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Cloning and Sequencing of Iranian Chicken Interleukine-2 Gene

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ABSTRACT

Background: : IL-2 is a cytokine that plays an active role in the activation and maintenance of both acquired and innate immune defenses. It is also capable of improving the protective immune responses that are generated by conventional vaccines against avian pathogens in the poultry industry when used as an adjuvant.

Objectives: The aim of this study was to extract and sequence Iranian chicken IL-2.

Materials and Methods: In this study, genomic DNA was extracted from Iranian chickens. Total RNA was isolated by culturing harvested splenocytesand lysing them with Trizol reagent per the manufacturer's instructions. mRNA was isolated and converted to cDNA using reverse-transcriptase (RT) and specific designed primers. Then, the PCR product was ligated into the PTZ57R/T plasmid (TA-cloning) and transformed into competent Top10 E. coli cells.

Results: A unique 668-bp band was obtained after RT-PCR. Restriction enzyme digestion and colony PCR analysis and direct sequencing confirmed the existence of the desired gene in transformants.

Conclusions: For first time in Iran, the chIL-2 gene was successfully extracted, cloned, and transferred into E. coli. The efficacy of recombinant or DNA vaccines can be modulated by codelivery of this cytokine gene.

Implication for health policy/practice/research/medical education:
The ability of chIL-2 to enhance T-cell activation and proliferation suggests that it augments the immune response when used as an adjuvant.

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1. Background

In the past 2 decades, the poultry broiler industry has grown astonishingly; almost 40 billion birds are hatched worldwide annually. This highly profitable industry provides approximately 40 % off all consumed meat, with a $150 billion per annum retail market, and supplies a multibillion dollar poultry health market. Since chickens are grown under forceful conditions, they are more susceptible to infectious diseases. These diseases can cause devastating consequencessuch as tremendous loss of productivity and further spread of the pathogen in the area. Unlike treatment with antibiotic drugs, which leads to further problems, such as appearance of antibiotic-resistance bacteria, vaccination against infectious disease is the most effective way of reducing or eradicating these diseases.

Cytokines are proteins that have a key role in stimulating the immune system. They can generate a protective immune response as an antibody-mediated (Th2) response or a cell-mediated (Th1) response, based on their combination (1). Therefore, the use of cytokines as novel vaccine adjuvants has been well investigated (2). Further, IL-2, a well-known cytokine, has attracted much interest

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due to its pleiotropic characteristics and vital role in activating T-cell proliferation (3).

The stimulation and differentiation of T cells, B cells, NK cells, lymphokine-activated killer (LAK) cells, monocyte/macrophages, and neutrophils (4-6) are wellknown. Many immunosuppressive avian pathogens, such as IBDV (7), Marek's disease virus (8), Newcastle disease virus (9), chicken anemia virus (10), and Eimeria tenella (11), interfere with the induction of chIL-2 in vivo or cause its abnormal production. This suggests that chIL-2 may be important for the control of these diseases (12). The aim of this study was to extract and sequence Iranian chicken IL-2.

2. Materials and Methods

2.1. Chicken

Outbreak inbred Iranian chickens were bred at the Razi Institute. Newlyhatched chickens were housed in wire-floor cages with free access to food and water. They were immunized against IBDV disease virus on the day of hatching. Thirty-day-old Iranian chickens were used for this study.

2.2. Isolation of Splenic Mononuclear Cells (SMCs)

Spleens from 4-week-old Iranian chickens were collected aseptically into RPMI 1640 medium (Invitrogen). Spleen tissue was minced using a pair of sterile scissors and passed through a steel mesh to obtain a homogeneous cell suspension. The cells were pelleted by centrifugation at 13,000 rpm for 5 min at 4°C and washed twice in RPMI 1640 medium (Gibco) with EDTA 2.5 %. Cell suspensions were then overlain on an equal volume of Histopaque-1077 (Sigma). The interface, rich in mononuclear cells, was recovered after centrifugation at 1500×g for 15 min at 4°C. Cells were washed twice in serum-free RPMI 1640 and centrifuged at 1300×g for 5 min at 4°C. After being assessed for cell viability by Trypan blue dye exclusion technique, the cells were resuspended at a concentration of 10^7cells/ml in RPMI 1640 (containing 1 % l-glutamine, 2 mg/ml fetal serum albumin, 1 U/ml penicillin, and 1 mg/ml streptomycin). Then, the cells were cultured in 6-well plates and stimulated with ConA at a final concentration of 10 μg/ml at 40°C in a humidified atmosphere with 5 % CO2.

