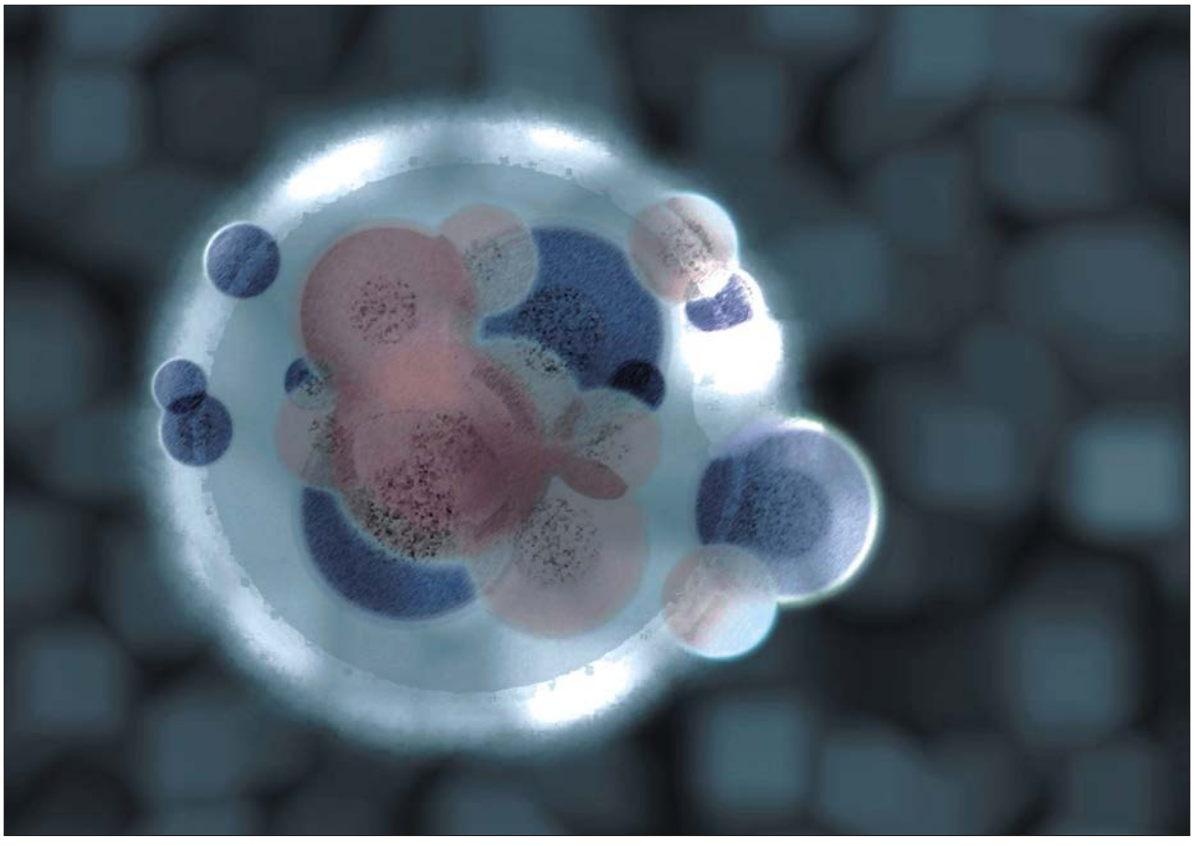

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Development of a Single Chain Variable Fragment Antibody Combinatorial Library Through a Simple Three Fragment Ligation Strategy

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Abstract: A strategy to construct a single chain variable fragment antibody combinatorial library was carried out through three fragment ligation using self-designed primers. Sequence information of antibody variable heavy and light fragments was used to design 2 sets of primers with the appropriate restriction endonuclease sites to facilitate the clone construction. This alternative approach allows construction of single chain variable fragment (scFv) phage display library through a simple three-fragment ligation process and allows inclusion of any desired detection marker at the same time.

Key words: Combinatorial library, scFv, primer design, PCR

INTRODUCTION

Recombinant phage antibody technology can be used for a reliable and cost effective antibody production. This technique can not only produce antibodies necessary for use in ELISA assays, but also can produce high specificity antibodies necessary for use in Western blot tests. Overall, this technique utilizes hybridomas or B lymphocytes as a primary source of specific antibody genes for cloning. In this study, the spleen tissue from a mouse immunized with *cucumber mosaic virus* coat protein was used to synthesize the variable heavy and variable light chains of the antibody.

