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Retroviral Transduction of Fluonanobody and the Variable Domain of Camelid Heavy-Chain Antibodies to Chicken Embryonic Cells

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ABSTRACT

Background: Single domain antibodies from camel heavy chain antibodies (VHH or nanobody), are advantages due to higher solubility, stability, high homology with human antibody, lower immunogenicity and low molecular weight. These criteria make them candidates for production of engineered antibody fragments particularly in transgenic animals. **Objective:** To study the development of transgenic chicken using a recombinant retrovirus containing fluonanobody. **Methods:** The retrovirus constructs containing nanobody genes along with secretory signals and GFP gene were established and packed. The virus particle containing the obtained fusion gene was injected into the eggs in stage X. Molecular detection and protein analysis was done in the G0 chickens. **Results:** The rate of hatched chicken after gene manipulation was estimated to be about 33%. Real-Time PCR assay showed that the nanobody along with GFP gene were integrated in cells of 1.2% of chickens. **Conclusion:** We conclude that although the rate of gene transfer by recombinant viruses in chickens is low, it would be possible to transfect the target camel immunoglobulin gene into chicken genome.

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Keywords: Fluonanobody, GFP, Nanobody, Retrovirus, Transgenic Chicken

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INTRODUCTION

Transgenic animals are becoming more important as they being used for disease models, gene therapy and production of high quality biological medicine. For example, transgenic chickens can be used for producing specific proteins which can be purified from the egg. This is economically more feasible and produces properly folded and glycosylated proteins which is not achievable in *E.coli* (1). The transgenic animals allow more effective treatments to be developed and help test the safety of new medicines and vaccines. Research on the transduction methods of recombinant camel variable domains of heavy-chain antibodies (nanobody) has potential value in the pharmacology industry (2). Additionally, transgenic chickens can be ideal bioreactors for producing large amounts of pure recombinant proteins (3-5). Transduction of chick embryo by injecting the recombinant virus near the blastoderm is a simple procedure and egg cell is easily accessible (6). However, in poultry egg cell is hidden and covered, thus opening of egg shell is necessary. So far, in the case of transgenic chicken successful and unsuccessful attempts have been reported (7-11). There are several procedures for injection of gene constructs into an animal egg. The advantages and disadvantages of each procedure are related to animal species (12-14)

Avian leucosis virus (ALV) with deficient replication system was derived from reticuloendotheliosis virus (REV) and used as a DNA vessel to produce transgenic chickens (10,15-16). Advantages in retroviral and lentiviral derived vectors over previously tested vector encouraged us to evaluate their use in production of transgenic chickens (7-11,16-17). Small size, specificity and ability of structural and functional manipulation made recombinant antibodies useful in a wide range of biotechnological applications, detection and therapeutic purposes (18-21). Fluorescent labeling of proteins has been employed in biochemical, immunological and clinical studies. However, in chemical labeling, efficient labeling of the target protein and control of specific sites are difficult and unwanted side reactions may occur (22). Therefore, recombinant green fluorescent protein (GFP) has become an efficient tag for studying intracellular proteins and their interactions in living cells. Fluorescent emitting proteins were originally isolated from pacific jellyfish named *Aquaria Victoria*. These proteins could be used to range of proteins including antibodies, fluorescent emitting antibodies have various applications including flowcytometry, fluorescent immunoassay, fluorescent microscopy, etc (23). Furthermore, these proteins could be used in targeting and imaging techniques (3, 24).

Single domain antibodies isolated from camel heavy chain antibodies are known as VHH or nanobody, are adaptable to screening techniques such as phage display which provides fast and easy isolation of specific antibody. VHHs have advantages over other antibody fragments such as scFv or Fab, these include higher solubility, lesser immunogenicity, lower molecular weight and higher stability (25-27).

Production of chimeric recombinant single domain antibody-green fluorescent protein fusion (fluonanobody) has already been reported (2). The purpose of this work was to study the development of transgenic chicken using a recombinant retrovirus containing fluonanobody.

