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RESEARCH ARTICLE

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Construction of a variable regions gene library of antibody against *Eimeria acervulian* merozoite of chicken

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Abstract Antigens of *Eimeria acervulina* merozoites were prepared and purified. BALB/C mice were inoculated with the merozoite soluble antigens of *E. acervulina*. The total RNA was extracted from the spleen cells of the immunized BALB/C mice. The first chains of cDNA were synthesized by reverse transcription with Oligo $(dT)_{15}$ Primer, using extracted RNA as a template. The variable regions of heavy chain (V_H) and light chain (V_L) genes were amplified by PCR with gene-specific primers. The results showed that the genes of amplified V_H and V_L were about 420 bp and 390 bp, respectively. PCR products were purified by agarose gel DNA purification kit, and then the V_H and V_L gene libraries were constructed, respectively. The purified products were cloned into PGM-T vectors. The cloned plasmids were transformed into *Escherichia coli* TOP10. The colonies were selected on ampicillin-containing agar plates and the plasmids were isolated from several independent clones. The selected colonies were sequenced using Sanger's dideoxy sequencing method. Nucleotide sequences of the V_H and V_L genes were compared with the published V_H and V_L sequences from the BALB/C mice. The sequences were confirmed with mouse antibodies. As expected, the sequence differences between V_H/V_L and the corresponding germline genes were identified and predominantly localized in complementary determining regions. The sequence analysis results of partial clones indicated that the constructed gene libraries of V_L/V_H had a good diversity.

Keywords *Eimeria acervulina* merozoite, variable region of heavy chain (V_H), variable region of light chain (V_L), gene library, chicken

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1 Introduction

Coccidiosis of the domestic chicken is one of the major global diseases involving severe enteritis with higher mortality and morbidity, which is caused by several species of protozoan parasites of the genus *Eimeria* (Levine, 1970; Biggs, 1982), making chicken producers and poultry industries suffer great losses annually because of its wide distribution and high frequency (Augustine, 2001). *Eimeria acervulina* is an intestinal protozoan that invades the duodenum, causing severe lesions and weight loss in poultry farming. *E. acervulina* infection can bring great economic losses to poultry industries worldwide (Bhogal et al., 1992). Although chemoprophylaxis is almost the exclusive means of control, over the years a number of methods have been explored, using genetic resistance of birds, nutritional techniques including vitamin supplements, sanitation and quarantine, and live vaccines (Rose and Long, 1980). In spite of the availability of a number of chemotherapeutic agents (Long, 1984), coccidiosis remains one of the major diseases confronting poultry industries and inflicting an estimated loss of over $250 million per annum (Long and Jeffers, 1986; Bhogal, 1992). Medication with several different anticoccidial drugs has been effective in preventing severe outbreaks of the disease. However, the life of most anticoccidial drugs is limited because of the emergence of resistant strains of coccidia, the high cost of synthesis and testing of new anticoccidial agents, the necessity for drug withdrawal periods, and the possibility of some medicines under restraint in feed. Therefore, there is a need for an alternative method of control, such as immunoprophylaxis. Although the detailed protective mechanism in avian coccidiosis is not well understood, considerable evidence suggests that cell-mediated immunity plays a predominant role in host defense against coccidiosis, thus prompting

human work toward the development of recombinant vaccines in many laboratories.

With the development of molecular biology technology, studies on *coccidia* have developed at a cellular and molecular level. A new method for the isolation and identification of merozoite provides a novel tool for the study of merozoite surface antigen of chicken coccidia (Xie et al., 1997). On the basis of the best excogitated method of purifying merozoites, the researchers performed their studies on antigenic characteristics, amino acid content, immunogenicity, and drug sensitivity of coccidium merozoite. It was reported that the surface antigen proteins from the second-generation merozoite of *Eimeria tenella* possessed certain immunogenicity (Han and Huang, 2001) and that schizonts played an important role in inducing protective immunity. Therefore, the research on merozoites is of academic significance, with a wide application potential for revealing the biological and immunological characteristics and the medicine prevention of coccidia.

The immunogenicity of coccidia in variety and structure of antigen as well as the mechanisms of antigen inducing strong protection immunity against coccidia were studied using hybridoma and molecular cloning techniques. In combination of combinatorial chemistry technology with engineering antibody techniques, phage display techniques were generated (Knappik, 2000; Ting et al., 2001; Braunagel et al., 2003; Abi-Ghanem et al., 2008), which provided a convenient and robust tool for the selection of high-quality antibodies, enabling the identification of antibodies in a fast and high-throughput mode. As engineering antibody techniques have now entered a new era of development, perfect phage display antibody library techniques were achieved based on the phage surface display techniques. The DNA expression and cDNA library of the coccidia were constructed by screening the DNA libraries. The ScFv antibody specific to chicken *Eimeria* was screened. However, no researches on the construction of a phage display antibody library against merozoites of *E. acervulina* were previously reported. It was reported that most of the 45 major antigens with molecular weight of 20 to 2 50 kDa of *E. acervulina* were located on the surface of merozoite (Jenkins, 1987). In our research, the variable region gene library of ScFv against the merozoites of *E. acervulina* was constructed using the merozoite soluble antigen, providing a basis for the study on chicken coccidiosis immunoprophylaxis at a molecular level.

