Saudi Journal of Biological Sciences 26 (2019) 1676–1681

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Development of Rift Valley fever (RVF) vaccine by genetic joining of the RVF-glycoprotein Gn with the strong adjuvant subunit B of cholera toxin (CTB) and expression in bacterial system

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ARTICLE INFO

Article history:
Received 31 July 2018
Revised 19 August 2018
Accepted 20 August 2018
Available online 22 August 2018

Keywords:
RVFV
Adjuvant
CTB
Subunit vaccine
Recombinant vaccine

ABSTRACT

One of the mosquito-borne zoonotic diseases is the Rift Valley fever virus (RVFV). Currently, there is no completely licensed vaccine that can be used to vaccinate animals or humans outside endemic areas. The aim of this work was to use the RVFV glycoprotein (Gn) and the subunit B of cholera toxin (CTB) at gene level and build up fused recombinant vaccine. The gene of CTB was joined to the gene Gn to work as an adjuvant in the resulting fusion protein. The designed merged genes (*CTB-Gn*) was tested for restriction sites, open reading frames, expected fusion protein tertiary structure and antigenicity using computer software. The insert sequence was submitted to the BioProject (GenBank). The insert was subcloned into the pQE-31 expression plasmid. The target recombinant protein (rCTB-Gn) was expressed in M15 bacteria, purified and identified by protein gel electrophoresis. The insert got the accession No: PRJNA386723. Analysis of the designed rCTB-Gn protein revealed that it had the right 3D structure, immunogenic and at the correct molecular weight. The presence of the CTB in the proposed vaccine will augment its immunogenicity. Doses and protection levels of the vaccine need to be manipulated.

1. Introduction

Rift valley fever (RVF) is one of viral zoonotic diseases that primary affect ruminant animals but also the virus has ability to infect humans (WHO, 2017). The RVF disease is caused by the arbovirus RVF virus (RVFV) which was first identified in Kenya's Rift Valley in 1931 when an investigation was done in a farm found there containing sheep (Daubney et al., 1931). RVFV, the single stranded RNA virus, belongs to phlebovirus under the Bunyaviridae family (Pepin et al., 2010). Till the year 2000, RVF was spreading only in the African continent, but in 2000 it was reported in Jizan region, Saudi Arabia as the first RVF infection outside Africa. At the same Human infectious can of the outbreak, there were thousands of animals affected including different species. The virus cause high rates of mortality and abortion (Ikegami and Makino, 2011). RVF is transmitted among livestock by mosquito's bites which act as biological vectors. In addition to infection can occur also vertically between animals (Antonis et al., 2013). On the other side, human infection is caused by the exposure through the contact (direct or indirect) with blood or organs of infected animals. Human infection can also result from drinking and using of milk that is not pasteurized milk of infected animals. In addition, bites of infected mosquitoes can infect human (Anyangu et al., 2010). The incubation period of the RVF virus in the human varies from 2 to 6 days. The symptoms of RVF ranged from mild to severe form. Most of the human RVF are mild cases. The symptoms of uncomplicated cases are usually nonspecific and include fever, headache, sudden onset

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2018.08.019
1319-562X/

of flu like fever, generalized weakness, myalgia, joint pain and back pain. Some few cases of patients develop more severe form of RVF disease. The severe form includes ocular form, meningoencephalitis form and hemorrhagic fever form (WHO, 2017).

The single-stranded genome of RVFV composed of a negative-sense RNA. This genome encodes four distinguished structural proteins (nucleoprotein (N), glycoprotein Gn, glycoprotein Gc and the L polymerase) non-structural proteins (NSs and NSm) and the 78-kDa protein which is unknown function (Gerrard et al., 2007; Gerrard and Nichol, 2002; Won et al., 2007). The non-structural proteins (NSm and NSs) play a very important role in the pathogenesis of RVFV where NSm prevents host cell apoptosis, while NSs inhibits innate immune responses of the host (Habjan et al., 2009; Ikegami et al., 2007). It was demonstrated that Gn and Gc function for virus attachment to host cell and these proteins carry epitopes that neutralize the virus (Besselaar and Blackburn, 1994). For these reasons, Gn and Gc are targeted to develop RVF subunit vaccines (Faburay et al., 2014; Kortekaas et al., 2012).