MATERIALS AND METHODS

GP2-293 Packaging Cell Line and GP-293 Luc Packaging Cell Line, were obtained from Clontech (USA) and cultured in DMEM (Dulbecco Modified Eagle Medium) containing 10% FCS (Gibco, USA), high glucose concentration along with sodium pyruvate, 4 mM glutamine and 1% penicillin/streptomycin antibiotic mixture. pLXRN, pLNHX, and pLLRN control vectors were also obtained from Clontech. Histoacryl tissue was obtained from Williams Medical Supplies. Endonoclease Free Plasmid purification kit was provided by QIAGEN. DNA extraction kit was provided by Bioneer Company. The InsTAclone™ PCR Cloning kit was obtained from Fermentas Company. Different primer sets were synthesized by MWG Company (Germany). Additional materials used in this study were purchased from Sigma and Merck Companies. Transfection procedures were carried out via calcium phosphate method. All primers used in this study were designed by Oligo 5.0 Software and are included in Table 1.

Table 1. List of primers used in this study.

Primer Name	Sequence (5´ → 3´)	Application
Ig-F1	CCCCTGGCTGCTCTGGGGGATGTGCAGCT	**VHH amplification**
Ig-R	CTAGCAAAGCTTTGAGGAGACGGTGACCTG	
Ig- F2	ATGAGGTCTTTGCTAATCTTGGTGCTTTGCTTCCTGCCCCTGGCTGCTGGG	**lysozyme signal-VHH construction by SOE-PCR**
Ig-F3	TCTAAGGGATCCATGAGGTCTTTGCTAATC	
Ig-R	CTAGCAAAGCTTTGAGGAGACGGTGACC	
Ig-F3	TCTAAGGGATCCATGAGGTCTTTGCTAATC	**lysozyme signal-VHH amplification**
Ig-R	CTAGCAAAGCTTTGAGGAGACGGTGACCTG	
Ig-F3	TCTAAGGGATCCATGAGGTCTTTGCTAATC	**lysozyme signal-VHH-GFP amplification**
GFP-R	GTGATAGATCTTTACTTGTACAGCTCGTCC	
GFP F	TTGATAAGCTCCACCGATGGTGAGCAAGGGC	**GFP amplification**
GFP R	GTGATAGATCTTTACTTGTACAGCTCGTCC	
F.GAPDH	CACCTTGCCTAGAATGATTCAG	**glyceraldehyde-3-phosphate dehydrogenase amplification**
R.GAPDH	GCAGTGCTCCCATCACAATC	
Forward R	GTTTATGCAAAGTCCGTGAGG	**Partial amplification of VHH-GFP**
Reverse R	GTCTTGTAGTTGCCGTCGTC	

VHH (Nanobody), Lysozyme Signal Sequence-VHH (LSS-VHH) and Lysozyme Signal Sequence-VHH-GFP Amplifications. Cloning of lysozyme-VHH-GFP (LSS-VHH-GFP) gene was performed as follows. The EGFP gene was amplified with "GFP F" and "GFP R" primers from pEGFP-C1 vector and cloned into *Hind III* and *Bgl II* sites of AAV-MCS viral vector. Obtained construct was named as "AAV-MCS-GFP". LSS-VHH encoding sequence obtained by SOE-PCR between synthetic LSS and VHH was cloned into *BamHI* and *HindIII* sites of pUC18 cloning plasmid. LSS-VHH gene was digested, gel purified and ligated upstream of GFP gene in AAV viral vector, product gene was named "AAV-MCS-LSS-VHH-GFP". The viral vector containing the desired gene (AAV-MCS-LSS-VHH-GFP) was used throughout the experiments (2).

Construction of Retrovirus Vectors Encoding Lysozyme Signal Sequence (LSS), Nanobody, and Green Fluorescent Protein (Fluonanobody). The viral vector containing LSS-VHH-GFP gene prepared in above section was digested and the product was ligated into the *BamHI* digested pLXRN vector. The construct was named pLXRN-LSS-VHH-GFP. In addition, AAV-MCS-LSS-VHH-GFP vector was furthermore digested and LSS-VHH-GFP product was cloned into *BglII* site of pLNHX vector. This product will be referred as pLNHX –LSS-VHH-GFP. The XL-Gold bacteria was transformed with the two above constructs separately and plated on selective LB medium containing 100 µg/ml ampicillin. Transformed bacteria were grown overnight at 37°C. Bacterial colonies were screened for the presence of gene by colony PCR. Positive colonies were cultured in LB medium containing 100 µg/ml ampicillin and incubated for 16 h at 37°C and then used for plasmid extraction. pLXRN-LSS-VHH-GFP vectors were digested with *XhoI* and *BamHI* enzymes, while *ClaI* and *Bgl II* enzymes were used for digestion of pLNHX-LSS-VHH-GFP vectors. The products were analyzed by electrophoresis to confirm the correct orientation of the gene cloning. The recombinant vectors were furthermore analyzed by sequencing.

