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# Development of Monoclonal Antibodies Against Cry1Ac/Ab Protein for Designing of Sandwich ELISA to Detect BT Toxin from Cotton Seeds and Leaves

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**Abstract**

The design of the study is to develop monoclonal antibodies against Cry1Ac/Ab protein for designing os sandwich ELISA(hybridoma technology). Hybridoma technology was invented by Cesar Milstein and Georges J.F Kohler in the year 1975 and is an unique method used to produce identical antibodies in maximum quantities. Monoclonal antibodies were developed by immunization of Balb/C mice with Cry1Ac/Ab Protein. Titer values of mice tail bleeds were checked and the best mice with higher titer value was used for fusion. Immunized mice spleen cells were fused with Myeloma cells (SP2-O), using a polyethylene glycol (PEG) and the fused cells were incubated with HAT medium for 12 days and initially 400 positive hybridoma clones were obtained, of which 13 potential clones were selected using indirect ELISA against Cry1Ac/Ab recombinant antigen. Cross reactivity was ruled out using indriet ELSA against cry proteins such as Cry2A, Cry1F and CP4EPSPS using. Cloning was carried out twice for all 13 clones by limiting dilution factor and pure single clones were selected. The class IgG/IgM/IgA and sub classes IgG1, IgG2, IgG3 antibodies are determined by isotyping. Determination of class and subclass of an antibody is very important for selecting proper purification methods. Commercially available rapid isotyping kits were used for isotyping which provides the information of 1) IgG, IgM, IgA, IgG2a, IgG2b or IgG3 2) Light chain identification as either kappa or lambda. All pure clones were preserved in Liquid Nitrogen for future use to develop immunological kits for detection of Cry1Ac/Ab present in the plant tissue.

**Keywords:** *Bacillus thuringiensis*, Hybridoma Technology, Cry1Ac/Ab, HAT medium, Monoclonal antibodies, Iso-typing, genetically modified crops and Immunization.

**Introduction:**

The use of entamo-pathogenic microorganisms for regulating insect pests was first proposed at the end of 19th century by several pioneering scientists. The undesirable effects of chemical pests on environment and human population triggered for a change, to utilize the benefits of these biopests which are remarkably nontoxic to humans and to a large extent of non target fauna (1).

Detection of insecticidal properties of *Bacillus thuringiensis* dates back to 1901 by a Japan scientist Ishiwata (2) and was rediscovered in 1911 by Ernst Berliner in Germany. *Bacillus*

*thuringiensis* is a gram positive, rod shaped, aerobic soil bacterium (3,4). During sporulation it produces crystalline parasporal inclusion bodies (3) which are classified as ä- endotoxin. The toxic effects of ä- endotoxin are restricted only to insects and observed to pose no potential threat to humans (5). The larvicidal activity of *Bacillus thiringiensis* is rapid, but sustained making it an attractive agent for pest control in agriculture. Advances in Genetic engineering resulted in several transgenic plants such as tobacco, cotton, maize, tomato, potato and rice, expressing insecticidal BT proteins that can control target chewing insects (Fearing et al., 1997). When a susceptible insect ingests BT the toxin gets activated by alkaline and enzymatic condition of insect gut and leads to anorexia and death of insect (3,6).

*Bacillus thiringiensis* was first available as a commercial pesticide in France in 1938 and entered the United States in 1950 (7). BT cotton is the most extensively grown transgenic crop in china and India. In 2005, China grew 3.3 million hectares of BT cotton, occupying about 66% of national cotton area (8).Use of genetically modified plants for agriculture and food production is regulated by national law and novel food regulation guide lines in European countries (6) and similar approach is practiced in other countries as well.

To enforce such regulations, reliable methods for the detection and quantification of GMO in plants and food products is required. Most frequently used method is the amplification of GMO specific DNA by polymerase chain reaction (PCR). Identification of specific amplification requires performing agarose gel electrophoresis, restriction fragment length analysis, or southern blotting (9,10). Even though results are highly sensitive, excuting PCR requires well-equipped laboratory, experienced investigators to optimize results and also time consuming and difficult to quantify (11).

Immunoassay provides an alternative means for the detection and quantification of GMO based on the protein product expressed in their tissues. Different types of ELISA's have been developed to indentify the *B. thiringiensis* toxins in different crops like maize and transgenic cotton (12). Majority of these assays use polyclonal antibodies raised in rabbits (13) or goats (12) Even though polyclonal antibodies are easy to obtain, quantity of polyclonal antisera is restricted from a specific host because antisera from different hosts vary in characteristics. Also polyclonal antibodies are a mixture of immunoglobulins with many different epitope specificities of antibodies. In contrast Monoclonal antibodies are produced specifically to a single epitope of their antigen and can be produced in unlimited quantities. Therefore the aim of the study is to produce monoclonal antibodies against Cry1Ac/Ab protein from *Bacillus thuringenesis.*

