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Production of Single Chain Fragment Variable (scFv) Monoclonal Antibody against Cry2B through Phage Display Technology

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Phage display technology was used to produce recombinant monoclonal antibody against *Bacillus thuringiensis* crystalline protein (Cry2B) from Tomlinson I antibody library. The polyclonal ELISA had revealed the fourth round of biopan shown highest specificity (1.480) and 92.5 folds greater than that of control. Total forty-five monoclonal antibodies were screened from fourth round of biopanning against Cry2B. Among forty-five clones, pscFvCry2B19 and pscFvCry2B43 had highest specificity against target. Further, the clones pscFvCry2B19 and pscFvCry2B43 were validated for their cross reactivity with other Cry proteins and the clones were sequenced with LMB3 forward and pHEN reverse primers. The scFv fragments of pscFvCry2B19 and pscFvCry2B43 were of 746 bp and 747 bp long respectively and almost similar to each other at DNA and amino acid level except at C' terminal end. The produced scFv monoclones have the potential to detect the Cry2B antigen.

Key words: Phage display technology; scFv; Biopanning; ELISA; Cry2B antigen; *Bacillus thuringiensis*.

Bacillus thuringiensis (*Bt*) is a Gram-positive spore forming soil dwelling bacteria with entomopathogenic properties. The bacteria produce toxic insecticidal proteins during the sporulation phase as parasporal crystals. These crystals are predominantly comprised of Cry proteins, also called δ-endotoxins which are highly selective against target insect, innocuous to human and plants[1]. Therefore, *Bt* spores are viable alternative to synthetic chemical pesticides for the control of insect pests in agriculture[2].

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The transgenic crops expressing *cry* gene product can be detected by use of molecular approaches such as polymerase chain reaction (PCR) and DNA hybridization. Immunological methods are very convenient and have the potential to be developed into a routine method for quantifying the presence of transgenes and its products in plants[3].

Immuno based diagnostic methods against Cry protein is simple, rapid and user friendly. The immunological methods include production of monoclonal antibody and/ or single chain fragment variable (scFv) against target molecule. The phage display technology is a path to develop the scFv antibody.

The phage display technology has been adopted for selection of proteins or peptides which has high affinity to almost any target molecules.

The phage display library was constructed using recombinant DNA technology where the scFv fused with one of phage coat protein (gIIIp) and displayed on phage surface[4]. One of the most successful applications of this technology is the isolation of monoclonal antibodies using phage antibody libraries[5]. A wide range of proteins and protein domains have been displayed on phage particles for carrying out directed evolution of molecules. For example, single binding domain for receptor[6], novel enzyme inhibitor[7], potential novel enzymes[8], targeting the liposomes[9], antibody specific for a enzyme[10,11] have been identified using phage display technology.

The scFv monoclonal antibody can be produced from phage display library by use of affinity selection process. During this process, the phage library is incubated with immobilized antigen. The unbound phages are discarded and the bound phages are eluted by triethylamine without impairing the phage property. The eluted phages are amplified by infecting an appropriate *E. coli* strain[12]. In each successive round of affinity process the proportion of number of strong binder is supposed to increase to reach the maximum concentration at the final round. The polyclones of each round were screened by polyclone phage ELISA with immobilizing antigen in microtiter plate. The scFv monoclonal antibodies are produced from randomly selected individual clones by affinity process which had indicated the highest polyclonal phage ELISA values[13, 14].

The present study the production of scFv antibody against one of the insecticidal protein, Cry2B by using phage display technology. The source scFv from Tomlinson library I was amplified and the scFv gene was expressed on the surface of phage with use of helper phage M13K07. Tomlinson library I was screened by series of affinity (biopanning) process and phages were revealed by ELISA test. This article reports the two monoclonal scFv antibodies for Cry2B was selected and their cross reactivity with other Cry proteins were analyzed with ELISA and the confirmed clones were selected and characterized.