2 Materials and methods

2.1 Design and synthesis of primers

Twenty-eight pairs of genus-specific primers were designed according to the published variable region sequences of heavy chain (V_H) and light chain (V_L) genes (de Haard et al., 1999; Mingyue et al., 2004; Imai et al., 2006) using Primer 5.0 software and synthesized by SunBiotechnology Co., Ltd. (Beijing, China). Primer sequences are listed below (Table 1).

2.2 Parasites

Large quantities of single *Eimeria coccidial* oocysts isolated from the mixed oocysts in chickens using single oocyst isolation were acquired by proliferation. After purification and extraction of genome DNA by PCR amplification with species-specific primers, the *Eimeria* strains separated from single oocyst were classified as monospecific *E. acervulina*.

Table 1 Primer sequences

primer name	primer sequences	length/bp
V_L—FORWORD	5′-cctttctatgcggcccagccggggcccagccggccGAYATTGTWCTCWCCCARTC-3′ 5′-cctttctatgcggcccagccggggcccagccggccccGAYATTGWGCTSACCCAATC-3′ 5′-cctttctatgcggcccagccggggcccagccggccccGAYATTSTGMTSACYCAGTC-3′ 5′-cctttctatgcggcccagccggggcccagccggccccGAYATTGTRATGACMCAGTC-3′ 5′-cctttctatgcggcccagccggggcccagccggcc GAYATTCAGATGAYDCAGTC-3′ 5′- cctttctatgcggcccagccggggcccagccggcc GAYATTGTDHTVWCHCAGTC-3′ 5′-cctttctatgcggcccagccggggcccagccggcccc GAYATTTTGCTGACTCAGTC-3′ 5′-cctttctatgcggcccagccggggcccagccggccccGAYATTGTGATGACBCAGKG-3′ 5′-cctttctatgcggcccagccggggcccagccggcccc GAYGCTGTTGTACTCAGGAATC-3′	55–59
V_L—REVERSE	5′-acc aga gcc gcc gcc gcc gct acc acc acc accCCGTTTSAGCTCCAGCTTGG-3′ 5′-acc aga gcc gcc gcc gcc gct acc acc acc acc CCGTTTGATTTCCARCTTKG-3′	53
V_H—FORWORD	5′-agc ggc ggc ggcggc tct ggt ggt ggt gga tccGAKGTRMAGCTTCAGGAGYC-3′ 5′-agc ggc ggc ggcggc tct ggt ggt ggt gga tccGAGGTGAASSTGGTGGARTC-3′ 5′-agc ggc ggc ggcggc tct ggt ggt ggt gga tccGAAGTGATGCTGGTGGAGTC-3′ 5′-agc ggc ggc ggcggc tct ggt ggt ggt gga tccGAGGTNCAGCTBCAGCAGTC-3′ 5′-agc ggc ggc ggcggc tct ggt ggt ggt gga tccGAVGTGAWGYTGGTGGAGTC-3′ 5′-agc ggc ggc ggcggc tct ggt ggt ggt gga tccGATGTGAACTTGGAAGTGTC-3′	53
V_H—REVERSE	5′-cggcaccggcgcacctgcggccgc YGAGGAGACGGTCACTGAGRT-3′ 5′-cggcaccggcgcacctgcggccgc YGCRGAGACASTGACCAGAGT-3′	45

2.3 Preparation of merozoite soluble antigen of *E. acervulina*

E. acervulina oocysts from the Baoding strain were propagated using 2-week-old broilers inoculated with 1×10^5 *E. acervulina* oocysts by oral infection. The second generation merozoites were extracted and purified 96 h after inoculation. The merozoite soluble antigens of *E. acervulina* were prepared from purified merozoites disrupted by ultrasonic and high-speed centrifugation. The protein content of merozoite soluble antigen estimated with the ultraviolet spectrophotometer was 2.72 $mg\cdot mL^{-1}$.

2.4 Immunization

The purified merozoite soluble antigens of *E. acervulina* were mixed with equal volume of Freund's complete adjuvant (FCA) and Freund's incomplete adjuvant (FIA), respectively, emulsified fully, and then inoculated to healthy 60-day-old BALB/C mice with 0.5 mL, and then intensified with FCA on the 1st day and 14th day and with FIA on the 24th day and the 31st day for four times. The 37th day after inoculation, blood was sampled and the serum was separated, inactivated at 56°C, and immune responses were measured using indirect ELISA. Mice with immune responses were sacrificed and their spleens were collected and stored in liquid nitrogen.

2.5 Extraction of total RNA from immunized mice genome

All the solutions and utensils were treated with diethylpyrocarbonate (DEPC) to eliminate RNase. By using the SV Total RNA isolation system according to the protocol of Reagent kit (Promega Co.), the total RNA of *E. acervulina* merozoite was extracted from the spleen cells of BALB/C mice immunized with the merozoite soluble antigen of *E. acervulina*, and then the total RNA quality was tested by formaldehyde denaturing gel electrophoresis. The purity and concentration of total RNA were assayed by spectrophotometer at 260 nm and 280 nm, and stored at −70°C.