## Materials and methods

***Animals, Chemicals and Reagemts :*** Balb/c mice used for immunization were obtained from National institute of Nutrition (NIN) Hyderabad, India. Lyophilized Cry1Ac/Ab Bt protein for mice immunization to develop monoclonal antibodies was provided by Amar Immunodiagnostics, Hyderabad. Cry1Ac/Ab positive cotton seed samples were purchased from the local market. Freund's complete, incomplete adjuvant, Hypoxanthine, Thymidine ,Glycine, Aminopterin, Penstrep fetal bovine serum, polyethylene glycol (PEG), Dimethyl sulfoxide (DMSO) solution and Goat anti mouse IgG labeled were purchased from Sigma Aldrich. Antibody isotyping lateral flow strip kit was purchased from Envirologix, USA. Sterile 96 well plates, T-24 plates, T25 flasks and T-75 flasks were purchased from Corning. SP2-O cells were provided by ICGEB. Ab-HRP conjugation kit(Lightning-Link Kit) purchased from Innova Bioscience.

***Media and buffers :*** Complete growth media was prepared by adding 1% RPM I-1640, 1% Pencillin-Streptomycin (10,000 U/mL), 0.2% Sodium bicarbonate $NaHCO_3$, 0.03% L-Glutamine,0.05% 2-Mercaptoethanol ($C_2H_6OS$), and 10% Fetal bovine serum to final volume of one liter of reagent grade water. HAT media was prepared by adding 10 ml of Hypoxanthine 100X, 5 ml of Thymidine

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Glycine 500X, and 5 ml of Aminopterin to 800 ml of complete growth media. Saline was prepared by adding 0.85g of sodium chloride to 100 ml of reagent grade water. 100 mM phosphate buffered saline with 0.005% Tween-20. Binding or wash buffer: 20 mM Sodium Phosphate with pH 7, and the elution buffer (EB): 0.1M Glycine-HCl with pH 2.7, 1M Tris Buffer and 1N HCL

***Mice Immunization :*** 6-7 weeks old female Balb/c mice were used for immunization. Prior to immunization 100µl control serum was collected from each mice which is also called as pre immune bleed. For primary immunization 120µg of Cry1Ac/Ab antigen in saline, was mixed with equal volume of complete Freund's adjuvant (CFA Sigma) and given intra peritoneal (IP). For the subsequent first and second boosters 120µg of Cry1Ac/Ab antigen in saline was mixed with equal volume of incomplete Freund's adjuvant (IFA Sigma) and injected intra peritoneal (IP). 10days before fusion, final boost was given without adjuvant. After 10 days of third immunization, test bleed was collected from each mice. All test bleeds were serially diluted at 1000 fold dilutions in normal saline and titer values were checked in Cry1Ac/Ab antigen coated plates by using secondary anti-mice antibody labeled with horse radish peroxidase and TMB. The best mice with highest serum antibody titer values was selected and given final booster of 25µg Cry1Ac/Ab antigen intravenously (IV).

***Growing SP2-O cells :*** SP2-O cells were kindly provided by ICGEB and were stored at -180ºC in liquid Nitrogen. The cell were thawed at 37 ºC and grown in standard tissue complete growth (CGM) culture media. After thawing, cells were diluted in Roswell Park Memorial Institute (RPMI) 1640 media and centrifuged at 1200 rpm for 5 minutes. Supernatant was discarded pellet was carefully resuspended in 10ml of CGM and transferred to T-25 flasks (NUNC). Viability of the cells was observed under inverted microscope (Olumpus). The Flasks were incubated in $CO_2$ incubator with 5% $CO_2$ at 37 ºC temperature for for 7 days. When the cell density reaches to 1 million per ml, the cells were transferred to T-75 (NUNC) flasks with cultural conditions remain standard. Flasks were observed periodically for contamination. Cell density in the flask should not exceed more than $1x10^6$/ml . After which trypan blue a staining dye is used to check the viability of the cells. Cells are diluted at the rate of 1:2 with Trypan blue solution and number of cells are counted using Haemocytometer counting chamber. The viability of the cells are decided by dye exclusion, and viability above 95% for a cell line is considered.

***Preparation of mice spleen cells :*** The spleen was collected aseptically from a hyper immunized Balb/c mice and chopped using a pair of autoclaved scissors, forceps and diluted in MEM minimal essential media. Single cell suspension was prepared by passing through sieve. The cells were centrifuged for 5 minutes at 1000 rpm in a table top centrifuge. Supernatant was discarded and pellet was washed with Tissue culture media, by repeating centrifugation step. Pellet was resuspended in 10 ml of RPMI solution and small aliquot was diluted at 1:10 with trypan blue solution. Total number of spleen cells were counted using haemocytometer counting chamber and the RBC present along with spleen cells are destroyed by lysis and removed by centrifugation (1200 rpm for 5 min) and the pellet was ready for fusion.