Production of Retrovirus Particles Containing Lysozyme Signal Sequence (LSS), Nanobody and Green Fluorescent Protein Gene (Fluonanobody Particles). Viral vectors were produced by transfection system as follows. The virus stocks were harvested 56 h post transfection and titrated on NIH3T3 cells in 6-well plates. 10 cm dishes seeded by GP-293 cells in the previous day with a maximum confluence of 70% were used for transfection by calcium phosphate treatment. Five micrograms of each vector (total of 10 µg of pLNHX-LSS-VHH-GFP, or 5 µg of pLXRN-LSS-VHH-GFP and 5 µg of pSVG) were used for each transfection procedure. The transfected cells were incubated for 5-6 h, after which the medium was replaced with 5 ml of fresh medium. Unless otherwise specified, the viral supernatants were harvested 56 h after transfection, filtered through 0.45 µm filters and tittered by infection on NIH 3T3 cells as follows. NIH 3T3 cells were plated onto six-well culture dishes at a density of 1-2×10^5 cells/well one day before infection. Serial dilution of recombinant virus was prepared from the original stock and after mixing one ml of dilution with 8 µg polybrene (Sigma) the mixture was added to each well. The dish was incubated at 37°C and 1 ml of fresh medium was added after 24 h. Virus titer was determined by selection in presence of 400 µg/ml of G418. The medium was replaced every 3 to 4 days and the resistant colonies were counted 10 to 15 days post infection by observing under light microscope. Virus titer was determined by multiplying the total number of G418-resistant colonies by dilution factors. pLLRN and pSVG control virus particle were made by GP-293 Luc Packaging Cell.

Injection of Eggs and Chicken Treatment. In a typical experiment (repeated few times in order to obtain proper transfected eggs and hatched chickens), hundred fertilized chicken eggs were purchased from a chicken care center. The chicken eggs were kept under 40 to 50% humidity and 22 to 25°C for at least one day before virus injection. In five experiments (stage X), the recombinant retrovirus were injected beneath the blastoderm of chicken embryos. Virus injection was carried out by two manners, air sac injection (vertical) and horizontal injection. Vertical virus injection was performed according to the following procedure: The egg shell was swabbed with 70 percent (v/v) ethanol; a window of 0.4 cm in diameter was made and carefully removed. Approximately 50 µl of high titer virus solution was injected into sub germinal cavity via air sac at 15°C angle. All injection procedures and embryo manipulations were carried out under sterile conditions. Each window was treated with antibiotics, sealed with Histoacryl tissue adhesive tape (tissue tape) or agarose, subsequently the window was covered with the removed piece of egg shell and fixed with cold-cure acrylic (AcroPARS). Injected eggs were kept in incubator under conditions of 37.5°C temperature and 70% humidity for 21 days. Eggs were rotated for the first 18 days and laid still for the last 3 days. The hatched chickens were kept under at 25°C and illumination cycles (17 h light and 7 h dark) and provided with food, water and supplements.

DNA Extraction. DNA samples were extracted from blood and different tissues of un-hatched (embryos) and 21 days old chickens. Tissues were chosen based on derivation of all three embryonic germ layers, namely the endoderm, mesoderm and ectoderm. DNA samples were extracted and purified from skin, brain, spinal cord, heart, liver, intestine, muscle and etc according to the kit manufacturer's procedure with some modifications for each tissue.

Primer design. Primers were specifically designed for each gene to minimize nonspecific interactions. All primers were designed by Oligo 5.0 software.

