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Full Length Research Paper

Cloning and expression of human immunoglobulin G4 (hIgG4) hinge region cDNA from blood sample

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Human immunoglobulin G4 (hIgG4) is increasingly being used for the detection of various infectious diseases and in allergy-related immunoassays, thus, anti-hIgG4 antibody is of interest in the development of diagnostic tests. The present study was conducted to produce a recombinant peptide antigen that can be used to generate monoclonal antibodies specific to hIgG4. The cDNA coding for the hinge region of hIgG4 was derived from mRNA from a human blood sample using reverse transcription polymerase chain reaction (RT-PCR). The cDNA was verified by sequencing and cloned into pQE-2 expression vector to construct pQE-2-IgG4. The latter was transformed *Escherichia coli* M15 cells, recombinant protein expression induced by IPTG and the expression was confirmed by SDS-PAGE and western blot analysis.

Key words: Human IgG4, hinge region, cDNA, peptide, expression.

INTRODUCTION

In recent years, there is an increasing use of human IgG4 (hIgG4) in diagnostics, such as for use in the detection of helminthic diseases (Anantaphruti et al., 2005). Towards this aim, the present study was conducted in an attempt to produce recombinant hIgG4 peptide.

Basic diagnostic tests, such as indirect hemagglutination, indirect immunofluorescence (IIF), radioimmunoassay and enzyme linked immunosorbent assay (ELISA), with relatively crude antigens were reported to be employed to detect infection status in human beings (Jacobson, 1996). Recent advances in immunochemistry and molecular biology have resulted in development of assays with markedly improved sensitivity and specificity.

However, most of the methods remain high cost and not readily available in many less developed countries where most parasitic diseases are found. It is therefore of great importance that approaches being taken to develop techniques of disease detection would combine the properties of user-friendly, easy accessibility and low cost without compromising the quality in terms of specificity and sensitivity of the detection. The use of hIgG4 as a marker of active helminthic infections could fulfill these requirements and the use has been widely documented. This includes reports on lymphatic filariasis, onchocerciasis, strongloidiasis, schistosomiasis, hydatid diseases, cyctercercosis and angiostrongyliasis (Rahmah et al., 2001).

Native proteins have traditionally been used to raise monoclonal antibodies and for some applications; there are advantages in using antibodies against native proteins. However, there are also distinct advantages of using peptides over native proteins, such as reduction of potential cross-reactivity between structurally homologous proteins, production of different antibodies for different epitopes of the same protein and production of antibodies for proteins with post-translational modifications (Geysen, 1985). Thus, peptides are now becoming more commonly used in antibody production. The hinge region of the hIgG4 was targeted for the peptide production since this region contains peptide sequences specific to hIgG4 (Lu et al., 2007). Thus, the use of this peptide as antigen for anti-hIgG4 monoclonal antibody (MAb) production would be expected to increase the specificity of the antibody**.** This in turn may increase the specificity of the test that utilizes the monoclonal

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antibody.

MATERIALS AND METHODS

Bacteria strains and medium

Escherichia coli Top10 was used for transformation of pGEM-T-IgG4 plasmid construct, whereas, *E. coli* M15 was used as a host for the expression of pQE-2-IgG4. Both bacterial strains were obtained from Qiagen, Germany. Luria-bertani (LB) containing 100 µg/ml ampicilin was used as the culture medium. *E. coli* M15 strain contains both the expression (pQE) and the repressor (pREP4) plasmids and can be used for the production of recombinant proteins. It was derived from *E. coli* K12 and have the phenotype NalS, StrS, RifS, Thi^-, Lac^-, Ara^+, Gal^+, Mtl^-, F^-, $RecA^+$, Uvr^+, Lon^+ (Murray, 2002).

Plasmid vectors

Plasmid vectors pGEM-T and pQE-2 were purchased from Qiagen, Germany. PQE-2 is used for the expression of N-terminally His-Tagged protein in *E. coli* and is based on the T5 promoter transcription-translation system.

