have to be present for activity against *Aedes aegypti*. Our findings compared to that speculated by Morse *et al*. (2001) could mean that these four amino acid changes within the N-terminus sequence might have resulted in some structural interactions that favoured the opening of the cavity and subsequent binding to the receptor. Thus, implying that structural interactions involving these four amino acids played a crucial role in specificity determination.

# 5. Studying the nature of interactions between the *Aedes aegypti* mid gut juice and Cry2A toxins.

## 5.1 Introduction

This chapter studied the nature of interactions between *Aedes aegypti* mid gut juice and the Cry2A toxins using a known protease (chymotrypsin) as a reference, to be able to answer some of the questions that arose from the results obtained in the previous chapter. We established that the N-terminal region is required for specificity determination in Cry2A toxins. This was arrived at through hybrid formation between a Cry2A toxin known to be toxic against *Aedes aegypti* (Cry2Aa) and another one known to be non-active (Cry2Ab), where we specifically discovered that the N-terminal 49-amino acids from Cry2Aa were enough to confer activity on Cry2Ab (Figure 4.2.64). Therefore, we wanted to investigate how the N-terminus could influence specificity.

Previous reports have shown that chymotrypsin and gut extracts from some insects (*B. mori, L. dispar*) cleave Cry2Aa toxin after the N-terminal 49th amino acid (Ohsawa *et al.*, 2012, Audtho *et al.*, 1999) whereas others (*P. xylostella*) do not (Xu *et al*., 2016).It is not known if and where *A. aegypti* gut enzymes cleave Cry2Aa. A complication is that since it is known that some gut enzymes cleave the N-terminal region this would act against the role of this in binding, unless the fragment stays attached after cleavage. The aim of this chapter was to establish what happens when Cry2A toxin is exposed to *Aedes aegypti* gut extract. Thus, answering the question "if Cry2A is cleaved by *Aedes aegyp*ti mid gut enzymes, how is it still toxic?".

Morse hypothesised that for Cry2Aa, removal of the 49 amino acid N-terminal through cleavage reveals a putative binding region (Morse *et al*., 2001). We believe that for toxicity against *Aedes aegypti*, Morse' hypothesis may not be true. This is because we believe based on our findings that the N-terminal 49-amino acid region of Cry2A is actually a putative binding region.

We hypothesised that the N-terminus sequence of Cry2A, after exposure to the protease in the mid-gut of *Aedes aegypti* larvae, must remain attached to the rest of the toxin for activity to be seen, or is not cleaved.

## 5.2 Results

### 5.2.1 In vitro activation of Cry2A toxins using chymotrypsin

To confirm previous reports' test conditions, Cry2A toxin was digested with chymotrypsin, since digestion with this protease mimicked the effect in various insect guts (Ohsawa *et al*., 2012, Audtho *et al*., 1999, Xu *et al*., 2016). We decided to carry out an in vitro activation of Cry2A toxins using chymotrypsin. The toxin used as representatives of this group of toxins was Cry2Aa; in that it was found to be active against *Aedes aegypti*, as such, it was a good reference toxin for use in activation and subsequent interaction studies involving both chymotrypsin and *Aedes aegypti* mid gut juice. The samples of Cry2Aa toxin employed in this initial study was expressed using *E.coli* expression system. Before activation, the proteins were solubilised using suitable buffers, 2 µl of each of the various concentrations of chymotrypsin shown in Figure 5.2.1 was added to 8 µl of Cry2Aa, giving a final chymotrypsin concentration of 2 mg/ml, 0.2 mg/ml and 0.02 mg/ml. Many optimisations were done through adjusting the pH and incubation periods of the crude samples before arriving at suitable conditions for solubilising the crude samples of Cry2Aa toxin expressed in *E.coli* following the procedure outlined in the materials and methods. The protein gel showing the activation of Cry2Aa toxin using chymotrypsin is depicted in Figure 5.2.1.

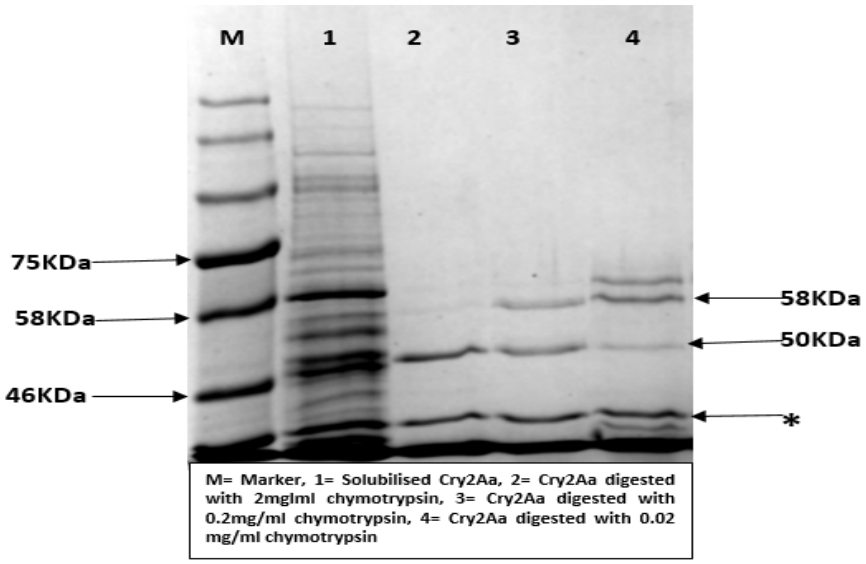


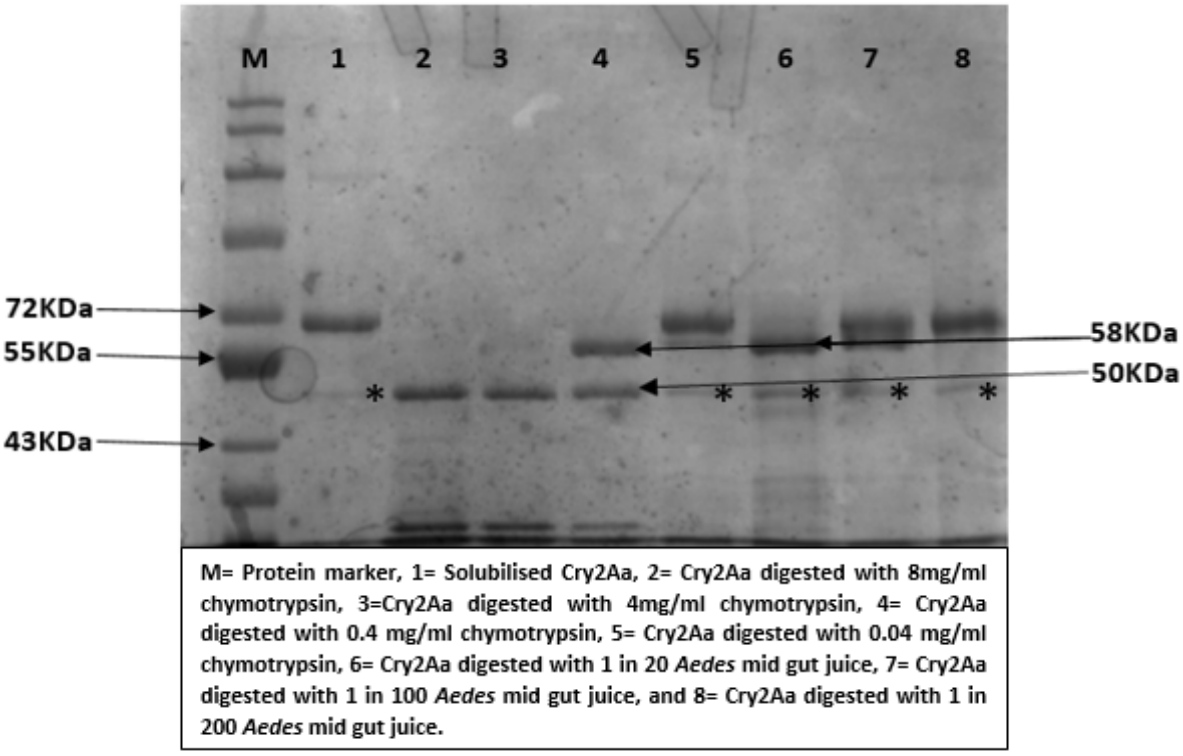

***Figure 5.2.1 Protein SDS-gel showing in vitro activation of Cry2Aa by chymotrypsin***

From the above gel (Figure 5.2.1), it was observed that full digestion of Cry2Aa2 toxin having an approximate molecular weight of 71KDa by chymotrypsin (lane 2), showed the formation of an approximately 50KDa band. However, partial digestion (lane 3 and 4) resulted in the formation of approximately 58KDa and 50KDa bands. The band indicated by an asterisk is probably from *E.coli* as it is also present in the solubilised sample. Though the incubation periods in this research differ from those of other researchers but the sizes of the bands resulting from both complete and partial digestion with chymotrypsin were consistent with those described in previous literature (Xu *et al*., 2016, Ohsawa *et al*., 2012, Audtho *et al*., 1999).

### 5.2.2 In vitro activation of Cry2A toxins using *Aedes* mid gut juice (AMJ)

On full digestion, a 50KDa protein was obtained because of the N-terminal cleavage of Cry2A toxin by chymotrypsin, which was reported by Xu *et al*. (2016) to occur around the 144$^{th}$ amino acid. In addition, the results of our bioassay showed that this 50KDa protein was not toxic against *Aedes aegypti*. Therefore, we want to find out what is happening in the case of *Aedes* mid gut juice activated Cry2A toxin, as cleavage after the region containing the amino acids E/RTD similar to what was obtained in the case of chymotrypsin would act against the role of the N-terminus in binding, unless the fragments stayed attached after cleavage.