There are different types of vaccines, formalin-inactivated RVFV vaccine which requires repeated regular booster doses to induce and maintain acceptable protection after repeated immunization schedule (Rusnak et al., 2011). Live attenuated vaccines rapidly induce immunity with no need for repeated booster doses is important. The live attenuated vaccine may cause some problems like birth defect and abortion in sheep and low immunization in cattle. MP-12, the live attenuated vaccine, is safe and immunogenic for human uses if adequate dose is applied (Pittman et al., 2016).

In this work, genetical joining of CTB to the glycoprotein Gn of RVFV was done. The purpose to use CTB was to increase the antigenicity of Gn protein.

2. Materials and methods

2.1. Design and analysis of the synthetic gene

(CTB) and Rift valley fever virus glycoprotein (Gn) from Saudi isolate coding sequences (accession No: KP037243.1 and KU978778.1 respectively) were obtained from GenBank.

The genes (*CTB* and *Gn*) were examined for the existing internal restriction enzyme sites as well as existing open reading frames using software (Gene Runner version 6.5.51). *Xho*I site sequence was used to link *CTB* (at 5′ end) and *Gn* genes (at 3′ end) together. The fused genes, the CTB-Gn (insert), were tested again for the new open reading frames. Before synthesis of CTB-Gn gene cassette, the codon preference was considered.

To the 5′ and 3′-ends, the restriction sites *Sph*I (GCATGC) and *Sma*I (CCCGGG) were added respectively of the insert. To get blunt ends, pUC57 plasmid was digested with the restriction enzyme EcoRV. By this way blunt-ended linearized pUC57 plasmid is ready to ligate with the blunt-ended synthesized CTB-Gn insert.

Through Gene Runner, all genes (*CTB*, *Gn* and *CTB-Gn*) were translated into their corresponding amino acid sequences taking into consideration the bacterial codon preference.

Using software (DNASTAR Lasergene 14.1) the proposed proteins were analyzed. The 3D configuration, presence of antigenic sites and different epitopes were analyzed for Gn protein, CTB protein and joined CTB-Gn protein using the program Protean 3D™ of Lasergene. The constructed gene cassette (CTB-Gn) was submitted to the GenBank.

2.2. Cloning of genes

2.2.1. Preparation of CTB-Gn gene in the expression vector

The final form of the insert (including the added restriction sites sequences) was produced at GenScript company (NJ, USA) and cloned into pUC57 to get pUC57-CTB-Gn (recombinant pUC57, Fig. 1).

The recombinant vector 1 (lyophilized) was dissolved in DNase/RNase-free water (Gibco, BRL) to a final concentration of 50 ng/μL. Top10 *E. coli* competent cells (Promega) were transformed with about 90 ng of the recombinant vector 1.

Qiaprep spin miniprep (Qiagen) kit was used to purify plasmids from transformed bacterial cells according to the protocol of the manufacturer. Plasmid concentration/purity was evaluated at 260/280 nm using spectrophotometer (GENESYS 10uv, Thermo Scientific). Plasmid containing the gene cassette (10 μg) was digested by *Sph*I and *Sma*I restriction enzymes (Invetrogen) simultaneously. After that, the products were run in agarose gel (1.2%) and the target insert (CTB-Gn) was excised from the run gel to be purified using Qiagen kit (Qiaquick gel extraction). In the same time, pQE-31(Qiagen) expression vector (2 μg) was cut and gel-purified.

2.2.2. Construction of the recombinant expression vector (CTB-Gn-pQE-31)

CTB-Gn gene was ligated to the linearized pQE-31 expression vector (in 1:5 ratio) using T4-DNA ligase enzyme (Promega) to get the expression recombinant construct (CTB-Gn-pQE-31). Ligation reaction (5 μL) was used for the transformation of competent Top10 bacteria (100 μL). From the grown bacteria on the antibiotic selection LB-agar plates, plasmids were purified as described above and checked for the existence of CTB-Gn insert by both restriction digestion using *Sph*I and *Sma*I restriction enzymes and polymerase chain reaction (PCR) using the sequencing primers recommended by Qiagen for pQE vectors. Forward Primer-1 (5′-CGG ATA ACA ATT TCA CAC AG- 3′ and reverse Primer-2 (5′ -GTT CTG AGG TCA TTA CTG G- 3′) were made at GenScript to be used in PCR amplification of *CTB-Gn* gene inside pQE-31.