***Fusion of myeloma cells with immunized mice spleen cells :*** Single spleen cells suspension from the immunized mouse are fused with the previously prepared myeloma cells. Fusion is accomplished by adding polyethylene glycol. PEG is a chemical substance that causes cell membranes to fuse. Mix 20 million spleen cells and 50 million myeloma cells in serum-free RPMI 1640. Centrifuge the mixture of cells at 1200rpm for 10 minutes. While the cells are centrifuging, set aside 12 ml of serum-free RPMI 1640 in sterile 50-ml tube. Keep 50% PEG and timer ready in the hood. Remove all the supernatant from the cell pellet. Overlay the pellet of cells with 0.5 ml of 50% PEG with a Pasteur pipette during 1 minute. Then add 5 ml of serum-free RPMI 1640 over 2 minutes followed by 7 ml

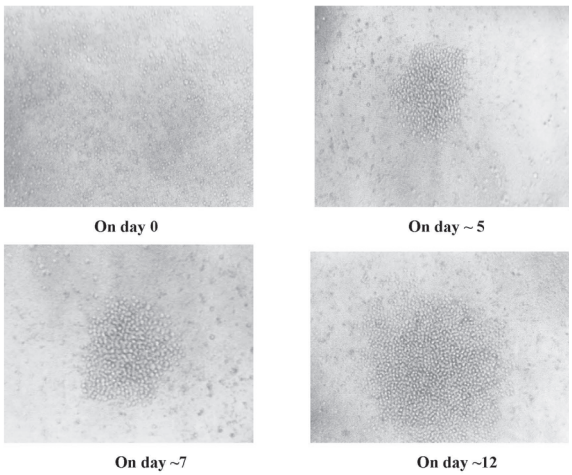


**Fig.1** Microscopic view of Hybridomas in different time periods

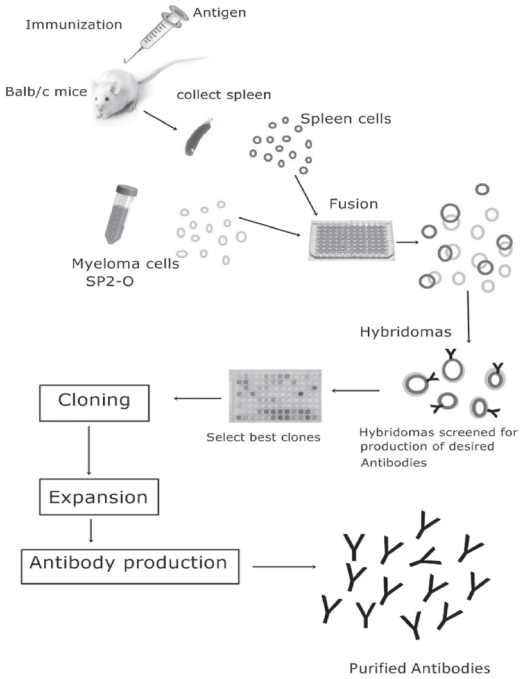


**Fig.2.** Schematic representation of development of monoclonal antibodies by Hybridoma technology

of serum-free RPMI 1640 over 3 minutes. Centrifuge cells at 1200rpm for 5 minutes and remove supernatant. Resuspend the pellet in HAT media. The cells are then distributed in 96 well plates containing feeder cells collected from saline peritoneal washes of normal BAlb/C mice. Feeder cells are used to supply growth factors that promote growth of the hybridoma cells (14) Fedder cells and fused cells are re-suspended in special selective media called HAT. Cells were allowed to grow for 10 days and tissue culture supernatant was collected and tested for presence of Cry1Ac/Ab antibodies.

***Antibody screening:*** 96 well microtiter ELISA plates were coated with Cry1Ac/Ab antigen in Tris saline at 1µg/ml and incubated over night at room temperature. Plates were washed 2 times with PBS-T and blocked with blocking buffer for 1 hour at room temperature. Decanted the blocking solution, dried the wells and used for antibody screening. 100µl of Hybridoma cell culture supernatant was added to each well of microtiter plate coated with Cry1Ac/Ab antigen and incubated for 60 minutes at room temperature on a shaker, followed by washing (4 times) with PBS-T (phosphate buffered saline with tween 20). Goat anti mouse IgG-HRPO is added and incubated for 30 minutes at room temperature on a shaker followed by washing (4 times with PBS-T) and the TMB (3,3',5,5'-Tetramethylbenzidine) solution is added and incubated for 15 minutes at room temperature.

All positive clones that secrete antibodies against Cry1Ac/Ab protein develop blue color and negative clones remain colorless. Reaction was stopped by adding 1N HCL. The intensity of color was measured by reading microtiter plates at 450-620 nm in ELISA reader (Thermo scientific) and OD values were recorded and the clones with OD values of more than 3 were selected, and those were cloned twice by doing limited dilution factor.

***Cloning*** **:** To obtain a monoclonal cell population from a mass of cells, the cells are abstained by executing a series of increasing dilutions of mother cell culture from master plate. Cloning of hybridomas by limiting dilution factor is widely used method which is fast and easy.