MATERIALS AND METHODS

For the production of scFv antibody, the Tomlinson library and host strain *E. coli* TG1 was taken from MRC, Gene Service, (Cambridge, UK). Helper phage (M13K07) purchased from New England Biolab, UK, Maxisorp Nunc immunotubes and Nunc ELISA plates were purchased from Nunc, (Denmark). HRP (Horse radish peroxidase) conjugated anti M13 secondary antibody was purchased from Pharmacia, UK, The adjuvant polyethylene glycol (PEG) - 6000, bovine serum albumin (BSA), bacterial media components, antibiotics, components of PBS and PBST (Phosphate buffer saline + 0.05 % Tween-20) were purchased from Hi Media, (Mumbai, India).

Production of polyclones

The scFv antibody was produced according to Tomlinson library protocol available at www.geneservice.co.uk/products/proteomic/tomlinsonIJ with slight modification and described below.

Library selection on immunotubes

The commercially available phage display Tomlinson library (library size: 1.47×10^8 with 96% insert) was screened against immobilized Cry2B antigen in immunotube. The immunotube was coated with 80 µg/ml antigen in 4 ml of carbonate buffer (15.9 g/l disodium carbonate, 29.3 g/l sodium bicarbonate, pH 9.6) at room temperature incubated overnight (12 h). The following day, immunotube was rinsed thrice with 1X PBS (14.5 g/l sodium carbonate, 0.27 g/l potassium di-hydrogen phosphate, 1.41 g/l di-sodium hydrogen phosphate, 0.20 g/l potassium chloride, pH 7.4) and blocked for 2 h at room temperature with blocking solution (3% BSA in 1X PBS). The tubes were washed with 1X PBS thrice and 10^{12} phage particles in 4 ml of 3% BSA were added to the immunotube. The immunotube was first incubated on a shaker (Rocker-100, Labtech, UK) for 30 min and then for 90 min standing upright at room temperature. Unbound phage were washed away by rinsing the immunotube ten times with 1X PBST (1X PBS - 0.05 % Tween-20) and three times with 1X PBS. The bound phages were eluted by means of incubation of 1 ml 100 mM triethylamine for 5 min and the reaction was neutralized by 0.7 ml 1 M Tris–HCl pH 7.4.

Amplification of phage

The 750 µl of eluted phage was used to infect 9.25 ml of exponentially grown *E. coli* TG1 for 30 min at 37 ^{0}C water bath. The phage-infected bacteria were centrifuged for 10 min at 3,700 rpm at

4 °C. The pellet was dissolved in 1 ml of 2X TY (16 g/l bacto tryptone, 10 g/l yeast extract, 5 g/l sodium chloride pH 7.2) and the cells were spread on petri dishes of size 200 mm containing TYE agar (10 g/l bacto tryptone, 5 g/l yeast extract, 8 g/l sodium chloride, 15 g/l agar agar type-I, pH 7.2), 100 µg/ml ampicillin, 1% glucose (TYE-Amp-Glu) and incubated for overnight at 37 °C. The cells were rescued with 3ml 2X TY, 1% glucose, 15% glycerol and stored at -80 °C. The 100 µl of rescued bacteria was grown to 0.4 OD stage in 100 ml of 2X TY with 100 µg/ml ampicillin and 1% glucose (2X TY-Amp-Glu). Of this culture, 10 ml was infected with M13K07 at the concentration of $0.5x10^{10}$ for 30 min at 37 °C. The cells were centrifuged at 3,500 rpm for 30 min at 4 °C and the pellet was resuspended in 100 ml of 2X TY broth with 100 µg/ml ampicillin, 1% glucose and 25µg/ml kanamycin. After overnight incubation at 37 °C, the supernatant was isolated at 10,800 rpm for 10 min at 4 °C and incubated on ice with 20 ml of 20% PEG/2.5 M NaCl for 1.5h. The mixture was centrifuged at 7,000 rpm for 20 min and the pellet was dissolved in 2 ml of 3% BSA in 1X PBS buffer. Again PEG/NaCl was added at one-fifth of the total volume and incubated on ice for 1 h, then centrifuged at 7,000 rpm for 20 min. The pellet was resuspended in 2 ml of 3% BSA in 1X PBS buffer and filtered using 0.45 µm syringe filter. These phages were used for further round of biopan at the $1x10^{12}$ concentration. So on up to four rounds of biopan were carried out.