The PCR cycles were as follow: preheating (95 °C for 5 min), 30 repeating cycles (denaturing (92 °C), annealing (50 °C) and extension (72 °C) for 1 min each) and post extension step (72 for 5 min). All products of the PCR were examined in 1.6% agarose gel. After PCR amplification completed, the insert was gel-purified and sent for the analysis of its nucleotides sequence at Macrogen (Korea) using the sequencing primers recommended by Qiagen.

2.3. Recombinant protein production

M15 bacteria included in the Qiaexpressionist kit (Qiagen) were prepared to be competent according to Chung et al. (1989) followed by transformation with 150 ng of the recombinant expression vector. Grown colonies after transformation process were analyzed for the getting of plasmids harboring the insert (CTB-Gn) through restriction enzyme digestion and PCR analysis as described above and sent for DNA sequence analysis. To prepare small-scale recombinant CTB-Gn (rCTB-Gn) protein, LBamp/kana (5 mL) was inoculated with the same test colony and incubated overnight at 37 °C with shaking (225 rpm). Negative control culture (M15 cells harboring empty pQE-31) was included under the same conditions. Into two 200-mL flasks, 75 mL of prewarmed LBamp/kana were added and 2.75 mL of overnight culture (separately) were added to each flask and grown with vigorous shaking (320 rpm) at 37 °C, until the OD_{600} was about 0.6 (approximately 50–60 min). Non-induced control (1-mL volume) from each culture were taken just before induction and their bacterial cells were collected by centrifugation for 5 min at 8,500 rpm, resuspended in 60 μL of 1× sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer (20% glycerol, 2% SDS, 0.1 M Tris.Cl, pH 6.8, 0.02% bromophenol blue, 0.1 M dithiothreitol (DTT), all from Sigma-Aldrich) and kept at −30 °C till use. Recom-