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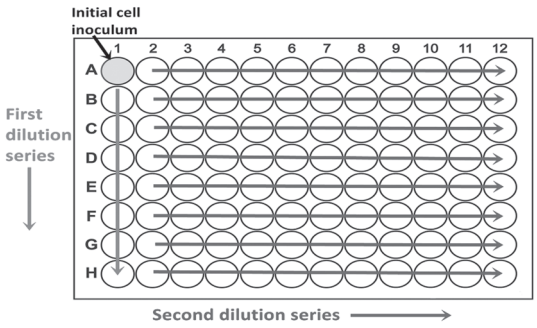


**Fig. 4.2.** Cloning hybridoma cell line by limiting dilution factor

96 well microtiter plates were used for cloning hybridomas. Sterile reservoir was filled with complete growth medium, using 8 channel pipette 100µl medium was added to all the wells. Approximately 2000-3000 cells of cell suspension from master fusion plate was added to well A1 using single channel pipette. Cell culture in A1 was mixed gently and transferred 100µl to B1 using same pipette, repeated 1:2 dilutions down the entire Column. 100 µl from H1 well was discarded. Then using 8 channel pipette transferred 100 µl from the wells in the first Coloumn (A1 through H1) to those in the second Coloumn (A2 through H2) using the same tips. 1:2 dilution was repeated across the entire plate. Final volume of all the wells were made up to 200 µl by adding 100 µl media to each well except (A12 to H12). All cloning plates were labelled and incubated in CO2 incubator at 37ºC and 5% CO2. Clones were visible under microscope after 4 to 5 days and were screened after 12 days. positive wells were observed under microscope and well contained single clones was marked, those single clones were expanded to T-24 well followed by T-25 flask and cloned 2nd time using Same procedure.

***Production of ascites:*** Monoclonal antibodies can be produced in large quantities by developing acites fluid in Balb/c mice. Injected 0.2 ml of pristine per mice into the peritoneum. After 5-7 days 2 million hybridoma cells per mice were injected intra peritoneal. Tapped the ascites fluid after 2 weeks following the injection of cells, centrifuged and stored clean supernatant at -20°C. Monoclonal antibodies were purified from ascites by using Protein G column (GE health care)

***ELISA Micro titer plate :*** Protein G purified monoclonal antibodies against Cry1Ac/Ab protein were diluted in coating buffer (100mM Tris Saline PH 7.2) at final concentration of 10µg/ml. Added 100µl of diluted antibody solution to microtiter plate, and incubated over night at room temperature. Empty the plate , tapped out the residual liquid and washed one time with 300µl wash buffer (10mM Tris saline with 0.005% Tween 20). 100µl blocking solution was added to each well and incubated 1 hour. Empty the plate , tapped out the residual liquid and packed in air tite pouch with desiccant.

***Antibody labeling with horse radish peroxidase enzyme (HRPO) :*** Antibodies labeled with HRP using lightning link HRP conjugation kit purchased from Innova biosciences. Lightning link technology works by targeting amine groups and can be used for most biomolecules including antibodies. horse radish peroxidase (HRP) conjugation kit allows HRP conjugation to be set up in seconds, simply by adding a solution of antibody to a activated HRP ligand lyophilized powder.10µl of LL modified provided in the kit was added to 100µl (100µg)Ab to be labelled and mixed gently. Remove screw cap from the vial of lightning link mix and antibody solution was pipette directly on to the lyophilized material resuspended gently by withdrawing and redispensing the liquid twice using a pipette. Placed the cap back on the vial and incubated 3 hours. Reaction was stopped by adding 10µl of LL quencher solution and incubated 30 minutes at room temperature. Finally conjugate was resuspended in proper diluent and stored at 2-4ºC.

**Protein Extraction from cotton seed and leaf samples**

***Extraction of protein from seed tissue :*** Single seed was crushed with hammer and transferred seed powder to 2ml centrifuge tube. 1ml of

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extraction buffer was added to the tube and mixed well. Sample was extracted for 5 minutes at room temperature.

***Extraction of protein from leaf tissue :*** Two leaf punches approximately 20mg tissue was transferred to 1.5ml centrifuge tube. Tissue was ground with pestle until leaf tissue is well ground. 0.5ml of extraction buffer was added and extracted for 5 minutes sample was ready to test.

**Results and Discussion:**

***Characterization of Cry1Ac/Ab antigen purity by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis) :*** The objective of the study was to immunize the mice with Cry1Ac/Ab pure antigen to develop monoclonal antibodies and to design and develop monoclonal antibody based immune assays for the detection of the Cry1Ac/Ab protein in Bt cotton. Before immunization the purity of the Cry1Ac/Ab protein was determined by SDS-PAGE. SDS results showed the presence of a single protein band with 55kd molecular weight indicating that the purity of the protein was more than 95% which is considered as high purity protein and is used as immunogen to immunize the mice.