Isolation of total RNA

The total RNA was isolated from fresh human blood using Tri-reagent (Fermentas, USA) according to the manufacturer's recommendations. The quantity and quality of RNA were analyzed by denaturing agarose gel electrophoresis, then, stored at -80°C. Informed consent was obtained before the human blood sampling by venepuncture, as required by USM human ethics committee.

Reverse transcription polymerase chain reaction (RT-PCR)

Reverse transcription was performed using a commercial kit (Fermentas, USA) 1 µg of the total RNA from the human blood was converted to single stranded cDNA using first strand cDNA-synthesis kit. Two-microlite of the product was used as a template to amplify human IgG4 hinge region of the cDNA sequence for 35 cycles. 1 µl of total RNA was added into 1 µl of oligo (dt) primer and the total volume was added to 12 µl with the addition of DEPC treated water. The mixture was incubated at 70°C for 5 min. 4 µl of 5 x reaction buffers, 1 µl of Ribolock Ribonuclease inhibitor and 2 µl of 10 mM dNTP were added into the tube. The tube was incubated at 37°C for 5 min followed by the addition of RevertAid M-Mulv reverse transcriptase to a final volume of 20 µl. The mixture was then, incubated at 42°C for 60 min. The reaction was stopped by heating at 70°C for 10 min and chilled on ice. The PCR was performed using the following conditions: Initial denaturation: 94°C for 5 min; denaturation: 94°C for 45 s; annealing: 65°C for 1 min; elongation: 72°C for 45 s; final hold: 72°C for 10 min.

After the last cycle of amplification, 10 µl of product was stained with ethidium bromide, analyzed by agarose gel electrophoresis and visualized under UV light.

Primers

The hinge region of hIgG4 consists of 20 amino acids. Among them, 14 were identified to be distinctive to hIgG4 (Tan et al., 1990). The human specific amino acid sequence was reported to be SYGPPSAPEFLGGP (Lu et al., 2007). Primers were designed to amplify the region around this hinge region. In addition, the primer design ensured that the amplification product would be flanked by 5'*Nde*I and 3' *Not1* restriction endonuclease cleavage sites to facilitate directional cloning into plasmid pQE-2 vector.

The sequences of primers were as follows: Forward primer 3: 5'-catatgagcttgggcacgaagacct-3' and reverse primer 4: 5'-gcggccgccacgcacgtgacctc-3'.

Cloning of PCR products

The human IgG4 cDNA was then used as a template for PCR cloning. PCR cycle comprised 30 cycles of heating at 94°C for 1 min, annealing at 62.5°C for 1 min and extension at 72°C for 2 min. The PCR product of approximately 218 bp product was then, cloned into the *Not1* and *Nde1* sites of pGEM-T cloning vector (Promega) to yield pGEM-T-IgG4 and subsequently transformed into *Top10 E. coli.* Blue-white colony screening on ampicillin plate was used to select positive clones. Plasmid from positive clones was extracted using plasmid purification kit (Promega, USA) and confirmed by *Nde1* and *Not1* restriction endonuclease digestions before sending for sequencing.

Recombinant sequence construction

Construction procedure is shown in Figure 1. The pGEM-T-IgG4 was subjected to digestion with *Nde1* and *Not1* restriction endonucleases, followed by gel purification of the 218 bp desired cassette. The pQE-2 vector was similarly digested with *Nde1* and *Not1* restriction endonucleases and gel purified. The insert cassette with the size of 218 bp was ligated to the linearized pQE-2 vector with T4 ligase in the presence of the desired buffer. The mixture was incubated at 37°C for 1 h. This yielded pQE-2-IgG4 with His-tag. The resulting construct was then transformed into *TOP10 E. coli* cells and the positive clones were screened by blue white colony screening on ampicillin plate, followed by analysis by *Nde1* and *Not1* restriction endonuclease digestions.

Expression of recombinant protein in M15 cells

The His-tagged pQE-2-IgG4 was transformed into *E. coli* M15 expression host. The transformants were cultured on the plates containing kanamycin (25 µg/ml) and ampicillin (100 µg/ml). Two isolated colonies were then, cultured in 1000 ml LB medium and induced for the protein expression with 1 mM IPTG and incubated for 4 h. A volume of 1 ml culture was collected at time zero (before IPTG induction) and the rest collected at the end of the incubation.