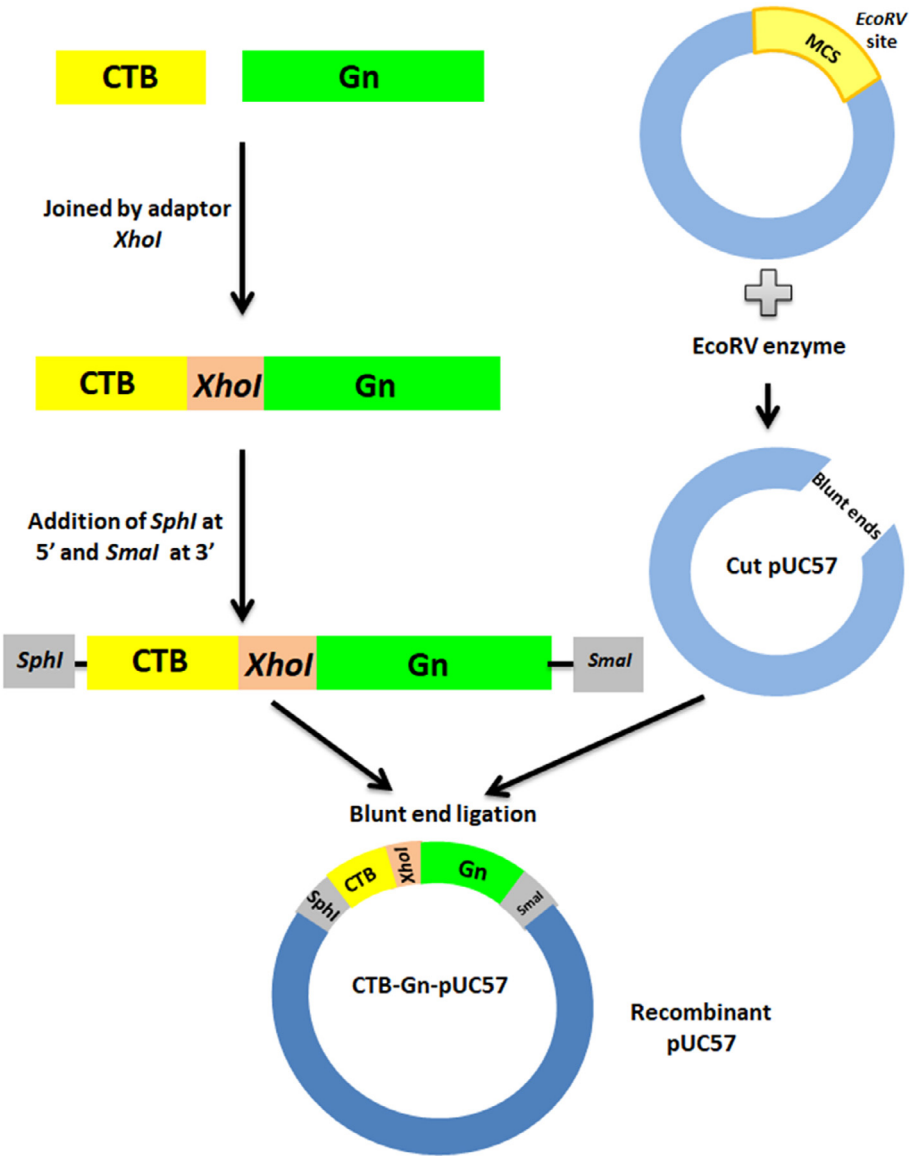

Fig. 1. Schematic representation showing cloning of CTB and Gn into pUC57.

binant protein expression was started by inducing bacterial cells with isopropyl β-d-1-thiogalactopyranoside (IPTG, Promega) at 1 mM final concentration. Throughout 5 h, 1-mL sample volume from each culture was collected hourly; cells were pelleted, lysed in 150 μL of 1× SDS-PAGE sample buffer. All samples were kept at −30 °C till used for analysis using SDS-PAGE. All collected cell lysates were analyzed using 13% SDS-PAGE and stained using Coomassie Brilliant Blue dye according to Laemmli (1970) and Green and Sambrook (2012).

Large-scale culture (1 L) preparation and purification of proteins tagged with the 6xHis were done using the Ni-NTA affinity chromatography were done following to the protocols recommended by QIAexpressionest kit. Purified proteins were run in 12% SDS-PAGE using Perfect Protein™ as a Marker (10–225 kDa, BioRad). The purified materials were subjected to dialysis in phosphate buffered saline and concentration using Amicon Ultra-15 Centrifugal Filter Unit (Cut-off value 3 KDa, Amicon) at 4 °C. The concentration of protein in all dialysed samples was estimated using BioRad kit according to Bradford (1976) method.

3. Results

All tested genes showed that it has many restriction sites. These restriction sites were taken into consideration not to be included in the cloning and sub cloning. Consequently, the restriction sites *Sph*I (at 5′) and *Sma*I (at 3′) were added to the target construct (*CTB-Gn*) as they are not found at any of the tested genes.

The corresponding amino acid sequences of *CTB*, *Gn* and *CTB-Gn* gene showed many antigenic sites and epitopes. The GenBank submitted construct was given an accession Number PRJNA386723.

The insert (*CTB-Gn*) was cloned into the vector pUC57 (after cutting using the restriction enzyme EcoRV for getting a blunt-ended cut pUC57 vector). The cut vector and the insert (both blunt-ended) were joined to each other to get the recombinant pUC57 vector (pUC57- CTB-Gn). The success of the ligation reaction was indicated by the successful Top10 bacterial transformation. In addition, restriction digestion confirmed that the target sequence (CTB-Gn) was at the expected size (1752 bp, Fig. 2). DNA sequence analysis proved that the insert within the pUC57 was at the right position.