Both of the immunoglobulin variable heavy (V_H) and light (V_L) chains are the main components for single chain variable fragment (scFv) construction. The DNA fragments of the two chains can be obtained via PCR using a framework region primer mix, which is relatively conserved for a particular animal used. Constructing an anti-plant-virus scFv antibody was the focus of this study which seeks to simulate the natural antibody folding and binding strategies by applying molecular techniques on synthesized V_H and V_L DNA fragments (Zeng *et al*., 2000). The V_H and V_L chain DNA fragments are joined together via synthetic peptide linkers that retain a similar functional structure as an antibody molecule. The smaller size of the scFv molecule compared to whole antibody molecule makes it easier to develop, manipulate and utilize for various applications. It can be easily inserted into various biological systems including plants as bio-factories for production of the novel antibodies (Chua *et al*., 2006). It can also be used as a delivery system for reporter genes to target cells (Gupta *et al*., 2001), for recognition of viruses (Nagesha *et al*., 2001; Long *et al*., 2001), for blocking of parasite transmission (Shigeto *et al*., 2001), neutralization of toxins (Cardoso *et al*., 2000; Devaux *et al*., 2001) and as anti-tumor agents (Rosenblum *et al*., 2000; Vitaliti *et al*., 2000).

Prior to screen for specific anti-CMV scFv that binds to CMV coat protein in present study, construction of a good scFv combinatorial library is very important. Generally, only the methodology of construction of a scFv combinatorial library through a simple fragment ligation bypassing standard overlapping Polymerase Chain Reaction (PCR) will be described here. Following that, analysis of the scFv combinatorial library to confirm the integrity of the scFv combinatorial library developed will also be discussed.

MATERIALS AND METHODS

V_H and V_L DNA fragment synthesis: A purified cucumber mosaic virus coat protein was used for mouse immunization. Approximately 1 mg of total RNA was extracted from 30 mg spleen tissue using the RNeasy Mini Kit (QIAGEN), followed by mRNA isolation using Oligotex™. Twenty five μg of the mRNA was obtained through this method. First strand cDNA synthesis was carried out in 2 separate microfuge tubes using approximately 500 ng of the mRNA as the template prior to amplification of the V_H and V_L chains by PCR. Initially the RNA was heated at 65°C for 10 min followed by

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Table 1: Sequences of designed primers

Primer 1: V_H (Forward)

5'-AAGGAAAAAAGGCCCAGCCGGCC $_{ATG}$ ***GTSMARCTGCASAGTCWGCAMCTGA-3'***

Primer 2: V_H (Reverse)

5'-CGGGCGGCGG TCCGGA TCCACCTCCGCCTGAACCGCCTCCACCT ***AGGAGACGGTGACCGTGGTCCCTTG-3'***

Primer 3: V_L (Forward)

5'-CGGGCGGCGG TCCGGA GGTGGCGGTTCG ***SAAAWTGTKCTCACCCAGTCTCCAGCAATC-3'***

Primer 4: V_L (Reverse)

5'-AAGGAAAAAAgcggccgc **GTCGAC CTTGTCATCGTCGTCCTTGTAGTC GACCCGTTTBAKYTCCAGCTTRGTSCCCCC-3'**

(Ten nonsense bases are shown in the black block. Bases in small font are the added start codon. Bases in the grey block are the synthetic peptide sequences. The single underlined bases contains *Sfi* I restriction endonuclease site. The double underlined bases contains *Bsp*E I endonuclease site while the bases in small letter contains the *Not* I restriction endonuclease site. The bases in bold are the FLAG-tag sequences for detection purposes. The bases in italic are the framework regions of the V_H and V_L chains, which were obtained from the sequence analysis)

immediate cooling on ice. To the template, 11 μL of the Primed First-Strand Mix (Amersham Pharmacia), 1 μL of the DTT solution and RNase-free water were added in a total volume of 33 μL. Duplicate mixtures in 2 separate microfuge tubes were then incubated at 37°C for 1 h to form the first-strand antibody cDNA. Following that, to the first microfuge tube, 2 μL each of the Heavy Primer 1 and Heavy Primer 2 (Amersham Pharmacia), 1 μL of Taq DNA polymerase (Roche) and 62 μL of sterile distilled water were added while to the second microfuge tube, 2 μL of the Light Primer Mix (Amersham Pharmacia), 1 μL of Taq DNA polymerase and 64 μL of sterile distilled water were added for heavy and light chain PCR amplifications, respectively. PCR was carried out in an Eppendorf 5330 thermocycler (95°C for 5 min, 1 cycle; 94°C for 1 min, 55°C for 2 min, 72°C for 2 min, 30 cycles; 72°C for 10 min, 1 cycle).

Joining variable heavy (V_H) and variable light (V_L) DNA fragments: An alternative approach using restriction digestion followed by ligation was carried out for linking of the two fragments. Basically, the V_H and V_L DNA fragments were ligated separately into pGEM®-T Easy vector (Promega) and then transformed into *E. coli* strain DH5α. Recombinant samples containing insert DNA were subjected to automated sequencing (ABI 377, AMCAL, University of Malaya, Malaysia). Based on the sequence information obtained, 2 sets of self-designed primers containing part of the linker peptide at the framework regions were synthesized (Table 1) for each chain. PCR was performed for V_H and V_L DNA fragment amplification separately using the appropriate set of primers. Again, the template used for the PCR was the first strand cDNA synthesized from mRNA of the immunized mouse spleen with CMV coat protein. After obtaining the new V_H and V_L DNA fragments, the V_H DNA fragments were digested with restriction endonucleases, *Sfi* I and *Bsp*E I (New England Biolabs) resulted in ≈400 bp fragments while the V_L DNA fragments were digested with *Not* I and *Bsp*E I resulted in ≈377 bp fragments (data not shown). Three DNA fragment ligation was performed in 20 μL solution containing 50 ng of each digested V_H DNA fragment, V_L DNA fragment and pCANTAB 5E vector. The ligation was carried out in a 16°C refrigerated water-bath for 16 h.