2.6 PCR amplification of V_H and V_L gene

The synthesis of the first-strand cDNA was carried out according to the protocol of the supplier with M-MLV Reverse Transcriptase (M-MLV-RT, Promega). Briefly, in a 1.5-mL Eppendorf tube, 4 μL RNA template mixed with 2 μL primer Oligo $(dT)_{15}$ (0.25 $\mu g\cdot\mu L^{-1}$) and DEPC-treated ddH_2O up to 8.5 μL were incubated at 70°C for 5 min to destroy the secondary structure, cooled immediately on ice for 5 min to prevent the secondary structure from reforming, and then transiently centrifuged, followed by adding 4 μL M-MLV 5× Reaction Buffer, 1 μL 10 $mmol\cdot L^{-1}$ deoxynucleoside triphosphate (dNTP), 0.5 μL RNAase inhibitor (40 $U\cdot\mu L^{-1}$), and 1 μL M-MLV RT (20 $U\cdot\mu L^{-1}$), with DEPC-ddH_2O up to 25 μL. The tube was incubated at 37°C for 5 min, 42°C for 60 min, and 70°C for 15 min, respectively, and was chilled on ice and stored at −20°C.

With the first-stand cDNA as a template, the cDNA fragment was amplified by Taq DNA polymerase adopting the 20 μL reaction system. The optimal reaction mixture of 2×PCR Master Mix (12.5 μL), cDNA template (2 μL), FORWARD primer (1 μL), REVERSE primer (1 μL), and DEPC-ddH_2O (up to 25 μL) were mixed gently by flicking the tube and centrifuged at 3000 $r\cdot min^{-1}$ for 3 to 5 s.

2.6.1 Amplification of V_H gene

Amplifications were performed as follows: predenaturation at 96°C for 5 min, denaturation at 96°C for 30 s, followed by 5 cycles of annealing 62°C for 30 s, and elongation at 72°C for 1 min; re-denaturation at 96°C for 30 s, followed by 25 cycles of annealing at 56°C for 30 s, elongation at 72°C for 1 min, prolonged elongation at 72°C for 7 min and remaining at 4°C. The PCR products were visualized under UV light on ethidium bromide–stained 1% agarose gel after electrophoresis.

2.6.2 Amplification of V_L gene

The amplifications were performed as follows: predenaturation at 96°C for 5 min, denaturation at 96°C for 30 s, followed by 5 cycles of annealing at 61°C for 30 s, and elongation at 72°C for 1 min; re-denaturation at 96°C for 30 s, followed by 25 cycles of annealing at 56°C for 30 s and elongation at 72°C for 1 min, prolonged elongation at 72°C for 7 min and remaining at 4°C. The PCR products were visualized under UV light on ethidium bromide–stained 1% agarose gel after electrophoresis.

2.7 Construction of Variable Regions Gene Library

PCR products are usually presented using individual electrophoretic bands. The amplified target fragments with the expected size were excised from the gel and purified using the PCR Fragment Agarose Gel DNA Purification Kit (TaKaRa Dalian Biotechnology Co., Ltd.) according to the manufacturer's instructions. The purified PCR products V_L and V_H variable regions were mixed equally respectively. The V_H gene library and the V_L gene library were constructed respectively.

2.8 Cloning and identification of amplified target fragment

Purified fragments were cloned into pGM-T Vector, and then transfected with *Escherichia coli* Top10, and cultivated at 37°C for filtration of blue and white clone. Original independent white bacterial colonies were selected and then cultured. The resulting recombinant plasmids were extracted by the Plasmid Small-scale

Extraction Kit (centrifugation columniation type, TIANGEN Beijing Biotechnology Co., Ltd.) and identified by PCR amplification and *Eco*RI enzyme digestion.

The recombinant plasmids were identified by sequencing the 200 randomly selected clones using Sanger's dideoxy sequencing method in SunBiotechnology Co., Ltd. (Beijing, China). Nucleotide sequences of the V_H and V_L genes were compared with the published V_H and V_L sequences from the BALB/C mice with the aid of DNAstar software.

3 Results

3.1 Result of immunization

The test result of ELISA showed that the serum antibody titer of the immunized mice with the merozoite soluble antigen of *E. acervulina* reached the highest dilution of 1∶12800. Therefore, their spleens could be used for RNA extraction. Mice with immune responses were sacrificed and their spleens were collected and stored in liquid nitrogen.

3.2 Total RNA of spleens of mice

OD_{260}/OD_{280} of extraction of total RNA was 2.1 by the ultraviolet spectrophotometer. It was between 1.9 and 2.0.

The 28 S and 18 S rRNA specific bands of mice were characterized on formaldehyde denatured electrophoresis (Fig. 1). The concentration of total RNA = $D_{260}\times 40\ \mu g\cdot mL^{-1}\times$ dilution multiple = $0.3880\times 40\ \mu g\cdot mL^{-1}\times 200 = 3.10\ \mu g\cdot \mu L^{-1}$. The results showed that the purity of total RNA was practicable for cDNA synthesis.