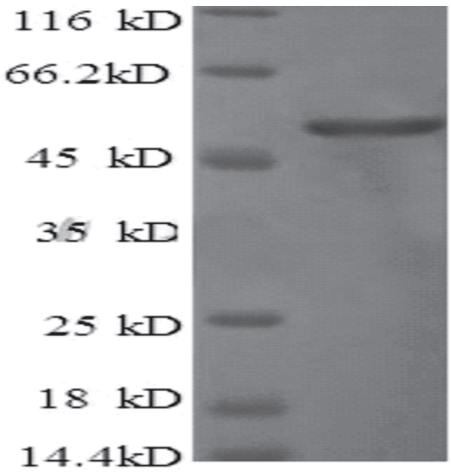


**Fig. 3.** Analysis of purity of Cry1Ac/Ab antigen by SDS-PAGE Lane 1: Marker. Lane 2: Cry1Ac antigen

**Graphical representation of mice bleed titer values :** A total of 4 mice were immunized using Cry1Ac/Ab antigen and each of the immunized animal responded differently. Mice with high titer value were selected for fusion. The titer of anti Cry1Ac/Ab specific IgG antibodies in sera of the immunized mice were monitored by collecting the mice bleed after 10 days of each immunization and antibody titer was checked against recombinant Cry1Ac/Ab antigen coated plates (Indirect ELSIA) throughout the immunization process to verify successful immunization and determine the titer values that would be useful for development of monoclonal antibodies. 100 µl of tissue culture supernatant was added Cry1Ac/Ab antigen coated plate and incubated for 60 min at room temperature. Plate was washed using PBS-T and to which 100 micro liters of secondary antibody labeled with HRPO was added and incubated for 30 minutes. Followed by washing with PBS-T. 100 micro liters of TMB substrate was added and incubated for 15 min. Reaction was stopped by adding 1N HCl. The intensity of the yellow colored product was measured at 450-620nm by reading the ELISA plate using ELISA reader. The intensity of the color is corresponded to the amount of specific antibodies in the serum of each animal. Titration curve was generated using dilution factor on X axis and O.D values on Y axis.

***Antibody screening methods and preservation of positive clones :*** Choosing the screening method for selecting the desired Ab secreting clones is very important and critical. In this study all fusion plates were individually screened for the production of Cry Ac/Ab antigen specific antibody using indirect ELISA against Cry Ac/Ab recombinant antigen. After initial screening against recombinant antigen a total of 40 positive clones were selected with OD of more than 2.5 in ELISA. To further conform, all the 40 positive clones were further screened for the production of antibodies using indirect ELSIA against Cry Ac/Ab recombinant antigen. Based on O.D values observed in indirect ELISA, number of clones in the well, size of the clone, cultural characteristics

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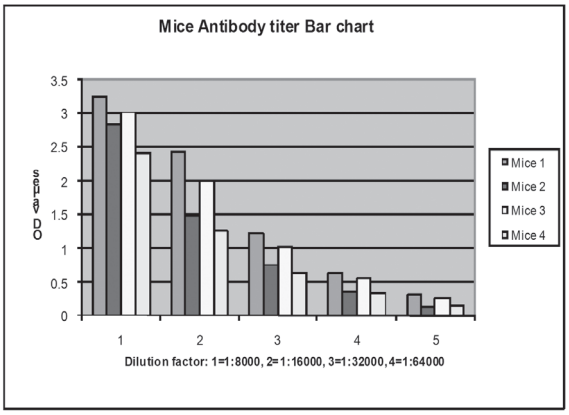
**Table:1** Titer values of 4 different mice bleeds checked by ELISA method

| S.No | Description | Mice 1 | Mice 2 | Mice 3 | Mice 4 |
|---|---|---|---|---|---|
| 1 | Mice test bleed at 1:4000 | 3.157 | 3.186 | 3.123 | 3.076 |
| 2 | Mice test bleed at 1:8000 | > 3.0 | 2.922 | 3.021 | 2.621 |
| 3 | Mice test bleed at 1:16000 | > 3.0 | 2.672 | 2.984 | 1.461 |
| 4 | Mice test bleed at 1:32000 | 2.816 | 2.452 | 2.201 | 1.118 |
| 5 | Mice test bleed at 1:64000 | 2.035 | 1.776 | 1.229 | 0.534 |
| 6 | Mice test bleed at 1:128000 | 1.173 | 0.944 | 0.659 | 0.271 |
| 7 | Mice test bleed at 1:256000 | 0.584 | 0.452 | 0.326 | 0.121 |
| 8 | Mice test bleed at 1:512000 | 0.293 | 0.215 | 0.163 | 0.062 |




Graph.1. Graphical representation of antibody titer values of mice tail bleed.

and rapid doubling time, 13 clones were selected and cultured in T-24 well plate. After 2 days of incubation, supernatant from T-24 well plate was tested against Cry1Ac/Ab protein then transferred and cultured in T-25 flask. Irrespective of single and multiple clone all 13 clones were sub cloned by limiting dilution to ensure monoclonality of the hybridoma. All 13 clones were protected by preserving them in LN2 for further use. After Cryo preservation tissue culture supernatant of 13 different clones were subjected to indirect ELSIA against Cry1Ac/Ab antigens to conform the presence of monocolonal antibodies specific for Cry1Ac/Ab antigen.