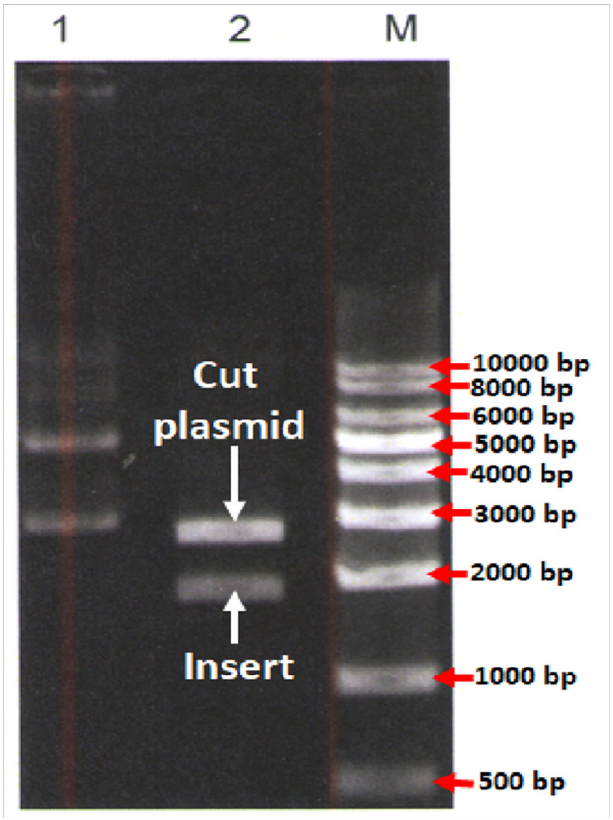

Fig. 2. Restriction enzyme digestion of recombinant pUC57 using SphI and SmaI enxymes. Lane 1: undigested recombinant pUC57; lane 2: digested recombinant pUC57 and lane M: KB ladder.

The linearized pQE-31 and insert were joined together to transform competent Top10 bacteria for the propagation of the recombinant vector. The resultant colonies were tested for the existence of *CTB-Gn* by restriction endonuclease enzymes digestion. Positive recombinant vectors (pQE31-insert) were used to transform competent M15 bacteria. Resulted colonies were checked for insert presence by PCR and its orientation by nucleotides sequence analysis. Results confirmed the presence of the insert in right orientation.

Time course for recombinant CTB-Gn (rCTB-Gn) protein expression revealed the presence of a characteristic protein band near the expected molecular weight (63.5 kd). The intensity of this band increased with time (Fig. 3). The most intense band was obtained at the 5th hour post induction. Consequently these conditions were used to prepare large-scale rCTB-Gn protein production.

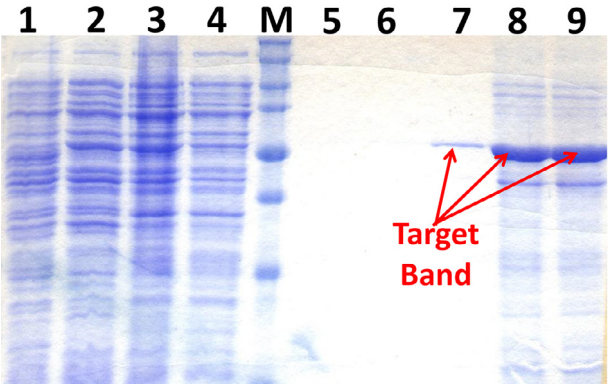

Fig. 3. CTB-Gn protein expression and purification. Lane 1: Uninduced culture, lane 2: induced culture, lane 3: crude lysate, lane 4: flow through, lane M: Perfect Protein™ Markers (10–225 kDa), lane 5–9: elution fractions.

4. Discussion

Infectious diseases have caused several problems at several levels causing a huge number of death cases. The transmission of diseases may be through animals or other routes. Vaccination is the most successful medical achievement ever in the history of human to prevent infection through animal contact or other routes (Andre, 2003). Till now, there are some obstacles facing both the methods of administration of vaccines and delivery. The essential problem that faces most of the vaccines is that the majority are injectable raising the danger of the reuses of their needles. Mucosal immune system-targeted vaccines overcome this problem (Sun et al., 2010). One of the practical and the inexpensive way is to transmit the vaccine using oral route to reach the lining of the gut (Streatfield, 2006). Regarding this approach of vaccine delivery, the present study was designed to produce a subunit candidate vaccine that can be formulated for the use either through oral or injection routes. Vaccines developed against RVFV can be categorized into conventional vaccines that are produced without using recombinant DNA technology approaches, and novel vaccines that include recombinant protein vaccines (Mackowiak et al., 1999).

