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Research Article

Construct appropriate source of recombinant heavy chain antibodies specific to CD19

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ABSTRACT
Nanobodies (Nbs) are the smallest, functionally integrated unit that has a special importance in the researchers in the field of immunotherapy and biotechnology. These Nbs are derived from heavy chain antibodies only which are found in members of the Camelids, family (llama, camel, alpaca). this paper aimed to establish molecular library of genes encoded to Nbs specific to CD19 ,The immune response was evaluated by ELISA assay ,which revealed a good immune response against the CD-19. Blood collection has performed at 71 days peripheral blood lymphocytes separated by density gradient centrifugation,
cDNA synthesized from immunized lama RNA used as templet for amplify the CD19 -VHH encoding region with the nested polymerase reaction, the first reaction using primers amplification product are 700 bp were detected in 1% agarose gel corresponding to all VH domains of immunoglobulins : IgG2 and IgG3, then fragment at 400 bp was amplified in second PCR reaction,
The second amplification product was cloned into vector pComb3Xss using E.coli Er2738 for transformation confirming the successful of cloning by colony PCR.

Keyword: CD19, Nanobodies, cloning, Nested PCR, and Lama pacos

INTRODUCTION

Camelids family, have become a common source for the development antibodies for biomedical research, all members of camelid family which includes, llamas, camels and alpacas[1].
This family have a special immune system that has been observe since 1990; it was discover that in addition to the typical mammalian type immunoglobulin (conventional antibodies)[2].
A different type of immunoglobulin which is simpler in structure, containing only heavy chain; That's why it's called heavy chain antibody (HCAb). Which used in the production of Nanobodies (Nbs).
Nbs also named single domain antibodies (sdAbs), derivative from variable domain of heavy chain antibody. Nbs are an invaluable tool that dazzled researchers with their potential in the field of biomedical research[3]
There are as effective as the conventional antibodies in terms of strong binding to the target are considered to be non-immunogenic due to their high similarity with human variable heavy chain sequences[2], [3]. Nbs is an antibody fragment consisting of a single monomeric variable antibody domain. Like a conventional antibody, with a molecular weight of only 12–15 kDa, single-domain antibodies are much smaller than conventional antibodies (150–160 kDa) and even smaller than antigen-binding fragments (Fab, 55 kDa) and single-chain variable fragments (ScFv, 28 kDa[4]
The conjugated Nbs are utilized for drug delivery regardless whether the conjugation is a single effector domain or containing antitumor drugs[5]–[7]. Physically, Nbs distinguished by long shelf life also by strong solubility and pH and temperature tolerance.[8]. They are stable at high pressure and chemicals such as thermos-fluorescence[9]–[11]. Heavy chain antibodies have become increasingly interesting by researchers, focusing on the part associated with the production called variable heavy chain antibodies, or so-called nanobodies.
Nbs are proteins with an independent and stable entity that have achieved outstanding success in several applications, the most important of which are for therapeutic and diagnostic purposes , It is unacceptable to assert that there are no therapeutic techniques before the discovery of Nbs, In fact, there are many techniques are important in B-cell depletion and mAbs antibodies combined with chemotherapy or radiation, or they are use independently. These techniques are use in the treatment of hematomas or in any attempt the patient's survival for a longer period of time, but one of the limitations of using these techniques, the researchers noticed the low response and resistance that they show towards them. Therefore, scientists resorted to using an

alternative technique represented by Nbs[12], [13].

CD markers can be defined as specific types of surface glycoproteins[10], [11], [13], [14]. It has the specificity of being present on surface of a specific type of cell and in a specific stage of cell development; they are consider an indicator of a defect in the functionality of the cell on its surface[14].

CD19 is the most vital surface biomarker for B cells; it is widely expressed during all stages of B cell development up to final differentiation in plasma cells, Because of the expression of CD19 on lineages of B-cells.