Enzyme linked immunosorbent assay

The affinity of each biopan against Cry2B was tested through polyclonal phage ELISA. The ELISA plate was overnight (12 h) incubated with Cry2B at the concentration of 80 µg/ml in 200 µl of carbonate buffer at 37 °C. The following day, the wells were rinsed thrice with 1X PBS and blocked with 300 µl of blocking solution for 2 h at 37 °C. They were later washed thrice with 1X PBS and the PEG-NaCl precipitated phage particles from each biopan added to microplate wells at $1x10^{12}$ concentration along with 200 µl of 3 % BSA in 1X PBS. After 90 min incubation at 37 °C the nonspecific phages were removed by washing thrice with 1X PBST and followed by washing thrice with 1X PBS. The 300 µl of anti M13 HRP conjugate was brought in contact with phage -Cry2B complexes and incubated for 90 min at 37 °C. The unbound secondary antibodies were removed by washing thrice with 1X PBST and excess detergent was removed by washing with 1X PBS. Later, 100 µl of 1X concentration of TMB substrate was added to each well and incubated for 60 min in the dark. The reaction was stopped using 50 µl of 1M H_2SO_4 and the reading was taken at 450 nm in a microtiter plate reader.

Production and screening of monoclonal scFv

The fourth biopan had highest ELISA value which was subsequently used to produce the monoclonal antibody. The forty-five clones were randomly picked from fourth biopan and the phages were isolated with the help of M13K07 helper phage (see amplification of phage section) and ELISA test was performed to detect phages with monoclonal ELISA (see ELISA section).

Cross specificity

The clones *viz*., pscFv*Cry*2B19 and pscFvCry2B43 gave the highest reading against Cry2B which were validated by cross specificity with mutant Cry2B proteins provided by Dr. P.U.Krishnaraj. The three Cry2B random mutant proteins *viz.*, Cry2B clone-10, Cry2B clone-14 and Cry2B clone-15 along with native Cry2B were diluted in 200 µl of carbonate buffer at 80 µg/ml concentration and overnight incubated in microtiter (ELISA) plate wells at 37 °C along with a batch of control. The cross reactivity was evaluated by ELISA as in polyclonal phage ELISA (see ELISA section).

Sequence analysis

The phagemid was isolated from the clones (pscFvCry2B19 and pscFvCry2B43) according to the Sambrook & Russell[15]. The insert scFv gene was sequenced from its phagemid vector pIT2 by using LMB3 forward and pHEN reverse primers. The homology at nucleotide level was analysed using BLASTn algorithm and conserved domains of proteins were searched in NCBI conserved domain search tool.

RESULTS

Production of polyclonal scFv using affinity process

The success of phage display selection mainly depends upon the quality of the original library used[16,17,18]. In this study, Tomlinson library I was used to screen against Cry2B antigen through biopanning process.

In present study, we used phage display technology for production of scFv monoclonal antibody. The concentration of phages ranged from 7.69 x 10^{10} to 1.03 x 10^{11} during biopanning stage first to fourth respectively (Table 1). During the affinity process the proportion of strong binder was expected to increase in each successive round. At the end of fourth biopan the concentration of phage increased about 13 times when compared to first biopan. A similar result was observed by Griep[19] in *Ralstonia solanacearum* race-3 during selection of LPS-binding clones from combinatorial library. The stringency of scFv to the antigen was gradually increased 100 to 1000 fold from biopan I to biopan IV[19]. In the present study, the fourth biopan showed maximum phage concentration which was subsequently used to produced scFv monoclones.

