IMMUNE RESPONSE OF BALB/C MICE AGAINST GENETIC VACCINATION WITH *LEISHMANIA MAJOR* GP63 GENE (*LMAJGP63*)

Wamedh Hashim Abbas* , Nadham Kadham Mahdi**
Selman A. Ali***

*Department of Microbiology, College of Veterinary Medicine, University of Basrah, Basrah, Iraq,
**Department of Microbiology, College of Medicine, University of Basrah, Basrah, Iraq,
***School of Science and Technology, Nottingham Trent University, Clifton, Nottingham, UK.

(Received 13 April 2014 ,Accepted 28 May 2014)

Keywords: *Leishmania*, VR1012 plasmid, vaccines.

ABSTRACT

Leishmania major glycoprotein 63 (*lmajgp63*) gene was used in this study as DNA vaccine candidates. Gene was inserted into VR1012 plasmid by using standard molecular biology protocols, resulting in preparation of *lmajgp63*/VR1012 plasmid. Vaccine either used as naked or gold particles coated DNA vaccine in immunization of females Balb/c mice. Animals were immunized at week 0, 2 weeks and 6 weeks. Dermojet needle free injector had been used to deliver gold particles coated DNA vaccine intradermally (I/D) while ordinary needle injection was used to deliver naked vaccine intramuscularly (I/M). Immune response for each vaccinated group were detected, two weeks after the third administration of the vaccines, by estimation of serum concentration of IL-2, IL-4, IL-10 and INF-γ, as well as anti-soluble *Leismania* antigen (anti-SLA) IgG titer, by ELISA test. The results demonstrated the effectiveness of DNA vaccines in induction immune response comparing to control groups ($P<0.05$). The highest serum concentrations of IL-2, INF-γ and anti-SLA IgG OD value observed in mice group which immunized with gold particles coated vaccine injected I/D (182.10 pg/ml, 1699.20 pg/ml and 0.6101 respectively), while the lowest titer was observed in group vaccinated with naked plasmids injected I/M (103.60 pg/ml, 1183.20 pg/ml and 0.3395 respectively). On the other hand group treated with naked plasmid I/M shows the highest titer of IL-4 and IL-10 concentration (85.30 pg/ml and 53.10 pg/ml respectively), while the lowest titer

observed in group injected I/D by gold coated plasmid (50.90 pg/ml and 41.88 pg/ml respectively). The results also demonstrated that I/D injection of gold particles coated DNA vaccine induced the highest Th_1/Th_2 response [IL-2/IL-10 (4.348) and INF-γ/IL-4 (33.383)], while I/M injection of naked vaccine gave the lowest results [IL-2/IL-10 (1.951) and INF-γ/IL-4 (13.879)].

INTRODUCTION

Genetic immunization is a relatively new tool for achieving specific immune activation with several advantages such as expression of concerned genes nearest to its native form, induction of cellular immune response, persistent expression of desired antigen (Ag) and induction of memory responses against the infectious disease [(1)]. Moreover, host cells take up coding plasmids, transcribe and translate the encoded gene, and produce protein that stimulates an immune response when presented to the immune system in the context of self-MHC [(2)]. Notably, vaccination with plasmid DNA has been shown to induce protective immunity through both MHC class I- and class II-restricted T cell responses in a variety of infections [(3)]. Therefore, the plasmid DNA encoding specific Ag induced both $CD4^+$ and $CD8^+$ T cells, which is essential for protection against intracellular diseases that require cell mediated immunity like leishmaniasis [(4)].

DNA vaccines may provide better protection against *Leishmania* than killed or live-attenuated vaccines as they can induce the expression of *Leishmania* antigens, which are unaltered in their protein structure and antigenicity. Furthermore, bacteria-derived DNA plasmids are naturally immunogenic as their backbones contain unmethylated cytosine-phosphate-guanosine (CpG) motifs which have been shown to readily induce Th_1 cytokine expression and enhance $CD8^+$ T cell responses [(5)].

However, at present, there is no effective vaccine available anywhere in the world for routine use against leishmaniasis[(6)]. The ineffectiveness of existed *Leishmania* vaccines is most likely due to the lack of consistent stimulation of helper T cells, a requirement for long-lived protection that usually occurs in response to natural and low profile persistent infections [(7)]. New antigens with such properties to stimulate memory T helper cells, a perquisite for a potent and lasting immunity, are still in need for constructing effective new vaccines [(8)].

The major surface glycoprotein of Leishmania major is a zinc metalloproteinase of 63 kDa referred to as leishmanolysin or GP63, which is encoded by a family of seven genes. GP63, a highly conserved protein, is abundantly expressed in promastigotes, and considered the major Ag determinant recognized by the serum samples of patients obtained from different clinical forms of leishmaniasis, moreover GP63 has an intrinsic ability to stimulate protective immunity and is promising vaccine candidate against leishmaniasis [(9)].

It has been reported that the mode of administration of the DNA vaccine can influence the type of immune response induced by the vaccine. Intramuscular injection of naked DNA was one of the first method described for gene immunization which has been reported to lead the immune response toward Th_1 type while application of gen gun-mediated delivery, gold particles covered with plasmid DNA is very effective at driving plasmid into the cells of the epidermis and requires far less DNA than needle injection [(10)].

MATERIALS AND METHODS

Animals and parasites

Fifty non pregnant female BALB/c mice (4-5 weeks old) were obtained from animal unit at College of Medicine/ University of Baghdad. Mice were bred in standard mice cages for ten weeks and fed on standard mice ration.

Ten ml vial frozen in liquid nitrogen contain *L. major* M379 strain (College of Science and Technology-Nottingham Trent University/ UK) was used in this study.

Parasite was grown in Drosophila Schneider media (Lonza), supplemented with 10% fetal calf serum (FCS) at 37ºC in CO_2 incubator for 24-48 hour. For parasite counting a volume of 10µl from the culture at specific time points was transferred to 1 ml eppendorf tube containing 90µl of 2% Paraformaldehyde. After mixing, 10µl of fixed parasites were transferred to a second eppendorf tube containing 90µl 1xPBS, and counted using Neubauer Hemocytometer.

Sampling procedure

After two weeks from the last vaccination, at least 1 ml blood sample was collected in1.5 micro-centrifuge tube from the heart of each mouse by using insulin syringe. Sera were obtained from blood samples by centrifugation and kept at -20℃ for further investigation.

Preparation of soluble *Leishmania* antigen (SLA)

Soluble *Leishmania* antigen (SLA) was prepared according to Dumonteil *et al* (2003) with slight modification [11].

Briefly, a number of 1~ $2*10^9$ late log *L. major* M379 (stationary phase) was washed 3 times with sterile 1xPBS (4000 g at 4˚ C). Parasite pellet was then re-suspended in *Leishmania* Buffer (a 100 mMTris pH 7.3 buffer with 1 mM EDTA, 0.5 mM Phenylmethanesulfonyl fluoride (PMSF) and 2.5 µg/ml Leupeptin - Sigma). The suspension was sonicated for 20 minutes. The sonicated pellet was centrifuged for 30 min at 13,000× rpm. Supernatant was dialysed against 3 litres of cold PBS for overnight with at least two changes of 1xPBS. SLA was sterilized by passing through 0.25 µm filters (Sartorius), and then kept at -20˚C for further investigations.

The total protein concentration of extracted protein was measured using Bicinchoninic Acid Protein Assay Kit according to manufacturer's protocol.