Colony hybridization: The bacterial colonies were transferred to a positive-charged nylon membrane (Roche) by placing the membrane directly on the colony surface. The membrane, colony side up, was then placed onto a piece of Whatman 3 MM paper wetted with solution A (0.5 M NaOH) for 5 min. After that, the membrane was transferred onto a fresh piece of Whatman paper wetted with solution B [1.5 M NaCl, 0.5 M Tris-Cl (pH 7.4)] for 5 min followed by being placing onto a new piece of Whatman paper wetted with solution C (1.5 M NaCl, 2X SSC) for 5 min. The membrane was then baked at 65°C for 30 min and the DNA on the membrane was fixed under 1.5 J cm^{-2} UV for 3 min. Hybridization was carried out using DIG-labeled VH and VL DNA probe as described in Sambrook *et al*. (1989).

PCR analysis: The PCR reaction was carried out with Taq DNA polymerase (Roche). Generally, the reaction mix of the PCR was prepared to a final volume of 50 μL in 0.5 mL thin wall tubes. The reaction mixture contained 1X reaction buffer, 0.3 mM dNTP mix, 1U Taq DNA polymerase, 1.0 mM $MgCl_2$, 1 μM upstream and downstream primers, 20 ng template DNA and nuclease-free water. The reactions were placed in a PCR thermocycler (Eppendorf 5330). The PCR program used was 94°C for 1 min (1 cycle); 94°C for 1 min; 55°C for 1 min; 72°C for 1 min (30 cycles); 72°C for 10 min (1 cycle).

RESULTS

Design of new VH and VL primers for scFv library construction: An approximately 356 bp V_H DNA fragment and 321 bp V_L DNA fragment were amplified when PCR was performed on cDNA from mouse spleen total RNA with heavy primer 1, 2 and light premix primer, respectively, the results are shown in Fig. 1. The V_H and V_L DNA fragments were cloned separately into pGEM T easy vector and transformed in to *E. coli* JM109. Nineteen of the V_H-pGEMT recombinant clones and 15 of V_L-pGEMT recombinant clones were randomly selected and subjected to confirmatory sequencing. Overall, no deletion was detected in all the sequenced V_H clones and the majority of the V_L clones have the complete DNA sequence which was ≈321 bp. However, some incomplete sequences were detected in the V_L clones in parts of the framework region. Samples with uncertain extra nucleotides and ambiguity nucleotides appearing in the framework region were not considered for primer design. The analysis results are shown in Fig. 2-5.

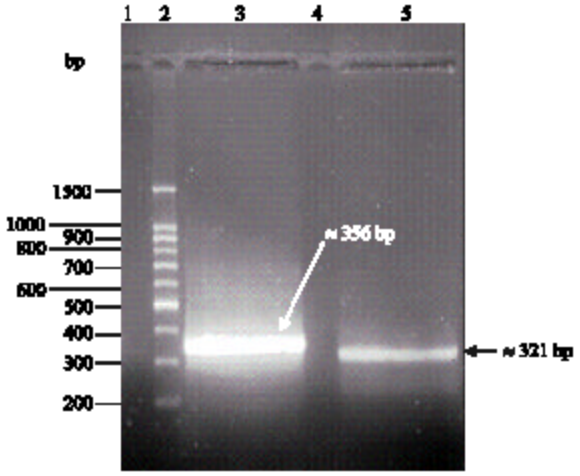

Fig. 1: PCR of variable heavy (V_H) and light (V_L) chain genes using first-strand cDNA as template. Negative control sample where PCR was performed in distilled water as the template using heavy primer 1 and 2 (lane 1), 100 bp DNA ladder (Promega) (lane 2), PCR product which was performed on cDNA converted from mouse spleen total RNA with heavy primer 1, 2 (lane 3), PCR sample in distilled water as the template using light primer mix (lane 4) and PCR product using cDNA from mouse spleen total RNA with V_L primer mix (lane 5) were separated on a 1% agarose gel followed by ethidium bromide staining. Approximate 356 bp amplified V_H DNA fragment is shown by the white arrow while black arrow shows the ≈321 bp amplified V_L DNA fragment

