Cloning and Expression of Truncated Haemoglobin Receptor of Leishmania tropica as a Vaccine Candidate

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Abstract

Cutaneous leishmaniasis is a tropical disease caused by Leishmania parasite and vaccination is the best way to control it. In this study, the truncated Haemoglobin receptor (HbR-N) gene of Leishmaniastropicawas cloned and expressed in Escherichia coli. The sequence of this gene was codon-optimized, synthetically synthesized and amplified using specific primers. pTYB21 plasmid as well as the amplified gene were digested by SapI and PstI and ligated using T4 DNA ligase. The recombinant plasmid was used to transform DH5α Escherichia coli. Transformants were confirmed using colony PCR, digestion of the plasmids using PvuII enzyme as well as DNA sequencing. The recombinant plasmid was used to retransform the T7 express E. coli and the transformants were confirmed using colony PCR. The cell carrying the plasmid was used to express HbR-N protein. The gene expression was analysed by SDS-page polyacrylamide gels. PCR has successfully amplified the HbR-N gene with a molecular size of 398 bp. Transformants carrying the pTYB21_HbR-N plasmid were obtained and designated as MYM1. DNA sequencing showed 100% similarity to HbR gene. The later plasmid was used to retransform T7 express E. coli, several transformants were obtained and they designated as MYM2. The MYM2 bacterial cell was used to express the gene. Finally, SDS-page polyacrylamide gel showed protein bands of the fused protein (intein-HbR-N) with a molecular size of about 70 kDa. In this study, the HbR-N gene was cloned and expressed in E. coli and could be used as a potential vaccine against several Leishmania spp.

Keywords: Cutaneous leishmaniasis, Leishmaniatropicawas, Haemoglobin receptor, vaccine

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Introduction

Leishmaniasis is a vector-borne disease caused mainly by Leishmania spp., which are obligate intracellular parasites that primarily infect macrophages replicating inside and colonizing as non-motile amastigotes to establish infection (Rogers et al., 2006; Bamorovat et al., 2018). About 88 countries around the world are under the risk of catching this disease, it affects 12 million individuals in the tropical and subtropical area and the prevalence of disease estimated to be one million new cases each year. The cutaneous form of leishmaniasis (CL) is considered as the most common form worldwide, which accounts for more than 50% of new cases. It is a zoonotic disease, which delivered from animals to humans and a self-healing lesion at the site of infection characterizes this form. In Iraq L. major and L. tropica are parasites causing this disease (Kedzierski et al., 2006; Miura et al., 2015; Whyte and Zufferey, 2017). Chemotherapy treatment to alleviate this threatening disease and vector control to reduce the transmission of protozoa is the only procedures to control leishmaniasis. Unfortunately, present chemotherapeutic drugs are unsuitable due to their high toxicity and the appearance of drug resistance. Recovery from this disease renders the host resistant to the next infection. Therefore in recent years, many efforts were made to identify novel immunological targets to produce safe and effective vaccines against leishmaniasis (Sharma and Madhubala, 2009; Domínguez-Bernal et al., 2015).
Haemoglobin receptor (HbR) of *Leishmania* is conserved across species of *Leishmania* and anti-HbR antibody was identified in kala-azar patients’ sera. Using the N-terminal of HbR-DNA as a vaccine in hamsters infected with *L. donovani* exhibited high protection and survived for eight months. It is believed to be a promising molecule as a vaccine candidate against leishmaniasis (Guha et al., 2013). In this study, an attempt to use DNA sequence coding for HbR (N-terminal) surface antigen from *L. tropica* to be cloned and expressed in *E. coli* as a vaccine candidate for *Leishmania*. Consequently, this project aims to develop a recombinant protein, which could be used as a prophylactic vaccine against *L. tropica*.