Preparation of *Leishmania major* cDNA

The *L. major* gp63 cDNA construct (PNUT), a kind gift by Dr. Selman Ali, Nottingham Trent University, Nottingham, UK, was bulked up by transformation of *Escherichia coli* followed by purification using Quia-gen EndoFree (West Sussex, UK) plasmid purification Maxi Prep Kits. The construct was sequenced by MWG Biotech using 5'-GTCTCCACCGAGGACCTCAC-3', 5'-GTTCAGCGGCCCATTCTT-3', 5'-TCTCCGCCTTCATGGACTAC-3',5'-CGTGTCCTTGGGTGACAAC -3' and 5'-CAGCACACCCTCCTCACTC-3' primers.

Cloning of *L. major* gp63 into VR1012

Leishmania major gp63 was cloned into VR1012 vector (kind gift by Dr. E. Dumonteil, Laboratorio de Parasitología Yucatan, Mexico.), which contained a mammalian antibiotic resistant gene. L. major gp63 was first amplified by PCR using 5'-TGTCGATATCCTATGCGTGGGCTGGA-3' and 5'-TCTGAGATCTGGGGAGGGGTCACAGG-3' forward and revers primers containing restrection site for *EcoRV* and *BgIII* restrection enzymes respectively. VR1012 vector and amplified gene were digested using the same restriction enzymes. Then, *lmajgp63* gene and the digested vector were ligated using a DNA ligase enzyme. The presence of the *lmajgp63* gene in VR1012 vector was first determined by PCR

amplification using 5'-CTATGCGTGGGCTGGAGC-3' (forward) and 5'-CAGCACACCCTCCTCACTC-3' (reverse) primers. Moreover, to ensure that the sub-cloned gene contains the correct gene sequence and no mismatches had occurred during the cloning procedure the whole gene was subjected to sequence analysis.

Preparation of gold particles coated DNA vaccine

Plasmid construct encoding *L. major* gp63 gene as well as empty vector control vaccine were coated onto 1.0µm gold microcarriers beads using manufacturer's instructions. Briefly, to help the DNA binding to the gold, 200µl of spermidine (Sigma) mixed with 16.6 mg of gold (Bio-rad), the solution was well mixed, followed by addition of 36 µg of construct DNA. After 10 seconds sonication, 200µl of 1mM CaCl2 was added drop wise to the mixture while sonicating. To precipitate the DNA, the mixture was allowed to stand for 10 minutes at room temperature. Precipitated gold-DNA was pelleted by centrifugation at 13,000 rpm for 5 minutes, and the pellet was washed three times with 1ml of 100% anhydrous ethanol (Sigma) with sonication for 30 second each time and spinning for 1 minute at 13.000 rpm, ethanol was removed by pipetting. After last wash gold-DNA pellet was re-suspended in 2ml of 0.025mg/ml Polyvinylpyrrolidone (PVP) in a sterile 15-ml conical tube. The gold PVP solution was then loaded into dried Tefzel tubes using 5 ml syringe. The solution was left to settle for 30 minutes on the Prep Station, so the gold particles settle to the bottom of the tube. The supernatant was gently poured off using the attached syringe. The tube was rotated for 5 second and then left to dry by turning the N2 on, for 5 minutes. The plastic tubing was removed from the Prep Station and re-suspended in PBS. Naked vaccine was prepared without coating with gold particles. DNA vaccines were kept at -20℃ until required.

Immunization with *lmajgp63/VR1012* vaccine

The animals were divided into 5 groups, each group contain 10 mice. Animals were immunized by using Dermojet free-needle injector JI-150 (Akra, France) with 50 µl gold particles coated vaccine , particles coated empty vector or gold particles at week 0, 2 weeks and 6 weeks intradermally, while 100 µl naked vaccine or naked empty vector was injected I/M by using ordinary insulin syringe, immunization protocol is shown in figure 1.

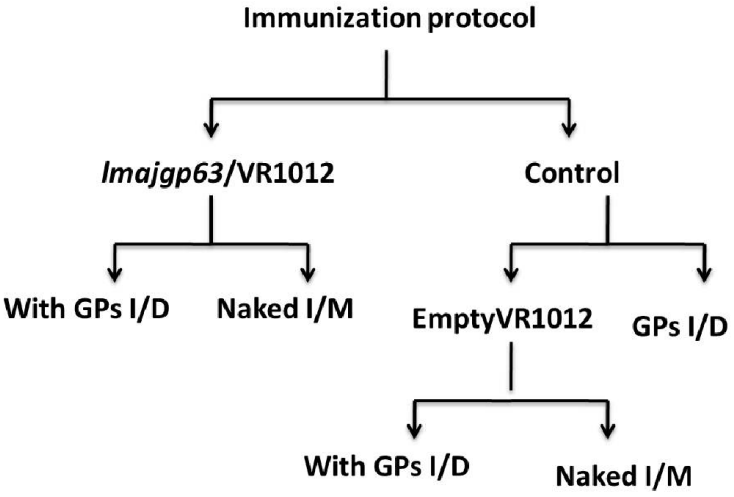

Figure 1: Immunization protocol

Estimation of IL-2, IL-4, IL-10 and INF-γ value in serum

Cytokines was measured in serum according to the instructions of eBioscience company. Briefly, microtiter plate was coated with 100 µl/well of capture antibody (pre-titrate purified anti- IL-2, IL-4, IL-10 or INF-γ antibody). The plate was sealed and incubated overnight at 4˚C. Cover film was removed and the plate was washed with 250 µl/well washing solution (1xPBS, 0.05 Tween-20) this procedure was repeated five times. Wells were blocked with 200 µl/well of 1x Assay Diluent and incubated at room temperature for 1 hour. Washing step was as mentioned above. 1x Assay Diluent was used to perform 2-fold serial dilutions of standards to make the standard curve. 100 µl/well of 1x Assay Diluent was added to the blank well. 100 µl/well of standards and serum samples were loaded to appropriate wells and the wells were covered and incubated at room temperature for 2 hours. Plate was washed as mentioned above. 100 µl/well of detection antibody (pre-titrated biotin-conjugated antibody) was added to each well. The plate was sealed and incubated at room temperature for 1 hour. Cover film was removed and the plate was washed as described previously. 100 µl/well of Avidin-HRP was added to each well and the plate was sealed and incubated for 30 minutes at room temperature. Plate was washed as in step 2 and repeated for total seven washes. 100 µl/well of substrate solution, tetramethylbenzidine (TMB), to each well and incubated for 15 minutes at room temperature. The reaction was stopped by adding 50 µl of stop solution to each well. The absorbance of each well was read at 450 nm using microplate reader.

Estimation of anti-SLA specific IgG titer in serum

Specific anti-SLA IgG antibody titer was measured according to the method of Rezvan *et al* (2011) with slightly modification. Briefly:

One hundred µl of SLA 1µg/well was coated on the flat bottom 96-well plates (Biorad, Hemel Hempstead, Hertfordshire, UK) and incubated overnight at room temperature. The plates were washed 4 times with PBS. 100µl of 1:100 diluted of the serum samples in dilution reagent (1% BSA, 0.05% Tween 20 in 20mM Trizma base, 150mM NaCl, pH 7.2-7.4) was added followed by 2h incubation at room temperature and 4 times washes with PBS. The plates were washed 4 times washes with PBS. 100µl of HRP conjugated goat anti-mouse IgG (Promega, UK) was added at 1:1000 followed by 1hour incubation at room temperature and 4 times washes. 100 µl/well of substrate solution to each well and incubated for 15 minutes at room temperature. The reaction was stopped by adding 100 µl of stop solution to each well. The absorbance of each well was read at 450 nm using microplate reader.