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VH1  : AGGTCAAGCTGCAGCAGTCTGGAACTGAAGTGG : 33
VH2  : AGGTGCAACTGCAGCAGTCTGGAACTGAAGTGG : 33
VH3  : AGGTGAAGCTGCAGCAGTCAGGACTGAGCTGG : 33
VH4  : AGGTGAAACTGCAGCAGTCTGGAACTGAAGTGG : 33
VH5  : AGGTCCAGCTGCAGCAGTCAGGACTGAGCTGG : 33
VH6  : AGGTGAAACTGCAGCAGTCAGGACTGAGCTGG : 33
VH7  : AGGTCAAGCTGCAGCAGTCAGGACTGAGCTGG : 33
VH8  : AGGTGCAGCTGCAGCAGTCTGGACTGAGCTGG : 33
VH9  : AGGTCAAACTGCAGCAGTCAGGAACTGAACTGG : 33
VH10 : AGGTCAAACTGCAGCAGTCTGGAACTGAAGTGG : 33
VH11 : AGGTGAAGCTGCAGCAGTCAGGAACTGAAGTGG : 33
VH12 : AGGTGAAGCTGCAGCAGTC GGACTGAGCTGG : 33
VH13 : AGGTCAAGCTGCAGCAGTC GGAACTGAAGTGG : 33
VH14 : AGGTCCAACTGCAGCAGTCAGGAACTGAAGTGG : 33
VH15 : AGGTGAAGCTGCAGCAGTCTGGACTGAGCTGG : 33
VH16 : AGGTCCAACTGCAGCAGTCTGGAACTGAAGTGG : 33
VH17 : AGGTGCAACTGCAGCAGTCAGGAACTGAAGTGG : 33
VH18 : AGGTGAAGCTGCAGCAGTCAGGACTGAGCTGG : 33
VH19 : AGGTGCAGCTGCAGCAGTCTGGACTGAGCTGG : 33
New VH degenerate forward primer sequence :
-- GTSMARCTGCAGSAGTCWGGAMCTGARSTGG-
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Fig. 2: Analysis of V_H chains forward sequence

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VH 1  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 2  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 3  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 4  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 5  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 6  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 7  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 8  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 9  : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 10 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 11 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 12 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 13 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 14 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 15 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 16 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 17 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 18 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
VH 19 : TGAGGAGACGGTGACCGTGGTCCCTTG : 27
New VH reverse primer sequence:
TGAGGAGACGGTGACCGTGGTCCCTTG
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Fig. 3: Analysis of V_H chains reverse sequence

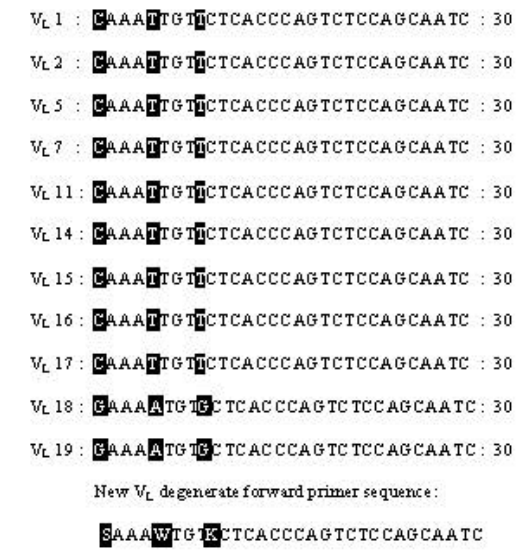

Fig. 4: Analysis of V_L chains forward sequence

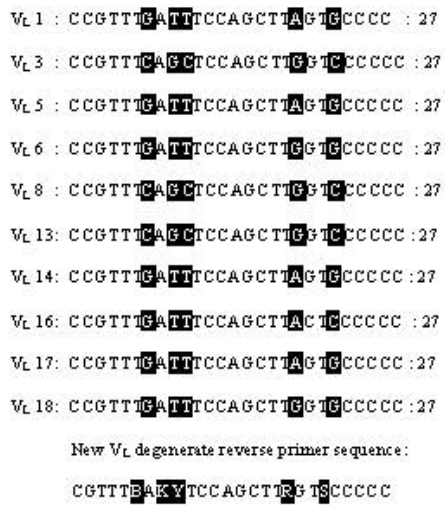

Fig. 5: Analysis of V_L chains reverse sequence

Translation analysis using TRANSLATE program shows that the 5' 3' frame 3 of the V_H and 5' 3' frame 1 of the V_L nucleotide sequence is in the correct frame without any pseudogenes detected (data not shown). The first 2 nucleotides at the 5' end of the V_H chain were not included in the primer design to maintain in-frame translation. Both the V_H, V_L and linker sequences were also subjected to Webcutter 2.0 for restriction endonuclease site analysis and the output shows that *Sfi* I and *Not* I sites could not be found in V_H and V_L chain nucleotide sequence (results not shown) and could be used for cloning. In addition, the *Bsp*E I restriction