2.2. RNA Isolation and RT-PCR

The cells were harvested, and total RNA was isolated with Trizol reagent (Invitrogen) at 18, 24, 36, and 48 h after ConA stimulation. Briefly, Trizol (750 μl) was added to cells, mixed thoroughly, and incubated for 5 min at room temperature (RT); 200 μl chloroform was added, mixed, and incubated for 5 min at room temperature. Then, centrifugation was carried out at 1200 g for 20 min at 4°C. Total RNA in the aqueous phase was precipitated with 800 μl isopropanol, and the RNA pellet was washed with 75 % ethanol, air dried, and resuspended in 50 μl nuclease-free water. To determine the full coding sequence of chIL-2, forward (5'- CTAGAATTCGATAACTGGGACACTG-3') (with EcoRI site underlined) and reverse primer (5'- GTCAAGCTTCAACGTACATTTTGAG-3') (with HindIII site underlined) were designed based on published sequences of chicken IL-2 (GenBank Accession No. AF000631), and cDNA was synthesized from total cellular RNA by one-step RT-PCR.

RT-PCR of chicken IL-2 (chIL-2) gene was performed in a 25-μl reaction for each mRNA sample containing 5 pmol of F-primer, 5 pmol of R-primer, 5 μl AMV/Tfl Buffer (5x), 3 μl MgSO4 (25mM), 0.5 μl dNTP Mix (10 mM), 0.5 polymerase (5 mμl-1) (Promega), 12 μl nuclease-free water, and 300 ng mRNA. The amplification program was as follows: 45°C for 45 min; 5 min at 95°C; 35 cycles of 30 s at 95°C for denaturation, 50 s at 55°C for annealing, and 1 min at 72°C for elongation; and 72°C for 10 min for prolonged elongation. The expected 668-bp PCR product was by electrophoresis of 4 μl of the total PCR reaction volume on a 1 % agarose (TAE) gel.

2.3. T/A Cloning of Chil-2 Gene

The desired 668-bp fragment was cloned into the PTZ57R/T vector using the TA cloning kit (Fermentas) with 3 μl PTZ57R/T vector, 6 μl Ligation Buffer (5x) (30 mM Tris-HCl pH 7.8, 10 mM MgCl2, 10 mN DTT, 200 μM ATP, 5 % polyethylene glycol), 1 μl T4 DNA Ligase (Promega), 18 μl nuclease-free water, and 200 ng PCR. Ten microliters of ligation mix was added to 150 μl of Escherichia coli Top10 competent cells and incubated on LB Agar plates (Merck) containing 50 μg/ml ampicillin, 100 mM IPTG, and 20 mg/ml X-Gal overnight at 37°C. White colonies were selected and checked for the presence of the insert by PCR.

2.4. Screening of Transformed Colonies

PCR was performed to confirmthe desired gene in the T/A vector in the selected colonies. PCR-positive colonies were grown overnight in 4 ml of LB medium containing 50 μg/ml ampicillin. Plasmid DNA was extracted and purified from 4 ml of bacterial suspension following the instructions of the Fermentase mini-prep kit. Also, the transformants were confirmed by RE analysis with EcoRI and HindIII at 37°C for 5 h. The recombinant plasmids were sequenced by Millegene (France). BLAST was used to compare the nucleotide sequences and the registered computational chicken sequence at NCBI (accession number AF000631).

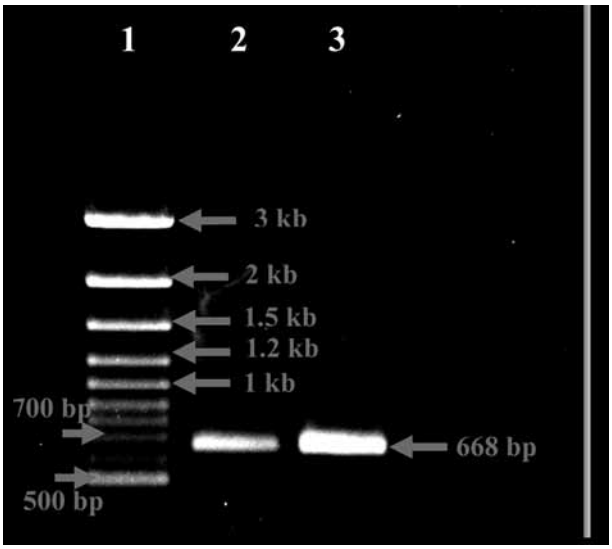
3. Results

3.1. Isolation SMC, RNA and RT-PCR

Based on the chIL-2 sequence published by Sundick and Gill-Dixon (1997) (3), 2 primers that spanned the coding region of chIL-2 were designed with restriction sites. Isolation of lymphocytes from the culture and ConA-activat-

ed spleens from Iranian chickens, followed by extraction of total RNA and RT-PCR, resulted in the synthesis of a 668-bp DNA fragment after 18 h of culture. After RT-PCR, a distinct 668-bp band appear that was related to the IL-2 gene *(Figure 1)*

Figure 1. RT-PCR Amplification of the chIL-2 Gene Using IL-2F and IL-2R Primers in 18 h of Culture.