Optical denisity less than 0.2 considered as resprocal end-point IgG titer (defined as the inverse of the highest seum dilution factor giving an absorbance >0.2) against SLA [(12)].

Statistical analysis

Data are expressed as the mean values ± standard deviation (SD) of samples. The statistical significance of the differences between various groups was determined by PostHoc test (LSD alpha 0.05) and one-way analysis of variance (ANOVA) using SPSS for windows. Differences were considered statistically significant for $p<0.05$.

RESULTS

Amplification of *lmajgp63* gene from original plasmid

forward and revers primers 5'-TGTCGATATCCTATGCGTGGGCTGGA-3' and 5'-TCTGAGATCTGGGGAGGGGTCACAGG-3' were used to amplify full length of *lmajgp63*, from *lmajgp63*/PNUT plasmid, with restriction sites for *EcoRV* and *BgIII* restriction enzymes by PCR. The result is shown in figure 2.

Digestion of amplified *lmajgp63* and *lmexgp63*/VR1012 by restriction enzymes

To obtain *lmajgp63* gene with sticky ends to insert into empty VR1012 plasmid, two restriction enzymes, *EcoRV* and *BgIII*, were used to digest both gene and plasmid. The result is shown in figure 3.

Ligation of first and second fragment of digested *lmajgp63* gene

Due to the presence of the sequence GATATC, which represent the restriction site for *EcoRV* restriction enzyme, inside *lmajgp63* gene after base number 626, *EcoRV* restriction enzyme digested the gene into two fragments. The re-ligation of the fragments was done successfully as shown in figure 4.

Confirmation of the newly prepared *lmajgp63*/VR1012 plasmid by PCR amplification

To make sure that the insertion of *lmajgp63* into VR1012 was successful and in the right direction, PCR amplification was done using two pairs of primers 5'-CTATGCGTGGGCTGGAGC-3' (forward) and 5'-CAGCACACCCTCCTCACTC-3' (reverse) primers and the results are shown in figure 5.

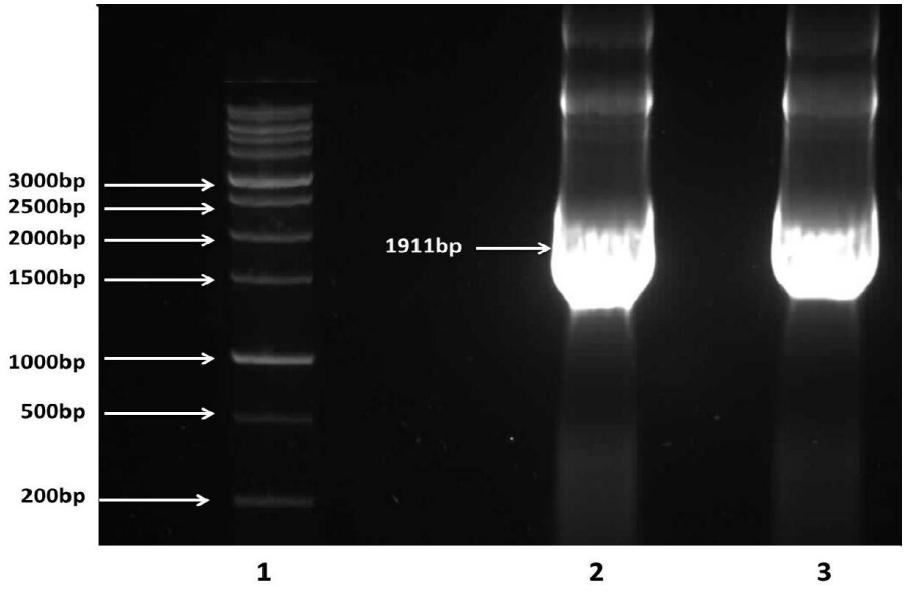

Figure 2: Agarose 1.5% gel electrophoresis shows the full length of *lmajgp63* amplified by PCR. Lane 1, 1Kp DNA ladder, Lane 2 and 3, amplified *lmajgp63*gene 1911bp.

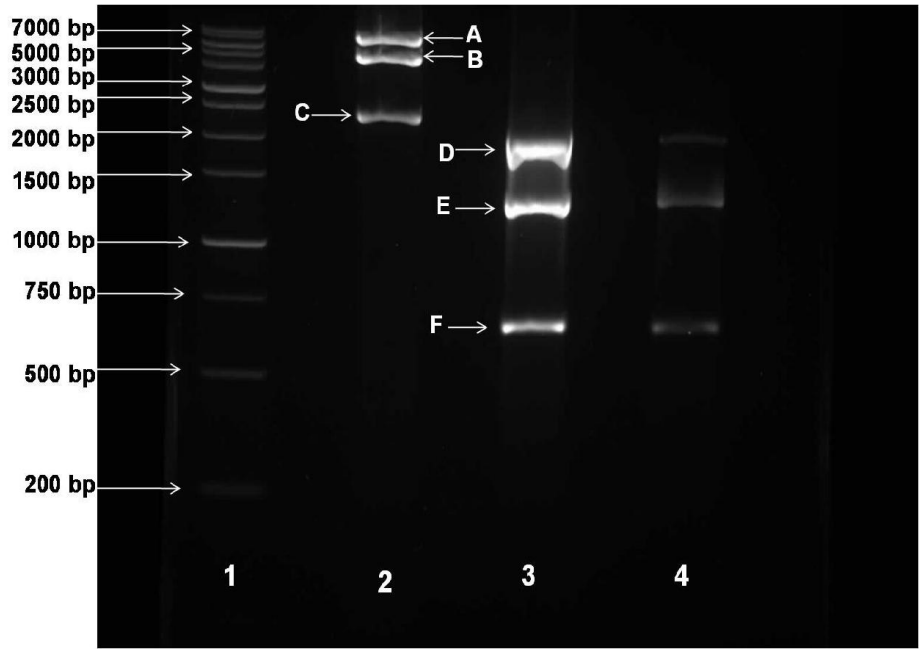

Figure 3: Agarose 1.5% gel electrophoresis shows the digested *lmajgp63* and VR1012. Lane 1, 1Kp DNA ladder; Lane 2, digested VR1012; Lane 3 and 4, digested *lmajgp63* gene. (A, undigested *lmexgp63*/VR1012 plasmid; B, empty VR1012 plasmid; C, *lmexgp63* gene; D, undigested *lmajgp63* gene 1911bp; E, first fragment of digested *lmajgp63* gene 1273bp; F, second fragment of digested *lmajgp63* gene 622 bp.).

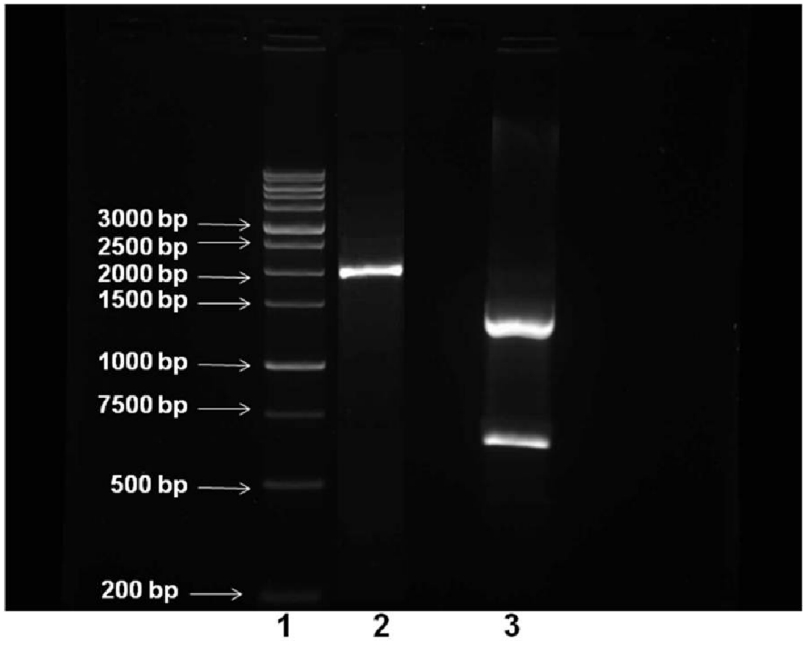

Figure 4: Agarose 1.5% gel electrophoresis shows the full length of *lmajgp63* after the re-ligation of digested fragments. Lane 1, 1Kp DNA ladder; Lane 2, the re-ligated *lmajgp63*gene 1897bp; Lane 3, the digested un-ligated *lmajgp63*gene.