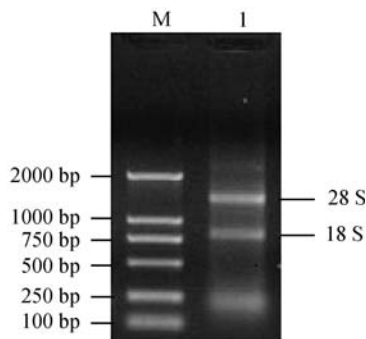

Fig. 1 Total RNA of spleen of mice
Note: M stands for DNA marker DL2000 and 1 for total.

3.3 Results of RT-PCR of V_L/V_H

A band of specifically variable region genes was visualized on the 1.0% agarose gel electrophoresis. The obtained specific bands of V_L and V_H reached approximately 390 bp and 420 bp, respectively (Figs. 2 and 3), which were consistent with the theory values.

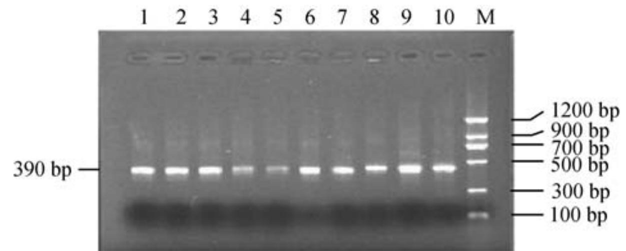

Fig. 2 PCR products of V_L repertoire analyzed by electrophoresis on 1.0% agarose gel
Note: M stands for DNA Marker DL2000 and 1 to 10 for PCR products of V_L repertoire of merozoites.

3.4 Identification of recombinant plasmid

PCR amplification with the recombinant plasmids as a template resulted in two specific bands. The target fragments of V_L and V_H reached approximately 390 bp and 420 bp, respectively (Fig. 4), which were consistent with our anticipation.

The recombinant plasmids pGM-V_L were digested with *Eco*RI, and then, the electrophoresis of the digested products showed fragments of 390 bp (V_L target fragment) and 3015 bp (T-vector; Fig. 5). When the recombinant plasmids pGM-V_H were digested with the restriction enzyme *Eco*RI, two DNA fragments of 420 bp (V_H target fragment) and 3015 bp (T-vector) appeared in the digested products (Fig. 6). The results were consistent with the theory values, revealing that the target V_L/V_H genes were cloned into pGM-T.

3.5 Sequence analysis of the V_H/V_L regions genes

The nucleotide sequences of the V_H/V_L genes of 200 clones were sequenced and compared with other sequence of V_H/V_L published in GenBank (data not shown). As expected, the sequence difference between V_H/V_L and the corresponding germline genes was identified and predominantly localized in complementary determining regions. The sequences representing independent clones designated as L1, L2, L3, L4, L5, L6, and L7 of light chains and H1, H2, H3, H4, H5, H6, and H7 of heavy chains are shown in Figs. 7 and 8. The sequences of V_L/V_H were different from each other (Figs. 9 and 10). Sequence analysis results of partial clones indicated that the constructed gene libraries of V_L/V_H had good diversity.

4 Discussion

The cloning technology of variable region genes of antibody is a key point in the phage antibody library technique. The

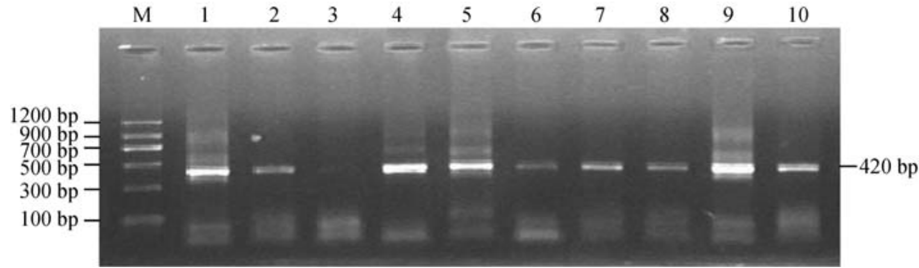

Fig. 3 PCR products of V_H repertoire analyzed by electrophoresis on 1.0% agarose gel
Note: M stands for DNA marker DL2000 and 1 to 10 for PCR products of V_H repertoire of merozoites.

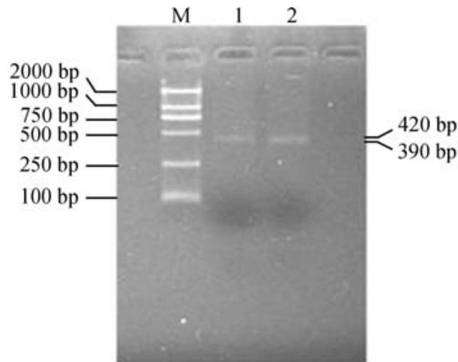

Fig. 4 PCR products of V_L and V_H repertoire analyzed by electrophoresis on 1.0% agarose gel
Note: M stands for DNA marker DL2000, 1 for PCR products of V_H and 2 for PCR products of V_L gene.