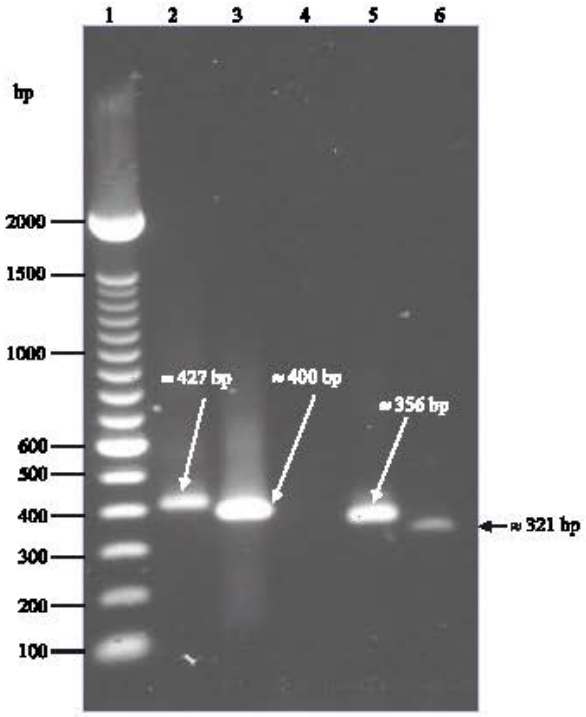

Fig. 6: PCR products obtained using commercial and self-designed primers. One hundred bp ladder (Gibco-BRL) (lane 1), V_H chain PCR product obtained through PCR with mouse spleen cDNA using V_H (Forward)/V_H (Reverse) new primers (lane 2), V_L chain PCR product obtained through PCR with mouse spleen cDNA using V_L (Forward)/ V_L (Reverse) new primers (lane 3), negative control where distilled water was used as the template and 4 self-designed primers V_H (Forward)/V_H (Reverse) and V_L (Forward)/V_L (Reverse) are used for PCR (lane 4), V_H PCR product obtained using V_H primer mix (Amersham Pharmacia) (lane 5) and V_L PCR product obtained using V_L primer mix (Amersham Pharmacia) (lane 6) were separated on 1% agarose gel. The sizes of V_H and V_L DNA fragments amplified using self-designed primers (lane 2 and 3) are slightly larger when compared to the V_H and V_L DNA fragments amplified using commercial primers (lane 5 and 6) as shown in this figure

endonuclease site, which could be found in the linker sequence was not detected in the V_H and V_L chain DNA fragments. Therefore, this restriction site could be included in the peptide linker to facilitate joining of the two chains. Overall, the new V_H and V_L fragments obtained after PCR (Fig. 6) followed by specific restriction enzymes digestions are summarized in Fig. 7.

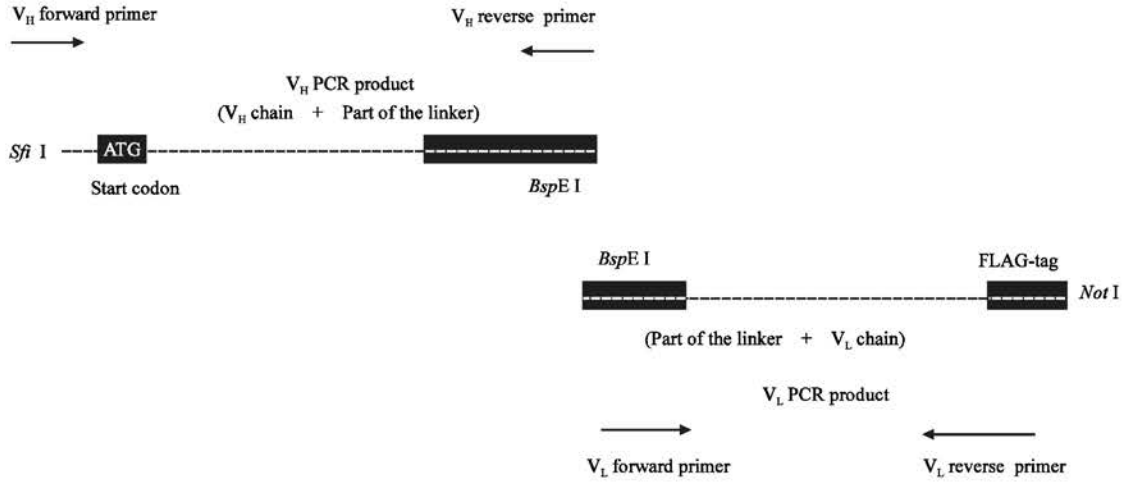

Fig. 7: Diagrammatic representation of expected V_H and V_L chain PCR products. Figure shows the expected V_H and V_L DNA fragments with part of the linker sequences when PCR is carried out using cDNA derived from mouse spleen mRNA and also shows the expected fragments obtained from both chains when particular restriction enzymes are used for digestion