The bacterial cells were spun down and the supernatant discarded. Half of the pellet was lysed in lysis buffer containing lysozyme; another half of the pellet was lysed in lysis buffer without lysozyme. For sample obtained before IPTG induction, only lysozyme-treated pellet was used. Lysates were incubated on ice for 30 min, followed by cell-breakage using French press and then centrifuged. Thereafter, both supernatant and pellet processed with and without lysozyme were analyzed using SDS-PAGE.

SDS-PAGE and western blot analysis of recombinant hIgG4 hinge region peptide

The samples was added to the SDS sample buffer, heated at 95°C for 5 min and analyzed on a 12% SDS-PAGE. The electrophoresed recombinant peptide was transferred onto nitrocellulose membrane using mini trans-blot apparatus (Bio-Rad, USA) according to the manufacturer's recommendations. The blotted membrane was blocked with casein (20%) for 1 h, washed five times with Tris-

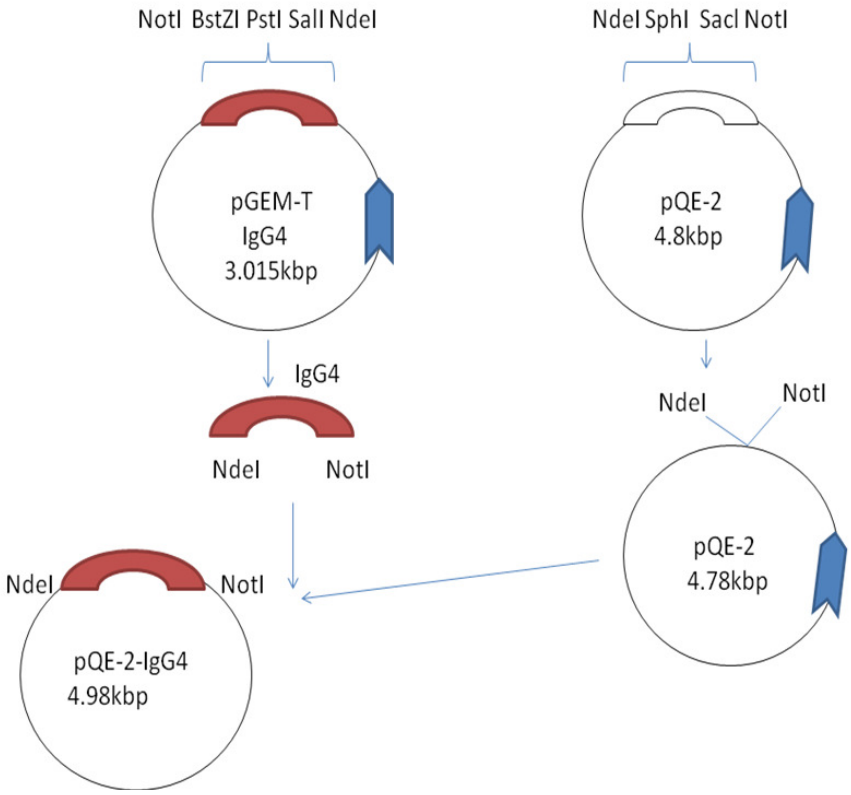

Figure 1. Flow chart for construction of pQE-2-IgG4.

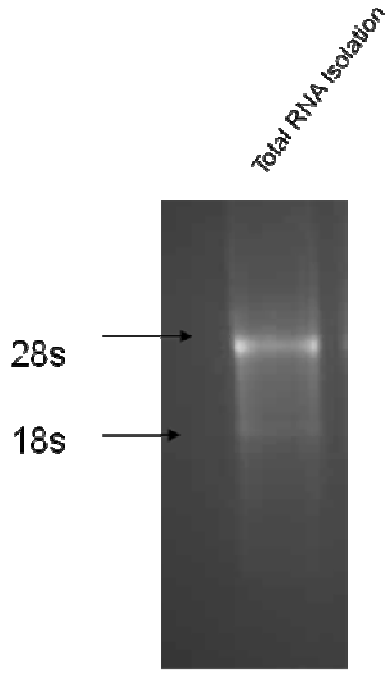

Figure 2. Total RNA isolated from human blood sample was subjected to agarose gel electrophoresis. The bands of 28s and 18s rRNA could be clearly observed.

