

Expression and characterization of codon-optimized Crimean-Congo hemorrhagic fever virus Gn glycoprotein in insect cells

Mehdi Rahpeyma^{1,2} · Alireza Samarbaf-Zadeh¹ · Manoochehr Makvandi³ · Ata A. Ghadiri⁴ · Stuart D. Dowall⁵ · Fatemeh Fotouhi⁶

Received: 19 August 2016 / Accepted: 22 February 2017 / Published online: 18 March 2017
© Springer-Verlag Wien 2017

Abstract Crimean-Congo hemorrhagic fever virus (CCHFV) is a major cause of tick-borne viral hemorrhagic disease in the world. Despite of its importance as a deadly pathogen, there is currently no licensed vaccine against CCHF disease. The attachment glycoprotein of CCHFV (Gn) is a potentially important target for protective antiviral immune responses. To characterize the expression of recombinant CCHFV Gn in an insect-cell-based system, we developed a gene expression system expressing the full-length coding sequence under a polyhedron promoter in Sf9 cells using recombinant baculovirus. Recombinant Gn was purified by affinity chromatography, and the immunoreactivity of the protein was evaluated using sera from patients with confirmed CCHF infection. Codon-optimized Gn was successfully expressed, and the product had the expected molecular weight for CCHFV Gn glycoprotein of 37 kDa. In time course studies, the optimum

expression of Gn occurred between 36 and 48 hours postinfection. The immunoreactivity of the recombinant protein in Western blot assay against human sera was positive and was similar to the results obtained with the anti-V5 tag antibody. Additionally, mice were subjected to subcutaneous injection with recombinant Gn, and the cellular and humoral immune response was monitored. The results showed that recombinant Gn protein was highly immunogenic and could elicit high titers of antigen-specific antibodies. Induction of the inflammatory cytokine interferon-gamma and the regulatory cytokine IL-10 was also detected. In conclusion, a recombinant baculovirus harboring CCHFV Gn was constructed and expressed in Sf9 host cells for the first time, and it was demonstrated that this approach is a suitable expression system for producing immunogenic CCHFV Gn protein without any biosafety concerns.

✉ Fatemeh Fotouhi
fotouhi@pasteur.ac.ir

¹ Infectious and Tropical Diseases Research Center, Health Research Institute, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

² Department of Virology, WHO Collaborating Center for Reference and Research on Rabies, Pasteur Institute of Iran, Pasteur Institute, Tehran, Iran

³ Department of Virology, Faculty of Medicine, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

⁴ Cellular and Molecular Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran

⁵ National Infection Service, Public Health England, Porton Down, Wiltshire, UK

⁶ Department of Influenza and Other Respiratory Viruses, Pasteur Institute of Iran, Pasteur Institute, Tehran, Iran

Introduction

Crimean-Congo hemorrhagic fever (CCHF), caused by Crimean-Congo hemorrhagic fever virus (CCHFV), represents a potential threat to global health. The virus belongs to the genus *Nairovirus* of the family *Bunyaviridae* and causes severe hemorrhagic fever in humans, with a mortality rate of 30% [1, 2]. The natural infection cycle of CCHFV has been linked to ticks of the genus *Hyalomma* [3]. Due to the climate requirements of the tick host, the potential risk spread of CCHFV to temperate regions is increased by global climate change [4].

CCHF has been reported in more than 30 countries around the world [5]. Evidence of CCHF seropositivity in the human population of Iran was first reported in 1970, and CCHFV was first isolated from a tick survey in Iran in

1978 [6]. CCHF is considered a health problem by Iranian health authorities, with many people becoming infected by tick bite and several losing their life each year to the resultant disease. Although CCHFV cases have been reported from most provinces of the country, the disease is endemic in the South-East region of Iran, and most positive cases are reported from Sistan-Baluchestan province. An outbreak in 1999 in Iran has led to official surveillance of the disease by health authorities [7–11].

Currently, there is no widely approved vaccine against CCHF disease. A formalin-inactivated vaccine obtained from CCHFV-infected mouse brain homogenate was introduced during the 1960s in Bulgaria [12], but its efficacy remains unclear, and it has not been widely used. Due to the nature of the virus preparation used in the Bulgarian vaccine, it is unlikely to gain international regulatory approval. The development of an effective and safe recombinant vaccine requires the identification and expression of immunoprotective viral proteins [13].