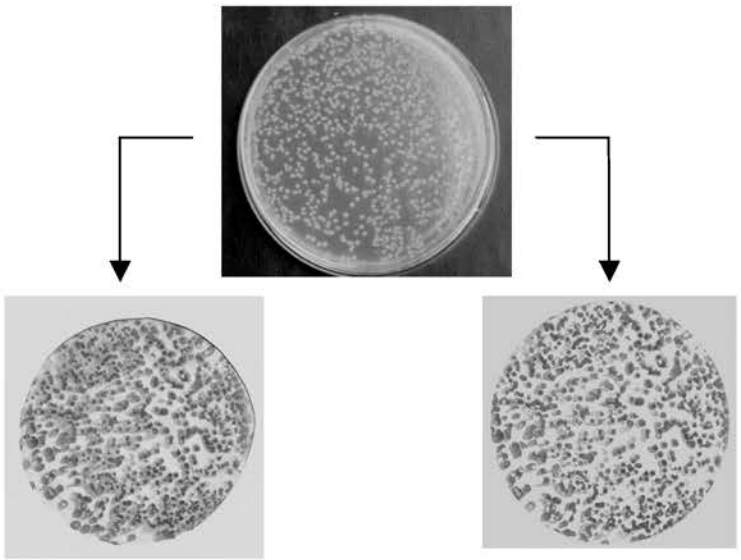

Fig. 8: Rapid analysis of combinatorial antibody library using colony hybridization. The 10^{-6} diluted scFv.pCANTAB 5E transformed *E. coli* TG1 was plated on SOBAG plate and the bacterial colonies were duplicated on 2 nitrocellulose membranes. The membrane on the left shows the colony hybridization result using DIG-labeled V_H as the probe while the membrane on the right shows the colony hybridization result using DIG-labeled V_L as the probe. The overall result shows that the majority of the *E. coli* strain TG1 in the scFv antibody combinatorial library carried the V_H and V_L genes

Analysis of the antibody combinatorial library: Following the 3 fragment ligation involving the V_H, V_L fragments and pCANTAB 5E vector, the reaction was transformed into *E. coli* TG1 cells. The estimation of transformed TG1 cells by direct colony counting was approximately 3×10^8 cfu mL^{-1}, indicating the estimated size of the antibody combinatorial library. Colony hybridization experiments carried out on the transformed TG1 colonies using both specific V_H and V_L DIG-labeled probes confirmed the successful transformation (Fig. 8). Nine

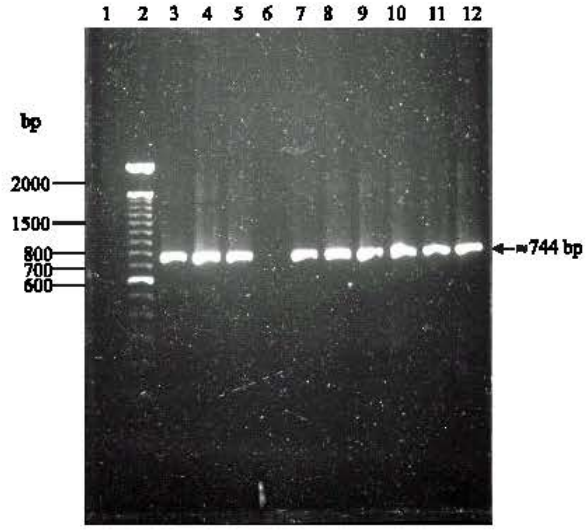

Fig. 9: Confirmatory analysis of a randomly selected ScFv clones by PCR analysis. Negative control where distilled water was used as the PCR template (lane 1), 100 bp DNA ladder (Gibco-BRL) (lane 2), negative control where DNA of the vector (pCANTAB 5E) transformed TG1 cell was used as PCR template (lane 6) and PCR products where DNA of the scFv transformed TG1 cell was used as the template (lane 3-5 and 7-12) were separated on a 1% agarose gel. Approximately 774 bp scFv bands were detected when the DNA of the scFv transformed *E. coli* was used for PCR amplification using the V_H (Forward) and V_L (Reverse) primers as indicated by the arrow

transformed TG1 colonies were also randomly selected for integrity confirmation using PCR analysis. Figure 9 shows that a ≈774 bp band was detected in the 9 randomly selected scFv transformed TG1 cells after PCR amplification using V_HF and V_LR primers and no PCR product was detected in the transformed TG1 cells with vector only. The phage display library was 2×10^8 cfu mL^{-1} in size after the bacterial library was infected with final titer of 4×10^{11} pfu mL^{-1} of M13K07 phage and analyzed by colony count.