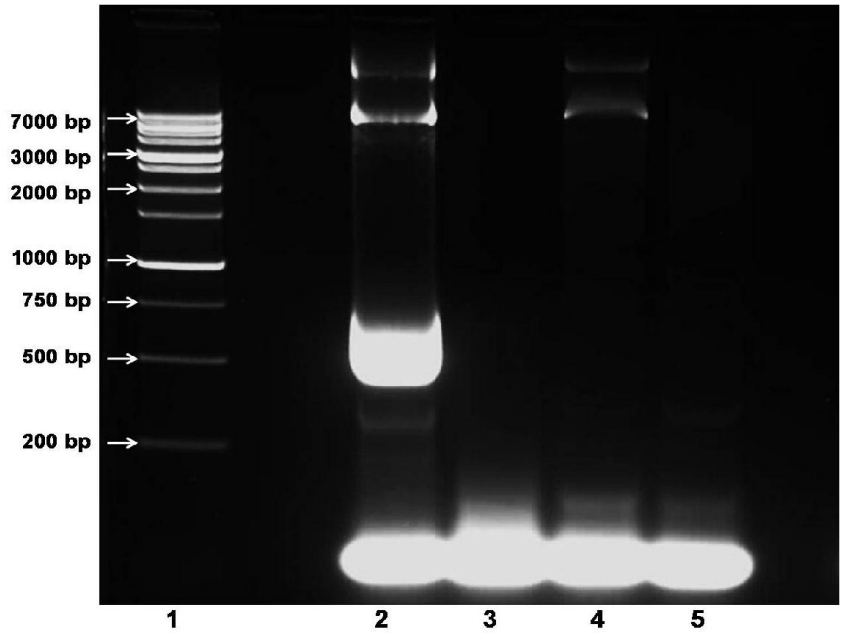

Figure 5: Agarose 1.5% gel electrophoresis shows *lmajgp63* amplified by PCR. Lane 1, 1Kp DNA ladder; Lane 2, amplified *lmajgp63* gene; Lane 3 and 5, negative control; Lane 4, empty plasmid.

Confirmation of the newly prepared *lmajgp63*3/VR1012 plasmid by sequencing

Five primers, 3 forwards and 2 revers primers were designed in this study to obtain the sequence of the full length of *lmajgp63* gene in the newly prepared *lmajgp63*/VR1012 plasmid. The sequences were edited using Chromas Lite software and were assembled and aligned with *lmajgp63* gene sequence from gen bank (Accession number Y00647) by Just bio software. Geneious Inspirational Software for Biologists was used for virtual protein translatiore and alignment.

Result is shown in figure 6.

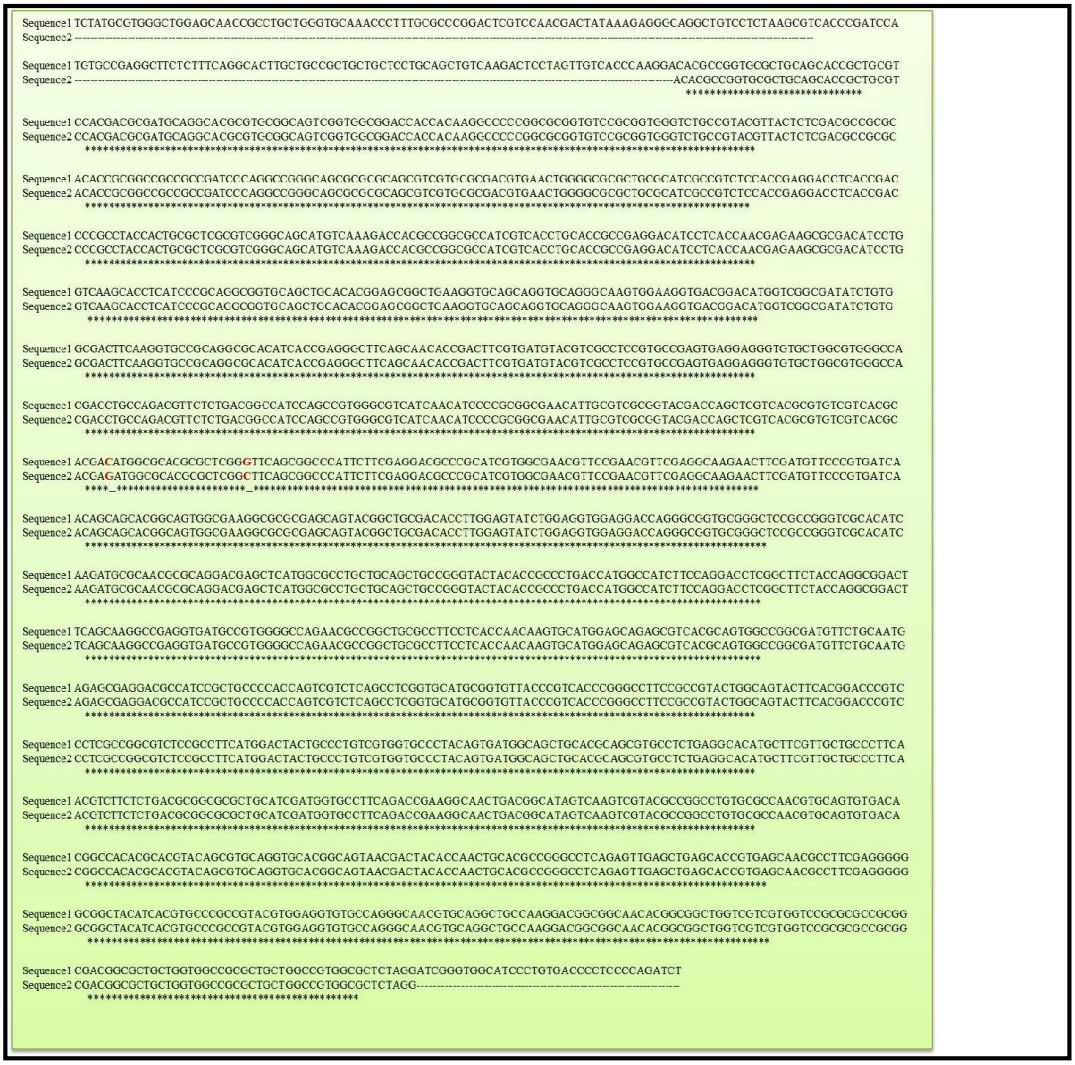

Figure 6: Alignment of *lmajgp63* gene sequence produced by Just bio software. Sequence 1 is the sequence produced by Source Bioscience LTD, UK; sequence 2 is the original sequence of *L. major* gp63 gene (Gene Bank, Accession number Y00647).