**Materials and Methods**

**Amplification of HbR-N**

Genomic DNA of *L. tropica* HbR-N (378 bp) was obtained from the NCBI GenBank database with the accession number (KE147312.1), codon-optimized using JCat program and sent to a gene synthesizing company (Macrogen, Korea). Restriction sites for SapI and PstI restriction endonuclease enzymes were incorporated in the forward (5'-GCCCTTGCTCTTTCAACATGG-3') and reverse (G2-R, 5'-GCCCTTCTGAGTTAGTCACCA-3') primers with additional random six bases. GoTaq® G2 Green Master Mix (Promega, USA) with Polymerase chain reaction (PCR), (Thermal cycler, Techne, UK) was used to amplify HbR-N. The reaction conditions were as follow: 95°C for 2 min followed by 35 cycles of 95°C for 30 s, 57°C for 30 s and 72°C for 30 s and one cycle of 72°C for 5 min. The amplified fragments were analysed on agarose gel (Promega, USA), electrophoresed on a gel electrophoresis system (BioRad, USA) and documented using Gel Doc XR+ Gel Documentation System (BioRad, USA).

**Cloning of HbR-N**

The amplified fragment (389bp) and pTYB21 plasmid (7514 bp), which was provided with IMPACT™ kit (New England Biolabs, USA), were digested sequentially with two restriction endonuclease enzyme SapI (New England Biolabs, USA) and PstI (Promega, USA) for 3 h at 37°C. Then, they purified using Wizard® SV gel and PCR clean-up system (Promega, USA). The two purified fragments were ligated using T4 DNA ligase (Promega, USA) at 16°C overnight and heat-inactivated for 10 min at 70°C.

**Transformation of Escherichia coli with pTYB21_HbR-N plasmid**

NEB® 5-alpha competent *E. coli* (New England Biolabs, USA) was used as a host to amplify the recombinant plasmid (pTYB21_HbR-N) using transformation protocol, which was done according to recommendations of the manufacturing company (New England Biolabs, USA). Shortly, *E. coli* cells were removed from the -80°C freezer and thawed completely until the last ice crystals disappear on ice. Fiftymicroliters of cells were pipetted into a transformation tube on ice. Fiftymicroliters of heat-inactivated ligation reaction were added to the cells and stirred briefly by flicking the tube 4-5 times. The mixture was placed on ice for 30 min. The mixture was transferred to a water bath at 42°C for 30 s. The mixture was placed again on ice for 5 min. Room temperature recovery medium (950 μl) was added to the mixture. The tubes containing the cells were incubated in a shaking incubator at 250 rpm and 37°C for 1 h. Transformation mixture (100 μl) was plated on LB plates containing 100 μg/ml of ampicillin and incubated overnight at 37°C.

**Screening and confirmation of real transformants**

Transformants were primary screened using colony PCR. In which, a well-isolated colony was picked using a sterile micropipette tip or inoculation loop and transferred into an Eppendorf tube containing 50 μl of sterile water. The tube was boiled for 10 min and centrifuged at 16,000xg for 5 min. Fiftymicroliters of the supernatant were used in a 50 μl PCR reaction. Positive colonies carrying pTYB21_HbR-N plasmid were subjected to plasmid purification using PureYield™ Plasmid Miniprep System according to its manufacturing company (Promega, USA). The plasmid was digested with PvuII-HF at 37°C for 3 h. Furthermore, this plasmid was sent for sequencing to Macrogen, Korea.

**Expression of HbR-N in E. coli**

HbR-N was expressed in T7 express competent *E. coli* according to the manufacturing company (New England Biolabs, USA) and it was as follow: pTYB21_HbR-N plasmid was transformed into T7 express competent *E. coli*. One hundred ml of LB medium, containing 100 μg/ml ampicillin, was inoculated with a freshly grown colony. The culture was incubated in a shaker at 37°C until the OD600 reaches 0.5-0.8. Isopropyl-β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.4 mM at 37°C for 3 h. The cells were spun down at 5000xg for 15 min at 4°C and the cell pellet was resuspended in 10 ml of ice-cold Column Buffer (20 mMTris-HCl, pH 8.5, 500 mMNaCl, 1 mM EDTA, 0.5% Triton X-100). The cells were lysed by sonication on ice using short pulses (15 s in
1 min out for 15 min) and centrifuged at 15,000xg for 30 min at 4°C. Samples were analysed on SDS-page polyacrylamide gel and banding patterns were documented using Gel Doc XR+ Gel Documentation System.