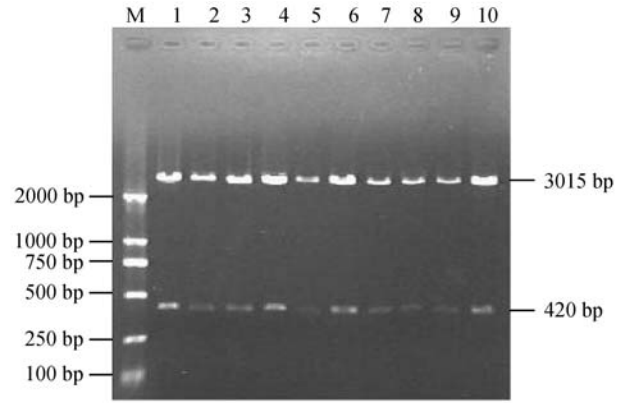

Fig. 6 Recombinant plasmid pGM-V_H digested by *Eco*RI
Note: M stands for DNA marker DL2000 and 1 to 10 for Enzyme digested products of pGM-V_H.

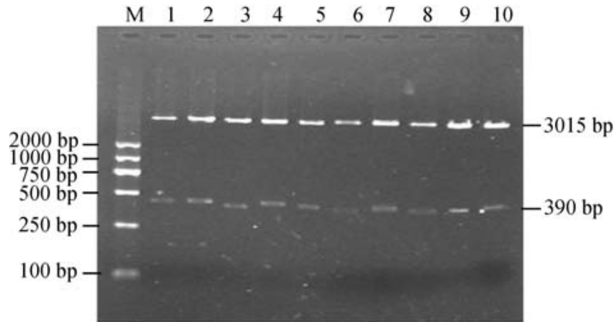

Fig. 5 Recombinant plasmid pGM-V_L digested by *Eco*RI
Note: M stands for DNA marker DL2000 and 1 to 10 for Enzyme digested products of pGM-V_L.

variable regions consist of hypervariable regions and framework regions. The variable regions of V_L/V_H possess three hypervariable regions (complementarity-determining regions), which constitute together the antigen-combining sites and also determine the characteristics of antibodies. Other structures are relatively conservative variable regions, which show β-sheet and play a role of framework in connecting variable regions, and hence they are called framework regions. Therefore, in order to obtain variable region genes, the PCR primers for amplification of V_L/V_H were designed and synthesized based on the nucleotide sequences of FR1 and FR4. The relatively conservative FR1 and FR4 sequences of variable region were synthesized by Orlandi et al. (1989). As universal primers were designed, the heavy and light variable region genes of antibodies in mice were amplified by PCR. Marks designed for six pairs of primers successfully amplified the V_H and V_K genes from lymphocytes in the peripheral blood of humans and the diversity of these genes was confirmed (Marks et al., 1991). The design of primers is related to the diversity of the variable region gene library of V_L/V_H and directly influences whether the antibody library can reflect faithfully the distribution feature of the original genes. In our research, pairs of genus-specific primers (containing degenerate primers) of variable region sequences of heavy chain (V_H) and light chain (V_L) were designed and synthesized according to the published genes encoding the antibody in mice. Thirty-three base pairs linker were designed respectively at the ends of the downstream primers of V_L and the upstream primers of V_H, and were used as foundation for constructing the variable region gene library of V_L/V_H of ScFv. In the previous studies, based on the objective of constructing antibody libraries, some scholars used to amplify V_L and

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    CCTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCCCCGACATTGTGCTGA Majority
             10        20        30        40        50
1   CCTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCC--GACATTGTTATCA L1
1   CCTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCCCCGACATTGTGCTCA L2
1   -CTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCCCCGACATTGAGCTGA L3
1   CCTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCCCCGACATTTTGCTGA L4
1   CCTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCC--GATATTCAGATGA L5
1   CCTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCCCCGACATTGAGCTGA L6
1   CCTTTCTATGCGGCCCAGCCGGGGCCCAGCCGGCCCCGATATTGTGCTCA L7

    CTCAGTCTCCAGCTTCCTTGGCTGTGTCTCTAGGGGAGAGGGTCACCATC Majority
             60        70        80        90       100
49  CTCAGTCTCCAGCACTCATAGCTGCATCTCCAGGGGAGAAGGTCACCATC L1
51  CCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACCATA L2
50  CCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACCATC L3
51  CTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGTTTC L4
49  TTCAGTCTCCAGCCTCCCTATCTGCATCTGTGGGAGAAACTGTCACCATC L5
51  CCCAATCTCCAGCTTCTTTGGCTGTGTCTCTAGGGCAGAGGGCCACCATC L6
51  CTCAGTCTCCAGCCATCCTGTCTGTGAGTCCAGGAGAAAGAGTCAGTTTC L7

    TCCTGCAGGGCCAGCGAAAGTATTGATA---------CTAGTTATATGCA Majority
            110       120       130       140       150
99  ACCTGCAGTGTCAGCTCAAGTATAAGTT---------CCAGCTACTTGCA L1
101 TCCTGCAGAGCCAGTGAAAGTGTTGATAGTTATGGCAATAGTTTTATGCA L2
100 TCCTGCAAGGCCAGCCAAAGTGTTGATTATGATGGTGATAGTTATATGAA L3
101 TCCTGCAGGGCCAGTCAGAGCATTGGCA---------CAAGC---ATACA L4
99  ACATGTCGAATAAACGAGAATATTTACA------------GTTATTTAGC L5
101 TCCTGCAGAGCCAGCGAAAGTGTTGATAATTATGGCATTAGTTTTATGAA L6
101 TCCTGCAGGGCCTGTCAGAGCATTGGCA---------CAAGC---ATACA L7