**Table.2.** List of final clones and OD values represents that all 13 clones develops monoclonal antibodies against Cry1Ac/Ab protein

| S.no | Clone ID | OD |
|---|---|---|
| **1** | **1F10F5** | 3.217 |
| **2** | **1G8D3** | 2.647 |
| **3** | **2F2C12** | 2.321 |
| **4** | **3AfE1** | 1.924 |
| **5** | **3B9C3** | 2.974 |
| **6** | **3D2B8** | 3.471 |
| **7** | **3E11B11** | 2.047 |
| **8** | **5C7F1** | 3.147 |
| **9** | **5F8F5** | 3.262 |
| **10** | **6H7E4** | 2.547 |
| **11** | **7D2E8H6** | 2.742 |
| **12** | **9G4H8** | 2.682 |
| **13** | **10A8F6** | 3.014 |

***Isotyping :*** The immunoglobulin Isotype refers to the slight phenotypic variations within the immunoglobulin gene family that encode for variant immunoglobulin heavy and light chains. Isotyping involves determining the class and subclass of monoclonal antibody production as it is mandate for selection of appropriate suitable purification method since the antibody obtained form the hybridoma clone is IgM, it can not be purified using protein G or protein A columns. Isotyping requires the use of specific anti immunoglobulin antibodies that are specific to for detecting the different

classes and sub classes of monoclonal antibodies encountered in antibody production. Isotyping of monoclonal antibodies were executed using commercial kit (Envirologix) as per the manufacturer instructions. The kit is used to determine the antibody classes and sub classes (IgG1, Ig2a, IgG2b, and Ig3, IgA or IgM and light chain identity {kappa or lambda}).

**Table.3.** Isotyping for Cry1Ac/Ab clones.

| S.no | Clone ID | Isotype |
|---|---|---|
| 1 | 1F10F5 | IgG2b k |
| 2 | 1G8D3 | IgG2b k |
| 3 | 2F2C12 | IgG1 k |
| 4 | 3AfE1 | IgG1 k |
| 5 | 3B9C3 | IgG2b k |
| 6 | 3D2B8 | IgG1 k |
| 7 | 3E11B11 | IgG1 k |
| 8 | 5C7F1 | IgG1 k |
| 9 | 5F8F5 | IgG2b k |
| 10 | 6H7E4 | IgG2b k |
| 11 | 7D2E8H6 | IgG2a k |
| 12 | 9G4H8 | IgG2b k |
| 13 | 10A8F6 | IgG2b k |

***Production of monoclonal antibodies in large quantities by developing Ascites :*** Ascites fluid is an intraperitoneal fluid collected from the mice peritoneal cavity due to development of peritoneal tumour. Antibodies are produced in large amount from ascites. The tumour is induced by injecting hybridoma cells into the peritoneum which acts as a growth chamber for the cells. The hybridoma cells grow to high densities and continue to secrete the antibody.

Adult male mice of the similar genetic background (Balb/c) were injected with 0.2ml of Pristane (2, 6, 10, 14 tetra methyl deconoic acid) into the peritoneum, as described by Liebermom et al, (1960). These solutions will act as irritants and initiate the mice to secrete nutrients, recirculating monocytes and lymphoid cells into the peritonial area that creates a supporting environment for the growth of the hybridoma cells.

After 7 days of pristine inoculation, the mice are ready to inject hybridomas for ascetic fluid production. The hybridoma cells to be injected in mice for ascites production were grown in tissue culture flask (T-75) to attain the highest cell density (1-1.2 × $10^6$/ml). Total number of cells was counted, centrifuge at 1400 rpm for 5 minutes and the supernatant was discarded. The pellet was resuspended in PBS. 2.3×$10^6$ to 2.8×$10^6$ hybridoma cells were injected in to each mice intra peritoneally. After 2 weeks following the date of injection of the hybridoma cells, the mice developed a large belly. 5 to 6 ml of fluid per mice was collected using 18-gauge needle attached to 5 ml syringe. After 4 days again 2-3 ml was collected/mice which were considered as second tapping. Ascites fluid was centrifuged at 5000 rpm for 10 minutes to remove cell debris and store at -20ºC.

***Purification of monoclonal antibodies through protein G column :*** Hi-trap protein 'G' HP 5ml column (GE-Health care) was used for antibody purification. Protein G column are made up of polypropylene which is bio compatible and non interactive with biomolecules. The top and bottom parts are made with porous polyethylene. Protein G Sepharose high performance is designed for purification and isolation of monoclonal and polyclonal IgG from ascites, serum and cell culture supernatants. Protein G a cell surface protein G of group G *Streptococci* is type III Fc receptor that binds to the Fc region of IgG by a non immune mechanism. 2 ml of ascites were loaded in 5ml of protein G column and 20 ml of albumin fractions were collected by applying wash buffer (20mM Sodium Phosphate, pH: 7.0) and subsequently IgG were eluted by adding elution buffer ( 0.1M Glycine-HCl pH 2.7) to the column.