**Results**

**Amplification of HbR-N**
Amplification of *L. tropica* HbR-N DNA was amplified by PCR. Successful result was obtained and DNA band of amplified HbR-N gene (389 bp) was appeared at the expected position between the fragments 300 and 400 bp of the 100 bp DNA ladder (Bioneer, Taiwan) on 1% agarose gel (figure-1).

![Figure 1: DNA amplification of HbR-N gene using specific primers (G1-F+G2-R). The amplified gene was electrophoresed on 1% agarose gel with 5 V/cm for about 90 min. Lane M: DNA marker ladder (100 bp), Lane 1: an amplified band of haemoglobin receptor gene (389 bp).](image)

**Cloning of the HbR-N gene**
HbR-N gene, as well as the pTYB21 plasmid (7514 bp), were digested with the SapI and PstI restriction endonucleases at 37 °C for 3 h. Digestion of the plasmid is seen in figure-2.

![Figure-2: Digestion of the plasmid pTYB21 with two restriction enzymes (SapI and PstI). Gel electrophoresed was done on 1% agarose gel with 5 V/cm for about 120 min. Lane M: DNA marker ladder (1 kb, Promega, USA), Lane 1: undigested plasmid, Lane 2: digested plasmid (7514 bp).](image)
Next, digested HbR-N and pTYB21 plasmid were ligated using T4 DNA ligase enzyme to generate a recombinant molecule designated as pTYB21_HbR-N with a total size of 7840 bp. The map drawing was done using SnapGene program as seen in figure 3.

![Figure-3: Drawing of cloning map of HbR-N gene into pTYB21 plasmid using Snapgene program.](image)

**Transformation of DH5α Escherichia coli with pTYB21_HbR-N plasmid**

pTYB21_HbR-N was transformed into a chemically competent DH5α *E. coli* to obtain a strain carrying pTYB21_HbR-N and the strain was designated as MYM-1. The transformation method was successful and too many colonies appeared on the LB agar plate containing 100 μg/ml of ampicillin as depicted in figure-4.

![Figure-4: Transformation of DH5α chemically competent *E. coli* (MYM-1) with a recombinant molecule (pTYB21_HbR-N).](image)

**Screening of *Escherichia coli* transformants**

Screening for transformants was achieved using two methods and confirmed by sequencing. The first one was colony PCR (primary screening) in which a well-isolated colony was picked up. Three picked colonies were tested and all of them were carrying HbR-N gene by showing of the amplified DNA bands at the expected position (Figure-5).

The second method was restriction endonucleases digestion (confirmative screening) using the enzyme PvuII. Theoretically, by using SnapGene program, PvuII should digest the pTYB21 plasmid in three positions (2662, 3409, and 3502) to give three bands with molecular sizes (93, 747, and 6674) bp. On the other hand, PvuII enzyme should digest the pTYB21_HbR-N in four positions (2662, 3409, 3502, and 7440) to give four bands with the molecular weight (93, 747, 3062, and 3938) bp. Practically, all DNA bands digested with PvuII enzyme of both pTYB21 and pTYB21_HbR-N plasmids appeared at the expected positions as shown in figure-7.

**Transformation and Screening of T7 express competent *Escherichia coli* with pTYB21_HbR-N plasmid**

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**Figure-6: Colony PCR for identification of true transformants carrying HbR-N gene. Gel electrophoresed was done on 1% agarose gel with 5 V/cm for about 90 min. Lane M: DNA marker ladder (100 bp), lanes 1-3: an amplified band of HbR-N gene (389 bp).**

**Figure-7: Digestion of plasmids using PvuII enzyme. Gel electrophoresed was done on 1% agarose gel with 5 V/cm for about 120 min. Lane M1: DNA marker ladder (1 kb), Lane 1: pTYB21 plasmid digested with PvuII to give two bands (93, 747 and 6674) bp, Lane 2: pTYB21_HbR-N plasmid digested with PvuII to give three bands (93, 747, 3062, and 3938) bp, lane M2: DNA marker ladder (100 bp).**