This was achieved by carrying out an in vitro activation of Cry2A toxin with *Aedes aegypti* mid gut juice. The mid gut juice of *Aedes aegypti* was prepared following the protocol outlined in the method section (Chapter 2). The *E. coli* expressed Cry2A toxin gave unclear results, so I decided to use a Bt expressed Cry2Aa toxin**.** I was able to arrive at a concentration for both the Cry2Aa and *Aedes* mid gut protease that worked well after several trials by changing their amounts and incubation periods. Different dilutions of the *Aedes* mid gut juice was used for digestion as indicated in Figure 5.2.2 by adding 5 parts of each dilution to 1 part of the Cry2Aa toxin. They were activated by incubating them for a period of 1 hour at 37$^0$C. The chymotrypsin-activated Cry2A toxin on this same gel (lane 2-5) were incubated for 5 minutes only. The gel showing the complete and partial activation of Cry2Aa by *Aedes aegypti* mid gut juice, run along with chymotrypsin for comparison, is shown in Figure 5.2.2.




*5.2.2* ***Protein SDS-gel showing the complete and partial activation of Cry2Aa by Aedes mid gut juice.***

The result above (Figure 5.2.2), showed that unlike chymotrypsin which digested the Cry2Aa to a 50KDa fragment on complete digestion, and 58KDa and 50KDa proteins on partial digestion, the *Aedes* mid gut juice digested Cry2Aa toxin to an approximately 58KDa band (lane 6). Partial digestion by *Aedes* mid gut juice (lanes 7 and 8), unlike chymotrypsin, does not result in the formation of two distinct bands. Thus, indicating that *Aedes aegypti* mid gut protease cut the toxin at only one location. The faint protein bands indicated by an asterisk, appeared to come from the expression host as this is also present in the solubilised (un-activated Cry2Aa toxin).

These results were consistent with chymotrypsin cleaving Cry2Aa toxin at around 144$^{th}$ amino acid position on complete digestion as speculated by Xu *et al*. (2016), whereas the first protein band arising from the partial digestion with chymotrypsin, approximately 58KDa, corresponding to its first cleavage site appeared to correspond to the *Aedes aegypti* mid gut protease cleavage site (Figure 5.2.2). Thus, this may signify that chymotrypsin and *Aedes* mid gut juice might share a cleavage site.

### 5.2.3 Bioassay results of Cry2Aa toxins digested using chymotrypsin and *Aedes* mid gut juice compared to the solubilised Cry2Aa.

Following the activation of Cry2A toxin with chymotrypsin, we discovered the formation of a 50kDa band, and this cleavage by chymotrypsin which has been shown to occur at the N-terminal portion of Cry2Aa was reported to occur around the 144$^{th}$ amino acid (Xu *et al*., 2016). Whereas, activation using *Aedes* mid gut juice resulted in the formation of an approximately 58kDa protein. We decided to test these on *Aedes aegypti,* since the 49 N-terminal region is known to play a significant role in the activity of Cry2A toxins against *Aedes aegypti*. This was achieved by carrying out bioassay experiment of solubilised Cry2A toxin, chymotrypsin activated Cry2Aa and *Aedes* mid gut juice activated Cry2Aa toxin against *Aedes aegypti*.

Bioassay was carried out using both the solubilised Cry2A, chymotrypsin activated and *Aedes* mid gut juice activated Cry2Aa at a concentration of 2 mg/ml following the general bioassay procedure reported in the Methods section (Chapter 2). In addition, the bioassay consisted of crude Cry2Aa crystals as positive control and deionised water as negative control, plus equal volumes of the two buffers (50 mM $Na_2C0_3$ and 1XPBS Buffer) used for preparing the toxin to be sure the buffers do not have any activity against *Aedes aegypti*. This was to be able to see if chymotrypsin cleaved Cry2Aa is still active against *Aedes aegypti* or not, which will shed more light on the requirement of the N-terminal region for activity against *Aedes aegypti*. More also, to see if the *Aedes* mid gut juice activated Cry2Aa protein is active against *Aedes aegypti* in which case it may suggest that the N-terminal portion remained attached to the rest of the toxin after cleavage. The graph of the bioassay experiment is shown in Figure 5.2.3.

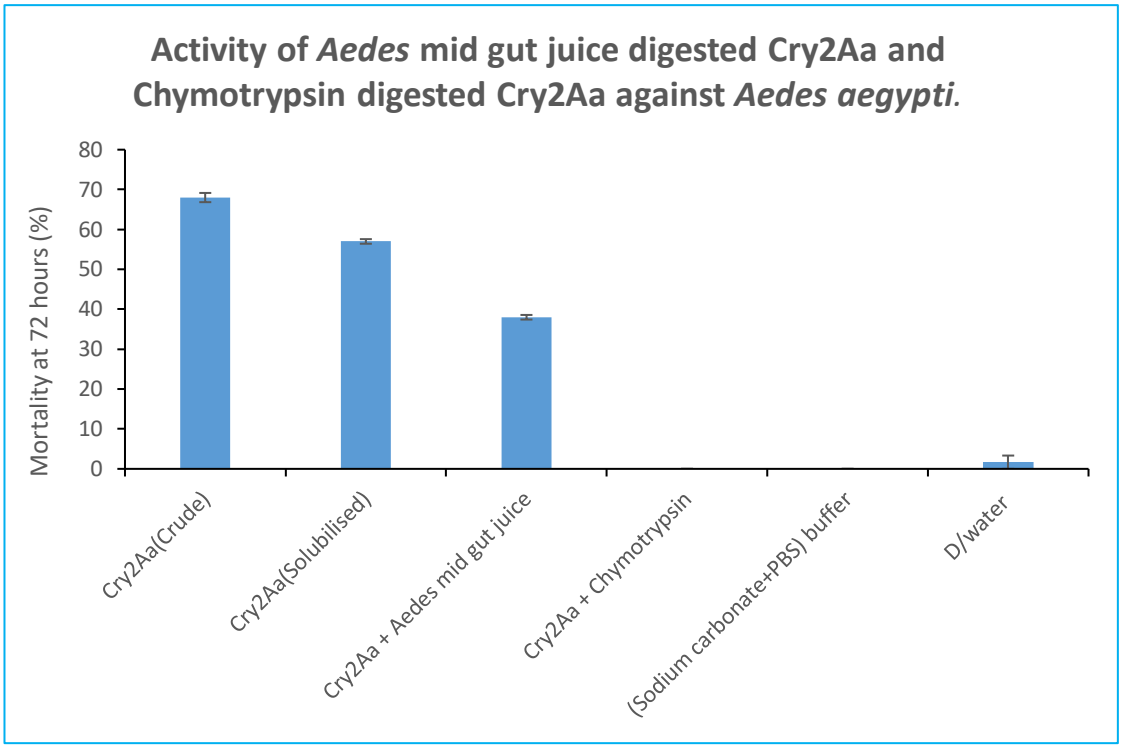



***Figure 5.2.3 Activity of chymotrypsin activated, Aedes mid gut juice activated and solubilised Cry2A toxin against Aedes aegypti. The percentage mortality values on the graph represent a pool value for three replicates per toxin, and then presented as a mean of three-repeated experiments. Error bars represent standard error of mean (SEM).***

From the results of the bioassay in Figure 5.2.3, it is clear that the crude, solubilised Cry2Aa and *Aedes* mid gut juice activated Cry2Aa toxin were active against *Aedes aegypti*. On the contrary, Cry2Aa activated by chymotrypsin showed no activity towards this insect. In addition, the buffers used have no effect on *Aedes aegypti*, as the negative control with deionised water and the two buffers showed no activity. Therefore, we speculated that the lack of activity seen in the chymotrypsin activated Cry2Aa toxin was due to loss of the N-terminus following cleavage, and that the activity seen in the *Aedes* mid gut juice activated Cry2Aa might indicate that the N-terminal portion remained attached to the rest of the toxin after cleavage by the *Aedes* mid gut juice.

### 5.2.4 Proposed mechanism of action of Aedes mid gut juice on Cry2A toxins.

Based on the results of the activation of Cry2A toxins using both chymotrypsin and *Aedes* mid gut juice along with the bioassay experiment performed we decided to critically analyse these results here so we could come up with a suitable model to describe the mechanism of action of *Aedes* mid gut juice activated toxin against *Aedes aegypti*.

The results showed that Cry2Aa on complete activation using chymotrypsin yielded a 50KDa band (Figure 5.2.1). However, on partial digestion with chymotrypsin, it yielded two bands of 58KDa and 50KDa respectively (Figure 5.2.1), which may correspond to cuts at Y49 and L144 respectively indicated by previous research (Audtho *et al*., 1999). However, complete digestion by the *Aedes* mid gut juice yielded only one band of approximately 58KDa (Figure 5.2.2). Our results also showed that the 50kDa Cry2Aa peptide from chymotrypsin digestion was non-toxic to *Aedes aegypti* while the 58kDa Cry2Aa peptide from digestion with *Aedes* mid gut juice was active against *Aedes aegypti* (Figure 5.2.3). This was instrumental in the formulation of our model as we earlier speculated that the lack of activity of this 50KDa protein from chymotrypsin digestion was due to the loss of the first 49 amino acids, comprising the region containing the amino acids E/RTD, from Cry2Aa essential for killing *Aedes aegypti*.

On activation of Cry2Aa with *Aedes* gut extracts we observed that Cry2Aa was cleaved to form approximately a 58kDa toxin, and this was found to be active against *Aedes aegypti*, which could suggest that the region containing the amino acids E/RTD essential for activity against *Aedes aegypti* remained attached to the rest of the toxin after cleavage. Therefore, based on our results, the first 49 amino acids are essential for killing *Aedes aegypti.* We therefore considered two possible models for the activation of Cry2Aa. The first one is that cleavage with *Aedes* mid gut juice might occur before the four specificity-conferring amino acids (E/RTD) found within the N-terminus, in which case the toxin remains active to bind to the insect's receptors and elicit toxicity. The second is that even after cleavage; the cleaved portion of the N-terminus might remain attached to the rest of the toxin due to hydrogen bonding or other forces of attractions. These forces might still hold the toxin intact. From our results for activation of Cry2A toxin with *Aedes aegypti* mid gut juice (Figure 5.2.2), the enzyme cuts at only one site (lane 6) giving an approximately 58KDa protein. This reduction in size and data from

previous studies (Xu *et al*., 2016, Ohsawa *et al*., 2012, Audtho *et al*., 1999) indicate that the 58KDa band arises from a cleavage around amino acid 49 (Y49), and all suggesting that the region containing E/RTD is upstream of the cleavage site. Therefore, the hypothesis suggesting that cleavage by *Aedes aegypti* mid gut juice might occur before the E/RTD amino acids within the N-terminal of Cry2A toxins could not stand. Hence, we considered the second model proposing that the fragment generated after cleavage of the N-terminal region still stays attached to the rest of the toxin, binds to the receptors, and elicits activity, a model we referred to as the "intact N-terminal model". This is depicted diagrammatically in Figure 5.2.4.