Several studies have recently concentrated on this antigen as a biomarker to cancer , Antibodies have the abilty to recognize the antigens that limited to leukemia, lymphoma, and normal hematopoietic cells represent a distinctive opportunity to evolve therapeutics Since they have the ability to handle them reasonably selectively treatment of the diseases[16].

Scientists observed that CD19 was overexpressed to a high degree in most B cell tumors in acute lymphocytic leukemia (ALL), chronic lymphocytic leukemia (CLL), B cell lymphomas, and autoimmune diseases, including rheumatoid arthritis and multiple sclerosis[17].

These discoveries led to an increase interest in CD19 as a potential target for immunotherapy and the use of this protein in diagnosing cancers that arise in this type of cell .

MATERIAL AND METHOD

Young female llama weighed almost 65kg was sub cutaneous injected with histidine tag CD19 using long immunization protocol for 64 days according to[18] .

CD19 antigen (Acrobiosystem \Newark) was prepared by dissolving 200 μg of the antigen powder in 1 ml of deionized water. The Ag solution was mixed with an equal volume of complete freund's adjuvant (CFA) as the first dosage. The second and third immunization dosages were prepared by mixing the Ag with incomplete freund "s adjuvant ((IFA) ,the last dosage was without IFA ,At the end of the last immunization dose in a few days, ranging from four to five days, blood was drawn and collected in anticoagulant tubes, the detection of immune response against CD19 was carried out by Enzyme linked Immunoassay (ELISA). using ELISA kit (Santa-Cruz / USA) according to[19]. the result has been detect by ELISA reader at 450 nm The control was used, PBS against the CD10 which coated the wells .

Peripheral blood lymphocyte were isolated from collected blood by density gradient centrifugation according to [19], Lymphocytes were kept in a 1 ml DMSO solution with 9 ml of serum bovine and kept at -80 ° C until use.

RNA was extracted from isolated lymphocytes arrange of ($1*10^5$-$1*10^6$) script ™ RT PreMix kit from Bioneer according to manufacturer's instruction. The RNA samples were convert to cDNA according to AccuPower® RocketScript™ RT PreMix kit from Bioneer Company.

To amplify Nbs gene fragment a nested PCR protocol was employed one set of specific primer of the first step of PCR are CAL001 forward including GTCCTGGCTGCTCTTCTACAAGG and CAL002 reverse GGTACGTGCTGTTGAACTGTTCC using lymphocyte cDNA as template. 700 base pairs in size, while specific set of primers of second amplification are VHH forward including CTAGTGCGGCCGCTGGAGACGGCCCAGGC GGCCTGGGT and VHH reverse including GATGTGCAGGGCCCAGGCGGCCGAGTCTGG RGGAGG with sfil enzyme sequence.

The first PCR product used as a template for the nested PCR product to give 400bp band. The PCR reaction was prepared to a total volume of 25 μl using a Genzol triRNA Pure Kit from Geneaid, cDNA first strand was synthesized according to AccuPower® Rocke including 50 ng (1ul) of template DNA ; 1 μl of each primer using GoTaq®Green Master Mix kit (Promega, USA) 12.5 μl and 8.5 μl free nuclease water,Thermal cycling conditions according to were as following in table no. 1

Table 1 the condition of PCR

Steps	Temperature/ºC	Time	Cycles
Initial turation	94	4 min	1
Denaturation	94	20 sec	35* 16**
Annealing	60.7* 55.8**	1 min	
Extension	72	40 sec	
Final extension	72	4 min	1
store	4		

*The first PCR ** the second PCR

A band of 400 bp detected at nested PCR using first PCR product as templet, which is the encoding gene to Nbs (VHH) fragment.