Estimation of IL-2, INF-γ and anti-SLA IgG concentration in mice sera

The highest serum concentrations of IL-2, INF-γ and anti-SLA IgG OD value observed in mice group which immunized with gold particles coated vaccine injected I/D (182.10 pg/ml, 1699.20 pg/ml and 0.6101 respectively), while the lowest titer was observed in group vaccinated with naked plasmids injected I/M (103.60 pg/ml, 1421.5 pg/ml and 0.498 respectively), results are shown in figure 7, 8 and 9 respectively.

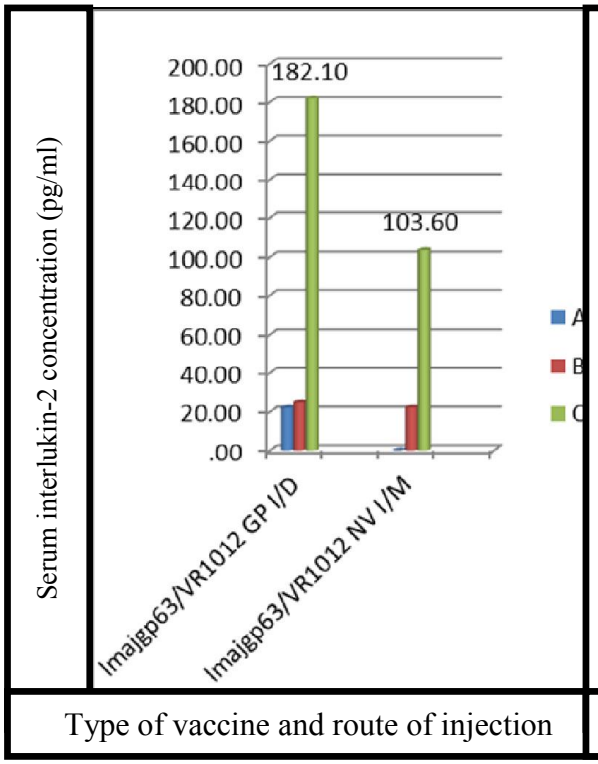

Figure 7: Differences between groups vaccinated with gold coated or naked *lmajgp63*/VR1012 vaccine according to serum IL-2 concentration, (A: Gold Particles; B: Empty VR1012 plasmid; C: DNA vaccine). $p<0.05$

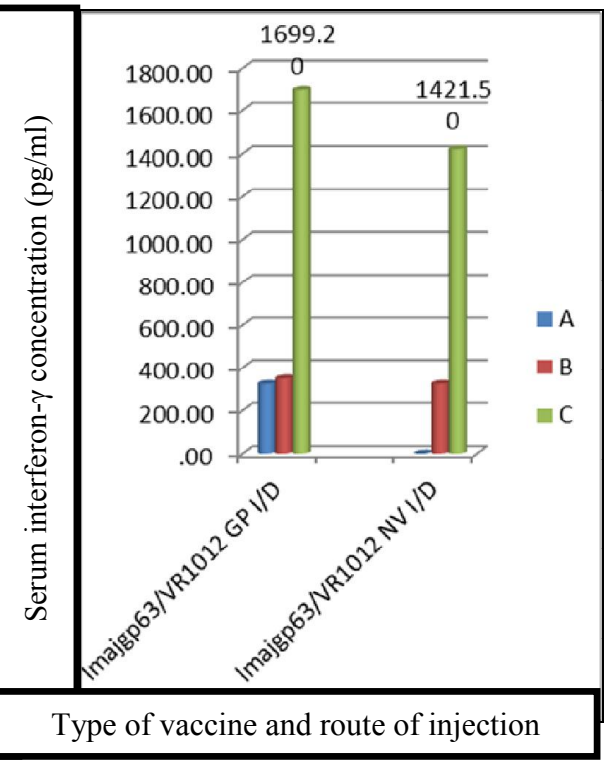

Figure 8: Differences between groups vaccinated with gold coated or naked *lmajgp63*/VR1012 vaccine according to serum INF-γ concentration, (A: Gold Particles; B: Empty VR1012 plasmid; C: DNA vaccine). $p<0.05$

Estimation of IL-10 and IL-4 concentration in mice sera

Mice group treated with naked plasmid I/M shows the highest titer of IL-4 and IL-10 concentration (85.30 pg/ml and 53.10 pg/ml respectively), while the lowest titer observed in group injected I/D by gold coated plasmid (50.90 pg/ml and 41.88 pg/ml respectively). Results are shown in figures 10 and 11.

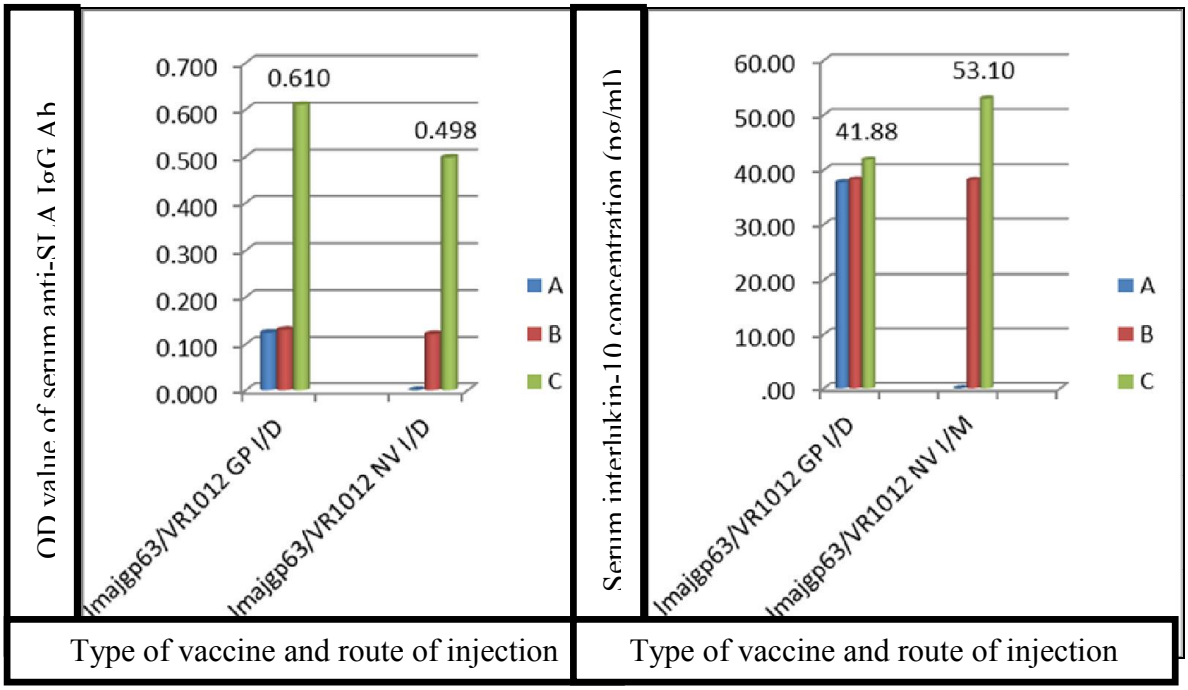

Figure 9: **Differences between groups vaccinated with gold coated or naked *lmajgp63*/VR1012 vaccine according to serum anti-SLA IgG antibody**, (A: Gold Particles; B: Empty VR1012 plasmid; C: DNA vaccine). $p<0.05$

Figure 10: **Differences between groups vaccinated with gold coated or naked *lmajgp63*/VR1012 vaccine according to serum IL-10concentration**, (A: Gold Particles; B: Empty VR1012 plasmid; C: DNA vaccine). $p<0.05$