    CTGGTACCAGCAGAAACCAGGACAGTCTCCCAAACTCCTCATCTATGATG Majority
            160       170       180       190       200
140 CTGGTACCAGCAGAAGTCAGGAATCTCCCCCAAACCCTGGATTTATGGCA L1
151 CTGGTACCAGCAGAAAACAGGACAGCCACCCAAACTTATCATCTATCGTG L2
150 CTGGTACCAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTATGCTG L3
139 CTGGTATCAGCAAAGAACAAATGGTTCTCCAAGGCTTCTCATAAAGTATG L4
137 ATGGTATCAGCAGAAACAGGGAAAATCTCCTCAGCTCCTGGTCTATAATG L5
151 CTGGTTCCAACAGAAACCAGGACAGCCACCCAAACTCCTCATCTATGCTG L6
139 CTGGTATCAGCAAAGAACAAATGGTTCTCCAAGGCTTCTCATAAAGTATG L7

    CATCCAACCTAGTATCTGGGATCCCTGCCAGGTTTAGTGGCAGTGGATCT Majority
            210       220       230       240       250
190 CATCCAATCTGGCTTCTGGAGTCCCTGCTCGCTTCAGTGGCAGTGGATCT L1
201 CATCCAACCTAGAATCTGGGATCCCTGCCAGGTTCAGTGGCAGTGGGTCT L2
200 CATCCAATCTAGAATCTGGGATCCCAGCCAGGTTTAGTGGCAGTGGGTCT L3
189 CTTCTGAGTCTATCTCTGGGATCCCTTCCAGGTTTAGTGGCAGTGGATCA L4
187 CAAAAACCTTAGCAGAAGGTGTGCCATCAAGGTTCAGTGGCAGTGGATCA L5
201 CATCCAACCAAGGATCCGGGGTCCCTGCCAGGTTTAGTGGCAGTGGGTCT L6
189 CTTCTGAGTCTATCTCTGGGATCCCTTCCAGGTTTAGTGGCAGTGGATCA L7

    GGGACAGATTTCACTCTCAACATCAACAGTGTGGAGGCTGAAGATGTTGC Majority
            260       270       280       290       300
240 GGGACCTCTTACTCTCTCACAATCAGCAGCATGAAGGCTGAAGATGCTGC L1
251 AGGACAGACTTCACCCTCACCATTAATCCTGTGGAGGCTGATGATGTTGC L2
250 GGGACAGACTTCACCCTCAACATCCATCCTGTGGAGGAGGAGGATGCTGC L3
239 GGGACAGATTTTACTCTTAGCATCAACAGTGTGGAGTCTGAAGATATTGC L4
237 GGCACACAGTTTTCTCTGAAGATCAACAGCCTGCAGCCTGAAGATTTTGG L5
251 GGGACAGACTTCAGCCTCAACATCCATCCTATGGAGGAGGATGATACTGC L6
239 GGGACAGATTTTACTCTTAGCATCAACAGTGTGGAGTCTGAAGATATTGC L7