Stringency of scFv-phage revealed by ELISA

All four biopan phages developed against Cry2B were subjected to polyclonal phage ELISA. The ELISA absorbance values indicated that the fourth biopan phage had higher affinity (1.480; Fig. 1) with antigen. Similarly, Goldman[20] (2000) had reported that the biopan selection was repeated two to three times to achieve higher affinity scFv phages. The forty-five scFv phage were randomly selected from fourth biopan and subjected for monoclonal phage ELISA. Among forty-five scFv clones, the two scFv clones *viz*., pscFvCry2B19 and pscFvCry2B43 had higher absorbance value 1.291 and 1.023 respectively (Fig. 2) than others. A similar result was observed by Francoise[21], who screened 120 scFv monoclonal phages against carrageenan polysaccharide. Of 120 scFv phages, the 25 scFv phage clones showed

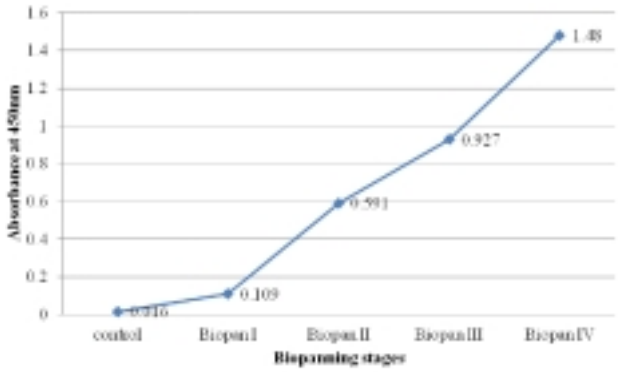

Fig. 1. The ELISA absorbance values of polyclonal scFv phages against Cry2B protein revealed that the stringency of scFv was steadily increased from biopan I to biopan IV for Cry2B-BL.

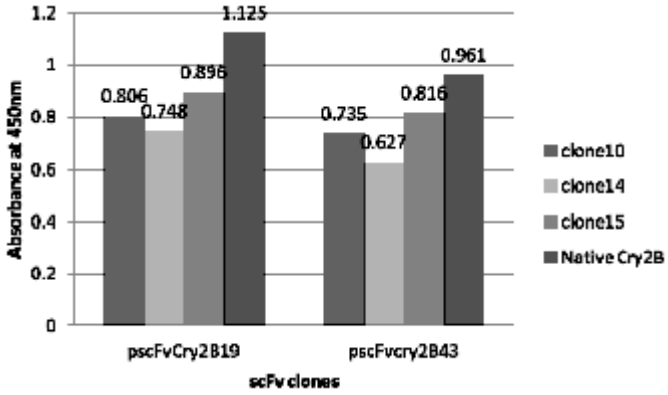

Fig. 2. Monoclonal scFv stringency revealed by monoclonal phage ELISA reading at 450nm for Cry2B. The clone number 19 and 43 had highest ELISA absorbance value (1.291 and 1.023 respectively)

ELISA value greater than 1.5 and 12 scFv phage clones showed ELISA value greater than 2.0.

Specificity of scFv monoclones

The ability of pscFvCry2B19 and pscFvCry2B43 monoclonal antibody to detect the Cry2B antigen was evaluated by ELISA with other Cry2B mutant proteins (mutant Clone-10, mutant clone-14 and mutant clone-15). The antigen Cry2B was detected at 80 µg/ml[22] concentration when compared to other Cry mutant proteins. In this assay, the monoclones pscFvCry2B19 and pscFvCry2B43 captured the Cry2B native protein at $1x10^{12}$ concentrations. The selected phages pscFvCry2B19 and pscFvCry2B43 have shown highest binding affinity of 1.125 and 0.961 respectively (Fig. 3) against native Cry2B than mutant Cry2B proteins. The results indicate that the selected scFv phages have higher affinity to native Cry2B.

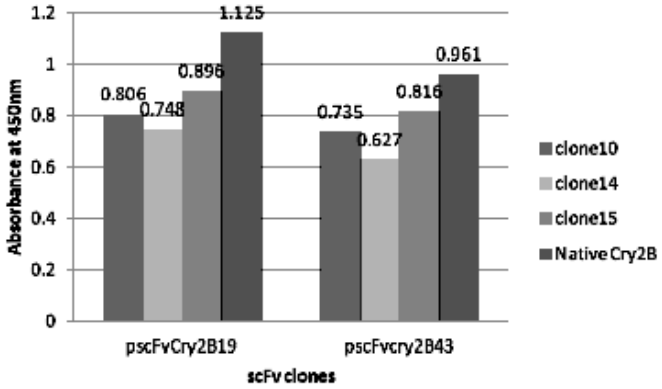

Fig. 3. Validation of pscFvCry2B19 and pscFvCry2B43 for Cry2B antigen by ELISA test absorbance at 450 nm. (The pscFvCry2B19 and pscFvCry2B43 had highest affinity with native Cry2B protein than Cry2B random mutant proteins *viz.*, clone-10, clone-14 and clone-15).