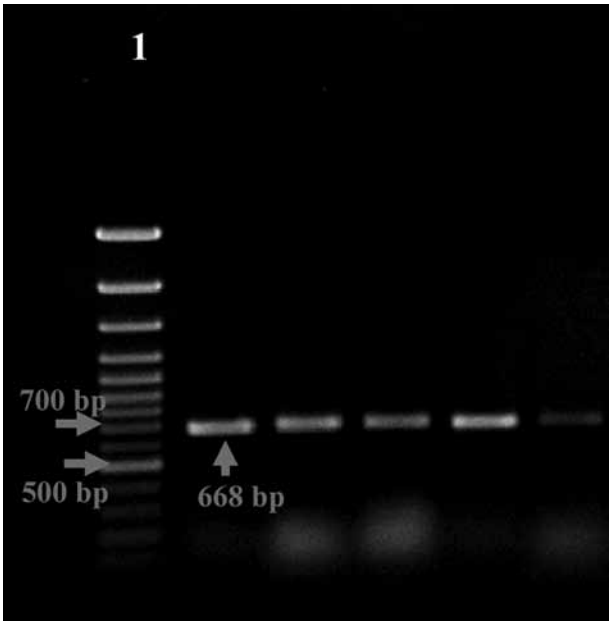


Lanes 2 and 3 are the Same PCR Products and Lane 1 is DNA Ladder of 1 Kb.

3.2 Screening of Transformed Colonies

The amplified PCR product was cloned into the T/A vector and transformed into competent *E. coli Top10*.White colonies (with the insert) were chosen and checked for the presence of the insert by colonyPCR. The amplification reaction contained 5 µl Tf1 Buffer, 3µl MgSO4, 0/5 µl dNTP, 5 pmol primer-F, 5 pmol primer-R, 0/5 µl Tf1 DNA polymerase, 10 µl H2O (nuclease-free), and transformed colony as DNA template. Figure 2 shows the results of the colonyPCR.

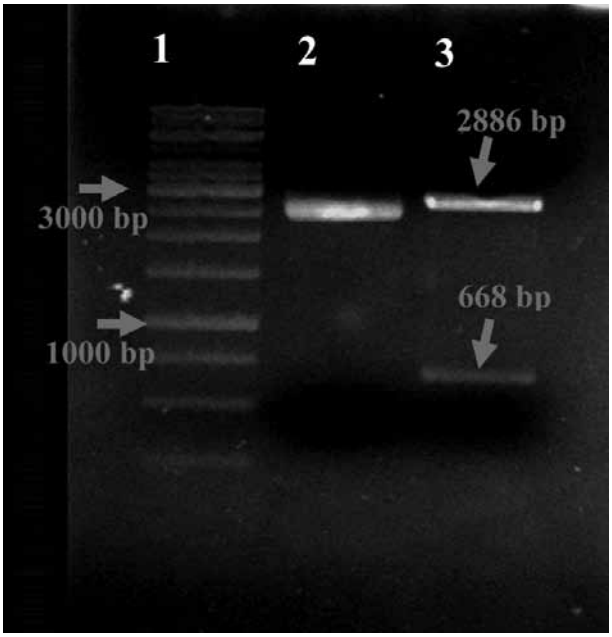
Figure 2. PCR Results of Five White Transformants After T/A Cloning and Transformation of T/A-chIL-2 Gene into Competent E. Coli.



Lane 1 is 100 bp DNA Ladder.

Extraction of T/A-IL-2 plasmid and agarose gel electrophoresis generated a band, and digestion of this purified recombinant plasmid with restriction enzymes showed 2 distinct bandschIL-2 gene and TA plasmid (*Figure 3*). By sequence comparison with chicken IL-2, the 668-bp PCR product was the intended gene, with 99 % identity. The entire sequenced Iranian chicken interleukin-2 gene is shown in *Figure 4.*

Figure 3. Digestion of TA/IL-2 Plasmid.



Lane 1: 1 kb DNA Ladder, lane 2 Uncut Extracted TA/IL-2, Lane 3: Digested TA/IL-2 With EcoRI and HindIII, yielding fragments of 2886 bp (TA) and 668 bp (chIL-2)

Figure 4. Sequence of Iranian Chicken Interleukin-2 Gene. Restriction Enzymes are Indicated in Bold Capital Letters.