Like all members of the family *Bunyaviridae*, the CCHFV genome comprises three single negative-stranded RNA segments: large (L), medium (M), and small (S), encoding the RNA-dependent RNA polymerase, structural glycoproteins and nucleoprotein, respectively [14, 15]. The structural glycoproteins Gn and Gc are expressed as a polyprotein precursor that is processed by cellular proteases during maturation [16]. The glycoprotein subunits Gn and Gc, form spikes on the virus particle and are involved in cell attachment and fusion. Both Gn and Gc are type I integral transmembrane (TM) proteins and are modified by glycosylation [8, 17, 18]. There are 78 to 80 cysteine residues in the CCHFV glycoproteins, which form a large number of disulfide bonds and a complex secondary structure. It has been shown that the structural glycoproteins of CCHFV elicit production of virus-neutralizing antibodies that are important for protective immunity [14].

The glycoproteins of viruses belonging to other genera of the family *Bunyaviridae* have been cloned and studied by several investigators using the baculovirus expression system, and the results have shown that the resultant recombinant proteins are useful in diagnostic or therapeutic applications [19–21]. Baculoviruses are enveloped viruses with rod-shaped nucleocapsids in which their double-stranded circular DNA genome of 88–135 kbp is encapsidated. Baculoviruses are widely used for the production of high levels of recombinant proteins and have the advantages that they are safe to use, allow correct posttranslational modification of proteins, and permit high expression levels of recombinant genes [22–25].

To date, there have been no reports of codon optimization and expression of recombinant CCHFV Gn protein in the baculovirus expression system. We report for the first time the production and purification of recombinant

CCHFV Gn antigen by using a baculovirus expression system and investigate the immunogenic and antigenic properties of the protein.

Material and methods

Vector construction

The recombinant plasmid containing the CCHFV Gn construct was constructed as described previously [26]. Briefly, to improve the expression of recombinant CCHFV Gn in an insect cell line, the gene construct was codon-optimized according to the specific codon usage of a cell line, derived from *Spodoptera frugiperda* (Sf9). The sequences of the native CCHFV Gn gene and the codon-optimized gene are shown in Fig. 1. The plasmid used was pFastBac HT B (Life Technologies), and the gene sequence encoding the CCHFV Gn_{519-803aa} Iran isolate (GenBank accession number DQ446216.1) was used to express the Gn protein (Fig. 2). This gene segment is flanked by the conserved cleavage sites RRLI and RKLI. Cleavage sites for BamHI and XhoI were designed at the 5' and 3' termini, respectively, of the Gn construct. A V5-tag sequence (GKIPNPLGLDST) was added at the 3' end of the construct to aid in detection of protein expression. As expression from pFastBac HT B generated a recombinant protein with a N-terminal histidine tag (HHHHHH), the recombinant protein was expressed as a His-tag-fused protein, enabling purification of recombinant Gn protein by affinity chromatography in the complete sequence of the construct (Gn sequence + all sequences that were added at the 5' and 3' ends of Gn), including 6 nt for the 5' cloning site (BamHI), and 3 nt for the initiation codon, the Gn sequence (852 nt), 42 nt for the V5 sequence, 3 nt for the stop codon, and 6 nt for the 3' cloning site (XhoI), was submitted to GenBank under accession no. KR921975.

Construction of recombinant bacmid

To generate a recombinant baculovirus expressing CCHFV Gn, the sequence encoding Gn was excised from a construct in which it had been cloned in the constructed vector pGEM-T Easy Vector (Bioneer, Korea) and then cloned into the pFastBac HT B vector (Life Technologies) using the BamHI and XhoI sites. Successful cloning was verified by sequencing. DH10Bac cells were transformed with recombinant pFastBac HT B-Gn by the CaCl₂ method [27], and the generation of a recombinant bacmid harboring the Gn gene was verified by PCR with M13 forward (5'-GTTTTCCAGTCACGAC-3') and reverse (5'-CAGGAAACAGCTATGAC-3') primers according to the kit protocol (Life Technologies). The high-molecular-weight