Fig. 4. Monoclonal antibody structure. The underlined aminoacid sequences are belongs to variable region of heavy (H) and light (L). (A) pscFvCry2B19 and (B**).** pscFvCry2B43. The bold and non-italic letters depicted in the figure is different aminoaicd sequences present at C' terminal end of the both clone.

Sequence analysis

Both the clones pscFvCry2B19 and pscFvCry2B43 were sequenced with LMB3 forward and pHEN reverse primer. The assembled sequence of pscFvCry2B19 and pscFvCry2B43 were 746 bp and 747 bp long respectively. At nucleotide level of both the clones were found almost similar except few nucleotides near 3' end and amino acid sequence of both the clones were similar except 10 amino acids at C' terminal end. The homology search was made at BLASTn algorithm. Both the clones shared maximum identity of 96% with *Homo sapiens* partial scFv gene with 0.0 E values and the conserved domains of both the proteins were analyzed. The pscFvCry2B19 protein have the conserved sequence domain in Ig super family (Ig immunoglobulin domain) between 47 and 119 position and immunoglobulin (Ig) light chain, kappa type variable (V) domain (IgV_L_kappa) from 139 and 243. Similarly the pscFvCry2B43 have shown homology between 47 and 119 of Ig super family (Ig immunoglobulin domain) and the region from 139 to 237 found homology in immunoglobulin (Ig) light chain, kappa type variable (V) domain (IgV_L_kappa).

DISCUSSION

Phage display technique has been successfully attempted in producing monoclonal antibodies against toxins, hormones, growth factors, chemical, protein, enzyme, viruses, fungi and bacteria. Thus, recombinant scFv antibodies are expected to serve as a powerful tool for a proteomic research[23].The success rate of monoclonal antibodies production is hundred per cent by using phage display technique in many research reports[24, 25, 21]. In this study, panel of single chain fragment variable monoclones against Cry2B and monoclonal antibody gene responsible for Cry2B were characterized. The monoclonal antibodies will be useful for developing a diagnostic kit for identification of transgenic crops carrying Cry2B gene.

In the present study, polyclonal antibody were generated against Cry2B protein. Four rounds of biopanning were carried out during the process, population of phage in each biopan contained strong and weak binders and the proportion of strong binder was expected to increase in each biopan. The yield and concentration of scFvCry2B obtained at the end of each round of biopanning ranged from 7.69×10^{10} to 1.03×10^{11}. During the process of biopanning week binders get eliminated and the concentration of strong binder antibodies increased. Use of pure form of antigen (Cry2B protein) is critical and essential to achieve monoclones with highest specificity. As the stage of biopan advanced the ELISA reading for Cry2B increased as expected .

Increase in the number of antigen specific scFv phages were identified by steady increase in absorbance after each round of panning. The titer of eluted phage was measured to monitor the efficiency of the selection process. In Cry2B almost hundred times increase in phage recovery after third round of panning when compared to second round of biopanning and a minimal increase in fourth round of panning over the third round. The phage population indicated that the fourth round of panning was almost equivalent population size as compared to third round because number of strong binders reached the highest population, which means scFv antibodies already enriched with specific antigen. These enriched scFv antibodies were confirmed by polyclonal phage ELISA in which the fourth panning showed higher value than previous panning process even when same

Table 1. Quantification of polyclonal phages by spectrophotometer at 269 nm and 320 nm at the end of each biopan for Cry2B antigen

Sl. No.	Biopan stages	Absorbance at 269 nm	Absorbance at 320 nm	Concentration Phage/µl
1	Biopan I	0.124	0.015	7.69×10^{10}
2	Biopan II	0.137	0.017	8.47×10^{10}
3	Biopan III	0.170	0.023	1.03×10^{11}
4	Biopan IV	0.164	0.021	1.00×10^{10}

concentration of phages ($1x10^{12}$/well) were used. Similar result was reported by Griep[19] for selected scFv antibody against lipopolysaccharide of *Ralstonia solanacearum* Race-3. The phages obtained from IV panning were used to generate the monoclones for Cry2B.