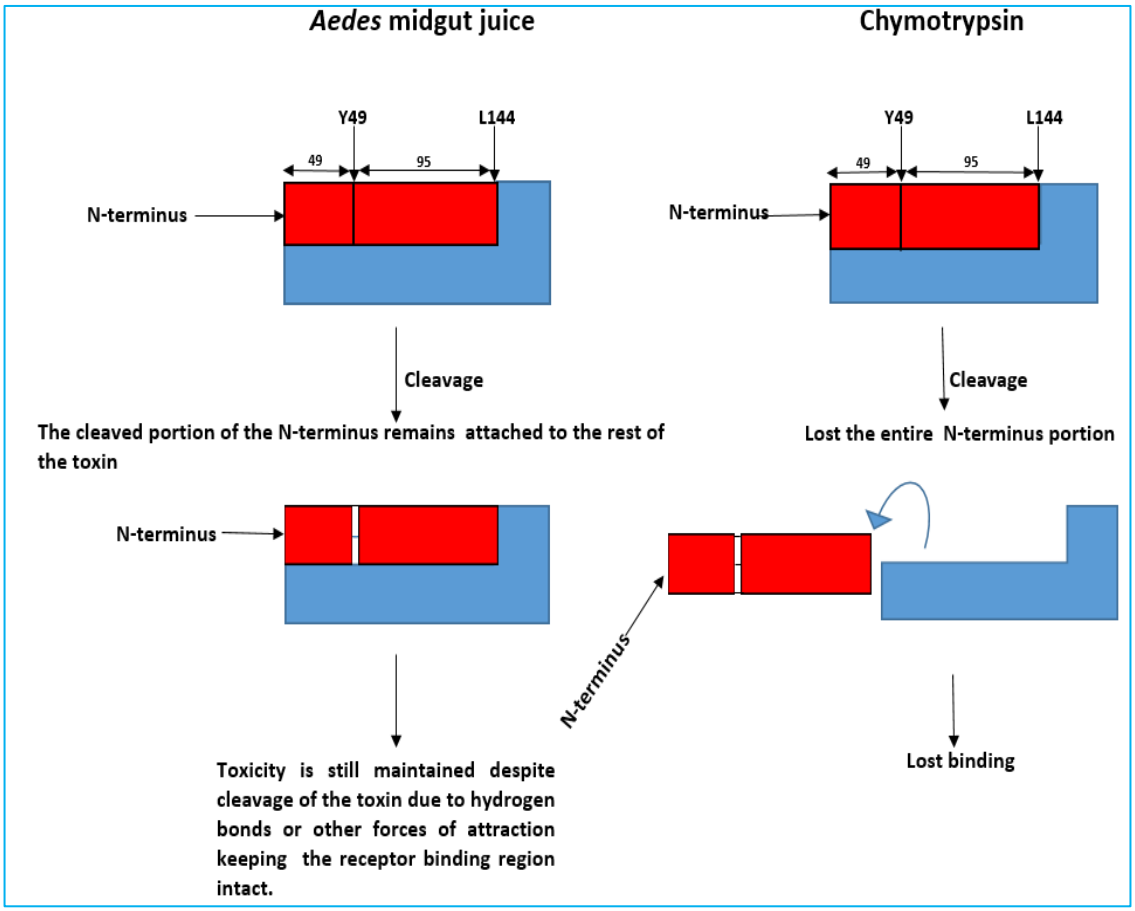



***Figure 5.2.4 Intact N-terminal model for Cry2Aa activation by Aedes mid gut juice.***

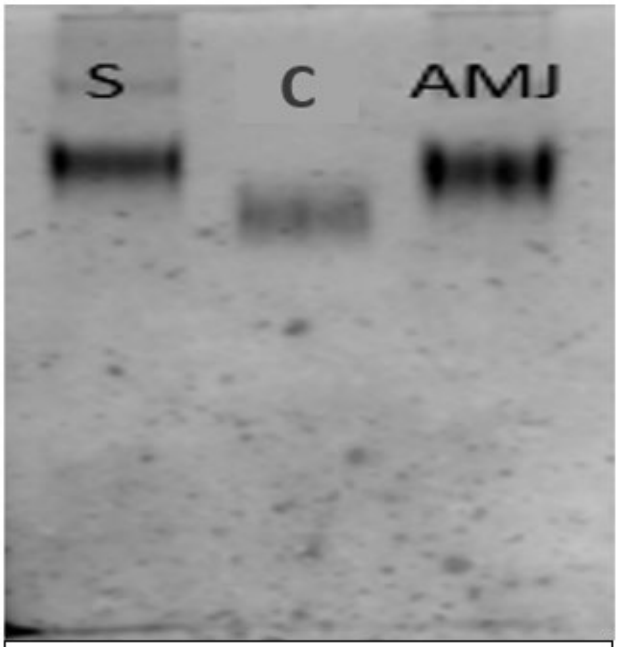
The intact N-terminal model for the mechanism of action of Cry2A against *Aedes aegypti* (Figure 5.2.4) suggested that after cleavage of the toxin by *Aedes* mid gut juice, the cleaved portion containing the amino acids E/RTD remained attached to the rest of the toxin by some forces of interaction. Thus, keeping the toxin intact, and maintaining its

receptor binding property, hence binds to the receptors in the insect's mid gut to exert its toxicity, hence the reason why this was found to be active against *Aedes aegypti*. On the other hand, the model proposes that on activation of Cry2Aa toxin with chymotrypsin (Figure 5.2.1) it resulted in two protein fragments of approximately 58KDa and 50KDa. However, after cleavage, the cleaved portion that contained the amino acids E/RTD is unable to remain attached to the rest of the toxin and thus detached from it. We speculate that the force holding the amino acids 1-144 to the rest of the toxin is weaker compared to the bond holding amino acids 1-49 to the rest of the toxin, hence the reason why it detached from the rest of the toxin as shown in Figure 5.2.4. Therefore, this result in the inability of the toxin to bind to the insect's receptor and hence consequent loss of activity as evident in our results in Figure 5.2.3.

### 5.2.5 Experiment to support the "intact N-terminal model" of Cry2A activity against *Aedes aegypti*.

Based on the intact N-terminal model for the activity of Cry2A toxin against *Aedes aegypti*, we proposed that the N-terminal portion might remain attached to the rest of the toxin after cleavage, which bind to the receptors and elicit activity. Therefore, we attempted to demonstrate the workability of this model experimentally.

We ran a native gel, which would hopefully maintain the 3D structure of the Cry2A as it is free of any denaturing conditions. The procedure used involved treating the solubilised Cry2Aa toxin each with the *Aedes* mid gut juice and chymotrypsin following the usual activation procedure described in the methods section (Chapter 2). The reaction was stopped after the specified durations by treatment with a protease inhibitor. The samples were not treated with SDS or boiled; instead, they were run on a native gel. This might enable us demonstrate if Cry2Aa cleaved by *Aedes aegypti* mid gut juice and/ or chymotrypsin remains attached to the rest of the toxin or not after cleavage. If the N-terminal fragment remains attached to the rest of the toxin after cleavage, as suggested by our model, it would give a similar band pattern to the solubilised Cry2Aa, which served as a control in this case.




S= Solubilised Cry2Aa, C= chymotrypsin activated Cry2Aa, AMJ= *Aedes aegypti* mid gut juice activated toxin.

***Figure 5.2.5 Native SDS-Page gel for Aedes mid gut juice and chymotrypsin activated Cry2Aa toxin.***

From the above gel (Figure 5.2.5), it could be deduced that the *Aedes aegypti* mid gut juice activated Cry2Aa toxin has a similar band pattern to the solubilised (un-activated Cry2A toxin (S)). This might indicate that after cleavage by *Aedes* mid gut juice; the two fragments generated i.e. from the N-terminal fragment, and the rest of the toxin, remain attached to each other as proposed by our model.

The chymotrypsin activated Cry2Aa toxin (Figure 5.2.5, lane C) ran lower on the gel compared to the other two (the solubilised and the AMJ activated toxin). Thus, this might indicate that after cleavage by chymotrypsin, the resulting two fragments probably do not remain attached to each other, and that the smaller fragment generated from the N-terminus might have detached from the rest of the toxin after cleavage. Hence, the reason for the differential migration of the protein compared to the both the solubilised and the *Aedes* mid gut activated toxin. Therefore, the above experiment does not unequivocally support our "intact N-terminal model" for the activity of Cry2A toxins against *Aedes aegypti* for the fact that the technique used (native gel) separates proteins based on charge and not molecular weight, we could not establish if Cry2Aa activated by the *Aedes* mid gut juice and the solubilised Cry2Aa are

of the same molecular weight even though they migrated at equal distance on the native gel depicted in figure 5.2.5.

## 5.3 Discussion

The mode of interaction of Cry2Aa and the *Aedes* mid gut was studied in this chapter. This was in an attempt to answer the question whether the whole or part of the 49-amino acids comprising the N-terminal of the Cry2A toxin is being cleaved by the protease in the *Aedes aegypti* mid gut or not, and if the cleaved portion remains attached to the rest of the toxin after cleavage. We discovered that *Aedes* mid gut juice digested Cry2Aa of approximately 68KDa in to an approximately 58KDa protein on complete digestion (Figure 5.2.3). This cleavage almost certainly occurred after the E/RTD region of the N-terminus, which we speculated to be responsible for receptor docking / binding. Hence, suggestive of the fact that the cleaved portion has to remain attached to the rest of the toxin for activity.

