

The Polyclonal Antibody Against Chicken Interleukin-6 Prepared by Immunization of Recombinant Eukaryotic Plasmid Can React Efficiently with its Prokaryotic Expression Protein

¹Min Li, ¹Yuanping Wang, ¹Nianli Zou, ¹Sanjie Cao, ¹Xintian Wen and ^{1,2}Yong Huang

¹College of Veterinary Medicine,

²Key Laboratory of Animal Disease and Human Health of Sichuan Province,
Sichuan Agricultural University, Ya'an 625014, China

Abstract: Interleukin-6 (IL-6) is a multifunctional cytokine which has a wide range of activity in animals and has an impact on almost all immune system cells. To produce specific antibody against chicken IL-6 and detect its activity, its complete Open Reading Frame (ORF) sequence was amplified and inserted into the eukaryotic expression vector pcDNA3.1 (+) to get recombinant eukaryotic plasmid pcDNA-ChIL-6 and BALB/c mouse were immunized with this plasmid to produce polyclonal antibody against Chicken IL-6. Then the mature protein coding genes of chicken IL-6 was cloned and inserted into the prokaryotic expression vector pET-32a (+) to get recombinant prokaryotic plasmid pET-ChIL-6, the expression of this fusion protein was successfully induced by Isopropyl 1- β -D-thiogalactoside (IPTG) and the recombinant protein was further purified by NTA His•Bind column. The purified recombinant protein could react positively with polyclonal antibody against pcDNA-ChIL-6 in Western-blot and indirect ELISA.

Key words: Chicken IL-6, eukaryotic expression, prokaryotic expression, polyclonal antibody, Western-blot, indirect ELISA

INTRODUCTION

Cytokines are small-molecule soluble regulatory peptide which secreted by immune cells. In the immune response, cytokines play an important regulatory role in the interaction between cells as well as modulating inflammatory responses (Poquan, 2001).

Interleukin-6 plays roles in regulating immune responses, acute phase reactions and haematopoiesis inducing proliferation and antibody production in hybridoma cells (Kishimoto *et al.*, 1995) and has the potential to become vaccine molecular adjuvant and alternatives to antibiotics (Hilton *et al.*, 2002). IL-6 is produced by many types of cells and can act on B cells (Hirano *et al.*, 1986), T cells (Houssiau *et al.*, 1988), hepatocytes (Gauldie *et al.*, 1987), haematopoietic progenitor cells (Ikebuchi *et al.*, 1987) and cells of the central nervous system (Satoh *et al.*, 1988).

In the immune system, IL-6 can induce differentiation of activated B cells to antibody producing cells and the activation, growth and differentiation of T-cell (Muraguchi *et al.*, 1988; Van Snick, 1990). It was also demonstrated to induce interferon- α (IFN- α) in spleens of chickens (Karaca *et al.*, 1996; Sick *et al.*, 1998). The complete open reading frame sequence of chicken IL-6

was cloned by Scheider (Schneider *et al.*, 2001) from spleens of chickens using suppression subtractive hybridization technology.

In this study, chicken IL-6 (ChIL-6) gene was cloned and expressed and the polyclonal antibody was obtained by inoculating BALB/c mice with eukaryotic plasmid DNA of chicken IL-6 gene. The mature protein of ChIL-6 was also expressed in *E. coli*. The activity of this polyclonal antibody was further verified by Western-blot and Indirect-ELISA.

MATERIALS AND METHODS

Bacterial strains, vector and grown conditions:

Escherichia coli DH5 α and BL21 (DE3) were purchased from invitrogen corporation (Madison, California, USA). The T-cloning vector pMD18-T plasmid were purchased from Takara biotechnology (Dalian, China) co., Ltd, Plasmid pcDNA3.1 (+) was purchased from invitrogen corporation (California, USA) and PET-32a (+) was purchased from Novagen (Darmstadt, Germany). *E. coli* was grown at 37°C in Luria-Bertani broth or on Luria-Bertani broth agar supplemented when necessary with ampicillin (50 μ g mL⁻¹) for selection and maintenance of recombinant plasmids.

Cloning of ChIL-6 and constructions of eukaryotic expression vector: About 4 weeks old chickens of Ya'an local species were killed and spleen was removed then the spleen was ground immediately and splenic lymphocyte were isolated by utilizing lymphocyte separation medium (Haoyang Biological Manufacture Co., Ltd. Tianjin, China) following the manufacturer's instructions. The splenocytes lymphocytes were stimulated with ConA ($10 \mu\text{g mL}^{-1}$) for 20 h and total RNA was extracted using Trizol reagent (Takara biotechnology, Dalian, China) according to the manufacturer's protocol. Purified RNA was resuspended in $30 \mu\text{L}$ RNase-free water and stored at -70°C until further use.