NNNNNNNGGATCCGATTCTAGAATTCGATAACTGGGACACTGCCATGA
TGTGCAAAGTACTGATCTTTGGCTGTATTTCGGTAGCAATGCTAATGAC
TACAGCTTATGGAGCATCTCTATCATCAGCAAAAAGGAAACCTCTTCA
AACATTAATAAAGGATTTAGAGATATTGGAAAATATCAAGAACAAGAT
TCATCTCGAGCTCTACACACCAACTGAGACCCAGGAGTGCACCCAGC
AAACTCTGCAGTGTTACCTGGGAGAAGTGGTTACTCTGAAGAAAGAA
ACTGAAGATGACACTGAAATTAAAGAAGAATTTGTAACTGCTATTCAA
AATATCGAAAAGAACCTCAAGAGTCTTACGGGTCTAAATCACACCGG
AAGTGAATGCAAGATCTGTGAAGCTAACAACAAGAAAAAATTTCCTG
ATTTTCTCCATGAACTGACCAACTTTGTGAGATATCTGCAAAAATAAG
CAACTAATCATTTTTATTTTACTGCTATGTTATTTATTTAATTATTTAATTA
CAGATAATTTATATATTTTATCCCGTGGCTAACTAATCTGCTGTCCATTC
TGGGACCACTGTATGCTCTTAGTCTGGGTGATATGACGTCTGTTCTAAG
ATCATATTTGATCCTTTCTGTAAGCCCTACGGGCTCAAAATGTACGTTG
AAGCTTACAATCTAGATGCNNNNNNNNNNN

The Translation Start Codon, ATG, and the Termination Codon, TAA, are Underlined.

4. Discussion

Immune cytokines, such as chIL-2, are being incorporated into vaccination regimens by the poultry industry and could potentially act as natural vaccine-enhancing molecules (13). IL-2 is a nonantigen-specific lymphokine produced by T lymphocytes following antigenic stimulation (14). This lymphokine is essential for T lymphocyte proliferation (15), differentiation of B lymphocytes into plasma cells (16), and cloning of antigen-specific T lymphocytes (17). IL-2 has come to be recognized as an important immunoregulatory molecule because of its various functions (14).

In the present study, based on the chIL-2 sequence published by Sundick and Gill-Dixon (1997), 2 specific primers were designed to span the coding region of chIL-2. Our group isolated lymphocytes from spleen cell cultures of 4-week chickens, versus other studies that used blood-extracted leukocytes, such as Hulse *et al.* (2004), who extracted chIL-2 from peripheral blood isolated by Histopaque 1077 by piercing the hearts of 8-week-old chickens (13). Also, Kumar *et al.* (2009) used white blood cells obtained by Histopaque 1077 (18). To isolate the leukocytes of peripheral blood, we used Ficoll. Our results are in good agreement with those of Kogut (2003) (19), Choi (2003) (11), and Li (2004) (12). However, Hulse (2004), Kumar (2009), and Zhou (2005) used Histopaque 1077 as an alternative for Ficoll (13, 18, 20).

The RT-PCR at a 55°C annealing temperature generated a 688-bp band of the chIL-2 gene after only 18 hours of culture, and no band was detected at 24, 36, or 48 hours. This is noteworthy, because the length of genes reported in other studies have varied, depending on primer design. We used Hindi III and EcoRI, unlike other reports.

Here, after performing gradient RT-PCR using specific primers and transferring the product to a 1 % TAE agarose gel, we observed a 688-bp band of chIL-2. To grow the PCR product, it was ligated into thePTZ57R/T vector, which is an appropriate system for direct one-step cloning of PCR-amplified DNA fragments, with a typical yield of greater than 90 %. Our sequencing results of Iranian chIL-2 show that full-length chIL-2 cDNA is 668bp, encoding a 143-amino-acid precursor. To determine whether this nucleotide sequence has shared identity with any known or characterized nucleotide sequences, sequence analysis was performed using GenBank. The analysis identified chIL-2 as a member of the IL-15 superfamily. Iranian chIL-2 shares 58 % homology with duck IL-2, 80 % homology with turkey IL-2, and 41 % homology with mammalian IL-2. Against other NCBI-published chIL-2sequences, our Iranian chIL-2 was 100 % identical to the Chinese breed broiler and UK chIL-2 genes. Furthermore, 99 % identity was seen with the US, Chinese chengren, and leghorn chIL-2 gene. Further analysis showed that Iranian chIL-2 shares 98 % homology with the Chinese breed Xianju and Indian morghi and 97 % with the Chinese breed silky. Analysis of the predicted amino acid sequence suggests that the overall protein structure is conserved.For the first time, in the present study, Iranian chIL-2 was extracted, and the similarities and differences were studied. Further studies should produce Iranian chIL-2 recombinant protein and study-potential in both DNA and recombinant vaccines. We conclude that systemic use of IL-2 enhances protection significantly, which correlates with intensified cellular responses, rather than antibody-mediated protection.

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