Polymerase Chain Reaction and Real-Time PCR. The amplifications were performed in 25.0 µl volumes with 1 unit of Taq DNA polymerase, 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2 mM MgCl2, 0.8 mM deoxynucleotide triphosphates (dNTPs: dATP, dCTP, dGTP, dTTP), 2 µl of primer mix for GAPDH or 2 µl of primer mix for VHH-GFP and DNA template at a concentration of approximately 250 ng. Thermal cycling conditions were as follows: (1) 95°C for 5 min, (2) 35 cycles: 94°C for 30 sec, 55°C for 30 sec, 72°C for 50 sec, and (3) 70°C for 10 min. Corbett RG3000 with Sybr-green were used for Real-Time PCR. All reactions were run in duplicate. The PCR reaction mixtures were prepared in 15 µl volumes and thermal conditions were same as above.

Sequencing and Alignments. The InsTAclone™ PCR Cloning Kit (Fermentas) was used for one-step cloning of PCR products with 3'-dA overhangs into pTZ57R/T vector. The products (VHH-GFP) obtained from PCR using extracted genome from different tissues of transgenic chickens as templates were cloned into TA vector according to the procedure instructions. The resulting recombinant vectors were used for sequencing. Obtained sequence results were blasted against NCBI nucleotide database. Subsequently sequence alignments were also accomplished with the MEGA3 software.

RESULTS

VHH, LSS-VHH and LSS-VHH-GFP Amplifications. VHH was amplified using Ig-F1 and Ig-R primers and resulted in 400 bp amplicon (Figure 1, lane 2).
VHH was joined to synthetic lysozyme signal by SOE-PCR using Ig-F3 and Ig-R primers. Resulted product was observed in 460 bp region (Figure 1, lane 3). GFP amplification with GFP F and GFP R primers resulted in a 720 pb PCR product.

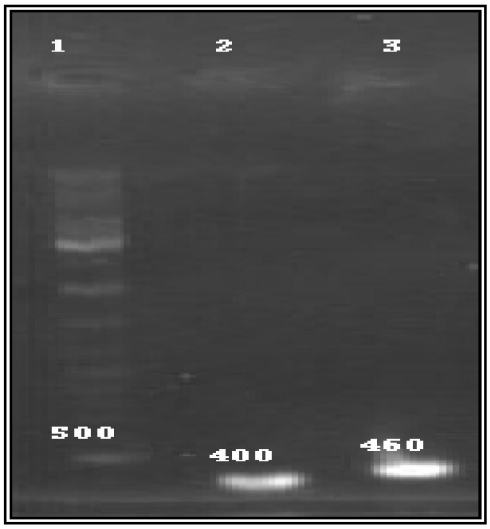

Figure 1. VHH was amplified (line 2) 400 bp, Lysozyme Signal (LSS) was attached to VHH by SOE-PCR (line 3) 460 bp. Lane 1: Marker, Lane 2 VHH and Lane 3 LSS-VHH.

LSS-VHH-GFP was amplified using Ig-F3 and GFP-R primers and resulted in an 1180 bp amplicon (Figure 2A, lanes 1-6). Schematics of constructed viral vectors are shown in Figure 2B.
Production of Retrovirus Particles Containing Fluonanobody Gene. The cell transfections were carried out with definitive vectors. The GP-293 packaging cell line were transfected with mixture of 5 µg pLNHX-LSS-VHH-GFP or pLXRN-LSS-VHH-GFP and 5 µg pSVG and changes in the cellular morphology were observed after few hours. No significant difference was observed between pLNHX-LSS-VHH-GFP and pLXRN-LSS-VHH-GFP titration. Additionally, the GP-293 Luc cells were transfected in a similar way. Concentration and titration of packed viruses were carried out.
Analysis of Transgenic Chickens. DNA was extracted from different tissues of transgenic chickens (hatched and un-hatched) and non transgenic chickens (as controls) that were derived from different embryonic layers. Genomic DNA extraction was carried out using Bioneer genomic DNA extraction kit, the results are shown in Figure 3.