Vaccines made through the production of recombinant protein technology depend on antigens that are generated by target genes cloning to generate large quantities of completely purified or semi-purified antigenic materials. In the case of RVFV, the target genes for the development of recombinant protein vaccine are Gn and Gc (the viral structural proteins) (Faburay et al., 2016; Kortekaas et al., 2012) and nucleocapsid protein (Jansen van Vuren et al., 2011; Kortekaas et al., 2012; Xu et al., 2013). In the current study we used *Gn* segment of the glycoprotein gene.

Many studies were done on ruminant models using recombinant subunit vaccine consisting of RVFV glycoproteins (Gn/Gc) produced using baculovirus system. Results of these studies showed a considerable neutralizing antibody reaction with conferring a full protection against virulent challenge (Faburay et al., 2016, 2014). The viral glycoproteins (Gn and Gc) production in glycosylated forms was shown to be more antigenic (Gavrilov et al., 2011). The host used in our study was *E. coli* that produces proteins without any form of glycosylation. To overcome the loss of glycosylation in our purified protein, CTB was added to enhance immunity against joined RVFV-Gn recombinant protein. In our study, CTB was ligated to *Gn* to benefit from its adjuvant properties to avoid the use of alum that is used in most vaccines. Aluminum compounds, as adjuvant, were shown to exhibit several drawbacks represented in the failure to induce good immune response against weak antigens, its carcinogenic effects and a bad responses in site of injection (Cameron et al., 1987). In Addition, it was previously reported that aluminum, as an immunostimulant, is weak to induce antibodies production and it causes shift in T-cell responses as it stimulates the response T_H2 instead of T_H1 (Wilde and Lumlertdacha, 2011).

We have chosen to the Gn protein and not nucleoprotein (N) because previous study indicated that in the time that the N is present in numerous amount with highly immunogenicity, partial immune protection was obtained when animals were immunized with nucleoprotein-based vaccines (Wallace et al., 2006). The same study showed that vaccination of the mice with the purified nucleoprotein did not raise specific antibodies and when challenged with RVFV only 60% survived, indicating the contribution of other factors.

In this study, a technical planning was done to set up the recombinant subunit vaccine. Cholera subunit B gene (375 bp) begins with the start codon (ATG) and ends with the stop codon (TAA) which are needed to be removed when cloning of CTB. The purpose of removing CTB start codon was to make the vector (pQE31) starts the expression using its own start codon to produce recombinant protein tagged with 6xHis-tag.

From the Genbank, the sequences of both *CTB* and *Gn* genes were retrieved and used for building up the recombinant CTB-Gn fusion protein. Before joining to each other, both genes were separately tested for internal restriction sites that may have to avoid using these sites in cloning into cloning and expression plasmids. *Gn* and *CTB* contained many restriction sites. The target to check types of restriction sites found plasmids and genes is to avoid using these restriction sites in cloning. The restriction site *Xho*I is not found in any used genes or plasmids.

The structure and 3D composition of the target fusion protein (CTB-Gn) was examined for the antigenic sites and epitopes. It was found that the restriction site *Xho*I (adaptor) connecting both CTB and Gn was positioned in a special way that kept the immunogenicity of CTB and Gn proteins, resulting in an effective recombinant protein (CTB-Gn).

When ligating the *CTB-Gn* into the cloning plasmid (pUC57), the EcoRV site needed to be cut to create blunt ends. *CTB-Gn* was ligated to the cut pUC57, so two possibilities are expected regarding the orientation of the insert; right or opposite direction. Regarding these mentioned possibilities, the orientation of ligated insert had to be checked out by analysis of nucleotide sequence. One of the good advantages of using blunt-ended DNA segments in cloning is the no need of adding specific restriction sites to the target DNA making this approach extremely versatile because it does not need any additional unwanted terminal DNA sequences making it ultimately easier and faster.