For the Reverse Transcription (RT) reaction, the Quantscript RT Kit (TIANGEN Biotech Co., LTD, Beijing, China) was used and the total reaction volume was $40 \mu\text{L}$, including $4 \mu\text{L}$ of $10\times\text{RT}$ buffer, $4 \mu\text{L}$ of random 6mers ($10 \mu\text{M}$), $4 \mu\text{L}$ of dNTP mix (2.5 mM each), $2 \mu\text{L}$ of Quant Reverse Transcriptase, $20 \mu\text{L}$ of template RNA and $6 \mu\text{L}$ RNase-free water. The reaction mixture was incubated at 37°C for 60 min and 94°C for 4 min. Then $5 \mu\text{L}$ of the RT product was used to amplify chicken IL-6 using the following primers: P1: $5' \text{ GCGTCGACTCAGGCACTGAAACTCCTGG } 3'$ with XhoI site (underlined); P2: $5' \text{ GCGTCGACTCAGGCACTGAAACTCCTGG } 3'$ with Sal I site (underlined). Amplification of the DNA was performed using $2\times\text{taq}$ PCR mixture $25 \mu\text{L}$ (TIANGEN, Beijing), forward primer $2.0 \mu\text{L}$, reverse primer $2.0 \mu\text{L}$, template DNA $4.0 \mu\text{L}$, $17 \mu\text{L}$ water comprising a total volume of $50 \mu\text{L}$. The optimum conditions for PCR were as follows: 94°C for 4 min, 30 cycles at 94°C for 30 sec, 53°C for 30 sec, 72°C for 40 sec and final elongation at 72°C for 10 min. The PCR product was analyzed in 0.9% agarose and extracted from the gels with E.N.Z.A.1 Gel extraction kit (Omega, USA) following the manufacturer's instruction. The PCR product (about 730 bp) was ligated with pMD18-T vector and then transformed into *E. coli* DH5 α . Confirmation of clones containing recombinant plasmid was achieved by PCR and Restriction Enzyme (RE) digestion. The correct clones were sequenced by Sangon (Shanghai, China) co., Ltd.

Both the recombinant pMD18-T-ChIL-6 and pcDNA3.1 (+) were digested with Xho I and Sal I, then ligated with T4 DNA ligase to yield eukaryotic expression plasmid pcDNA-ChIL-6. Confirmation of clones containing recombinant plasmid was achieved by PCR and RE digestion.

Production of polyclonal antibody against chicken IL-6: After large-scale isolation of recombinant eukaryotic plasmid pcDNA-ChIL-6 DNA using plasmid Maxi kit (OMEGA) following the manufacturer's instructions. The concentration of plasmids was determined by Smartspec

Plus spectrophotometer (BIO-RAD). Twelve BALB/c mice obtained from (the experimental animal center of Sichuan University (Chengdu, China) were injected intramuscularly with $200 \mu\text{L}$ Tris-EDTA buffer (TE) containing $200 \mu\text{g}$ of pcDNA-ChIL-6 on days 0, 14 and 28, respectively. Another twelve mice received $200 \mu\text{g}$ of the pcDNA3.1 (+). Mice were bled from eyes 10 days following the final boost and sera were collected.

Construction of recombinant prokaryotic expression vector: A pair of specific primers was designed to amplify the mature peptide of ChIL-6 gene. The primers are given as follows: $5' \text{ CATGGATCCCCGCTGCCCGCCGCC } 3'$ with BamHI site (underlined); $5' \text{ AGCTCGAGTCTCAGGCAC TGAAACTCC } 3'$ with Xho I site (underlined). Amplification of the DNA was performed following the procedure above except the template DNA was pcDNA-ChIL-6. Amplification of the DNA was performed using $2\times\text{taq}$ PCR mixture $25 \mu\text{L}$ (TIANGEN, Beijing), forward primer $2.0 \mu\text{L}$, reverse primer $2.0 \mu\text{L}$, template DNA $1.0 \mu\text{L}$, $20 \mu\text{L}$ water comprising a total volume of $50 \mu\text{L}$. The optimum conditions for PCR were as follows: 94°C for 4 min, 30 cycles at 94°C for 30 sec, 60°C for 30 sec, 72°C for 30 sec and final elongation at 72°C for 10 min. The PCR product (about 603 bp) was inserted into pMD18-T vector and then introduced into *E. coli* DH5 α . The recombinant plasmid was sequenced by Sangon (Shanghai, China). The recombinant pMD18-T plasmid containing the target gene and pET-32a (+) were digested with BamHI and XhoI, then ligated with T4 DNA ligase. The ligated products were transformed into *E. coli* DH5 α and then transformed into BL21 to generate recombinant prokaryotic expression plasmid pET-ChIL-6. Confirmation of clones containing recombinant plasmid was achieved by PCR and RE digestion.