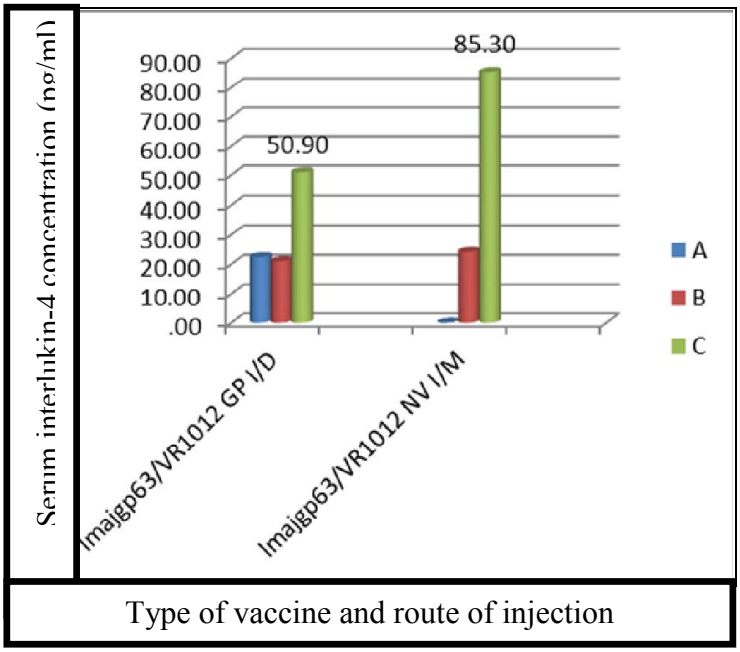

Figure 11: Differences between groups vaccinated with gold coated or naked *lmajgp63*/VR1012 vaccine according to serum IL-4 concentration, (A: Gold Particles; B: Empty VR1012 plasmid; C: DNA vaccine). $p<0.05$

Table 1: Th_1/Th_2 immune response induced by each type of DNA vaccination.

Type of DNA vaccine	Th_1/Th_2 Immune response	
	IL-2/IL-10	INF-γ/IL-4
I/D injected Gold coated *lmajgp63*/VR1012	4.348	33.383
I/M injected naked *lmajgp63*/VR1012	1.951	13.879

DISCUSSION

Leishmania major glycoprotein 63 is a characterized immunogenic protein highly expressed to all developing stages of the *Leishmania* parasites, where it has been demonstrated that the administration of DNA-encoding *Leishmania* gp63 protein can generate immunity and partially protect Balb/c mice from infection [3, 11].

PCR cloning was carried out after PCR amplification of coding region of gp63 gene of *L. major* presence in PNUT plasmid (obtained as a kind gift from Dr. Dumonteil, Laboratorio de Parasitología Yucatan Mexico) using following pairs of primers: forward primer with 6 base pair represent the restriction site for *EcoRV* endonuclease restrection enzyme 5` TGTCGATATCCTATGCGTGGGCTGGA 3` and reverse primer 5` TCTGAGATCTGGGGAGGGGTCACAGG 3` containig restrection site for *BgIII* enzyme. Four base pair was added to each primer as stabilizer.

The gene size obtained after PCR amplification was 1919 base pair in compare to DNA ladder including 20 base pair at each end to make sticky end for ligation with the plasmid in the right direction (Figure 2).

Using *EcoRV* restrection enzyme for digestion of gp63 resulting in split the resrection site at the forward end and devided the gene into two segaments (1273 bp and 622bp) due to the presence of the sequence GATATC at the site 627 which recognized by *EcoRV* enzyme as restriction site (Figure 3) and because of that another step of re-ligation was needed to religated the gene by using ligase enzyme (Figure 4).

The re-ligated *lmajgp63* was inserted into VR1012 plasmid successfully using ligase enzyme and it was confirmed by PCR amplification using pair of primers mentioned above (Figure 5).

Five primers were designed in this study by Primer 3 design plus sfotware for the sequencing of *lmajgp63* gene in the newly prepared plasmid. The needing for that number of primers was due to the size of the gene. Results produced by Source Bioscience LTD, UK, were assembled using Just bio software and compared with the sequence of L. major gp63 gene in gene bank (Accession number Y00647) and they were identical except in base number 847 (C inspit of G) and base number 865 (G inspite of C) which represent minor mutation during PCR amplification (Figure 7).

Sharifian H. 2010, found that the ratio of mutants depends on the multiplication of the sequence length and mutation rate. The mutation rate is determined by the performance of the DNA polymerase. So using an accurate polymerase, about 98% of the sequences are amplified without mutation when they are not so long (e.g. 100 bp). But the probability to have mutants is higher for longer sequences (e.g. 1000 bp). Her results showed that about 12% of the generated sequences have one mutation even by using an accurate enzyme [(13)].

To analyse the mutations result during amplification, allignment between amino acids produced by virtual translation of the sequence created in this study by using of Geneious Inspirational Software for Biologists and amino acid sequence published in gene bank for *L. major* gp63 protein (Accession number Y00647) was done. The result was both amino acid sequences were identical, i.e, the mutations were silent mutation (result is not shown). Point mutations were said to be "silent" when they do not affect the amino acid sequence of the protien. For example, a codon change from CGA to CGG dose not affect protien because both of these codons specify arginine [(14)].

Many studies have been reported that resistant to *Leishmania* parasite infection is contributable to Th_1 immune responses [(15, 16, 17)]. In this study IL-2 and INF-γ concentration in mice sera were estimated to detect Th_1 response against DNA vaccines. According to IL-2 and INF-γ serum concentration, there were significant differences (p<0.05) between mice groups vaccinated with *lmajgp63*/VR1012 plasmid, administrated via I/D or I/M routes as DNA vaccines, and control groups (Figures 8 and 9), that mean there are significant differences between vaccinated groups and control groups in stimulation of Th_1 cells.

These findings are in line of Dey *et al.* (2008), who found that the using of *Leishmania* parasites genes codding to certain protein as DNA vaccines can evoke immune responses against *Leishmania* parasite antigens and can stimulate Th_1 cells leading to increase in the secretion of IL-2 and INF-γ [18].

The routes of administration played significant role in the stimulation of Th_1 cells. I/D injection were better than I/M injection in evoking type one immune response by DNA vaccine. According to present results gold coated plasmid injected via I/D route stimulate Th_1 to produce the highest levels of IL-2 for group vaccinated with *lmajgp63*/VR1012 (182.10 pg/ml), and same findings were observed for INF-γ serum concentration (1699.20 pg/ml), while I/M injection of naked plasmid showed the lowest results for both IL-2 and INF-γ in group vaccinated with *lmajgp63*/VR1012 (1183.20 pg/ml).

However there are no available data about using Dermojet needle-free injector as delivery system for DNA vaccination in mice to compare our results with them, needle-free vaccine delivery has been studied in numerous species including cats [19], dogs [20], cattle [21] and pigs [22]. The vast majority of needle-free studies demonstrated that needle-free intradermal DNA vaccine delivery resulted in an enhanced immune response when compared to traditional needle-and-syringe vaccine delivery for example Aguiar *et al*. (2001) found that rabbits vaccinated with 3 doses of plasmid encoding malarial antigen (*Plasmodium falciparum* circumsporozoite protein) by needle-free injection had 8 to 50 fold greater Ab titers than those injected intramuscularly with traditional needle-syringe device[23]. This discrepancy can be explained by that the skin is an active immune surveillance site and is rich in very potent antigen presenting dendritic cells (DCs) such as Langerhan's cells (LCs) in the epidermis and the immature DCs in the dermis [24]. DCs are thought to play at least three distinct roles in genetic immunization: (1) MHC class II-restricted presentation of antigens secreted by neighbouring, transfected cells, (2) MHC class I-restricted ''cross'' presentation of antigens released by neighbouring, transfected cells, and (3) direct presentation of antigens by transfected DCs themselves [25].