    AACTTATTACTGTCAACAAAGTAATAAGTGTCCGTTCACGTTCGGTGGTG Majority
            310       320       330       340       350
290 CACTGATTACTGTCAACAGTGGAGTAGTTCCCCACCCACGTTCGGTGGAG L1
301 AACCTATTACTGTCAGCAAAGTAATGAGGATCCGTGGACGTTCGGTGGAG L2
300 AACCTATTACTGTCAGCAAAGTAATGAGGATCCGTACACGTTCGGAGGGG L3
289 AGATTATTACTGTCAACAAAGTTATACCTGGCCGCTCACGTTCGGTGCTG L4
287 GAGTTATTACTGTCAACATTTTTGGACTACTCCGTTCACGTTCGGTGCTG L5
301 AATGTATTTCTGTCAGCAAAGTAAGGAGGTTCCGTGGACGTTCGGTGGAG L6
289 AGATTATTACTGTCAACAAAGTAATAGCTGGCCGCTCACGTTCGGTGCTG L7
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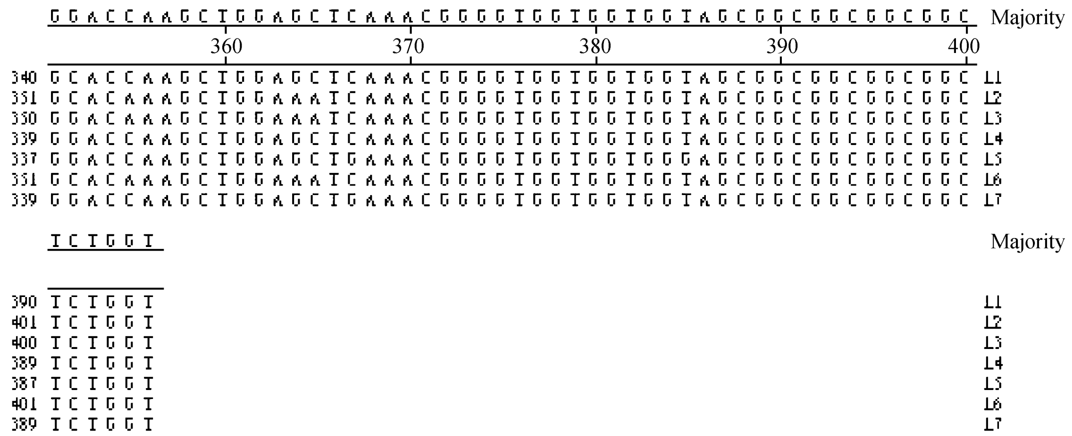
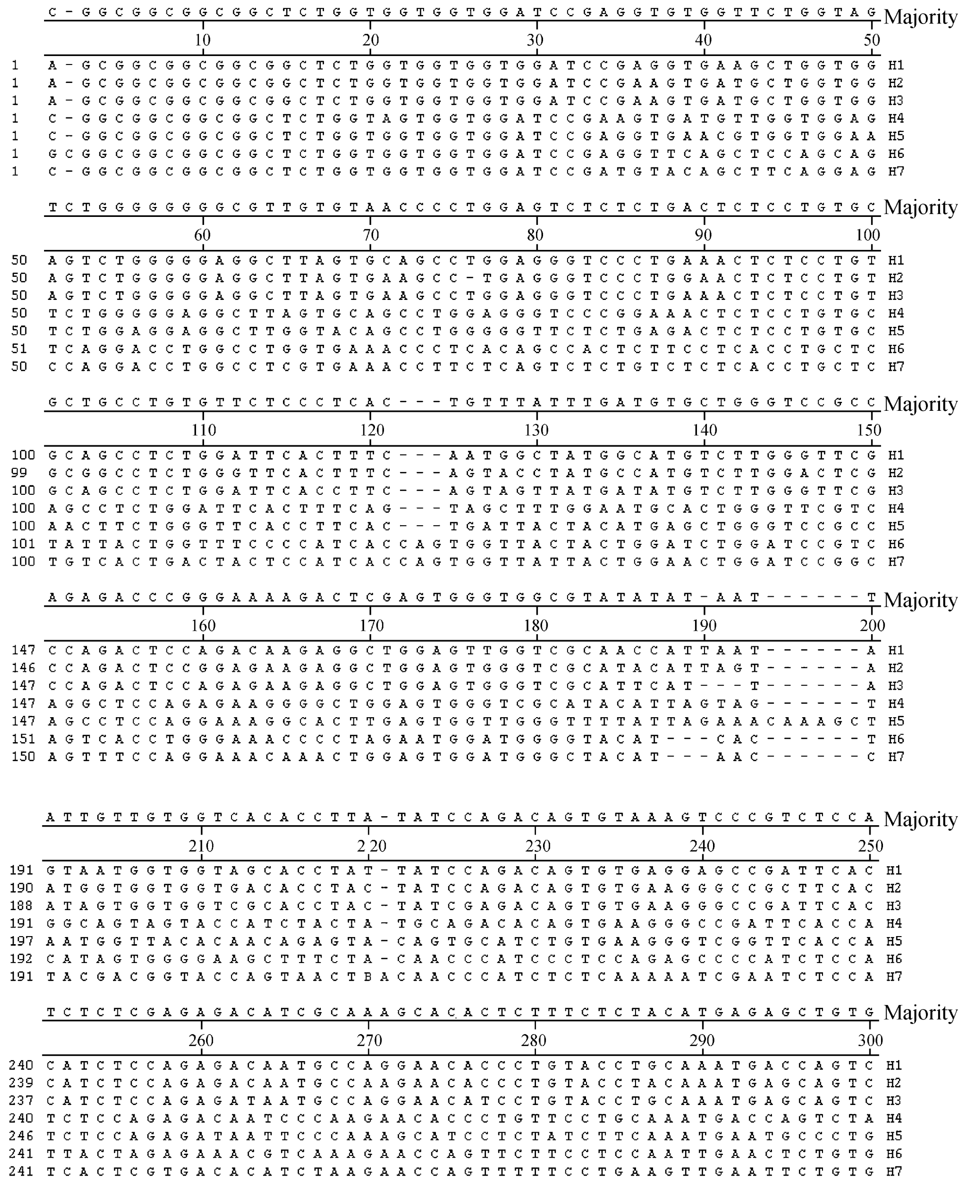


Fig. 7 The nucleotide sequences of the partial clones of V_L genes
Note: "-" stands for the position where there is no acid residue.