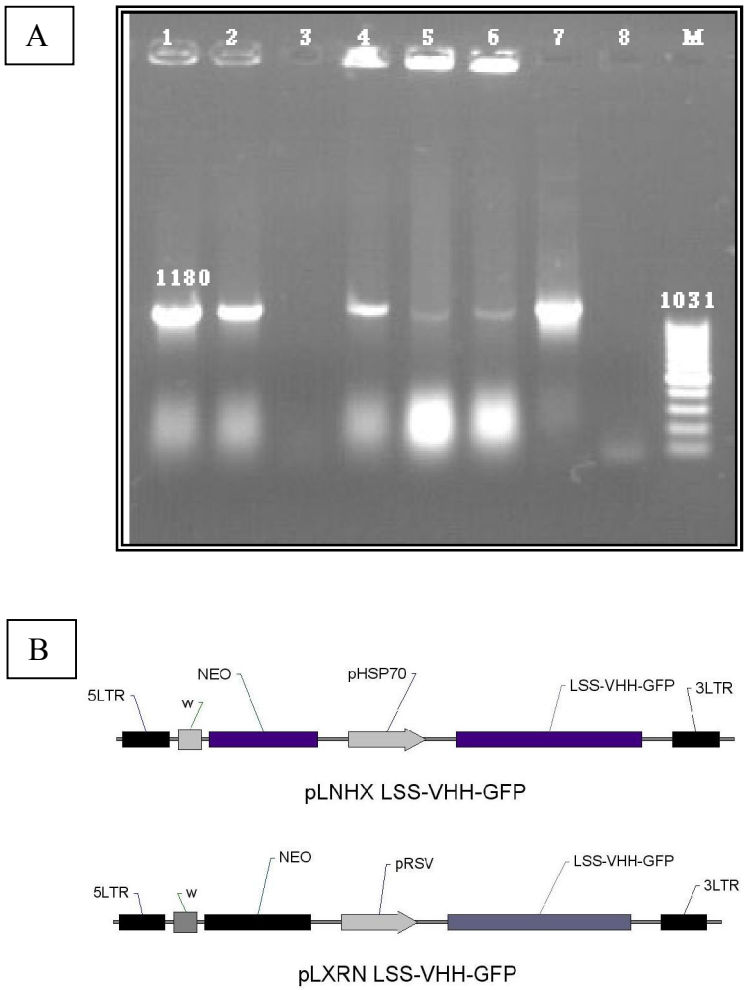

Figure 2. A)The LSS-VHH-GFP with 1180 base pair in length is shown in lane 1, 2,4,5,6 and 7. Line M indicates the DNA ladder. This fragment (1180) was inserted into the viral vectors. B) Schematic representation of viral vectors that contains LSS-VHH-GFP. pLXRN LSS-VHH-GFP was digested with XhoI and BamHI. pLNHX-LSS-VHH-GFP was digested with Bgl II and ClaI. Sequence analysis was performed and the results confirmed recombinant vectors contained the desired gene.

Control PCR on GAPDH housekeeping gene was performed on all samples and resulted in 200 bp PCR product. Presence of LSS-VHH-GFP gene in chicken's genomic DNA was confirmed with PCR on extracted genomic DNA using forward R and reverse R primers mentioned in Table 1.

Positive control PCR was carried out at the same conditions on plasmid containing target gene. In addition, negative control reaction using extracted genomic DNA from control chickens as a template was included in all steps. An amplicon with a 600 bp in length was observed in positive transfections which indicated the presence of LSS-VHH-GFP gene. These results are shown in Figure 4.