However, digestion of Cry2Aa by chymotrypsin yielded approximately a 58KDa band and 50KDa on partial digestion whereas complete digestion resulted in the formation of only the 50Da band protein, which is not further digested with increased concentration of chymotrypsin (Figure 5.2.3). Though the incubation periods differ but the position of the bands resulting from both complete and partial activation with chymotrypsin were consistent with previous literatures. For instance, Ohsawa *et al*. (2012) demonstrated that Cry2Aa3 was hydrolysed with chymotrypsin to yield two peptides 58KDa and 50KDa by cleaving the linkages 49Y/V50 and 144L/S145 respectively in the protoxin. In addition, the larger fragment of 58KDa disappeared after a long incubation period and/or treatment with increased concentration of chymotrypsin while the smaller fragment of 50KDa did not. Xu *et al*. (2016) studied the role of proteolysis in the activation and toxicity of Cry2Ab against *Plutella xylostella.* They discovered that both trypsin and chymotrypsin cleaved Cry2Aab at R139 and L144 respectively, resulting in the production of an activated toxin of 50KDa in each case, similar to what they obtained when activated by the mid gut juice of *Plutella xylostella*. Comparing our findings with theirs, it is likely that the two bands obtained (58KDa and 50KDa) from partial digestion with chymotrypsin (Figure 5.2.1), may likely correspond to the cuts at Y49 and L144

though this is yet to be proven. Whereas the single band obtained (50KDa) on complete digestion (Figure 5.2.1) might likely resulted from the cut at L144.

The results for the bioassay for chymotrypsin activated Cry2Aa, *Aedes* mid gut juice activated Cry2Aa and the solubilised Cry2Aa toxin depicted in Figure 5.2.3 showed that the solubilised Cry2Aa and *Aedes* mid gut juice activated Cry2Aa toxin were active towards *Aedes aegypti* whereas the chymotrypsin activated Cry2Aa toxin showed no activity. These bioassay results led to the speculation that the lack of activity of the chymotrypsin activated toxin was due to the loss of the N-terminal portion, which we had demonstrated to be essential for the activity of Cry2A toxins towards *Aedes*. More also, that the activity seen in the *Aedes* mid gut juice activated Cry2Aa might be as a result of the N-terminal portion remaining attached to the rest of the toxin after cleavage. These results are in line with the intact N-terminal model, which we proposed depicted in Figure 5.2.4 showing that after cleavage of Cry2Aa by chymotrypsin the N-terminus portion of the Cry2Aa detaches from the rest of the toxin thereby losing its ability to bind to the receptor.

The results in Figure 5.2.2 demonstrating the effect of digesting Cry2A toxin with *Aedes aegypti* mid gut juice showed that both *Aedes aegypti* mid gut juice and chymotrypsin might share a similar cleavage site around Y49. Audtho *et al*. (1999) showed that cleavage of Cry2Aa1 by the mid gut juice of gypsy moth (*Lymantria dispar*) fourth instar larvae resulted in the formation of two major fragments after one minute; a 58KDa fragment and a 49KDa fragment. N-terminal sequencing revealed that the protease cleavage sites are at the C terminal of Y49 and L144. These results are similar to ours in terms of digestion with chymotrypsin but differ in terms of digestion with the *Aedes* mid gut juice, which yielded only one band on full digestion. Our results showed that the *Aedes aegypti* mid gut juice and chymotrypsin might share a cleavage site around (Y49) for Cry2Aa.

We supported our “intact N-terminal model” for the mechanism of action of Cry2A toxin, which we proposed as opposed the partial cleavage of the N-terminal portion before the E/Q region, by running both *Aedes* mid gut juice and chymotrypsin activated Cry2Aa on a native gel (which maintained the 3D structure of the toxin intact) depicted in Figure 5.2.5. It was discovered that the *Aedes aegypti* mid gut activated Cry2Aa had the same

band position with the solubilised (non-activated) toxin. However, the chymotrypsin activated toxin resulted in a protein at a different band position compared to the other two, probably due to the loss of the detached part of the N-terminus. This, therefore, supports our model that the portion of the N-terminal region of Cry2Aa cleaved by the *Aedes aegypti* mid gut juice stays attached to the rest of the toxin, binds to the receptors and elicit toxicity. In addition, contrary to the speculation by Morse *et al*. (2001) that the entire N-terminus portion has to be cleaved in order to expose the receptor binding motif for the activation of Cry2Aa toxin and hence activity to occur. Our results, therefore, supports the idea that the N-terminal region is actually the receptor-binding motif and therefore required to remain attached to the rest of the toxin for the activity of Cry2A toxins against *Aedes aegypti*.

We considered the possibility of H-bonds holding the cleaved fragments of the N-terminal to the rest of the toxins by observing the H-bonds within the structure of Cry2Aa since it has already been resolved through X-ray crystallography (Morse *et al*., 2001). The structure, which is depicted in Figure 5.3.1, revealed that there is likely one H-bond found between L5 and L369, which connects the first 49 amino acids (coloured orange) to the rest of the toxin. There are no H-bonds between the α-helix from the first 49 amino acids and those from amino acids 50-144 (coloured red), which we could have considered to be holding the two helices together even after cleavage. In addition, we found only one H-between G66 and L264 connecting amino acids 50-144 and the rest of the toxin. Therefore, with only 2 H-bonds between amino acids 1-144 and the rest of the toxins, this could explain why the region containing amino acids 1-144 could detach after cuts at amino acids 49 and 144 by chymotrypsin. However, it cannot be explained how the region containing amino acids 1-49 remains attached. We could only presume that there are other forces such as Van der Waals or hydrophobic interactions involved.

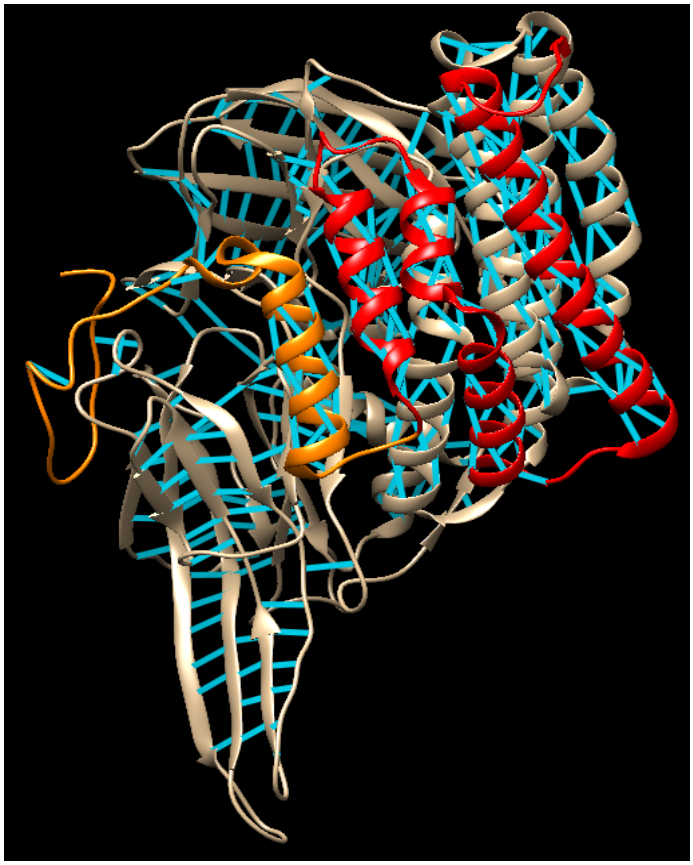


***Figure 5.3.1 Structure of Cry2Aa showing the first 49 amino acids coloured orange, amino acids 50-144 coloured red, and the rest of the toxins coloured gold and potential H-bonds between the molecules coloured blue.***

These results indicate the significance of the N-terminus region in the activity of Cry2A toxins suggesting that the region containing the amino acids E/RTD, after being cleaved, must remain attached to the rest of the toxin for activity to occur.

This requirement for the N-terminal region for activity in Cry proteins was also observed by a previous work on Cry4D protein, which showed that while the full-length protein was highly toxic to mosquito larvae, the truncated protein with a 9.6kDa deletion at the N-terminus was non-toxic to mosquitoes (Pang *et al*., 1992).

# 6. General discussion

This study sought to understand the mechanism for the mosquitocidal activity of Cry2A toxins against *Aedes aegypti*. It involved confirmation of some of the available Cry2A toxins used in this study by sequencing the genes and comparing the results of the gene sequencing obtained to those of the sequences of known Cry2A toxins from the Bt nomenclature database (Crickmore *et al.*, 1998). The toxins were further characterised by expressing and harvesting the crystal toxin protein and assaying them against *Aedes*.

The results of the bioassay for the wild type toxins indicated that all the Cry2Aa toxins with the exception of Cry2Aa17 and mCry2Aa17 were toxic against *Aedes aegypti* mosquito whereas all the Cry2Ab were nontoxic against this insect. These results agreed with those of previous researches where these two toxins were tested against *Aedes aegypti* (Widner and Whiteley, 1990, Liang and Dean, 1994). The toxicity profile of Cry2Aa17 against three order of insects was reported to be like those of Cry2Ab toxins thus implicating domain I as a specificity-determining region (Shu *et al.*, 2017).

We discovered that the domain I of Cry2A toxins is responsible for specificity determination against *Aedes aegypti* after creating hybrids through domain I swaps. However, these results contradict those of previous researchers who alluded specificity determination in Cry2A toxins against *Aedes aegypti* to amino acid residues in the middle of domain II (D-block) of Cry2A toxins (Widner and Whiteley, 1990, Liang and Dean, 1994). But, it was discovered that the activities of our domain I hybrids were much closer to their wild type counterparts compared to their hybrids, which they created through some amino acids swaps within the D-block located in domain II. For instance, Hyb513 was 20-fold less toxic than Cry2Aa. This suggests that the role of the D-block located in domain II was overestimated relative to that of domain I in determining specificity to *Aedes aegypti*.