Expression and purification of recombinant protein: The BL21 strains containing the recombinant prokaryotic plasmid were grown overnight with shaking at 37°C . The overnight cultures were diluted in the proportion of 1:100 and grown at 37°C with vigorous shaking for 3-4 h until the optical density (OD600) of the cultured cells reached 0.6, expression of the fusion protein was induced with 1 mM IPTG for 4 h at 37°C (Zou *et al.*, 2010). The cells of 1.0 mL were harvested by centrifugation at 8000 rpm for 5 min and washed once with 1 mL distilled water at 8000 rpm for 5 min.

Then cells were resuspended with $100 \mu\text{L}$ distilled water and $25 \mu\text{L}$ $5\times\text{SDS}$ loading buffer after heating at 100°C for 5 min, recombinant protein was analyzed by SDS-PAGE. For purification of recombinant ChIL-6, the strains were harvested by centrifugation and then

suspended in Phosphate-Buffered Saline (PBS) (pH 8.0). After repeated freezing and thawing for 3 times, the suspension were sonicated, centrifuged and resuspended with PBS containing 6 mol L⁻¹ guanidine-HCl for overnight at 4°C. After centrifugation at 10000 rpm for 60 min, the supernatant was filtrated through 0.45 µm filtration membrane.

Then the solution was purified on a column packed with Ni-NTA His•Bind superflow according to the manufacture's instruction (Merck, Darmstadt, Germany). The eluate from each imidazole concentration were collected and analyzed by SDS-PAGE and then treated with different concentrations of guanidine-HCl (from 5-0 M) in renaturation buffer (50 mM Tris-Hel (PH8.0) (0.1) mM oxidized glutathione(1 mM reduced glutathione) for 24 h at 4°C, respectively.

Western blot: The purified proteins were separated by SDS-PAGE gels and then transferred to Polyvinylidene Difluoride (PVDF) membrane with 0.45 µm pore size (Millipore Corp., USA) at 15 V for 1.5 h using a Transblot Cell (Bio-Rad) according to the manufacturer's instructions. To prevent non-specific binding of antibodies, the membrane was blocked for 60 min with milk buffer (20 mM Tris-HCl pH 8.0, 150 mM NaCl, 0.05% Tween 20, 5% skimmed dry milk) at 37°C. Then the membranes were washed by tris-buffered saline with Tween 20 (TBST) buffer (100 mM Tris-HCl pH8.0, 150 mM NaCl, 0.05% Tween 20) for 3 times and incubated with mouse anti-ChIL-6 polyclonal antibody diluted 1:100 in 0.5% Bovine Serum Albumin (BSA)/PBS for 60 min at 37°C. After washed by TBST for three times, the membrane was incubated with a HRP-labeled sheep anti-mouse IgG (zhongshan Goldenbridge Biotechnology Co., LTD, Beijing, China) for 60 min at 37°C. After washed by TBST for three times, target proteins were visualized using 3,3'-diaminobenzidine (DAB) (Tiangen, Beijing, China).

Polyclonal antibody titer analysis by ELISA: Antibody titer was measured using an indirect Enzyme Linked Immunosorbent Analysis (ELISA). Checker board method was used to determine the optimal antigen coating concentration. The purified recombinant antigen diluted to 0.025 g mL⁻¹ in carbonate solution (pH 9.6) was coated on 96 well microtiter plate (Costar, USA) at 100 µL per well incubated at 4°C overnight.

The wells were then washed three times by PBST buffer (0.05% Tween 20 in PBS) and blocked for 60 min with 5% milk buffer at 37°C. After washed, the wells were incubated with 100 µL mouse anti-ChIL-6 polyclonal antibody at different dilutions (from 1:40-

1:5120) and incubated at 37°C for 60 min. Then the wells were washed and incubated with 100 µL of sheep anti-mouse IgG diluted 1:5000 in 0.1% BSA/PBS at 37°C for 60 min washed again and detected with 50 µL of 3,3',5,5'-Tetramethyl Benzidine (TMB) for 10 min at room temperature. The reaction was stopped by the addition of 35 µL of 2M H₂SO₄. The Optical Density (OD) value was read at 450 nm using a Bio-Rad model 860 plate reader (Bio-Rad, CA, USA) and mouse anti-pcDNA3.1 (+) were added.