The fragment purified by Kit from Geneaid /Korea inserted into a plasmid pComb3Xss (Addgene, US). The insertion was done by restriction enzyme sfil and ligase enzyme T4 (England/ bio Lab company), and has been proven by electrophoresis comparing it with proper size of plasmid,

the bacterial transformation process was done by melt freezing method, to introduce recombinant DNA (pcom3ss-VHH fragment) to competent ER2738 (NEB Company, Germany), then cultured in Luria Bertani (L .B)agar medium treated with ampicillin 100 mg / mL for 24 hrs at 37 ° C.

the successful transformed Er2738 colonies which carried of recombinant DNA was confirmed by colony PCR technique according to[20].where the results of the gel electrophoresis of the colonized bacteria, colonies encoded for the desired gene using the same set primer that were used in the second stage of amplification,

UV imaging showed the amplification of a size of 400 base pair encoded for the gene of nanobodies

The results

In this study, The experimental animal used was a llama (Lama pacos) as it was raised successfully and adapted into its new environment, The immune response was detect by the ELISA assay demonstrated positive result at dilution titer 1: (Fig .2)

Peripheral blood lymphocytes were separated by the density gradient centrifugation method where the blood was drawn periodically, gradual increase in the number of lymphocytes observed after each immunization dosage the highest value of the number of cells after day 64, When completing the last immunization dose (figure 1).

The extraction of Total RNA were from 10^6 cells and subsequently synthesis cDNA by reverse transcription (Fig. 3) was used as PCR template the amplify fragment was at 700 bp corresponding to variable region of heavy chain antibodies -hinge region and ,CH2 domain of both IgG2 and IgG3 , the specific primer VHH - forward and VHH-reverse were used to amplify fragment with 400 bp corresponding to Nbs (.Fig .4 :A ,B) respectively ,nested PCR product and pComb3xSS vector digested with sfil enzyme then ligated together by ligase T4 enzyme (Fig. 5) the recombinant DNA fragments (VHH fragment and pComb3xSS fragment) has been ready for cloning inside electrocomptent E.coli Er2738 cultured on L.B agar contain ampicillin incubated for **24 hr. at 37℃**. (Fig.6) the successful transformation confirmed by colony PCR producing on 1% agarose gel (Fig. 7).

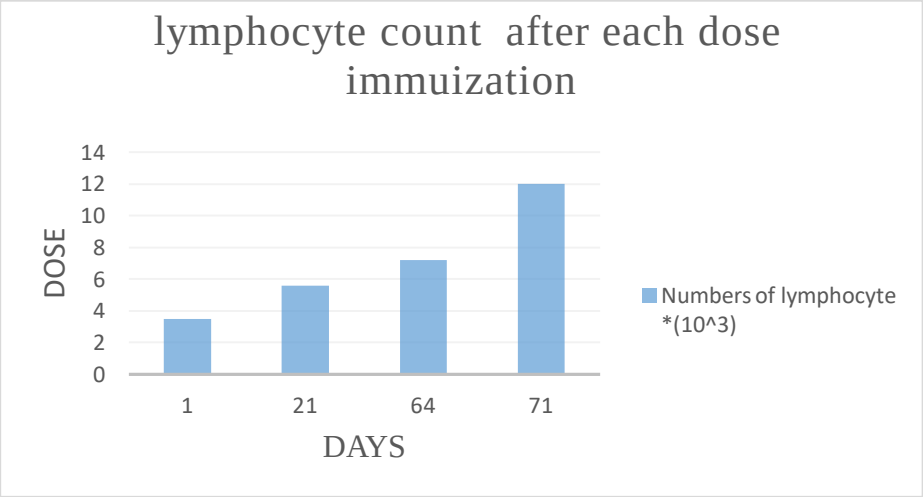


Fig.1. Gradual increase in the number of lymphocytes in accordance with the immunization dose