We got some reports implicating the N-terminus region as playing a crucial role in specificity, as well as toxicity determination in Cry toxins. A conclusion arrived at after the researchers discovered that N-terminal swaps between an active Cry toxins known to possess a pore forming activity and a non-active Cry toxin not known with such property brought pore forming activity and hence toxicity to the non-active Cry toxin (Hu *et al.*, 2014). In addition, another researcher reported that some amino acids

deletions and substitutions along the N-terminal portion of domain I of Cry2A sequence led to the formation of a mutant with more toxicity relative to the wild type (Mandal *et al*., 2007).

Following the findings of (Hu *et al*., 2014, Mandal *et al*., 2007), along with Widner and Whiteley (1990), and Liang and Dean (1994) mentioned above. We created hybrids through N-terminal 49 amino acid swaps among the Cry2A toxins to find the connection between domain I and II, which based on the hypothesis we postulated was that it "folds back and becomes a functional part of domain II, and thus influencing toxin's binding and specificity". Hybrids were also created through the deletion of the N-terminal region of Cry2Aa in trying to achieve this goal. These results showed that the N-terminal 49 amino acid residues were required for the activity of Cry2A toxins against *Aedes aegypti*.

These results contradict the speculation made by Morse *et al*. (2001) that the cleavage of the N-terminal region of Cry2Aa toxin exposes the binding epitope, as our findings indicated that the N-terminal region, actually, constitutes the binding motif.

The results of mutagenesis carried out within the N-terminal region of Cry2Aa showed that four amino acids E, R, T and D at positions 27, 43, 44 and 45 of Cry2Aa might be responsible for the specificity of Cry2Aa toxin against *Aedes aegypti*.

Results from modelling suggested that the amino acid E27 is associated with the opening of a cavity within Cry2A toxins as changing the amino acid glutamine (Q) at position 27 from a non-toxic Cry2Ab to glutamic acid (E), led to the formation of a hydrophobic pore (opening). Likewise, changing glutamic acid (E) at the same position from the toxic Cry2Aa to glutamine (Q) led to the closure of the opening and a consequent loss of activity. In addition, the results showed that even if this hole is open by the presence of the amino acid E at position 27 and any of the triad (RTD) is changed, there is consequent loss of activity. Thus, it clearly showed that all these four amino acids (ERTD) have to be present for activity to occur in Cry2A toxins. This, therefore, made us to propose some roles for these four amino acids; that the hole formed by the amino acid E is the cavity required for receptor docking whereas the other three amino acids (RTD) are thought to be directly involved in binding to the receptor. These results contradict that of Widner and Whiteley (1990) in which part of domain II of Cry2Ab was swapped with Cry2Aa and

showed some activity despite having Cry2Ab in domain I, a hybrid they referred to as hybrid 513. However, modelling showed that the cavity had opened in this hybrid.

Previous reports have shown that chymotrypsin and gut extracts from some insects (*B. mori, L. dispar*) cleave Cry2Aa toxin at both the N-terminal 49th and 144$^{th}$ amino acids (Ohsawa *et al.*, 2012, Audtho *et al.*, 1999). Whereas others (*P. xylostella*) do not cleave Cry2Aa, but was reported by Xu *et al*. (2016) to cleave Cry2Ab at the 144$^{th}$ amino acid position only. It was not known if and where *Aedes aegypti* gut enzymes cleave Cry2Aa. The implication of which is since it is known that some gut enzymes cleaved the N-terminal region in which case it would act against the role of the N-terminus in binding, unless the fragment stays attached after cleavage.

Therefore, we attempted to answer the question whether the 49-amino acids comprising the N-terminal region of Cry2A toxin is being cleaved by the protease in the *Aedes aegypti* mid gut or not, and if it stays attached to the rest of the toxins after the cleavage to elicit toxicity. This was achieved through studying the mechanism of interaction of Cry2Aa toxin with *Aedes* mid gut juice (AMJ), and chymotrypsin as a reference protease. The results showed that on full digestion of Cry2Aa with AMJ an approximately 58KDa protein was produced, which may indicate a cut after the amino acid E/RTD that we earlier speculated to play a role in receptor docking/binding. On the other hand, chymotrypsin produced a band at approximately 50KDa position on complete digestion, and two bands of approximately 58KDa and 50KDa on partial digestion; similar to what was obtained by previous studies (Audtho *et al*., 1999, Xu *et al*., 2016). These researchers speculated that the cleavage site for the complete digestion with chymotrypsin was L144 when they sequenced the protein. However, we are yet to establish the exact location where AMJ cleaved Cry2Aa toxin. It is likely that *Aedes* mid gut juice and chymotrypsin might share a cleavage site (the one yielding a 58KDa peptide), which may be Y49, the higher band formed on partial digestion with chymotrypsin (Audtho *et al*., 1999) . In addition, that the region containing the amino acids E/RTD is perhaps completely digested by chymotrypsin, but rather cleaved in the case *Aedes* mid gut juice, hence explaining why it may still get attached to the rest of the toxin. The 50KDa protein produced by chymotrypsin digestion was found to be nontoxic against *Aedes aegypti*, which we speculated was perhaps because of the loss

of the N-terminal containing E/RTD. Leading us to propose a model, we referred to as the “intact N-terminal model” which proposed that after cleavage of the toxin by *Aedes* mid gut juice, the cleaved portion of the N-terminal remained attached to the rest of the toxin by some forces of interaction. Thus, keeping the toxin intact, and maintaining its receptor binding property. On the other hand, the model proposes that the two fragments resulting from chymotrypsin cleavage are unable to remain attached to the rest of the toxin and thus detach from it. Therefore, this results in the inability of the toxin to bind to the *Aedes* receptor and hence consequent loss of activity.

We tested this model experimentally by running a native gel, which retained the 3D structure of the toxin intact after activation by the respective proteases. Using this we found that the protein from the solubilised toxin migrated the same distance with that of the AMJ activated as the two bands from these proteins occurred on similar location on the gel. In addition, both of them differ from the chymotrypsin-activated toxin, which differed probably because of the loss of the N-terminal portion of the toxin. However, we could not establish if the Cry2Aa toxin activated by the *Aedes* mid gut juice and the solubilised Cry2Aa are of the same molecular weight using this technique as proteins are being separated based on charge and not molecular weight, though they migrated the same distance on the gel (Figure 5.2.5).

The proposal by Morse *et al*. (2001) that the cleavage of the N-terminal region of Cry2Aa toxin exposes the binding epitope of the toxin, which enable binding of the toxin to the insect receptors could work for targets other than *Aedes*.

Cry2Aa toxin has a broad spectrum of activity against various insects; the same approach used in this current study could be applied towards understanding the nature of the specificity of other related Cry toxins against other insects. This will help in designing a recipe for generating a more potent and broad-spectrum biological insecticides that could withstand insect resistance through mutagenesis, genetic engineering and bioassay studies.

Future studies might focus on excising the bands resulting from the Cry2Aa activated by *Aedes* mid gut juice and the solubilised Cry2Aa obtained from the native gel or separate the two proteins using size exclusion chromatography and obtain their amino acids

sequences to see if they are of the same molecular weight for the intact N-terminal model to be fully demonstrated experimentally. More also, PISA software could be used to understand the forces of attraction and/ or bonds that might be helping the cleaved portion of the N-terminal to remain attached to the rest of the toxin after cleavage. Future studies might seek to understand the cleavage sites of both *Aedes* mid gut juice and chymotrypsin activated Cry2Aa toxins through excising the bands, purifying them, and sequencing the protein, which will give an idea of the specific amino acids found within these cleavage sites and hence much more insight in to the type of bonds and /or interactions therein. In addition, it will be very interesting to study the activity of other Cry toxins, different from Cry2A, against *Aedes aegypti* via N-terminal modifications.

# 7. References


ABDULLAH, M. A., ALZATE, O., MOHAMMAD, M., MCNALL, R. J., ADANG, M. J. & DEAN, D. H. 2003. Introduction of *Culex* toxicity into *Bacillus thuringiensis* Cry4Ba by protein engineering. *Appl Environ Microbiol,* 69**,** 5343-5353.

ADANG, J. A., CRICKMORE, N., JURAT-FUENTES, J.L. 2014. Diversity of *Bacillus thuringiensis*Crystal Toxins and Mechanism of Action. . *In:* GILL, T. S. D. A. S. S. (ed.) *Advances in Insect Physiology,*. Oxford: Academic Press.

ALOUT, H. & WEILL, M. 2008. Amino-acid substitutions in acetylcholinesterase 1 involved in insecticide resistance in mosquitoes. *Chem Biol Interact,* 175**,** 138-141.

ALPHEY, L., MCKEMEY, A., NIMMO, D., NEIRA OVIEDO, M., LACROIX, R., MATZEN, K. & BEECH, C. 2013. Genetic control of *Aedes* mosquitoes. *Pathog Glob Health,* 107**,** 170-179.

ALPHEY, N., BONSALL, M. B. & ALPHEY, L. 2011. Modeling resistance to genetic control of insects. *J Theor Biol,* 270**,** 42-55.

ARAUJO, A. P., ARAUJO DINIZ, D. F., HELVECIO, E., DE BARROS, R. A., DE OLIVEIRA, C. M., AYRES, C. F., DE MELO-SANTOS, M. A., REGIS, L. N. & SILVA-FILHA, M. H. 2013. The susceptibility of *Aedes aegypti* populations displaying temephos resistance to *Bacillus thuringiensis israelensis*: a basis for management. *Parasit Vectors,* 6**,** 297.

ARENAS, I., BRAVO, A., SOBERON, M. & GOMEZ, I. 2010. Role of alkaline phosphatase from *Manduca sexta* in the mechanism of action of *Bacillus thuringiensis* Cry1Ab toxin. *J Biol Chem,* 285**,** 12497-12503.