For determine the cut-off value, twenty sera samples from mice infected with PBS were used as control group sera, the cut-off value was calculated using the formula: mean of the negative serum values plus three Standard Deviations (SDs).

RESULTS

Cloning of ChIL-6 and constructions of eukaryotic expression vector: The ChIL-6 cDNA was acquired by RT-PCR from splenocytes lymphocytes of chicken. The PCR products were analyzed on 1% agarose gel electrophoresis then one band of 734 bp DNA fragment was observed (Fig. 1). Based on the sequencing result, the ORF of ChIL-6 was 726 bp long, containing 241 amino acid residues and having a signal peptide of 47 amino

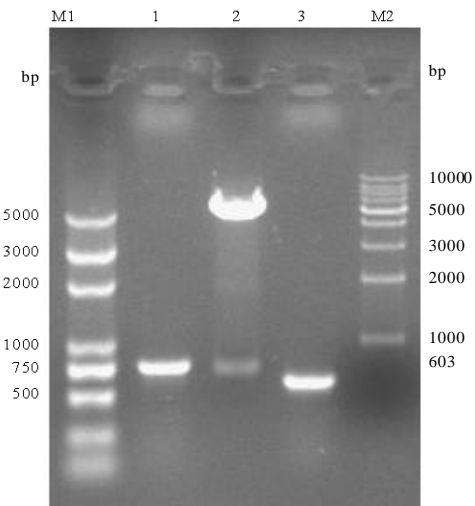


Fig. 1: RT-PCR result of ChIL-6 and Identification of ChIL-6 eukaryotic expression vector Lane M1: DL5000 DNA Marker. Lane 1: RT-PCR result of ChIL-6 complete reading frame gene. Lane 2: Products from pcDNA-ChIL-6 digested by XhoI and SalI. Lane 3: PCR result of ChIL-6 mature protein encoding gene. Lane M2: DL10000 DNA Marker

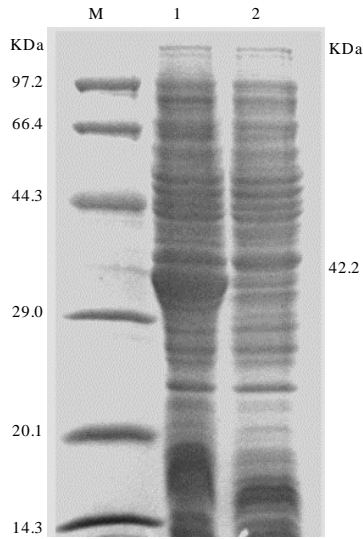


Fig. 2: SDS-PAGE results of expression of recombinant protein, Lane M: Protein marker, Lane 1: Induced BL21 stains containing recombinant plasmid for 4 h. Lane 2: Non-induced BL21 stains

acids. The nucleotide sequence has been sent to the GenBank (accession number: HM367074). The BLAST network server of the National Center for Biotechnology Information (NCBI) were used to compare the homology of chicken IL-6 genes in GenBank, the results showed that the nucleotide sequence identities between sequence HM367074 and other four chicken IL-6 genes NM204628.1, AB191038.1, EU170468.1, AB302327.1 were 99.0, 99.0, 98.0 and 98.0%, respectively. No significant similarity was found between sequence of chicken IL-6 and the human IL-6 genes. The complete ORF of chicken IL-6 was cloned into pMD-18 and then pCDNA3.1, RE analysis showed that eukaryotic expression plasmid pcDNA-ChIL-6 was successfully constructed (Fig. 1).

Construction of recombinant prokaryotic expression vector, expression and purification of ChIL-6 protein:

The mature peptide ChIL-6 gene of 603 bp long was amplified by PCR (Fig. 1) and cloned into pET-32a (+) to achieve the recombinant prokaryotic plasmid PET-32-ChIL-6. The PET-32-ChIL-6 was expressed to produce a fusion protein band of approximately 42 kDa size by SDS-PAGE (Fig. 2). The fusion protein was purified using NTA His•Bind column and the optimal wash concentration of imidazole was 100 mM. The purified protein showed a single target band as shown by SDS-PAGE (Fig. 3). The concentration of purified protein reached 1.5 mg mL⁻¹.