SDS-PAGE was run on 5% ~10% gel, followed by Coomassie blue staining. sample description as follows. **Lane 1**: Cry1Ac Mab clone ID:1F10-F5, **Lane 2**: Cry1Ac Mab clone ID 1G8-D3, **Lane 3**: Cry1Ac Mab clone ID 2F2-C12, **Lane 4**: Cry1Ac Mab clone ID 3A5-E1, **Lane 5**: Cry1Ac

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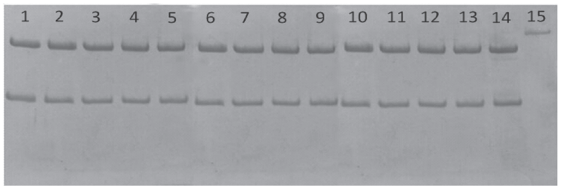


**Fig.4.** Checking monoclonalantibody purity by SDS-PAGE

Mab clone ID 3B9-C3, **Lane 6**: Cry1Ac Mab clone ID 3D2-B8, **Lane 7**: Cry1AcMab clone ID 3E11-B11, **Lane 8**: Cry1Ac Mab clone ID 5C7-F6, **Lane 9**: Cry1Ac Mab clone ID 5F8-F5, **Lane 10**: Cry1Ac Mab clone ID 6H7-E4, **Lane 11**: Cry1Ac Mab clone ID 7D2-B8, **Lane 12**: Cry1Ac Mab clone ID: 9G4-H8, **Lane 13**: Cry1Ac Mab clone ID: 10A8-F6, **Lane 14** : IgG control, **Lane 15**: Bovine serum albumin (BSA) control.

***Sandwich-ELISA for Cry1Ac/Ab Protein:*** By crosswise testing of the 13 antibodies in a sandwich-type ELISA, clone 1F10F5 was selected as solid phase antibody and 3D2B8 as conjugate antibody.

To determine the limit of detection for Cry1Ab protein, an assay was performed by diluting Cry1Ac/Ab lyophilized protein at different concentration range from 1, 2.5, 5 and 10ng in PBS. PBS was used as a 0nglml control. The results are shown in Figure 5. From these results, the limit of detection was determined as 0.51ngml–1 with a linear range from approximately 1 to 10ngml–1 protein (the limit of detection was calculated as average of the zero values ± three standard deviations of the zero values)

***Cross reaction with other Bt Cry proteins :*** ELISA kit developed was checked for cross reactivity with other Cry protein such as Cry2A, CP4EPSPS (RUR),and Cry1F. These three proteins provided in lyophilized form were initially reconstituted in phosphate buffered saline and then diluted to get concentration range from 100 ng/ml(nanogram) to 2 ng/ml. Cry1Ac/Ab recombinant antigen was used as positive control. OD values at 450nm were recorded. The OD values for Cry1Ac/Ab protein were observed at more than 2.0 and the OD values with other Cry proteins were observed below 0.1 which is similar to PBS blank. Therefore the monoclonal antibodies were Cry1Ac/Ab specific and there is no cross reactivity with other Cry proteins such as Cry2A,CP4EPSPS and Cry1F.

Limit of detection (LOD) was determined by plotting the O.D using standard curve.

| Standard value in ng/ml | Absorbance |
|---|---|
| 0 | 0.009 |
| 1.0 | 0.339 |
| 2.5 | 0.835 |
| 5.0 | 1.646 |
| 10.0 | 2.965 |

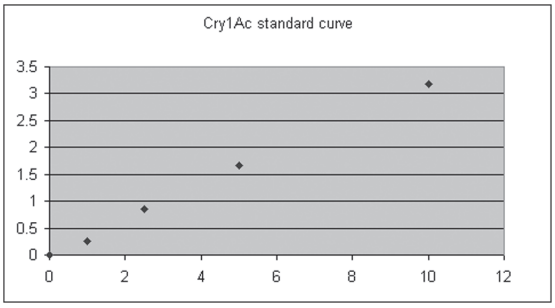


**Fig. 5**. Calculation of LOD

**Discussion:**

The objective of this study was to develop mAbs against the insecticidal protein Cry1Ac/Ab from B. thuringiensis and to design sandwich ELISA for the detection of Cry1AC/Ab proteinin in cotton seed and leaf samples.

Although many Bt proteins have been isolated and described (15), the protein which was selected for this study was of special interest in agriculture as biopesticides, particularly due to their use in transgenic plants (EPA, 2001). Only a limited number of insects are targeted whereas soil organisms (16, 17) and beneficial arthropods such as predators (18) or parasitoids (19) remain mostly unaffected. The Purity of the Cry1Ac/Ab

protein was checked by SDS-PAGE. SDS results showed that there is only one protein band visible indicating that the purity of the protein was more than 95%. It was considered as high purity and was used as immunogen to immunize the mice. Total of 4 mice were immunized using Cry1Ac/Ab antigen. The number of booster injections should be limited for the interests of the animal. Usually, a maximum of two or three booster injections are recommended (20). FCA should be used only once because repeated injection of FCA *(Mycobacteria* proteins) may lead to severe tissue reactions. FIA should be used for booster injections Mice with high titre value were selected for fusion.