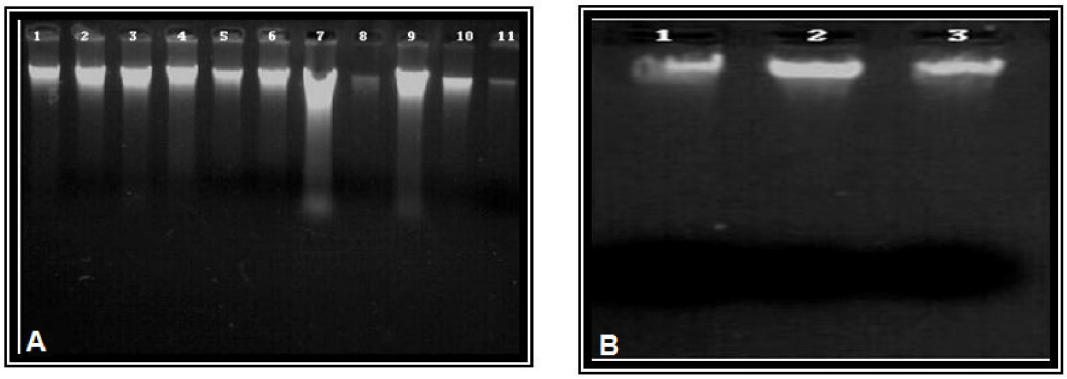

Figure 3. A: DNA in different tissues was purified in a large amount and proper quality which has been shown in lanes 1 to 11. The purification of DNA from tissues, such as skin and heart (lanes: 8, 11) was not as same as other tissues since the pulverization of these tissues was more difficult. B: DNA was extracted from blood tissue (lanes: 1, 3).

The Real-Time PCR results are shown in Table 2. The Real-Time PCR results confirmed the previous results and the presence of VHH-GFP gene.

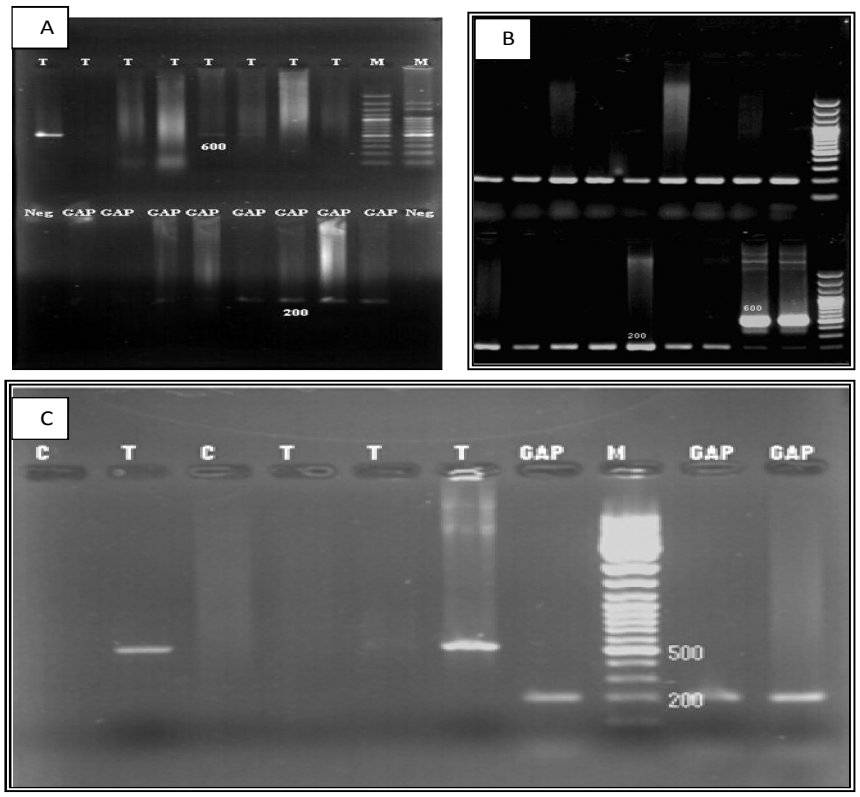

Figure 4. In figure A, PCR of tissues were carried out with GAPDH primers (amplicons used as control housekeeping gene) and a band of 200 8 b.p related to GAPDH on chicken genome was observed. Figures B and C, PCR was also performed with VHH-GFP primers and a band of approximately 600 b.p. related to VHH-GFP on the same tissue were observed which is denoted by a band at 600 bp in the region of T.

Real-Time PCR confirmed the presence of GAPDH gene in all tissues, for VHH-GFP gene most tissues which were derived from ectoderm were also positive.
These results suggest that integration of VHH-GFP gene into genomic DNA mostly takes place on tissue derived from ectoderm. However, VHH-GFP gene was not detected in the blood tissue of manipulated chickens. Finally the rate of gene transfer in this experiment was calculated to be about 1.2 percent.

Table 2. The results from PCR and Real-Time PCR on Tissue form eggs that couldn't hatch.

Tissue	GAPDH Gene	VHH-GFP Gene
Brain	(33/33)	(5/33)
Spinal cord	(33/33)	(2/33)
Skin	(33/33)	(1/33)
Muscle	(33/33)	(33/33)
Heart	(33/33)	(1/33)
Liver	(33/33)	(33/33)
Lung	(33/33)	(33/33)
Colon	(33/33)	(33/33)
intestine	(33/33)	(3/33)

N=33 Hatched chicken.