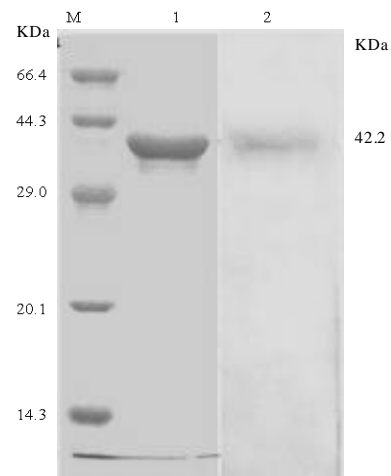


Fig. 3: Results of purification and western-blot of recombinant protein. Lane M: Protein marker. Lane 1: purified PET-32-ChIL-6. Lane 2: western-blot result of purified recombinant protein with pcDNA-ChIL-6 infected anti-sera

Table 1: ELISA detection of sera from mice immunized with pcDNA-ChIL-6 plasmid (OD450 nm)

ELISA OD of values (450 nm)	Serum dilutions							
	1:40	1:80	1:160	1:320	1:640	1:1280	1:2560	1:5120
Anti-pcDNA-ChIL-6 serum	1.541	1.422	1.215	1.037	0.998	0.831	0.548	0.396
Anti-pcDNA3.1 (+) serum	0.218	0.177	0.109	0.097	0.081	0.066	0.051	0.050

Western blot analysis: The western blot analysis showed a strap of interest protein in the transformed membrane (Fig. 3), it suggested that the rPET-32-ChIL-6 can react positively with mouse anti-ChIL-6 polyclonal antibody by immunoblot analysis.

Titer analysis by ELISA: About 20 control group mice sera were detected by ELISA, the average χ was 0.156 and Standard Deviation (SD) was 0.032. The critical value was $\chi+3SD = 0.252$. This means that when the OD value of sample ≥ 0.252 and the ratio of positive sera and negative sera (P/N) ≥ 2.5 , the result was positive while the OD value of sample < 0.252 , the result was negative.

The results of indirect ELISA experiment revealed that the mouse anti-ChIL-6 polyclonal antibody could react positively with the rPET-32-ChIL-6 protein but serum from the mice immunized by anti-pcDNA3.1 (+) react negatively with the rPET-32-ChIL-6 protein. The antibody titer of all the twelve sera could reached 1:5120 in ELISA and ELISA results of one representative positive serum and one negative serum were shown in Table 1.

DISCUSSION

Interleukin-6 is an important cytokine mainly mediating humoral immunity but its role in animal vaccination and infection is not clear now. It is reported that the biological activity of recombinant chicken IL-6 (rChIL-6) was similar to that of recombinant human IL-6 (rhuIL-6) with regard to *in vitro* proliferation of the IL-6-dependent murine hybridoma cell line 7TD1 and induced synthesis of corticosterone *in vivo* in chickens (Van Snick *et al.*, 1986) this cross-species biological activity is an exception of cytokines in chicken (Xie *et al.*, 2008). It had shown that rChIL-6 eukaryotic expression vector could improve the immune effect of LaSota vaccine of Newcastle disease vaccine significantly (Liu *et al.*, 2009). IL-6 can be used as an indicator of immune status preparation of antibodies against chicken IL-6 will make the basis for the development of serological method for the detection of chicken IL-6.

The comparison of published four sequences of chicken IL-6 on GenBank showed IL-6 gene of different kinds of chickens have high homology of no <98% while having no significant similarity with those of the mammalian. In this study, IL-6 gene of chickens of Ya'an local species was cloned, its nucleotide and amino acid sequence identities with other chicken IL-6 genes were 98-99%. Bioinformatics analysis showed that this sequence contained 241 amino acid residues and had a long signal peptide sequences as mammals. The GC content of the DNA sequences of the signal peptides was high and reached 80%.

In this study, we constructed a recombinant expressing plasmid pcDNA-ChIL-6 by inserting the ChIL-6 gene into eukaryotic expression vector pcDNA3.1 (+) under the control Cytomegalovirus (CMV) promoter, polyclonal antibodies was obtained by inoculating BALB/c mouse. Then prokaryotic expression plasmid PET-ChIL-6 was constructed and expressed in *E. coli* BL21 effectively. The result of Western-blot and indirect ELISA showed that plasmid pcDNA-IL-6 can be expressed in eukaryotic cells. The production of mouse anti-ChIL-6 polyclonal antibodies will help in developing an antigen-capture ELISA for the detection of chicken IL-6 which will be highly useful in deciphering the role of chicken IL-6 in immunological or pathological processes in chickens during vaccination or infections.

CONCLUSION

In conclusion, the polyclonal antibody against chicken Interleukin-6 prepared by immunization of recombinant eukaryotic plasmid can react efficiently with its prokaryotic expression protein.

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