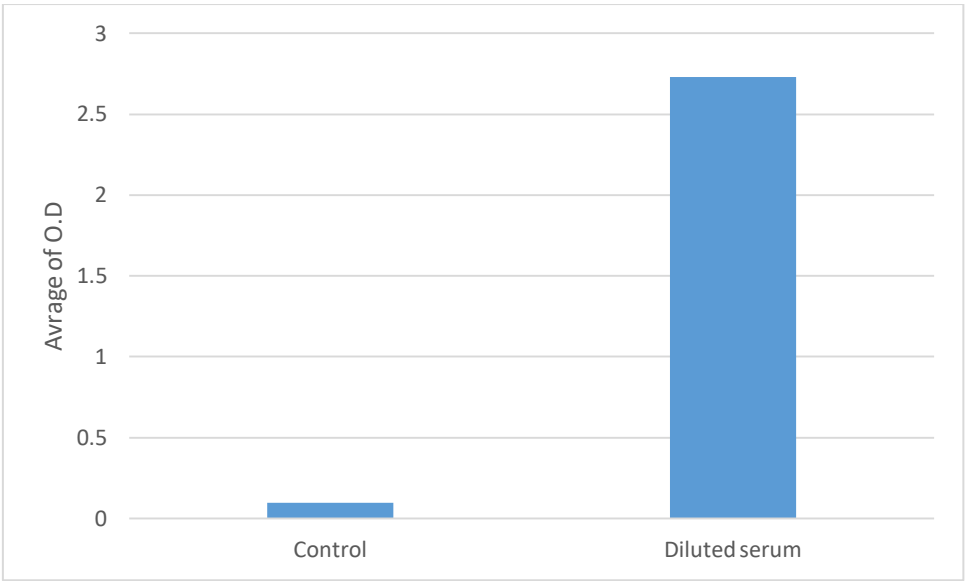
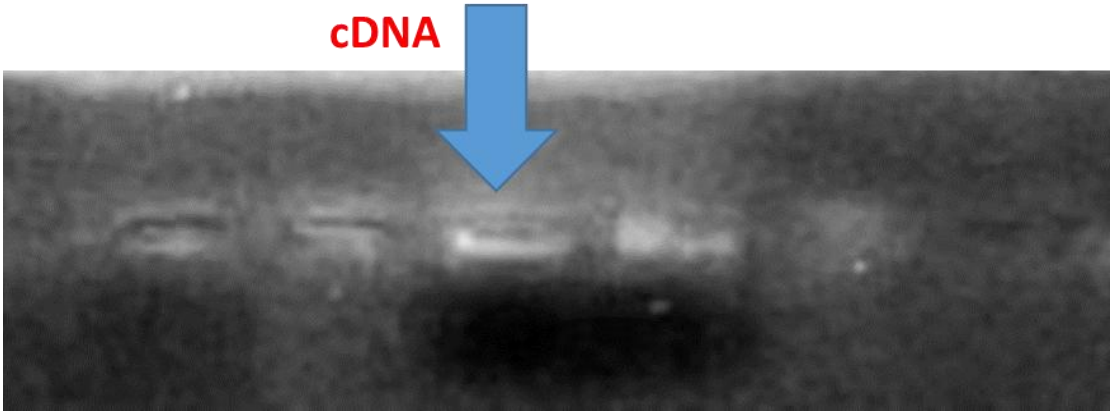


Fig.2 detection of immune response of immunized llama toward CD 19 by ELISA assay reported as demonstrated at O.D 450



cDNA synthesis by reverse transcription on 0.8% agarose gel electrophoresis Figure3