ATSUMI, S., INOUE, Y., ISHIZAKA, T., MIZUNO, E., YOSHIZAWA, Y., KITAMI, M. & SATO, R. 2008. Location of the *Bombyx mori* 175kDa cadherin-like protein-binding site on *Bacillus thuringiensis* Cry1Aa toxin. *FEBS J,* 275**,** 4913-4926.

AUDTHO, M., VALAITIS, A. P., ALZATE, O. & DEAN, D. H. 1999. Production of chymotrypsin-resistant Bacillus thuringiensis Cry2Aa1 delta-endotoxin by protein engineering. *Appl Environ Microbiol,* 65**,** 4601-4605.

BEL, Y., SIQUEIRA, H. A., SIEGFRIED, B. D., FERRE, J. & ESCRICHE, B. 2009. Variability in the cadherin gene in an *Ostrinia nubilalis* strain selected for Cry1Ab resistance. *Insect Biochem Mol Biol,* 39**,** 218-223.

BERRY, C., O'NEIL, S., BEN-DOV, E., JONES, A. F., MURPHY, L., QUAIL, M. A., HOLDEN, M. T., HARRIS, D., ZARITSKY, A. & PARKHILL, J. 2002. Complete sequence and organization of pBtoxis, the toxin-coding plasmid of Bacillus thuringiensis subsp. israelensis. *Appl Environ Microbiol,* 68**,** 5082-5095.

BRAVO, A., GILL, S. S. & SOBERON, M. 2007. Mode of action of *Bacillus thuringiensis* Cry and Cyt toxins and their potential for insect control. *Toxicon,* 49**,** 423-435.

BRAVO, A., GOMEZ, I., CONDE, J., MUNOZ-GARAY, C., SANCHEZ, J., MIRANDA, R., ZHUANG, M., GILL, S. S. & SOBERON, M. 2004. Oligomerization triggers binding of a *Bacillus thuringiensis* Cry1Ab pore-forming toxin to aminopeptidase N receptor leading to insertion into membrane microdomains. *Biochim Biophys Acta,* 1667**,** 38-46.

BROWN, N. P., LEROY, C. & SANDER, C., 1998. MView: a web-compatible database search or multiple alignment viewer. *Oxford University Press,* 14**,** 380–381.

BUTTLER, D. 2011. Mosquitoes score in chemical war: Growing resistance is threatening global malaria-control efforts. *Nature,* 475, .

CADAVID-RESTREPO, G., SAHAZA, J. & ORDUZ, S. 2012. Treatment of an *Aedes aegypti* colony with the Cry11Aa toxin for 54 generations results in the development of resistance. *Mem Inst Oswaldo Cruz,* 107**,** 74-79.

CARVALHO, D. O., MCKEMEY, A. R., GARZIERA, L., LACROIX, R., DONNELLY, C. A., ALPHEY, L., MALAVASI, A. & CAPURRO, M. L. 2015. Suppression of a Field Population of *Aedes*

*aegypti* in Brazil by Sustained Release of Transgenic Male Mosquitoes. *PLoS Negl Trop Dis,* 9**,** e0003864.

CASIDA, J. E. & DURKIN, K. A. 2013. Neuroactive insecticides: targets, selectivity, resistance, and secondary effects. *Annu Rev Entomol,* 58**,** 99-117.

CDC 2019. Lifecycle and information on *Aedes aegypti* mosquitoes. www.cdc.gov/dengue/resources/factSheets/MosquitoLifecycle. 1-2.

CHEN, J., AIMANOVA, K. G., PAN, S. & GILL, S. S. 2009. Identification and characterization of *Aedes aegypti* aminopeptidase N as a putative receptor of *Bacillus thuringiensis* Cry11A toxin. *Insect Biochem Mol Biol,* 39**,** 688-696.

CHILCOTT, C. N. & ELLAR, D. J. 1988. Comparative toxicity of *Bacillus thuringiensis* var. israelensis crystal proteins in vivo and in vitro. *J Gen Microbiol,* 134**,** 2551-2558.

CLEMENTS, A., N., 1999. *The Biology of Mosquitoes, Vol. II. Egg laying. Cabi, Wallingford.*

COLE, L. M., NICHOLSON, R.T., CASIDA, J.E. 1993. . Action of phenylpyrazole insecticides at the GABA-gated chloride channel. . *Biochem. Physiol,* 46**,** 47–54.

CRICKMORE, N., BAUM, J., BRAVO, A., LERECLUS, D., NARVA, K., SAMPSON, K., SCHNEPF, E., SUN, M. AND ZEIGLER, D.R. 2018. Bacillus thuringiensis toxin nomenclature

CRICKMORE, N., ZEIGLER, D. R., FEITELSON, J., SCHNEPF, E., VAN RIE, J., LERECLUS, D., BAUM, J. & DEAN, D. H. 1998. Revision of the nomenclature for the *Bacillus thuringiensis* pesticidal crystal proteins. *Microbiol Mol Biol Rev,* 62**,** 807-813.

DANKOCSIK, C., DONOVAN, W. P. & JANY, C. S. 1990. Activation of a cryptic crystal protein gene of *Bacillus thuringiensis* subspecies kurstaki by gene fusion and determination of the crystal protein insecticidal specificity. *Mol Microbiol,* 4**,** 2087-2094.

DE MAAGD, R. A., BAKKER, P., STAYKOV, N., DUKIANDJIEV, S., STIEKEMA, W. & BOSCH, D. 1999. Identification of *Bacillus thuringiensis* delta-endotoxin Cry1C domain III amino acid residues involved in insect specificity. *Appl Environ Microbiol,* 65**,** 4369-4374.

DE MAAGD, R. A., BRAVO, A. & CRICKMORE, N. 2001. How *Bacillus thuringiensis* has evolved specific toxins to colonize the insect world. *Trends Genet,* 17**,** 193-199.

DONOVAN, W. P., GONZALEZ, J. M., JR., GILBERT, M. P. & DANKOCSIK, C. 1988. Isolation and characterization of EG2158, a new strain of *Bacillus thuringiensis* toxic to coleopteran larvae, and nucleotide sequence of the toxin gene. *Mol Gen Genet,* 214**,** 365-372.

DU, W., AWOLOLA, T. S., HOWELL, P., KOEKEMOER, L. L., BROOKE, B. D., BENEDICT, M. Q., COETZEE, M. & ZHENG, L. 2005. Independent mutations in the Rdl locus confer dieldrin resistance to Anopheles gambiae and An. arabiensis. *Insect Mol Biol,* 14**,** 179-183.

DUSFOUR, I., ZORRILLA, P., GUIDEZ, A., ISSALY, J., GIROD, R., GUILLAUMOT, L., ROBELLO, C. & STRODE, C. 2015. Deltamethrin Resistance Mechanisms in *Aedes aegypti* Populations from Three French Overseas Territories Worldwide. *PLoS Negl Trop Dis,* 9**,** e0004226.

ELLEUCH, J., ZRIBI ZGHAL, R., LACOIX, M. N., CHANDRE, F., TOUNSI, S. & JAOUA, S. 2015. Evidence of two mechanisms involved in *Bacillus thuringiensis israelensis* decreased toxicity against mosquito larvae: Genome dynamic and toxins stability. *Microbiol Res,* 176**,** 48-54.

EVANS, J. 2014. A meta-analysis of mosquitocidal activity of the Cry2A family of toxins: Does toxin specificity allow for identification of putative specificity-conferring amino acids in the protein sequence? MSc dissertation, School of Life Sciences, University of Sussex, UK, 27- 52

FABRICK, J., OPPERT, C., LORENZEN, M. D., MORRIS, K., OPPERT, B. & JURAT-FUENTES, J. L. 2009. A novel *Tenebrio molitor* cadherin is a functional receptor for *Bacillus thuringiensis* Cry3Aa toxin. *J Biol Chem,* 284**,** 18401-18410.

FAUCON, F., GAUDE, T., DUSFOUR, I., NAVRATIL, V., CORBEL, V., JUNTARAJUMNONG, W., GIROD, R., POUPARDIN, R., BOYER, F., REYNAUD, S. & DAVID, J. P. 2017. In the hunt for genomic markers of metabolic resistance to pyrethroids in the mosquito Aedes aegypti: An integrated next-generation sequencing approach. *PLoS Negl Trop Dis,* 11**,** e0005526.

FERNANDEZ, L. E., AIMANOVA, K. G., GILL, S. S., BRAVO, A. & SOBERON, M. 2006. A GPI-anchored alkaline phosphatase is a functional midgut receptor of Cry11Aa toxin in *Aedes aegypti* larvae. *Biochem J,* 394**,** 77-84.

FFRENCH-CONSTANT, R. H., ANTHONY, N., ARONSTEIN, K., ROCHELEAU, T. & STILWELL, G. 2000. Cyclodiene insecticide resistance: from molecular to population genetics. *Annu Rev Entomol,* 45**,** 449-466.

FOSTER, W., A, WALKER, E,D., 2002. *Mosquitoes (Culicidae) Medical and Veterinary Entomology*Academic press, San Diego, CA, 203-262

GEORGHIOU, G. P. & WIRTH, M. C. 1997. Influence of Exposure to Single versus Multiple Toxins of *Bacillus thuringiensis* subsp. *israelensis* on Development of Resistance in the Mosquito Culex quinquefasciatus (Diptera: Culicidae). *Appl Environ Microbiol,* 63**,** 1095-10101.

GJULLEN, C. M., PETERS, R.F., 1952. Recent studies of mosquito resistance to insecticides in California. . *Mosq. News* 12**,** 1-7.

GOMEZ-DANTES, H. & WILLOQUET, J. R. 2009. Dengue in the Americas: challenges for prevention and control. *Cad Saude Publica,* 25 Suppl 1**,** S19-31.

GROCHULSKI, P., MASSON, L., BORISOVA, S., PUSZTAI-CAREY, M., SCHWARTZ, J. L., BROUSSEAU, R. & CYGLER, M. 1995. *Bacillus thuringiensis* CryIA(a) insecticidal toxin: crystal structure and channel formation. *J Mol Biol,* 254**,** 447-464.