DISCUSSION

In this study, a molecular approach for a scFv combinatorial library construction was utilized to bypass the tedious and time-consuming hybridoma development prior to antibody clone screening. Initially, antigen immunized mouse spleen tissue was used to construct the scFv fragment for antibody production. In this study, *cucumber mosaic virus* (CMV) coat protein was used for mouse immunization. The mouse immunization method was necessary as it was shown that no desired scFv binding clones were found when a random combinatorial library constructed from unimmunized mouse was used for affinity selection (Clackson *et al*., 1991). The immunization step is to create or enrich the particular V_H and V_L domains of the antibody molecule in the animal immune system, therefore ensuring that selecting the genes encoding the desired antigen-specific antibody by PCR method would become possible and easier. After that, the construction of the scFv was initiated by selecting the variable heavy chain (V_H) and light chain (V_L) from the pool of genes in the immunized mouse spleen RNAs. First strand cDNA synthesis was carried out to prepare the template for the V_H and V_L DNA production. The expected sizes of the V_H and V_L DNA fragments were approximately 356 and 321 bp, respectively and confirmed by electrophoresis. Although the PCR amplification conditions and the cDNA template used remained the same, the ethidium bromide-stained V_H DNA fragment shows slightly higher intensity compared to the V_L DNA fragment possibly due to the mouse lambda light chain gene in light chain gene pool not being amplified using the kappa specific primers provided in the RPAS kit (Amersham Pharmacia). Instead of both primers, only the kappa specific primers were provided as the product obtained from lambda specific chains primers are very unstable (justification by the manufacturer). Absence of the lambda specific chain primer will cause the reduction of variability in development of scFv antibody libraries. However, exclusion of the unstable lambda specific chains primer in PCR amplification can reduce errors or mutations during the scFv antibody library construction. As a result, only V_L kappa light chain was amplified using this system.

The approach of assembling of the V_H and V_L chains together after obtaining the V_H and V_L chain DNA fragments to construct the scFv library with the Fv portion should consistently maintain the binding specificity and affinity of the antibody (Glockshuber *et al*., 1990). The initial strategy to join the chains using commercial primers and PCR assembly as described by Horton *et al*. (1989) failed to join the two V_H and V_L chain fragments together. This might be due to unsuitable or unoptimized chain assembly conditions. Therefore, an alternative approach using restriction endonuclease digestion followed by 3 fragment ligation was carried out. This method included ligation of V_H, V_L chains with part of the peptide sequence fragment at the *Bsp*E I restriction endonuclease site and *Sfi* I and *Not* I restriction endonuclease sites with the vector to create the scFv combinatorial library. This method was similar to

that used by Chaudhary *et al.* (1990) but with differences in the primer design approach. Since there was no information on the framework region, the V_H and V_L chains were cloned and sequenced to obtain the framework region sequence information in order to design new primers for the linking process. The 5' and 3' ends of the genes encoding the V_H and V_L chains of the antibody structure are believed to be conserved within an animal species (Jiang *et al.*, 1994; Lilley *et al.*, 1994). This was also shown in this study where the overall sequencing results showed that the 3' ends of the V_H chain were identical and the rest of the framework region sequences for both chains were relatively conserved among a number of randomly screened recombinant clones. Although deletions were encountered in some of the V_L clones, the sequencing data obtained for the other end was still useful for analysis and PCR primer design. The deletions seen in the V_L clones might be due to degradation of the clones during isolation or other experimental procedures in this study. Analysis on the sequences to ensure correct frame to be used was carried out for proper scFv library construction. Ten base pair extra of nonsense nucleotide sequences were added to the 5' end of each primers in order to increase the digestion efficiency when restriction endonuclease was used. A FLAG-tag sequence was also incorporated into the V_L reverse primer so as to introduce an additional hydrophilic DYKDDDDK octapeptide at the COOH-termius of the scFv. This was to improve detection of the scFv during screening especially in other vectors or systems. The addition of the FLAG-tag detection marker to the scFv was important for reconstruction of the clone in the future as the original E-tag detection marker is located on the pCANTAB 5E vector. Reconstruction of the complete scFv in other expression vectors by digesting the scFv out from the vector at the *Sfi* I and *Not* I restriction endonuclease sites would cause loss of the E-tag detection property but not the FLAG-tag marker. The V_L reverse primer was also designed in such a way so as to easily allow the removal of the FLAG-tag region when necessary using Hinc II restriction endonuclease. As a result, dual detection properties were included in the scFv construct. The Complementarity-Determining Region (CDR) sequences were not included in the primer design to avoid bias amplification of single binding specificity of either the V_H or V_L genes. The size of the V_H and V_L chain PCR products amplified using the self-designed primers were slightly larger when compared to the V_H and V_L chain fragments that were amplified by the commercial primers (Fig. 7). This was due to the incorporation of extra nucleotides including the peptide linker sequence and FLAG-tag sequence.

The inclusion of *Sfi* I, *Not* I and *Bsp*E I restriction endonuclease sites in the V_H and V_L chains facilitated successful ligation to the *Sfi* I/*Not* I linearised pCANTAB5E vector and precise assembly of the two chains with a short peptide $(Gly_4Ser)_3$. This allowed the scFv combinatorial library to be produced through a one step cloning approach, although a 2 step cloning is commonly used for library construction (Lake *et al.*, 1994). Basically, 2 step cloning involves cloning of the V_H and V_L genes separately into a cloning vector, followed by screening, digestion and final ligation into a bacterial expression vector. The advantage of one step cloning not only avoids loss of important genes, but also directly increases the size and diversity of the scFv combinatorial library, although 2 step cloning have been claimed to increase the cloning efficiency by 10-100 folds (Lake *et al.*, 1994).