buffered saline with 0.05% of Tween-20 (TBST), incubated with anti-His-HRP antibody (1:1000 in PBS pH 7.3, Santa Cruz, USA) for1 h at room temperature then washed three times with TBS-T. Binding of the antibody to the antigen was detected using supersignal west Pico chemiluminescent substrate (Thermo Scientific, USA) according to the procedure suggested by the manufacturer.

RESULTS

RNA isolation and RT-PCR

Figure 2 shows the gel electrophoresis picture of the total RNA isolated from human blood sample, clear bands of 28s and 18s rRNA can be observed. The result also showed that, there was no obvious degradation of the isolated RNA that was used in the reverse transcription to cDNA. RT-PCR reaction mix was analyzed using a 1% agarose gel electrophoresis. A specific band with the approximate expected size of amplicon of interest (218 bp) was shown in the gel (Figure 3). The sequence of the end product, pGEM-T-IgG4 was as expected with no base mutation and is as follows: 5'catatgagcttgggc-acgaagacctacacctgcaacgtagatcacaagcccagcaacaccaaggt ggacaagagagttgagtccaaatatggtcccccatgcccatcatgcccagca cctgagttcctgggggaccatcagtcttcctgttccccccaaaacccaagga cactctcatgatctcccggacccctgaggtcacgtgcgtggcggccgc 3'.

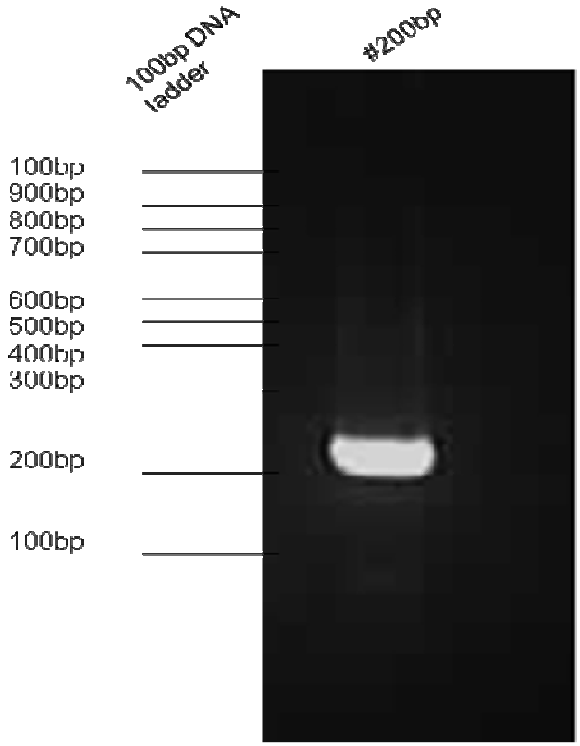

Figure 3. Product of the RT-PCR reaction ran on a 1% agarose gel showed a single band at approximately 200 bp.