HAIDER, M. Z. & ELLAR, D. J. 1989. Mechanism of action of *Bacillus thuringiensis* insecticidal delta-endotoxin: interaction with phospholipid vesicles. *Biochim Biophys Acta,* 978**,** 216-222.

HARRIS, A. F., RAJATILEKA, S. & RANSON, H. 2010. Pyrethroid resistance in *Aedes aegypti* from Grand Cayman. *Am J Trop Med Hyg,* 83**,** 277-284.

HAYAKAWA, T., SHITOMI, Y., MIYAMOTO, K. & HORI, H. 2004. GalNAc pretreatment inhibits trapping of *Bacillus thuringiensis* Cry1Ac on the peritrophic membrane of *Bombyx mori*. *FEBS Lett,* 576**,** 331-335.

HILBERT, D. W. & PIGGOT, P. J. 2004. Compartmentalization of gene expression during *Bacillus subtilis* spore formation. *Microbiol Mol Biol Rev,* 68**,** 234-262.

HODGMAN, T. C. & ELLAR, D. J. 1990. Models for the structure and function of the *Bacillus thuringiensis* delta-endotoxins determined by compilational analysis. *DNA Seq,* 1**,** 97-106.

HOFTE, H. & WHITELEY, H. R. 1989. Insecticidal crystal proteins of *Bacillus thuringiensis*. *Microbiol Rev,* 53**,** 242-255.

HU, X., LIU, Z., LI, Y., DING, X., XIA, L. & HU, S. 2014. PirB-Cry2Aa hybrid protein exhibits enhanced insecticidal activity against *Spodoptera exigua* larvae. *J Invertebr Pathol,* 120**,** 40-42.

IBRAHIM, M. A., GRIKO, N., JUNKER, M. & BULLA, L. A. 2010. *Bacillus thuringiensis*: a genomics and proteomics perspective. *Bioeng Bugs,* 1**,** 31-50.

KELLEY, L. A., MEZULIS, S., YATES, C. M., WASS, M. N. & STERNBERG, M. J. 2015. The Phyre2 web portal for protein modeling, prediction and analysis. *Nat Protoc,* 10**,** 845-858.

KNOWLES, B. H. A. E., D. J. 1987. Colloid-osmotic lysis is a general feature of the mechanism of action of *Bacillus thuringiensis* δ-endotoxins with different insect specificity. *Biochimica et Biophysica Acta* 924**,** 509-518.

LAMBERT, B., BUYSSE, L., DECOCK, C., JANSENS, S., PIENS, C., SAEY, B., SEURINCK, J., VAN AUDENHOVE, K., VAN RIE, J., VAN VLIET, A. & PEFEROEN, M. 1996. A *Bacillus thuringiensis* insecticidal crystal protein with a high activity against members of the family *Noctuidae*. *Appl Environ Microbiol,* 62**,** 80-86.

LEE, M. K., YOUNG, B. A. & DEAN, D. H. 1995. Domain III exchanges of *Bacillus thuringiensis* CryIA toxins affect binding to different gypsy moth midgut receptors. *Biochem Biophys Res Commun,* 216**,** 306-312.

LI, J. D., CARROLL, J. & ELLAR, D. J. 1991. Crystal structure of insecticidal delta-endotoxin from *Bacillus thuringiensis* at 2.5 A resolution. *Nature,* 353**,** 815-821.

LI, T. & LIU, N. 2010. Inheritance of permethrin resistance in *Culex quinquefasciatus*. *J Med Entomol,* 47**,** 1127-1134.

LIANG, H., LIU, Y., ZHU, J., PENG, G., LI, S., WANG, S., ZHENG, A., LIU, H. & LI, P. 2011. Characterization of cry2-type genes of *bacillus thuringiensis* strains from soilisolated of sichuan basin, china. *Braz J Microbiol,* 42**,** 140-146.

LIANG, Y. & DEAN, D. H. 1994. Location of a lepidopteran specificity region in insecticidal crystal protein CryIIA from *Bacillus thuringiensis*. *Mol Microbiol,* 13**,** 569-575.

LIGON, B. L. 2006. Reemergence of an unusual disease: the chikungunya epidemic. *Semin Pediatr Infect Dis,* 17**,** 99-104.

LIMA, G. M. S., AGUIAR, R. W. S., CORREA, R. F. T., MARTINS, E. S., GOMES, A. C. M., NAGATA, T., DE-SOUZA, M. T., MONNERAT, R. G., AND RIBEIRO, B. M. 2008. Cry2A toxins from *Bacillus thuringiensis* expressed in insect cells are toxic to two lepidopteran insects. *World Journal of Microbiology and Biotechnology, ,* 24**,** 2941-2948.

LIU, X. S. & DEAN, D. H. 2006. Redesigning *Bacillus thuringiensis* Cry1Aa toxin into a mosquito toxin. *Protein Eng Des Sel,* 19**,** 107-111.

LUMJUAN, N., RAJATILEKA, S., CHANGSOM, D., WICHEER, J., LEELAPAT, P., PRAPANTHADARA, L. A., SOMBOON, P., LYCETT, G. & RANSON, H. 2011. The role of the *Aedes aegypti* Epsilon glutathione transferases in conferring resistance to DDT and pyrethroid insecticides. *Insect Biochem Mol Biol,* 41**,** 203-209.

MANDAL, C. C., GAYEN, S., BASU, A., GHOSH, K. S., DASGUPTA, S., MAITI, M. K. & SEN, S. K. 2007. Prediction-based protein engineering of domain I of Cry2A entomocidal toxin of *Bacillus thuringiensis* for the enhancement of toxicity against lepidopteran insects. *Protein Eng Des Sel,* 20**,** 599-606.

MARCOMBE, S., MATHIEU, R. B., POCQUET, N., RIAZ, M. A., POUPARDIN, R., SELIOR, S., DARRIET, F., REYNAUD, S., YEBAKIMA, A., CORBEL, V., DAVID, J. P. & CHANDRE, F. 2012. Insecticide resistance in the dengue vector *Aedes aegypti* from Martinique: distribution, mechanisms and relations with environmental factors. *PLoS One,* 7**,** e30989.

MCNEIL, B. C. & DEAN, D. H. 2011. *Bacillus thuringiensis* Cry2Ab is active on *Anopheles* mosquitoes: single D block exchanges reveal critical residues involved in activity. *FEMS Microbiol Lett,* 325**,** 16-21.

MILNER, R. J. 1994. History of *Bacillus thuringiensis*. *Agric Ecosyst Environ* 49**,** 9–13.

MORSE, R. J., YAMAMOTO, T. & STROUD, R. M. 2001. Structure of Cry2Aa suggests an unexpected receptor binding epitope. *Structure,* 9**,** 409-417.

MUNOZ-GARAY, C., PORTUGAL, L., PARDO-LOPEZ, L., JIMENEZ-JUAREZ, N., ARENAS, I., GOMEZ, I., SANCHEZ-LOPEZ, R., ARROYO, R., HOLZENBURG, A., SAVVA, C. G., SOBERON, M. & BRAVO, A. 2009. Characterization of the mechanism of action of the genetically modified Cry1AbMod toxin that is active against Cry1Ab-resistant insects. *Biochim Biophys Acta,* 1788**,** 2229-2237.

NARAHASHI, T. 1988. Molecular and cellular approaches to neurotoxicology: past, present and future. . *In:* LUNT, G. G. (ed.) *Neurotox'88: Molecular Basis of Drug and Pesticide Action, .* Newyork: Elsevier.

NELSON, M., J., 1986. Biology and Ecology. Pan American Health Organization. Washington, D.C. .

NICHOLLS, C. N., AHMAD, W. & ELLAR, D. J. 1989. Evidence for two different types of insecticidal P2 toxins with dual specificity in *Bacillus thuringiensis* subspecies. *J Bacteriol,* 171**,** 5141-5147.

OBATA, F., KITAMI, M., INOUE, Y., ATSUMI, S., YOSHIZAWA, Y. & SATO, R. 2009. Analysis of the region for receptor binding and triggering of oligomerization on Bacillus thuringiensis Cry1Aa toxin. *FEBS J,* 276**,** 5949-5959.

OHSAWA, M., TANAKA, M., MORIYAMA, K., SHIMAZU, M., ASANO, S., MIYAMOTO, K., HAGINOYA, K., MITSUI, T., KOUYA, T., TANIGUCHI, M. & HORI, H. 2012. A 50-kilodalton Cry2A peptide is lethal to Bombyx mori and Lymantria dispar. *Appl Environ Microbiol,* 78**,** 4755-4757.

PACHECO, S., GOMEZ, I., ARENAS, I., SAAB-RINCON, G., RODRIGUEZ-ALMAZAN, C., GILL, S. S., BRAVO, A. & SOBERON, M. 2009. Domain II loop 3 of *Bacillus thuringiensis* Cry1Ab toxin is involved in a "ping pong" binding mechanism with *Manduca sexta* aminopeptidase-N and cadherin receptors. *J Biol Chem,* 284**,** 32750-32757.

PANG, Y., FRUTOS, R. & FEDERICI, B. A. 1992. Synthesis and toxicity of full-length and truncated bacterial CryIVD mosquitocidal proteins expressed in lepidopteran cells using a baculovirus vector. *J Gen Virol,* 73 ( Pt 1)**,** 89-101.

PASTEUR, N. & RAYMOND, M. 1996. Insecticide resistance genes in mosquitoes: their mutations, migration, and selection in field populations. *J Hered,* 87**,** 444-9.

PETTERSEN, E. F., GODDARD, T. D., HUANG, C. C., COUCH, G. S., GREENBLATT, D. M., MENG, E. C. & FERRIN, T. E. 2004. UCSF Chimera--a visualization system for exploratory research and analysis. *J Comput Chem,* 25**,** 1605-1612.

PONLAWAT, A., SCOTT, J. G. & HARRINGTON, L. C. 2005. Insecticide susceptibility of Aedes aegypti and Aedes albopictus across Thailand. *J Med Entomol,* 42**,** 821-825.