The titer of anti Cry1Ac/Ab specific IgG antibodies in sera of the immunized mice were monitored throughout the immunization process to verify successful immunization and determine the titer values that would be useful for development of monoclonal antibodies. Titers were performed on the collected bleed from each animal using an indirect non competitive ELISA by coating recombinant antigen on solid phase. The intensity of the yellow coloured product was measured at 450-620nm directly corresponded to the amount of specific antibodies in the serum of each animal. Titration curve was generated using dilution factor on X axis and O.D values on Y axis. Mice with high titer values was selected for fusion. The hybridization or fusion process involves the fusion of mice splenic B cells with histocompatible myeloma cells, such as Sp2/0. Once these hybrid cells are formed and plated into tissue culture wells, the un fused myeloma cells are removed by using a selective medium containing hypoxanthine, aminopterin, and thymidine, known as “HAT. Some hybridomas are unstable and regress. Hence, careful attention should be given to the visual examination of hybridomas using an inverted microscope (21,22). Once Hybridomas are stabilized colony will grow unlimitedly in culture medium (such as RPMI- 1640 with antibiotics and fetal bovine serum) and produce antibody. After 15 days of fusion hybridomas can be propagated in “HT” medium (hypoxanthine and thymidine only) because aminopterin is no longer required (21,22).

Choosing the screening method for selecting the positive clones is very important and critical otherwise numerous unwanted hybridomas will compete for time and nutrients in terms of culture plates and medium (21,22). In this study all fusion plates were individually checked against recombinant antigen. After initial screening against recombinant antigen total 40 positive clones were noted which gave OD of more than 2.5 in ELISA. Those 40 clones were again checked against Cry protein. Based on O.D values observed in ELISA ,13 clones were selected and were expanded to T-24 folled by T-25 and T75 flasks. All clones were cloned twice to get pure single clone.

Isotype determination serves not only to define the immunoglobulin class or subclass but also helps identify the presence of a single isotype for example, IgG1 or a mixture, such as IgM and IgG2b (21).If an antibody is determined to be IgM, in isotyping it can not be purified through protein G or protein A coloumns. Isotyping requires the use of specific anti immunoglobulin antibodies that are capable of detecting the different classes and sub classes of monoclonal antibodies encountered in antibody production.

The up-scaling of MAb production is accomplished by injections of MAb-producing hybridoma cells in the abdominall cavity of mice and then by collecting the ascites that develops after the next 7 to 14 days. The abdominal cavity is serves as an optimal growth chamber for the hybridoma cells because it guarantees a constant temperature, an optimal nutrient and oxygen supply, and the optimal removal of CO2 and metabolic waste products (23). Ascites fluid is an intraperitoneal fluid collected from the mice peritoneal cavity due to development of peritoneal tumour. The hybridoma cells grow to high densities and continue to secrete the antibody. Mab’s were purified from ascites through protein G column and purity was checked by SDS-PAGE. Results indicates that the purity of all mAb’s were more than 95%.

In order to select appropriate mAb for solid phase and to make conjugates all were coated

on solid phase and labeled with HRP. It was conformed that a sandwich ELISA method could be more readily developed for detection of transgenic Bt cotton plants. ELISA developed is Simple qualitative ELISA where presence or absence of Cry1Ac protein can be detected in cotton seed and leaf samples. Results can obtained in 60 minutes. There was no issue of false positive and false negative with the results. To prove that certified known Cry1Ac positive and negative samples were tested by simultaneous comparison with commercially available kit.

The linear range of the assay is approximately 1 to 10ngml–1 protein concentration and the detection limit for purified Cry1Ac/Ab protein was determined as 0.51ngml–1. Since the limits of detection of other published immunoassays for Bt toxins are given in other units, for example ng toxin per microgram soluble protein (Vazquez et al., 1996) or ng of toxin perg dry weight soil (24) they are difficult to compare. However, the detection limit of the assay is in the range which can be expected for an sandwich-type enzyme-linked immunoassay. Based on the determination of three different Cry proteins,

Cross reactivity of all mAB's were checked against other Cry proteins like Cry2A and CP4EPSPS. No cross reactivity was observed.

**Summary**

In this study 13 different monoclonal antibodies against Cry1Ac protein were developed. When tested in ELISA all antibodies bound to recombinant Cry1Ac protein from *Bacillus thuringeneis* and natural Cry1Ac protein present in cotton leaf & seed. Isotyping of each antibody was done by using commercially available lateral flow strips from Envirologix and all were observed to be IgGk and IgG2bk.

In conclusion,monoclonal antibody based an ELISA for the detection of the B. thuringiensis toxin Cry1AC/Ab was developed. Furthermore, it was demonstrated that this assay can be used for the determination of the Cry1Ac/Ab protein in Bt cotton. No cross reactivity was observed with other Cry proteins such as Cry2A, CP4EPSPS and Cry1F.

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