The fourth round polyclone of Cry2B was subsequently used to generate the monoclones antibodies. Forty-five clones were randomly picked from IV round panning. The phage particle from each clone was isolated. The concentrations of all forty-five clones were measured by use of spectrophotometer. The phage concentration of $1x10^{12}$ was added to ELISA plate wells in which the antigen was overnight coated with carbonate buffer. Out of forty-five, two clones namely pscFvCry2B19 and pscFvCry2B43 had shown the highest value of 1.291 and 1.023, respectively. Similar result was observed by Francoise[21], who screened monoclonal antibodies against carrageenan which is a polysaccharide. The phage isolated from 120 clones was subjected to monoclonal ELISA and each scFv showed different absorbance against the carrageenan. Lower reading has indicated the lower affinity to the antigen whereas higher absorbance value showed the stronger binding to target molecule (carrageenan). The selected clones (pscFvCry2B19 and pscFvCry2B43) were cross checked with three random mutant Cry2B proteins. The result indicated the developed scFv monoclonal antibody identified the Cry2B at higher level, simultaneously it also identified the random mutant version of Cry2B because there is very small difference between the mutant version and native Cry2B protein. It confirms us that the developed scFv monoclonal antibody has strong affinity to the Cry2B protein.

The both clones were sequenced and result had indicated that the conserved domain of both proteins has similar domains that bind to Cry2B. The amino acid sequence of both protein was almost similar expect C-terminal 10 amino acid sequences (Fig.4a and Fig. 4b). Based on this, it is predicted that the both scFv antibody bind to a single epitope.

In conclusion, the isolated scFv antibodies were able to detect the Cry2B protein expression. Both the clones pscFvCry2B19 and pscFvCry2B43 have shown higher affinity with target Cry2B antigen. As a future line of work, the protein scFv antibody can be expressed and isolated in soluble form in suitable host. This protein will be used for development of Cry2B diagnostic kit for rapid, economy and user friendly detection of Cry2B protein in plant samples.

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REFERENCES

1. Bravo, A., Sarjeet, S. G., Mario, S., Mode of action of *Bacillus thuringiensis Cry* and *Cyt* toxins and their potential for insect control. *Toxicon,* 2007; **49**: 423–435.
2. Bravo, A., Gill, S. S., Soberon, M., *Bacillus thuringiensis* mechanisms and Use. *Compre. Mol. Insec. Sci.,* 2006; **6**:175-206.
3. John, F., Monitoring GMOs released into the environment and the food production system. *Biosafety I.* Traavik, T. and Lim, L.C. (eds.), (Tapir Academic Publishers), 2007, 1-27.
4. Smith, J., Kontermann, R. E., Embleton, J., Kumar, S., Antibody phage display technologies with special reference to angiogenesis. *FASEB J.*, 2005; **19**: 331-341.
5. Winter, G., Griffiths, A. D., Hawkins, R. E., Hoogenboom, H. R., Making antibodies by phage display technology. *Annu. Rev. Immunol.,*1994; **12**: 433-455.
6. Lowman, H. B., Steven, H. B., Nancy, S., James, A. W., Selecting high-affinity binding proteins by monovalent phage display. *Biochem.*, 1991; **30**: 10832–10838.
7. Roberts, B. L., Markland, W., Ley, A. C., Kent, R. B., White, D. W., Guterman, S. K., Ladner, R. C., Directed evolution of a protein: selection of potent neutrophil elastase inhibitors displayed on M13 fusion phage. *Proc. Natl. Acad. Sci.*, 1992; **89**: 2429-2433.
8. Soumillion P, Jespers L, Bouchet M, Marchand-Brynaert J, Sartiaux P, Jacques, F., Phage display of enzymes and in vitro selection for catalytic activity. *Appl. Biochem. Biotechnol.*, 1994; **47**(2-3): 175-190.
9. Jayanna, P. K., Torchilin, V. P., Petrenko, V. A., Liposomes targeted by fusion phage proteins.