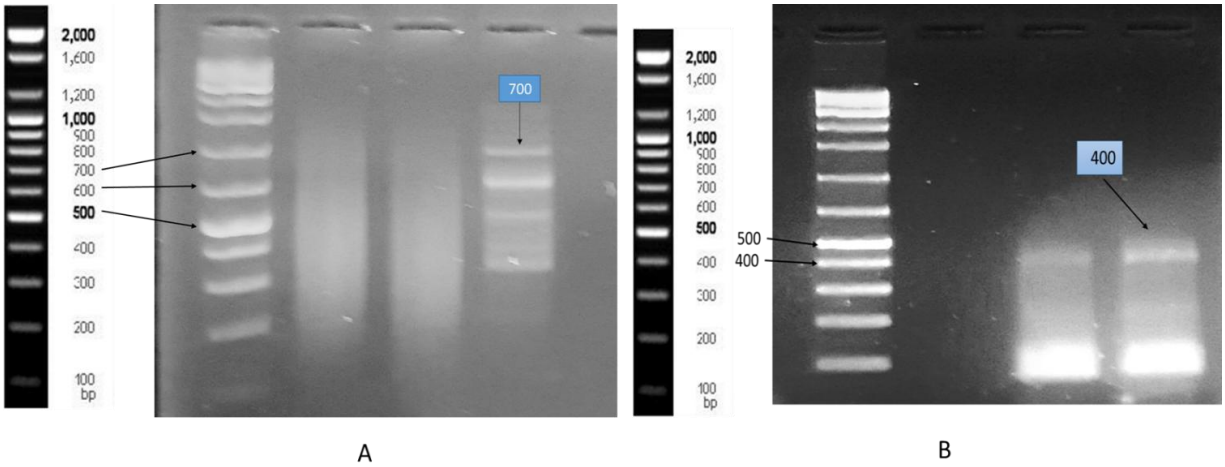


Fig.4:Amplification of VHH through two stage of reaction shown on1% agarose gel Electrophoresis A) first PCR reaction using CAL001 and CAL002 primer amplify fragment at 700bp on 1% agarose gel Electrophoresis ;B) amplification of nested PCR for Nbs fragment at 400bp using VHH forward and VHH reverse .

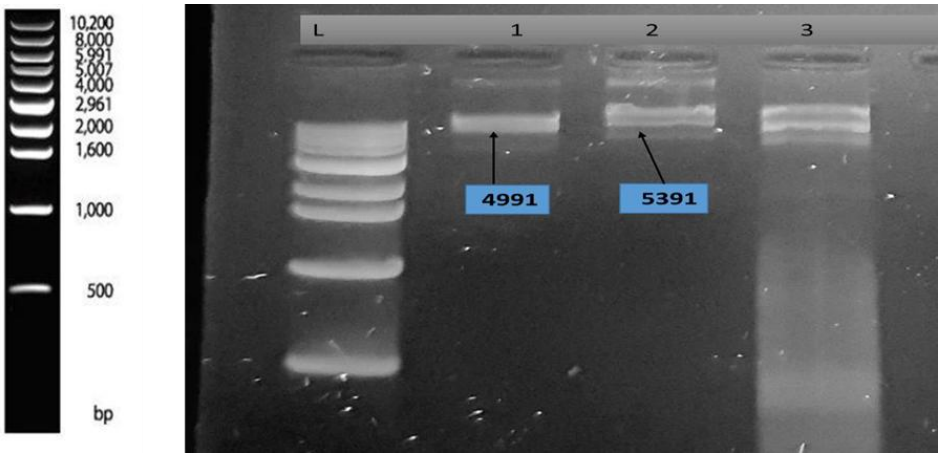


Fig.5:Agarose gel electrophoresis of pComb3xSS vector: lane 1 DNA marker Lane 2 the vector pComb3xSS at 4991 bp, lane 3 and lane 4 recombienent DNA fragment (VHH fragment and DNA fragment of pComb3xSS) ligated with T4 ligase at 5391 bp .