PCR and Real-Time PCR were done on genomic DNA of chicken that could not hatch because the embryonic growth was stopped in the end and they had abnormality of body especially in gastro-intestinal system. The rate of gene transferring was higher than hatched chicken.

DISCUSSION

Since the 1970s DNA manipulation techniques have created a significant and economically efficient method for genetic engineering and gene transfer. Scientists have achieved remarkable success in the production of drugs and recombinant products which can be used in the field of therapy and detection. The recombinant products from transgenic animals introduced a great interest for many research groups and pharmaceutical companies. Low cost, high efficiency and the production of a functional protein are the main advantages of transgenic animals (1-3). Antibodies have been applied for diagnosis and treatment of numerous diseases. However, due to the disadvantages of monoclonal and polyclonal antibodies, such as their immunogenicity and production of human anti-mouse antibody (HAMA), many research groups have

tried to replace them with affibody, aptamer, humanized antibody or recombinant antibodies (28-29). VHH, an antibody with higher solubility, stability, lower immunogenicity and molecular weight can be an ideal candidate for production of recombinant antibodies, particularly in the transgenic animals (25,27,30-31). In the present study we have developed an avian transgenic model containing VHH fused to GFP (fluonanobody). Eukaryotic expression of chimeric fluonanobody was already reported in our previous studies (2).

Manipulation of the oocyte or zygote to incorporate the target gene into chicken genomic DNA is not applicable. So far, several methods have been developed and tried in order to increase the efficiency of transgenesis. Various viral systems have been tested for introduction of conventional immunoglobulin into the chicken germinal cells. Among them, retroviral system has several advantages, such as stable expression by integration of the gene into the chromosome especially with 22 internal integration sites in chicken (32). Reports indicate that the type of virus used in the experiment, its titer, sealing of injection site and shell substitution are very important factors in obtaining higher rate of transgenesis (11-12). In our study a retrovirus model containing VHH gene fused to GFP gene was used for developing transgenic chickens. Eggs in stage X were prepared and viruses were injected with two procedures. Injection through the air sac resulted in more viable embryos compared to horizontal position injection, in this way the rate of viable embryos was taken to be 33 percent, which was in accordance with other reports (20-40 percent) (7,9). The injected site was sealed with tissue glue. However, agarose and histoacryl tissue adhesive tape was also used which exhibiteda significant decrease in the number of hatched chicken. DNA extraction was accomplished with some modifications to increase the extraction efficiency from different tissues which were derived from ectoderm, mesoderm and endoderm. PCR and Real-Time PCR analysis confirmed integration of fluonanobody gene in chicken genome. The fluonanobody gene was detected in 1.2 percent of G0 chickens. Generally mosaicism phenomena are commonly reported in transgenic animals. The rate of mosaicism is about 30 percent for animal such as mice and etc (33). This phenomena is higher in chickens, because the number of cells that injection is carried out on are much higher and are about 50000 to 60000 cells in fertilized eggs. Obtained results from this study indicate that there is no significant difference between the efficiency of the pLXRN-LSS-VHH-GFP and pLNHX-LSS-VHH-GFP transfection. PCR products of transgenic chicken genome were cloned into T/A vector and used for sequencing. Results confirmed the presence of VHH and GFP gene and their correct cloning. Our results also indicated that the gene of interest was integrated into tissues derived from ectoderm. The frequency of producing a transgenic chicken was low (1-10 percent) (9,34) which indicates that the transgenic technology is very inefficient with the present knowledge and techniques in hand especially when considering chicken transgenesis. We conclude that although the rate of transfection in chickens is low, it would be possible to transfect the target camel immunoglobulin gene into chicken genome which could end up in production and secretion of the produced immunoglobulin into the blood stream and subsequently concentration into the egg compartment. Currently more studies are being done in our laboratory in order to observe the protein expression status in G1 and G2 transgenic chicken. This strategy may revolutionize large scale antibody production in living organisms. According to our knowledge, this is the first report on VHH or VHH-GFP gene transfer into chicken.

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