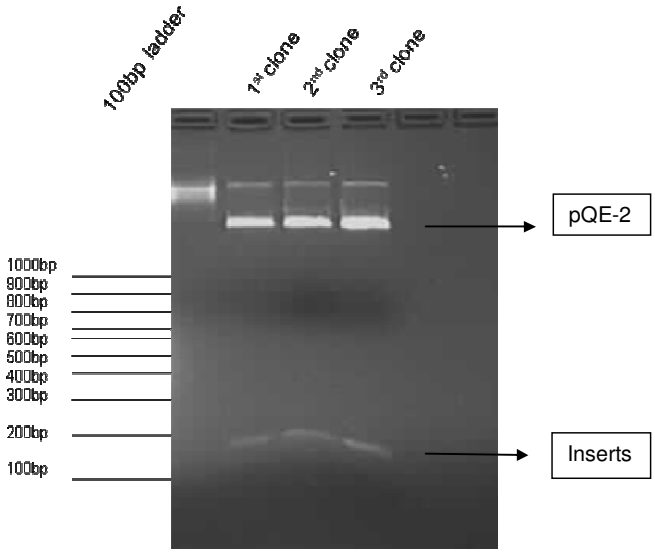

Figure 4. Plasmid DNA isolated from *E. coli* M15 transformed with pQE-2-IgG4 was digested with restriction endonucleases *Not*1 and *Nde*1. Agarose gel electrophoresis result showed the band of pQE-2 (4.8 kb) and the insert (about 204 bp).

Based on the earlier mentioned, the corresponding peptide comprised 68 residues of the expected sequence of amino acids.

Construction of pQE-2-IgG4 expression plasmid DNA

Figure 4 shows the gel picture of the result of restriction digestion of pQE-2-IgG4. Two bands representing the vector pQE-2 (4.8 kb) and the insert (218 bp) in each lane of DNA extracted from clones 1, 2 and 3 can be seen. The digestion was not complete and hence, the undigested DNA construct could be observed at the top of each lane. These pQE-2-IgG4 constructs were sent for sequencing and the results reconfirmed that, the correct sequence has been inserted (data not shown).

Recombinant peptide expression of hIgG4 hinge region

The expected size of the expressed protein was approximately 12 kDa. As shown in Figure 5, there is a little expression of the recombinant protein at 0 h of induction (1st and 2nd lanes). With IPTG induction, the amount of recombinant protein obtained was much higher. The use of lysozyme results in higher amounts of the recombinant protein than without the use of the enzyme (lanes 5 and 3, respectively). As the protein of interest was tagged with six histidines at the N-terminal, the presence recombinant protein in the samples was confirmed by western blot analysis (WBA) using anti-His-tag-HRP (Figure 6). Similar to that seen in SDS-PAGE, under non-induced condition there was some leaky protein expression as shown in lane 1. However, the signal detected was relatively low compared with that seen in lane 3 from supernatant of cell pellet lysate from IPTG induced culture.

DISCUSSION

The results of the present study confirmed the expression of the desired recombinant His-tagged peptide hinge region specific to hIgG4. With this peptide, the immunization of mice to produce specific anti-hIgG4 MAb can be performed. However, the removal of histidine tag would first be required to eliminate the production of antibody against the His-tag.

The capability to produce this hIgG4 specific peptide in unlimited amounts in the laboratory will be very useful in the MAb production and for use in other applications. MAb produced against human IgG4 antibody is useful for the development of immunoassays to detect helminthes infections for use in in-house assays and for diagnostic kit production. An example of a detection kit that uses anti-human IgG4 is Brugia Rapid^{TM}, which is based on the principle of binding of the *Bm*R1 recombinant antigen to a filarial-specific human IgG4 antibody in patient sample, followed by binding of the complex to mouse anti-human IgG4 MAb (Rahmah et al., 2001). In the process of the diagnostic kit production, a major cost