In this study, the integrity of the scFv combinatorial library was proven using colony hybridization on duplicated membranes with DIG-labeled V_H and V_L probes separately and PCR analysis of randomly selected clones. No self-ligated vector was detected in the scFv library and the size of the scFv combinatorial library was estimated at 3×10^8 cfu mL^{-1} bacteria carrying single scFv constructs. The size of the phage library was maintained at 2×10^8 cfu mL^{-1} after conversion of the bacterial library with the help of M13K07 phage. Overall, the phage display scFv combinatorial library was sufficient in size for biopanning experiments.

REFERENCES

Cardoso, D.F., F. Nato, P. England, M.L. Ferreira, T.J. Vaughan, I. Mota, J.C. Mazie, V. Choumet and P. Lafaye, 2000. Neutralizing human anti-crotoxin scFv isolated from a nonimmunized phage library. Scand. J. Immunol., 51: 337-344.

Chaudhary, V.K., J.K. Batra, M.G. Gallo, M.C. Willingham, D.J. Fitz Gerald and I. Pastan, 1990. A rapid method of cloning functional variable-region antibody genes in *Escherichia coli* as single-chain immunotoxins. Proc. Nat. Acad. Sci. USA., 87: 1066-1070.

Chua, K.H., K. Norzulaani and Y.O. Rofina, 2006. Expression of a functional anti-cucumber mosaic virus single-chain variable fragment antibody in Tobacco plant (*Nacotiana tabacum*). J. Plant Biotechnol., 8: 9-14.

Clackson, T., H.R. Hoogenboom, A.D. Griffiths and G. Winter, 1991. Making antibody fragments using phage display libraries. Nature, 352: 624-628.

Devaux, C., E. Moreau, M. Goyffon, H. Rocha and P. Billiald, 2001. Construction and functional evaluation of a single-chain antibody fragment and neutralizes toxin AahI from the venom of the scorpion *Androctonus australis* hector. Eur. J. Biochem., 268: 694-702.

Glockshuber, R., M. Malia, I. Pfitzinger and A. Pluckthun, 1990. A comparison of strategies to stabilize immunoglobulin Fv fragments. Biochemistry, 29: 1362-1367.

Gupta, S., J. Eastman, C. Silski, T. Ferkol and P.B. Davis, 2001. Single-chain Fv: A ligand in receptor-mediated gene delivery. Gene Ther., 8: 586-592.

Horton, R.M., H.D. Hunt, S.N. Ho, J.K. Pullen and L.R. Pease, 1989. Engineering hybrid genes without the use of restriction enzymes: Gene splicing by overlap extension. Gene, 77: 61-68.

Jiang, W., T.P. Bonnert, K. Venugopal and E.A. Gould, 1994. A single chain antibody fragment expressed in bacteria neutralizes tick-borne Flavivirus. Virology, 200: 21-28.

Lake, D.F., K.S. Lam, L. Peng and E.M. Hersh, 1994. Molecular cloning, expression and mutagenesis of an anti-insulin single chain Fv (scFv). Mol. Immunol., 31: 845-856.

Lilley, G.G., O. Dolezal, C.J. Hillyard, C. Bernard and P.J. Hudson, 1994. Recombinant single-chain antibody peptide conjugates expressed in *Escherichia coli* for the rapid diagnosis of HIV. J. Immunol. Methods, 171: 211-226.

Long, M.C., K.E.J. Marshall, B.J. Kearney, G.V. Ludwig, J.P. Wong and L.P. Nagata, 2001. Pharmacokinetics study of a novel chimeric single-chain variable fragment antibody against western equine encephalitis virus. Hybridoma, 20: 1-10.

Nagesha, H.S., L.F. Wang, B. Shiella, G. Beddome, J.R. White and R.A. Iraving, 2001. A single chain Fv antibody displayed on phage surface recognises conformation group-specific epitope of bluetongue virus. J. Virol. Methods, 91: 203-207.

Rosenblum, M.G., S.A. Horn and L.H. Cheung, 2000. A novel recombinant fusion toxin targeting HER-2/neu-over-expressing cells and containing human tumor necrosis factor. Int. J. Cancer, 88: 267-273.

Sambrook, J., E.F. Fritsh and T. Maniatis, 1989. Molecular Cloning: A Laboratory Manual. New York: Cold Spring Harbor Laboratory.

Shigeto, Y., I. Daisuke, M. Hiroyuki, E. Hitoshi and I. Akira, 2001. Bacteria expressing single-chain immunotoxin inhibit malaria parasite development in mosquitoes. Mol. Biochem. Parasitol., 113: 89-96.

Vitaliti, A., M. Wittmer, R. Steiner, L. Wyder, D. Neri and R. Klemenz, 2000. Inhibition of tumor angiogenesis by a single-chain antibody directed against vascular endothelial growth factor. Cancer Res., 60: 4311-4314.

Zeng, J.Z., Z.Y. Zhou, Y.Q. Wu, Z.P. Liu, W.X. Wang, H.L. Huang, Z.N. Cai and J.L. Yu, 2000. Expression of single-chain Fv antibody for anti-beet necrotic yellow vein virus in *Escherichia coli*. Acta Genet. Sin., 27: 1006-1011.