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To express the cloned gene (HbR-N), T7 express competent *E. coli* was used to achieve this goal. The purified plasmid (pTYB21_HbR-N) from the previous step was used to transform T7 express chemically competent *E. coli*. Transformed colonies appeared on LB plate as seen in figure-8. Screening for a true transformant carrying the pTYB21_HbR-N plasmid was achieved using colony PCR. Six colonies were picked up and used for colony PCR. All tested colonies were carrying HbR-N gene, which was confirmed by appearing of the amplified DNA bands at the expected position (389 bp) between two bands of standard DNA ladder bands 250 and 500 bp as shown in figure-9.

Expression of HbR-N in *E. coli*

The final step in gene cloning is the expression of a certain gene in a certain host. In this study, HbR-N gene was cloned into pTYB21 plasmid under the control of T7 strong promoter. This gene was also fused to *Saccharomyces cerevisiae* vacuolar membrane ATPase intein 1 gene containing chitin-binding domain (CBD). The recombinant plasmid (pTYB21_HbR-N) was transformed into T7 express competent *E. coli*. The expression of this gene inside bacterial cells was induced using IPTG to give the fused protein intein-CBD-HbR-N. The molecular weight of the intein tag is 56 kDa and molecular weight of HbR-N protein that composed of 126 amino acids should be about 14kDa. Therefore, the theoretical molecular weight of the fused protein should be about 70 kDa. Results showed in figure-10 demonstrated that the uninduced cells do not express the protein whereas the induced bacterial cells using IPTG (Isopropyl-β-D-thiogalactopyranoside) expressed the fused protein to give a protein band lied at the expected position between the 80 and 60 kDa of the standardmarker protein bands.
Discussion

*Leishmania* spp., kinto-plastid protozoan parasites, are the causative agents of different clinical forms of leishmaniasis. The clinical form manifestations ranging from a simple ulcer to a fatal disease. Consequently, there are three main forms of leishmaniasis, which are cutaneous (CL), mucocutaneous (MCL), and visceral leishmaniasis (VL) (Srivastava et al., 2016). In Iraq, leishmaniasis is widely spread across almost all provinces where both cutaneous and visceral forms are established. Cutaneous leishmaniasis (CL) is prevalent in Iraq except in three northeastern provinces (Salam et al., 2014). Two kinds of CL were registered in Iraq, which caused by *L. major* and *L. tropica* (AlSamarai and AlObaidi, 2009).

For any antigen to be developed as a possible vaccine, two methods were searched by either inhibiting main parasite transporters or stalling vital metabolic pathways of a parasite. Significant efforts to determine potential antigen that can provide protection against Leishmaniasis. Many antigens were produced as recombinant proteins and tested experimentally including A2 protein (Ghosh et al., 2001), cysteine proteinase (Rafati et al., 2006), nucleoside hydrolase (Al-Wabel et al., 2007) and KMP-11 (Agallou et al., 2011). *Leishmania* lacks a complete heme biosynthesis pathway and thus has to acquire it from the external environment by Haemoglobin receptor protein (HbR). HbR is also an essential enzyme of the parasite glycolytic pathway (Sengupta et al., 1999; Singh et al., 2003; Krishnamurthy et al., 2005). A previous study from Guha et al (2013) had shown the prophylactic potential of HbR antigen as a DNA vaccine by eliciting both T helper type 1 cytokines and multifunctional T cells. They concluded that the truncated HbR (N terminus) provide the same results. Furthermore, it is conserved among various *Leishmania* spp. Therefore, HbR can be considered as a possible standalone therapeutic target vaccine against different types of *Leishmania* spp. (Guha et al, 2013, Dean et al, 2014).

In this study, we cloned and expressed the truncated form of HbR (HbR-N) in E. coli as a fused protein with a molecular weight of 70 kDa. Yet, this protein needs to be purified and tested in experimental mice.

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