RANSON, H., N'GUESSAN, R., LINES, J., MOIROUX, N., NKUNI, Z. & CORBEL, V. 2011. Pyrethroid resistance in African anopheline mosquitoes: what are the implications for malaria control? *Trends Parasitol,* 27**,** 91-98.

RASKO, D. A., ALTHERR, M. R., HAN, C. S. & RAVEL, J. 2005. Genomics of the *Bacillus cereus* group of organisms. *FEMS Microbiol Rev,* 29**,** 303-329.

RAUSELL, C., GARCIA-ROBLES, I., SANCHEZ, J., MUNOZ-GARAY, C., MARTINEZ-RAMIREZ, A. C., REAL, M. D. & BRAVO, A. 2004. Role of toxin activation on binding and pore formation activity of the *Bacillus thuringiensis* Cry3 toxins in membranes of Leptinotarsa decemlineata (Say). *Biochim Biophys Acta,* 1660**,** 99-105.

READ, T. D., PETERSON, S. N., TOURASSE, N., BAILLIE, L. W., PAULSEN, I. T., NELSON, K. E., TETTELIN, H., FOUTS, D. E., EISEN, J. A., GILL, S. R., HOLTZAPPLE, E. K., OKSTAD, O. A., HELGASON, E., RILSTONE, J., WU, M., KOLONAY, J. F., BEANAN, M. J., DODSON, R. J., BRINKAC, L. M., GWINN, M., DEBOY, R. T., MADPU, R., DAUGHERTY, S. C., DURKIN, A. S., HAFT, D. H., NELSON, W. C., PETERSON, J. D., POP, M., KHOURI, H. M., RADUNE, D., BENTON, J. L., MAHAMOUD, Y., JIANG, L., HANCE, I. R., WEIDMAN, J. F., BERRY, K. J., PLAUT, R. D., WOLF, A. M., WATKINS, K. L., NIERMAN, W. C., HAZEN, A., CLINE, R., REDMOND, C., THWAITE, J. E., WHITE, O., SALZBERG, S. L., THOMASON, B., FRIEDLANDER, A. M., KOEHLER, T. M., HANNA, P. C., KOLSTO, A. B. & FRASER, C. M. 2003. The genome sequence of *Bacillus anthracis* Ames and comparison to closely related bacteria. *Nature,* 423**,** 81-86.

RODRIGUEZ, M. M., BISSET, J. A. & FERNANDEZ, D. 2007. Levels of insecticide resistance and resistance mechanisms in *Aedes aegypti* from some Latin American countries. *J Am Mosq Control Assoc,* 23**,** 420-429.

ROMERO, M., GIL, F. M. & ORDUZ, S. 2001. Expression of mosquito active toxin genes by a Colombian native strain of the gram-negative bacterium *Asticcacaulis excentricus*. *Mem Inst Oswaldo Cruz,* 96**,** 257-263.

SCHNEPF, E., CRICKMORE, N., VAN RIE, J., LERECLUS, D., BAUM, J., FEITELSON, J., ZEIGLER, D. R. & DEAN, D. H. 1998. *Bacillus thuringiensis* and its pesticidal crystal proteins. *Microbiol Mol Biol Rev,* 62**,** 775-806.

SCHNEPF, H. E., WONG, H. C. & WHITELEY, H. R. 1985. The amino acid sequence of a crystal protein from *Bacillus thuringiensis* deduced from the DNA base sequence. *J Biol Chem,* 260**,** 6264-6272.

SCHWARTZ, J. L., LU, Y. J., SOHNLEIN, P., BROUSSEAU, R., LAPRADE, R., MASSON, L. & ADANG, M. J. 1997. Ion channels formed in planar lipid bilayers by *Bacillus thuringiensi*s toxins in the presence of *Manduca sexta* midgut receptors. *FEBS Lett,* 412**,** 270-276.

SHAPOVALOV, M. V. & DUNBRACK, R. L., JR. 2011. A smoothed backbone-dependent rotamer library for proteins derived from adaptive kernel density estimates and regressions. *Structure,* 19**,** 844-858.

SHU, C., ZHANG, F., CHEN, G., JOSEPH, L., BARQAWI, A., EVANS, J., SONG, F., LI, G., ZHANG, J. & CRICKMORE, N. 2017. A natural hybrid of a *Bacillus thuringiensis* Cry2A toxin implicates Domain I in specificity determination. *J Invertebr Pathol,* 150**,** 35-40.

SIMS, S. R. 1997. Host activity spectrum of the CryIIA Bacillus thuringiensis subsp.

kurstaki protein: effects on Lepidoptera, Diptera, and non-target arthropods.Southwest. *Entomol. ,* 22,**,** 395–404.

SOBERON, M., LOPEZ-DIAZ, J. A. & BRAVO, A. 2013. Cyt toxins produced by *Bacillus thuringiensis*: a protein fold conserved in several pathogenic microorganisms. *Peptides,* 41**,** 87-93.

STALINSKI, R., TETREAU, G., GAUDE, T. & DESPRES, L. 2014. Pre-selecting resistance against individual Bti Cry toxins facilitates the development of resistance to the Bti toxins cocktail. *J Invertebr Pathol,* 119**,** 50-53.

SUTER, T., CRESPO, M. M., DE OLIVEIRA, M. F., DE OLIVEIRA, T. S. A., DE MELO-SANTOS, M. A. V., DE OLIVEIRA, C. M. F., AYRES, C. F. J., BARBOSA, R. M. R., ARAUJO, A. P., REGIS, L. N., FLACIO, E., ENGELER, L., MULLER, P. & SILVA-FILHA, M. 2017. Insecticide susceptibility of *Aedes albopictus* and *Ae. aegypti* from Brazil and the Swiss-Italian border region. *Parasit Vectors,* 10**,** 431.

TABACHNICK, W. J., MUNSTERMANN, L. E. & POWELL, J. R. 1979. Genetic Distinctness of Sympatric Forms of *Aedes Aegypti* in East Africa. *Evolution,* 33**,** 287-295.

TOMORI, O. 2004. Yellow fever: the recurring plague. *Crit Rev Clin Lab Sci,* 41**,** 391-427.

VAN FRANKENHUYZEN, K. 2009. Insecticidal activity of *Bacillus thuringiensis* crystal proteins. *J Invertebr Pathol,* 101**,** 1-16.

WANG, L., NOMURA, Y., DU, Y., LIU, N., ZHOROV, B. S. & DONG, K. 2015. A mutation in the intracellular loop III/IV of mosquito sodium channel synergizes the effect of mutations in helix IIS6 on pyrethroid resistance. *Mol Pharmacol,* 87**,** 421-429.

WHO 1997. Dengue haemorrhagic fever : diagnosis, treatment, prevention and control, 2nd ed. Geneva : World Health Organization. .

WHO 1998. Expanded Programme on Immunization & World Health Organization. Division of Emerging and Other Communicable Diseases Surveillance and Control. Yellow fever : technical consensus meeting, Geneva, 2-3 March 1998. Geneva : World Health Organization.

WHO 2005a. Dengue, dengue haemorrhagic fever and dengue shock syndrome in the context of the integrated management of childhood illness. Geneva : World Health Organization.

WHO 2005b. Disease Control, Prevention and Eradication WHO Pesticide Evaluation Scheme. Guidelines for Laboratory and Field Testing of Mosquito Larvicides. World Health Organisation.

WHO 2005c. Disease Control, Prevention and Eradication WHO Pesticide Evaluation Scheme. Guidelines for Laboratory and Field Testing of Mosquito Larvicides. World Health Organisation.

WHO 2006. Pesticides and their application for the control of vectors and pests of public health importance.

WHO 2013. World Malaria Report: National Press Club in Washington, DC on 11 December 2013.

WIDNER, W. R. & WHITELEY, H. R. 1990. Location of the dipteran specificity region in a lepidopteran-dipteran crystal protein from *Bacillus thuringiensis*. *J Bacteriol,* 172**,** 2826-2832.

WU, D. & ARONSON, A. I. 1992. Localized mutagenesis defines regions of the *Bacillus thuringiensis* delta-endotoxin involved in toxicity and specificity. *J Biol Chem,* 267**,** 2311-2317.

WU, D., CAO, X. L., BAI, Y. Y. & ARONSON, A. I. 1991. Sequence of an operon containing a novel delta-endotoxin gene from *Bacillus thuringiensis*. *FEMS Microbiol Lett,* 65**,** 31-35.

XU, L., PAN, Z. Z., ZHANG, J., LIU, B., ZHU, Y. J. & CHEN, Q. X. 2016. Proteolytic Activation of *Bacillus thuringiensis* Cry2Ab through a Belt-and-Braces Approach. *J Agric Food Chem,* 64**,** 7195-7200.

YAMAMOTO, T., MCLAUGHLIN,R.E., 1981. Isolation of a protein from the parasporal crystal of Bacillus thuringiensis var Kurstaki toxic to the mosquito larva, Aedes taenior hynchus. *Biochem. Biophys. Res. Commun., ,* 103 414-421.

YANOLA, J., SOMBOON, P., WALTON, C., NACHAIWIENG, W., SOMWANG, P. & PRAPANTHADARA, L. A. 2011. High-throughput assays for detection of the F1534C mutation in the voltage-gated sodium channel gene in permethrin-resistant Aedes aegypti and the distribution of this mutation throughout Thailand. *Trop Med Int Health,* 16**,** 501-509.

ZHANG, Q., HUA, G. & ADANG, M. J. 2017. Effects and mechanisms of Bacillus thuringiensis crystal toxins for mosquito larvae. *Insect Sci,* 24**,** 714-729.

ZHANG, X., CANDAS, M., GRIKO, N. B., ROSE-YOUNG, L. & BULLA, L. A., JR. 2005. Cytotoxicity of *Bacillus thuringiensis* Cry1Ab toxin depends on specific binding of the toxin to the cadherin receptor BT-R1 expressed in insect cells. *Cell Death Differ,* 12**,** 1407-1416.

# 8. Appendix

## Multiple sequence alignment for the N-terminal sequence of Cry2A toxins