Previous studies reported that plasmids are large molecules, and cell's cytoplasmic and nuclear membranes represent barriers to delivery of plasmids to the

eukaryotic nuclei where plasmids are expressed. One promising mechanism to deliver DNA vaccines is the use of particle-mediated epidermal delivery (PMED) [1, 26, 27, 28]. In PMED, the DNA plasmid is deposited onto a gold particle, and the gold particles are then accelerated through the skin using a compressed gas. Some of particles come to rest in the nuclei of cells, and the plasmids then elute off the particles and are expressed. The advantage of PMED for delivering plasmid DNA vaccines is the evident in that microgram quantities of plasmid can achieve strong immune responses, whereas much larger quantities of plasmid are typically needed to elicit a similar immune response using naked plasmid by intramuscular injection [29, 30, 31, 32, 33].

Type two immune response (Th_2) was reported to be the main cause of susceptibility to *Leishmania* parasite infection in Balb/c mice [6], therefor activation of Th_2, by DNA vaccines, was detected by estimation of IL-10 and IL-4 serum concentration (Figures 10 and 11)

However there were significant elevation of IL-10 and IL-4 serum concentrations in vaccinated group, it's easily to note that INF-γ/IL-4 and IL-2/IL10 ratio increased in all vaccinated groups, i.e., Th_1/Th_2 ratio of $CD4^+$ population was significantly increased in vaccinated mice. As shown in table 1, the highest Th_1/Th_2 ratio was observed in group injected I/D with gold coated plasmids by using Dermojet needle-free injector (33.383 and 4.348 respectively). While the lowest rates of INF-γ/IL-4 and IL-2/IL10 were induced *lmajgp63*/VR1012 naked plasmids injected intramuscularly vaccines, INF-γ/IL-4 (13.879) and IL-2/IL10 (1.951) respectively, indicating that the best method of vaccination was the using of gold coated vaccine injected intradermally.

These findings are in line with Ali *et al* (2009), whose found that however using of both intradermally injection of gold coted *lmexgp63*/VR1012 plasmid, by using another device "gen gun" as a delivery approach, and injection of naked plasmid intramuscularly increase Th_1/Th_2 immune response, immunization of Balb/c mice with low dose (1µg) of gold coated plasmid intradermally can induce strong type one immune response after one week of injection, while 100 µg of naked plasmid introduced intramuscularly needed to induce Th_1 activation in the same level after seven week of last injection[6].

Immunoglobulin-G reacted to SLA derived from *L. major* were detected as indicator to humeral immune response against DNA vaccines results are shown in figure 9. There were significant diffrences between immunized mice groups ($p<0.05$) and control groups. These fidings are in line with Ramirez *et al*., 2010, who found that immunizion of Balb/c mice by DNA vaccine induced the increasing of anti-SLA IgG titre as a result of stimulation of humeral immune response[12]. Glod coated particle vaccine induced the highest IgG level for groups vaccinated I/D (OD mean 0.6101 ±0.018083). This discrepancy explained by Aberle *et. al*., 1999, who found that however, the induction of Th_1/Th_2 immune response is influenced by type of DNA vaccine (gold coated or naked) and by route of administration (I/D by gen gun or I/M by needle injection) but it is strongly influenced by type of Ag encoded by that DNA vaccine[34].

الاستجابة المناعية لفئران Balb/c ضد التطعيم الجيني بجين *Lieshmania major* gp63 (*lmajgp63*)

وميض هاشم عباس* ناظم كاظم مهدي** سلمان عبد الحليم علي***
*فرع الأحياء المجهرية ، كلية الطب البيطري، جامعة البصره ،البصره،العراق.
** فرع الأحياء المجهرية،كلية الطب، جامعة البصرة، البصرة، العراق،
***كلية العلوم والتكنولوجيا، جامعة نوتنغهام ترينت، نوتنغهام، المملكة المتحدة

الخـلاصـــة

استخدم في هذه الدراسة جين البروتين السكري (*lmajgp63*) من طفيلي *L. major* كلقاح جيني مناسب. ادخل الجين الى بلازميد VR1012 لتحضير بلازميد *lmajgp63*/VR1012 باستخدام طرق التقنيات الحيوية القياسية. استخدمت البلازميدات اما عارية او حملت على جزيئات الذهب كلقاح جيني لتمنيع اناث فارمختبري نوع Balb/c بعمر 4−5 اسابيع. لقحت الفئران في الاسبوع الاول للتجربة ثم بعد اسبوعين من اللقاح الاول ثم بعد ستة اسابيع من اللقاح الثاني. استخدم جهاز Dermojet الخالي من الابرة لحقن اللقاح المحمل على جزيئات الذهب في الادمة واستخدمت المحقنة العادية لزرق اللقاح العاري في العضلة. قيست الاستجابة المناعية للمجاميع الممنعة بعد اسبوعين من اخر جرعة لقاح من خلال قياس تراكيز IL-2 و IL-4 و IL-10 و INF-γ في مصل الدم و كذلك الاجسام المضادة من نوع γ لمستضد الليشمانيا الذائب (anti-SLA IgG) (بواسطة اختبار ELISA. اظهرت النتائج كفاءة اللقاحات الجينية المحضرة لاحداث الاستجابة المناعية مقارنة بمجاميع السيطرة ($P<0.05$). لوحظ ان اعلى تراكيز IL-2 و INF-γ و anti-SLA IgG كانت في امصال الفئران في المجموعة الملقحة بالقاح المحمل على جزيئات الذهب والمحقون في الادمة (182.10 pg/ml و1699.20 pg/ml و 0.6101 على التوالي)، بينما لوحظت اقل التراكيز في المجموعة الملقحة باللقاح العاري والمحقون في العضلة (103.60 pg/ml و 1183.20 pg/ml و 0.3395 على التوالي). من جانب اخر لوحظ ان اعلى التراكيز IL-4 و IL-10 في المصل كان في امصال المجموعة الملقحة بالقاح العاري

والمحقون في العضلة (85.30 pg/ml و 53.10 pg/ml على التوالي) بينما لوحظ ان اقل التراكيز كان في المجموعة الملقحة باللقاح المحمول على جزيئات الذهب والمحقون بالادمة (50.90 pg/ml و 41.88 pg/ml على التوالي). كما اظهرت النتائج ان الحقن في الادمة للقاحي المحمل على جزيئات الذهب احدث النسبة الاعلى لاستجابة الخلايا اللمفاوية المساعدة من النوع الاول الى الخلايا اللمفاوية المساعدة من النوع الثاني (Th_1/Th_2) {IL-2/IL-10 (4.348) و INF-γ/IL-4 (33.383)}، بينما الزرق في العضلة للقاح الجيني العاري اعطى النتائج الاقل{IL-2/IL-10 (1.951) و INF-γ/IL-4 (13.879)}.