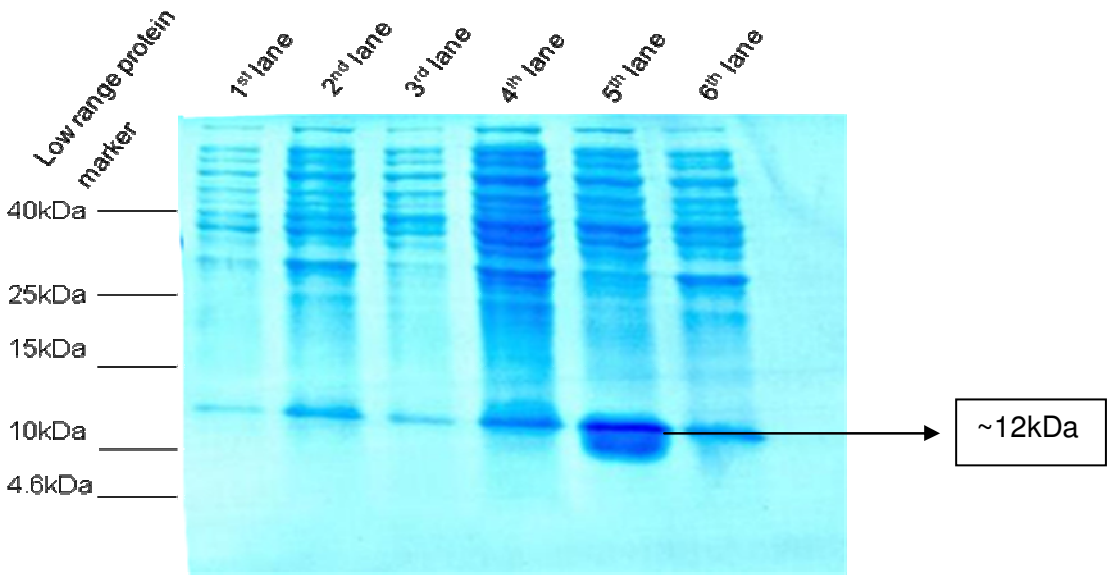

Figure 5. Equal amounts of proteins from supernatant (medium) and cell pellet lysate were subject to SDS-PAGE. The expected size of the protein of interest was 12 kDa.

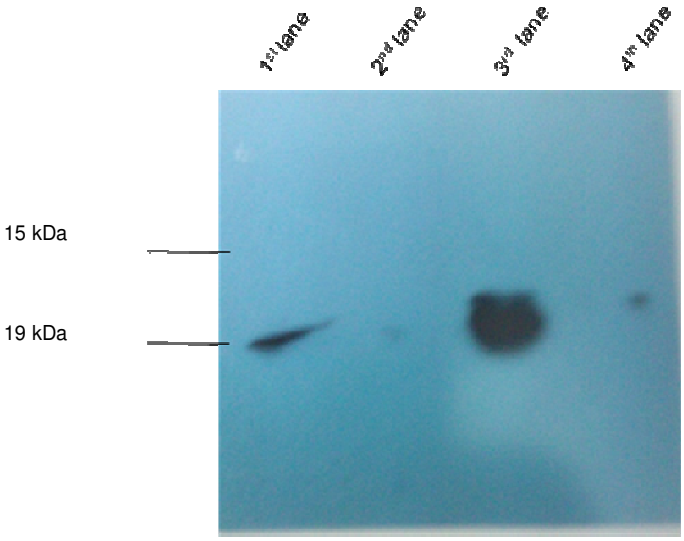

Figure 6. Western blot analysis using HRP-conjugated anti-His antibody. A thick band at ~12 kDa is seen in the lane from the supernatant of lysozyme-treated pellet.

incurred is the purchase of anti-human IgG4. Since this kit is for detection of brugian filariasis, a neglected tropical disease endemic in poor countries, the ability to produce IgG4 MAb would enable the kit to be available at a much lower price. Specific IgG4 antibody has also been used as a marker of antigen exposure in allergy, including those caused by food allergy (Tomee et al., 1996; Homburger et al., 1986). The levels of specific IgG4 antibodies have been measured in clinical studies of allergic diseases such as asthma, rhinitis, urticaria, aspergillosis (Till et al., 004). In addition, allergen-specific IgG4 have also been used in monitoring immunotherapy (Aalberse et al., 1993).

MAbs raised against Fab and Fc regions of the antibody has been previously reported, such as for cancer research (Li et al., 1998). However, to the best of our knowledge, there is no report this far on MAb against the antibody hinge region. The peptide of the hinge region is the most specific region for IgG4 molecules, thus, this was cloned in for future use to produce anti-human IgG4 MAb. Antibody raised against this region would be expected to be more specific than that raised against the whole antibody.

Although, the primary sequence of the peptide corresponded to the specific hinge region of hIgG4, the folding of the peptide may not mimic the one in the whole hIgG4 molecule and hence, the antibody produced from the peptide may not be able to recognize the native hIgG4. This can only be ascertained after the MAb to the recombinant peptide is produced and tested.

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