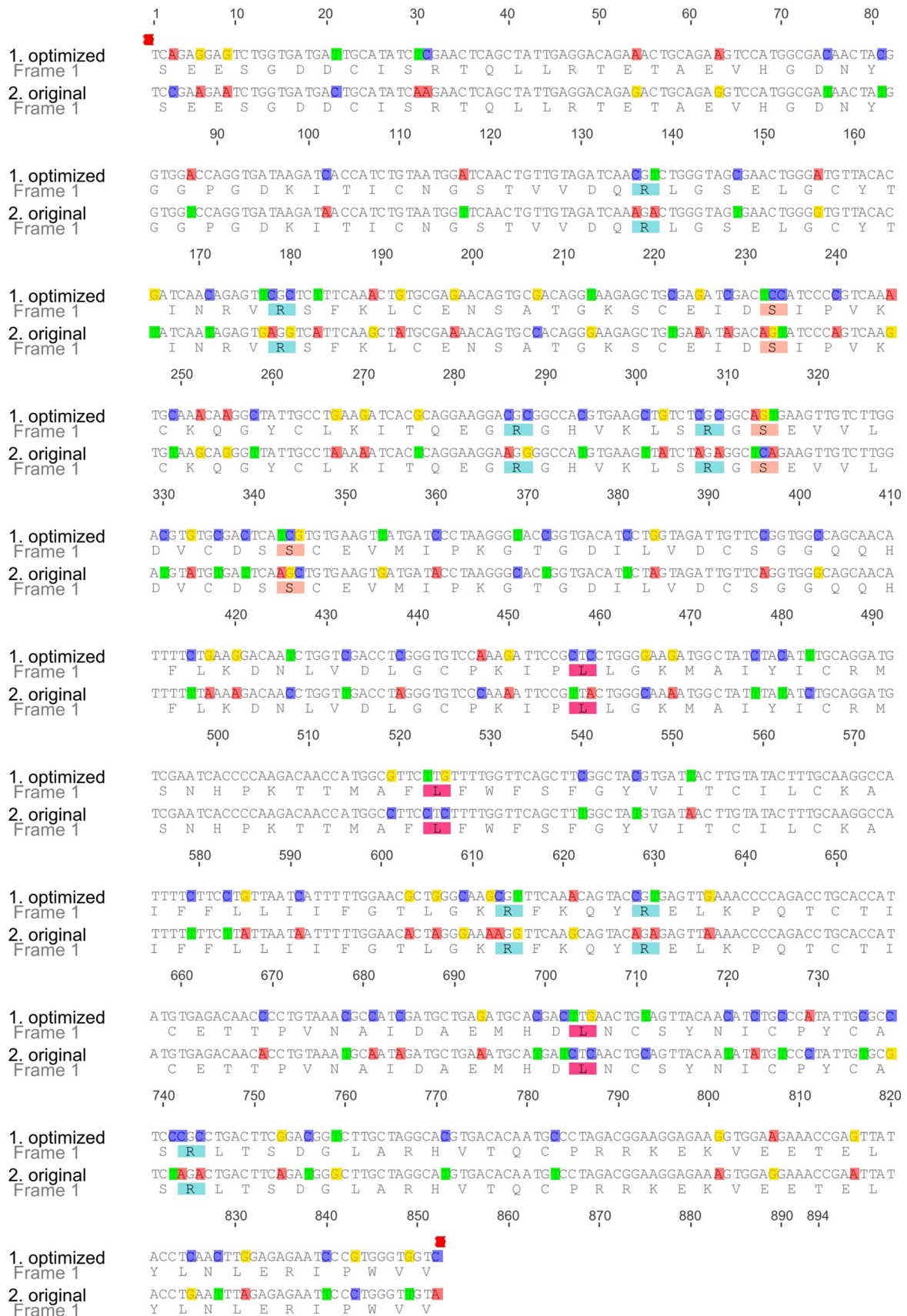


Fig. 1 Alignment illustrating the sequences of the native and codon-optimized coding sequences of the CCHFV Gn gene. The optimized codons are marked with a colored box. Yellow is for guanine (G), green is for thymine (T), blue is for cytosine (C) and red is for adenine (A). The amino acids with two- or three-nt codon optimization are indicated by a large colored box. R is for arginine, S is for serine and L is for leucine (color figure online)

bacmid DNA was isolated according to the instructions supplied by the manufacturer (Life Technologies).