REFERENCES

1. Tang, DC, DeVit, M, Johnston, SA (1992) Genetic immunization is a simplemethod for eliciting an immune response. *Nature,* 356, 152–154.
2. Mazumder, S, Maji, M, Das, A, Ali, N. (2011). Potency, efficacy and durability of DNA/DNA, DNA/protein and protein/protein based vaccination using gp63 against *Leishmania donvani* in Balb/c mice. *J. Plos One*, 6 (2).
3. Walker, PS, Scharton-Kersten, T, Rowton, ED, Hengge U., Bouloc A., Udey M. C.,Vogel J. C. (1998). Genetic immunization with glycoprotein 63 cDNA results in a helper T cell type 1 immune response and protection in a murine model of leishmaniasis. Hum Gene Ther, 9, 1899–1907.
4. Campos-Neto A, Porrozzi R, Greeson K, *et al*. (2001). Protection against cutaneous leishmaniasis induced by recombinant antigens in murine and nonhuman primate models of the human disease. *Infect Immun,* 69, 4103–4108.
5. Sachdeva, R, Banerjea, AC, Malla, N, Dubey ML. (2009) Immunogenicity and efficacy of single antigen Gp63, polytope and polytope HSP70 DNA vaccines against visceral Leishmaniasis in experimental mouse model. *PLoS One,* 4, e7880.
6. Ali, SA, Rezvan, H, McArdle, S E, Asteal FA, Rees RC (2009). CTL responses to *Leishmania mexicana* gp63-cDNA vaccine in a murine model. *Parasite Immunol, 31*(7), 373-383.
7. Awasthi, A, Mathur, RK, Saha, B (2004). Immune response to *Leishmania* infection. *Indian J Med Res*, 119, 238-258.
8. Dunning, N (2009). *Leishmania* vaccines: from leishmanization to the era of DNA technology. Bioscience Horizons. 2, 73-82.

9. Joshi, PB, Saks, DL, Modi, G, McMaster, WR (1998). Targeted gene deletion of *Leismania major* genes encoding developmental stage-specific leishmanolysin (gp63). Molecular Microbiology, 27 (3), 519-530.
10. Razvan, H, Rees, R, Ali, SA (2011). *Leishmania Mexicana* gp63 cDNA using gene gun induced higher immunity to *L. Mexicana* infection compared to soluble *Leishmania* antigen in Balb/c. *Iranian Parastol*, 6 (4), 60-75.
11. Dumonteil, E, Ramirez-Sierra, MJ, Escobedo-Ortegon, J, Garcia-Missl, MR (2003). DNA vaccines induce partial protection against Leishmania mexicana. Vaccine, 21, 2161-2168.
12. Ramirez, L, Iborra, S, Cortes, J, Bonay, P, Alonso, C, Barral-Netto, M, Soto, M (2010). BALB/c mice vaccinated with *Leishmania major* ribosomal proteins extracts combined with CpG oligodeoxynucleotides become resistant to disease caused by a secondary parasite challenge. *J Biomed Biotechnol.* 181, 690.
13. http://e-collection.library.ethz.ch/eserv/eth:1397/eth-1397-01.pdf.
14. Mark, A, Lieberman, M, Peet, A (2004). Marks' Basic Medical Biochemistry: a clinical approach. 4th edition, Lippincott Williams & Wilkins, a Wolters Kluwer business, Philadelphia PA 19106, section III: P: 252.
15. Scott, P (1991). IFN-gamma modulates the early development of Th1 and Th2 responses in a murine model of cutaneous leishmaniasis. *J Immunol*, 147(9), 3149-3155.
16. Wang, ZE, Reiner, SL, Zheng, S, Dalton, DK, Locksley, RM (1994). CD4+ effector cells default to the Th2 pathway in interferon gamma-deficient mice infected with *Leishmania major*. *J Exp Med,* 179, 1367–1371.
17. Roberts, MTM (2006) Current understandings on the immunology of leishmaniasis and recent developments in prevention and treatment. *Br Med Bull,* 75–76, 115–130.
18. Dey, A, Sharma, P, Redhu, NS, Singh, S (2008). Kinesin motor domain of *Leishmania donovani* as a future vaccine candidate. *Clin. Vccine Immunol*., 15(5), 836.
19. Grosenbaugh, DA, Leard, T, Pardo, MC (2004). Comparison of the safety and efficacy of a recombinant feline leukemia virus (FeLV) vaccine delivered

transdermally and an inactivated FeLV vaccine delivered subcutaneously. *Vet. Ther*. 5, 258-262.

20. Anwer, K, Earle, SA, Shi, KA, Wang, J, Russell, Mumper,J, Proctor, B, Jansa, K, Harry, J, Ledebur, C, Davis, S, Eaglstein, W, Rolland, AP (1999). Synergistic effect of formulated plasmid and needle-free injection for genetic vaccines. *Pharm*. *Res*. 16, 889-895.
21. Van Drunen, S (2006). Rationale and perspectives on the success of vaccination against bovine herpesvirus 1. Vet. Microbiol., 113, 275-282.
22. Jones, GF, Rapp-Gabrielson, V, Wilke, R, Gary, F, Jones, D, Vicki Rapp-Gabrielson, P, Wilke, R, Thacker, EL, Thacker, B, J, Gergen, L, (2005). Intradermal vaccination for Mycoplasma hyopneumoniae. *J*. *Swine Health Prod*. 13, 19-27.
23. Aguiar, JC, Hedstrom, RC, Rogers, WO, Charoenvit, Y, Sacci, JB (2001). Enhancement of the immune response in rabbits to a malaria DNA vaccine by immunization with a needle-free jet device. Vaccine, 20, 275-280.
24. Cui, Z, Mumper, RJ (2002). Topical immunization using nanoengineered genetic vaccines. *J Control release*,81, 173-184.
25. Takashima, A, Morita, A (1999). Dendritic cells in genetic immunization. *J*. *Leukoc*. *Biol*., 66, 350–356.
26. Haynes JR, Fuller DH, McCabe D, Swain, WF, Widera, G (1996). Induction and characterization of humoral and cellular immune responses elicited via gene gun-mediated nucleic acid immunization. *Adv drug devilry Rev*, 21, 3–18.
27. McCluskie, MJ, Brazolot Millan, CL, Gramzinski, RA, Robinson, RH, Santoro, JC, Fuller, JT, Widera, G, Haynes, JR, Purcell, RH, Davis, HL (1999). Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. *Mol Med*, 5, 287–300.
28. Payne, LG, Fuller, DH, Haynes, JR (2002). Particle-mediated DNA vaccination of mice, monkeys and men: looking beyond the dogma. *Curr Opin Mol Ther*, 4, 459–466.

29. Ugen, KE, Nyland, SB, Boyer, JD, (1998). Route and method of delivery of DNA vaccine influence immune responses in mice and non-human primates. Vaccine, 16, 1818–1821.
30. Boyer JD, Cohen AD, Vogt S (2000). Induction of CD4+ T cell-dependent CD8+ type 1 responses in humans by a malaria DNA vaccine. *J Infect Dis* 181, 476–483.
31. MacGregor, RR, Boyer, JD, Ciccarelli, RB, (2000). Safety and immune responses to a DNA-based human immunodeficiency virus (HIV) type I env/rev vaccine in HIV-infected recipients: follow up data. *J Infect Dis*, 181, 406.
32. Wang, R, Epstein, J, Baraceros, FM (2001) Induction of CD4(+) T cell-dependent CD8(+) type 1 responses in humans by a malaria DNA vaccine. *Proc Natl Acad Sci* USA, 98, 10817–10822.
33. MacGregor, RR, Ginsberg, R, Ugen, KE, et al. (2002). T-cell responses induced in normal volunteers immunized with a DNA-based vaccine containing HIV-1 *env* and *rev*. AIDS, 16, 2137–2143.
34. Aberle, JH, Aberle, SW, Allison, SL (1999). A DNA immunization model study with constructs expressing the tick-borne encephalitis virus envelope protein E in different physical forms. *J Immunol* 163, 6756–6761.