Following the cloning of insert (CTB-Gn) into cloning plasmid (pUC57), it was mandatory to analyze the nucleotide sequence choose colonies that harbor the right directed insert joined to the plasmid.

After successful transformation, the cloning plasmid (pUC57) which may contain the insert was digested with *Sph*I and *Bam*HI restriction enzymes to get pure *CTB-Gn* with unique restriction sites on both ends. Many researchers used the same view for getting pure insert and pure cut plasmid (Ibrahim et al., 2018; Selleck and Tan, 2008). In the present work, the insert was gel-purified to get pure DNA segment without digestion reaction constituents.

The expression vector (pQE-31) was cut with the enzymes *Sph*I and *Bam*HI which led to the creation of identical sticky ends the same as in the digested insert. This approach enabled the direct ligation of the sticky-ended insert to the sticky-ended pQE-31 leading to right oriented ligation (Bakhshi et al., 2013). To propagate the ligation reaction (insert + pQE-31), it was used to transform DH5-α bacteria. DH5-α bacteria were transformed with ligation reaction first and we did not use M15 expression bacteria directly because cloning bacteria focus on synthesizing multiple copies of the strange plasmid rather than concentrating on protein expression for cloned insert as expression bacteria do.

After the transformation of the DH5-α bacteria with the pQE31-CTB-Gn, the resulted colonies were examined for the harboring of the insert. Harboring of the target gene (*CTB-Gn*) was checked by restriction endonuclease digestion, PCR and the analysis of DNA sequence. The analysis of nucleotide sequence is very important at this point to check the orientation of insert within the expression vector and to check for insert open reading frame (Areas et al., 2002). Results confirmed that *CTB-Gn* was at right orientation and no shift in the open reading frame. Testing of the open reading frame is important to avoid any construct with shifted open reading frame which could result during the processes of cloning and subsequent sub cloning steps.

For recombinant protein expression the bacteria M15 were transformed with recombinant expression vector. M15 bacterium is supplied containing the plasmid pREP4 which is important for helping pQE vectors in protein expression and making the bacteria resistance to the antibiotic kanamycin. If not induced, M15 bacteria produce the least amount of recombinant protein, but upon induction with IPTG, it starts production of large amount of target protein (Dvorak et al., 2015).

To optimize the best protein synthesis conditions, time course strategy was used. The optimal time for protein production was monitored by checking bacteria lysates for specific band using SDS-PAGE. It was found that the best time for protein production is 4 h post IPTG which lead to use this condition in large-scale protein production.

Vaccines produced by recombinant proteins approaches have great advantages regarding the safety and the cost sides if compared to traditional vaccines. But the only drawback of this type of vaccine that they have weak immunogenicity resulting in the need for an adjuvant for helping them to provide a longer protection. In many functional and important vaccines like those protecting against the viruses like hepatitis B and human polio virus, an adjuvant, like aluminum salt, should be used to result in an effective immune response, the side effects of this adjuvant is unknown till this day. In this current study, we used an alternative approach regarding adjuvant where a naturally occurring protein adjuvant (CTB) replaced the chemical one.

Finally, the trend to prevent infectious diseases still a challenge till this century, licensed RVFV vaccine for human and animals still not found.

This study hoped to develop an advanced subunit vaccine alternative to overcome the problems of the currently used vaccines, lessening of the side effects, decrease production costs, making it safer, more immunogenic adjuvant.

5. Conclusions

Linking of DNA sequences encoding for RVFV Gn structural glycoprotein to the highly non-toxic immunogenic cholera toxin subunit B gene in one reading frame was done. Production of rCTB-Gn double protein vaccine in a single expression system will afford a vaccine which is save and has economic feedback. The produced protein can be used to vaccinate targets and to prepare hyper immune anti-RVFV sera.

Acknowledgement

This research was supported by the Research Deanship, King Khalid University, Project No. KKU-SCI-173.

Conflict of interest

The authors disclose that there are no any commercial associations that might create a conflict of interest in connection with submitted manuscripts.

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