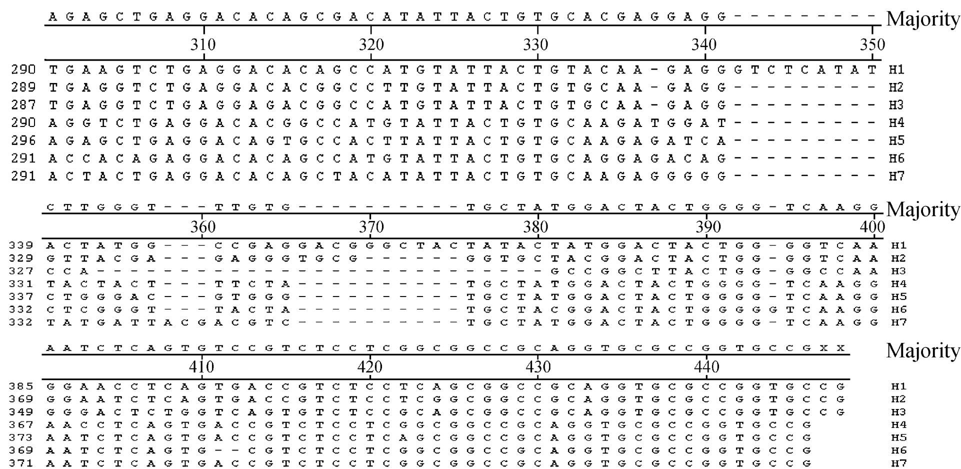


Fig. 8 The nucleotide sequences of the partial clones of V_H genes
Note: "-" stands for the position where there is no acid residue.

percent identity (upper triangle); divergence (lower triangle)

	1	2	3	4	5	6	7		
1	■	75.7	73.1	74.2	72.7	74.4	73.4	1	L1
2	31.3	■	75.9	91.1	70.4	91.6	75.9	2	L2
3	37.3	31.4	■	74.6	72.7	72.6	98.2	3	L3
4	32.9	9.5	33.1	■	70.7	92.6	73.6	4	L4
5	37.3	41.2	35.6	40.6	■	69.9	72.7	5	L5
6	34.7	8.9	35.2	7.8	40.3	■	72.8	6	L6
7	36.9	31.5	1.8	33.9	36.4	36.1	■	7	L7
	1	2	3	4	5	6	7		

Fig. 9 The nucleotide sequence homogeneity of the partial clones of V_L genes

percent identity (upper triangle); divergence (lower triangle)

	1	2	3	4	5	6	7		
1	■	89.2	88.6	87.6	76.5	64.9	67.5	1	H1
2	11.9	■	89.6	88.6	75.2	67.0	67.6	2	H2
3	12.5	11.3	■	86.3	75.4	63.4	64.1	3	H3
4	13.6	12.5	15.2	■	74.1	65.4	67.1	4	H4
5	28.5	30.4	30.0	32.2	■	68.1	68.3	5	H5
6	47.4	43.5	50.2	46.3	41.5	■	79.1	6	H6
7	42.7	42.4	49.0	43.5	41.4	24.7	■	7	H7
	1	2	3	4	5	6	7		

Fig. 10 The nucleotide sequence homogeneity of partial clones of V_H genes

V_H, and join the genes of V_H and V_L by a DNA linker of flexile15 peptide sequence (GGGGS) coding hydrophilicity amino acid–rich "connection peptide gene" to form a single-chain fragment (ScFv) with SOE-PCR. Besides, the Fd sections and the complete light chains could be also connected to form the Fab sections, and then connected with corresponding phagemid vector, transforming *E. coli* cells to construct the phage antibody library (Martsev et al., 2000; Takemura et al., 2000; Wang et al., 2000; Feng et al., 2003). In our experiment, the linkers were added to the ends of the V_L and V_H primers, respectively, which could not influence the amplification of target genes. Our experimental operation was quicker, with higher efficiency, so that there was no need for connection with linkers.

Another important parameter of primer is the melting temperature (Tm). In an ideal state, the lower anneal temperature may guarantee the annealing of primers and target genes effectively. Simultaneously, in order to reduce nonspecific binding, it is necessary to maintain a high enough annealing temperature. Generally, the reasonable annealing temperature may be 55°C to 70°C, 5°C lower than the Tm of primers. To obtain optimum results of PCR amplification, a pair of primers should possess an approximate Tm value. If the Tm value difference of primer pairs preponderates over 5°C, the primer will use the low annealing temperature in the circulation and display an obvious wrong start. In our experiment, the design of primers was based on the need for splicing and transcription and translation, so it was necessary that the primer sequences had larger differences among each other, as with the Tm value of primer. In order to increase the specificity of PCR, the amplifications were performed at a relatively higher annealing temperature of 61°C, designed according to higher Tm value in the first five cycles, and then at a relatively lower annealing temperature of 56°C, designed according to lower Tm value in the second cycles. As a result, a great deal of objective DNA was obtained.

In our experiment, the V_H and V_L genes were cloned by a TA cloning method that was convenient to operate, sensitive, rapid, and accurate with a high positive rate. Thermostable DNA polymerase (*Taq* polymerase) in PCR possesses a non-template-dependent activity. Due to this activity, a single A was added to the 3′ends of product (Jiang et al., 2000) and a single T was added to the 5′ ends

of the PGM-T vector with Taq DNA polymerase, respectively. A single T tail of vector makes the vector connecting highly effective directly with the PCR product (Jia et al., 2001). This TA clone technology has a higher reorganization efficiency compared with other PCR clone methods and generally is larger than 90% masculine clone to include the goal DNA fragments. More than 90% positive clones contain target DNA fragments. Recycling TA vector clone technology makes it more convenient and much cheaper to clone PCR products.

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