Nanomed., 2009; **5**: 83-89.

10. McCafferty, J., Griffiths, A. D., Winter, G., Chiswell, D. J., Phage antibodies: filamentous phage displaying antibody variable domains. *Nature*, 1990; **348**: 552-554.
11. Clackson, T., Hoogenboom, H. R., Griffiths, A. D., Winter, G., Making antibody fragments using phage display libraries. *Nature*, 1991; **352**: 624-628.
12. Paola, N., Naoko, S., Susumu, H. T., Kentaro, T., Hideyuki, A., Toshiyasu, S., Yasushi, A., Yoshikazu, K., Takao, T., Single chain variable fragment antibodies against Shiga toxins isolated from a human antibody phage display library. *Vaccine*, 2001; **29**: 5340– 5346.
13. Kretzschmar, T., von Rüden, T., Antibody discovery: phage display. *Curr. Opin. Biotechnol.*, 2002; **13**: 598-602.
14. Smith, G. P., Petrenko, V. A., Phage display. *Chem. Rev.*, 1997; **97**: 391-410.
15. Sambrook, J., Russell, D. W., Molecular cloning. A laboratory manual, 3rd edn. cold spring harbor laboratory press, *Cold Spring Harbor*, New York, 2001.
16. Zwick, M. B., Shen, J., Scott, J. K., Phage-displayed peptide libraries. *Curr. Opin. Biotechnol.*, 1998; **9**: 427-436.
17. Brown, K. C., New approach for cell-specific targeting: identification of cell-selective peptides from combinatorial libraries. *Curr. Opin. Chem. Biol.*, 2000; **4**: 16-21.
18. Romanov, V. I., Phage display selection and evaluation of cancer drug targets. *Curr. Cancer Drug Targets*, 2003; **3**: 119-129.
19. Griep, R. A., Van Twisk, C., Beckhoven, V. J., Wolf, J. M., Schots, A., Development of specific recombinant monoclonal antibodies against the lipopolysaccharide of *Ralstonia solanacearum* Race 3. *Phytopatho.*,, 1998; **88**: 795-803.
20. Goldman, E. R., Pazirandeh, M. P., Mauro, J. M., King, K. D., Frey, J. C., Anderson, G. P., Phage-displayed peptides as biosensor reagents. *J. Mol. Recognit.*, 2000; **13**: 382-387.
21. Francoise, L., William, H., Pierre, V. C., Production and characterization of a phage-display recombinant antibody against carrageenans: evidence for the recognition of a secondary structure of carrageenan chains present in red algae tissues. *Glycobiol.*, 2005; **15**: 849-860.
22. Zu-Quan, H., Jin-Long, L., He-Ping, L., Shu, X., Sheng, X., Jing-Bo, Z.., Jian-Hua, W., Greta, N., Yu-Cai, L., Generation of a highly reactive chicken-derived single-chain variable fragment against *Fusarium verticillioides* by Phage Display. *Int. J. Mol. Sci.*, 2012; **13**: 7038-7056.
23. Geuijen, C. A., Clijsters-van der, H. M., Cox, F., Rood, P. M., Throsby, M., Jongeneelen, M. A., Backus, H. H., van, D. E., Kruisbeek, A. M., Goudsmit, J., de Kruif, J., Affinity ranking of antibodies using flow cytometry application in antibody phage display based target discovery. *J. Immunol. Methods*, 2005; **302**: 68-77.
24. Griffiths, A. D., Williams, S. C., Hartley, O., Tomlinson, I. M., Waterhouse, P., Crosby, W. L., Kontermann, R. E.,Jones, P. T., Low, N. M., Allison, T. J., Isolation of high affinity human antibodies directly from large synthetic repertoires. *J. EMBO*, 1994; **13**: 3245-3260.
25. Anderson, M. R., Jinnny, L., Hayhurst, A., Ellen, R., Goldman, Multiplexed fluid array screening of phage displayed anti-ricin single domain antibodies for rapid assessment of specificity. *Biotechniques*, 2007; **43**: 806-811.