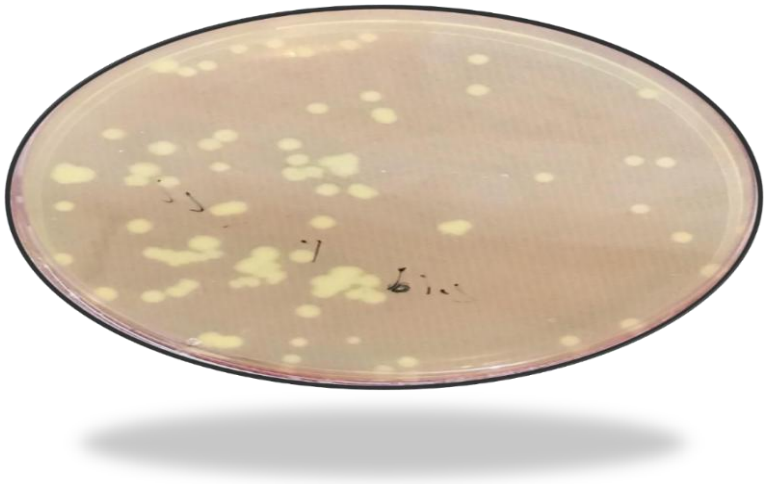


Fig.6:Transformed colonies of Er2738, which contain VHH gene, cultured on L.B agar with ampicillin for 24 h. at 37°C

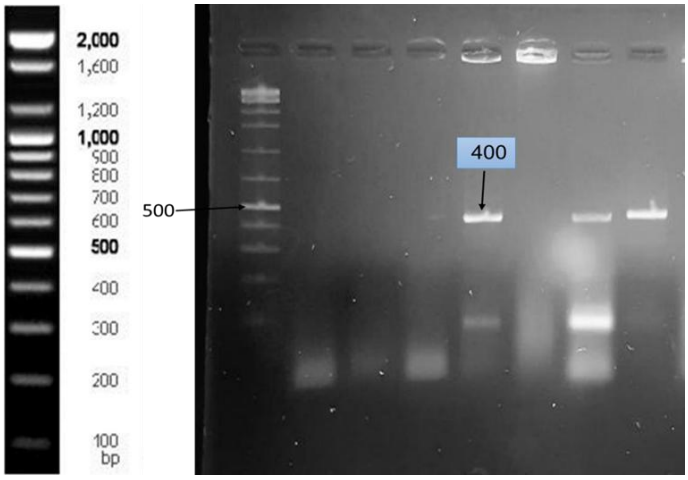


Fig.7:PCR product for VHH fragment in transforming E.coli ER 2738 M: 100 bp DNA marker. 4, 6, 7 transformed colonies with VHH gene.lane 1.2,3 untransformed colonies (without VHH gene) lane

DISCUSSION

The construct of an immune molecular library of VHH gene consider as repertoire of VHH gene Prepared for future research productivity of Nbs, also called VHH, it is predicted that nanobodies would present a low immunogenicity in humans and its convenient for theraptic outlook to humanize which have it gained importance by researchers and their use in the fields of diagnosis and treatment In this study, Lama pacos were used, even though the previous researchers[21](used camel in native country in the Middle East countries, the small size of the llama (75-90) kilo, This has many positive benefits which are less food than the camel needs, the ease of handling the immunization program, Breed, and care for an animal in the yard or garden of the house, where it is possible to provide All the requirements of being in a habitat that is not its original homeland,

Peripheral blood lymphocytes isolation is an important step in order to obtain a good percentage of RNA, so there was an opinion on the use of animal's lymph nodes to prepare a repertoire of RNA[22] the evaluation of RNA integrity is a crucial first step of the successful implementation of modern molecular biological, Nested PCR is a method developed by conventional PCR require two primers set, the first one amplifies part of the gene where as the second set amplifies a part of the gene within the first part[24]. the specific primers were used according to[24] which are adequate for amplifying gene encoded to Nbs of all members of camelid family. CALL002 primer annealing region of (CH2) for all camelids immunoglobulins isotypes, whereas CALL001 annealing all variable elements of camelids family. While the specific VHH primers anchored with sfil restriction sites as a correct start to the cloning. molecular cloning techniques is very suitable for making many copies of VHH fragment With simple and inexpensive capabilities, a repertoire of transforming colonies with Nbs (VHH) gene, ready for future research in the production and purification process[25].

CONCLUSION

Creating a prokaryotic expression system for Nbs by :- immunization of llama with interest antigen to obtain huge of VHH-encoded RNA - constriction a molecular library of gene encoded to Nbs .

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