Generation of recombinant baculovirus and expression of CCHFV Gn protein

Sf9 insect cells (purchased from the Cell Bank, Pasteur Institute of Iran) were cultured in Grace Medium (Life Technologies) supplemented with 10% fetal bovine serum (FBS) and 100 U of penicillin, 100 µg of streptomycin, and 1 µg of fungizone (Life Technologies) per ml. The cells were transfected with purified recombinant bacmid using Cellfectin II Reagent (Life Technologies/Invitrogen) according to the manufacturer's instructions. Passage 4 (P4) recombinant baculovirus expressing CCHFV Gn was used to infect Sf9 cells at a multiplicity of infection (MOI) of 10. The virus titer was determined by endpoint dilution assay, and the MOI was calculated based on the number of cells when seeded. Infected cells were incubated at 27 °C for 36 h and then harvested by centrifugation, washed with phosphate-buffered saline (PBS), and pelleted by centrifugation. To visualize expression of recombinant proteins, standard protocols were followed. Briefly, Sf9 cells infected with baculovirus-CCHFV Gn were lysed by three freeze-thawing cycles and then boiled for 5 min in sample buffer (62.5 mM Tris-HCl, pH 6.8, 1% SDS, 10% glycerol,

and 5% β-mercaptoethanol). Twenty µl of the resulting extract was separated on a 12% SDS-polyacrylamide gel, which was subsequently subjected to either Coomassie staining or Western blotting. For Western blotting, after nitrocellulose membrane electro-transfer, the membrane was blocked and proteins were detected using a monoclonal antibody recognizing the V5 tag (Life Technologies), followed by an anti-mouse antibody conjugated with horseradish peroxidase (HRP) (Sigma). Antigen-antibody complexes were visualized using DAB-chromogenic substrate (3,3-diaminobenzidine tetra hydrochloride).

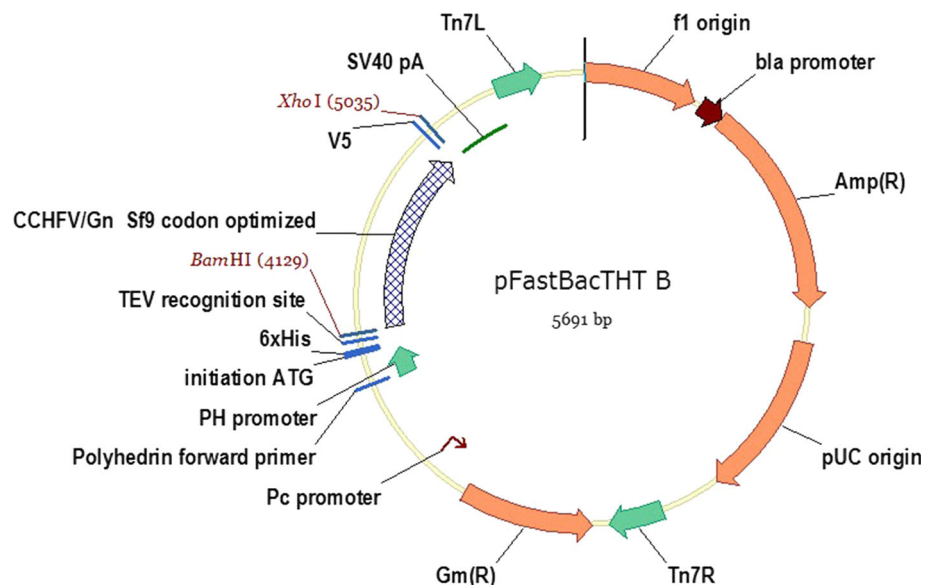
Time course study of Gn expression

To determine the optimal time point for protein expression, Sf9 cells in a 6-well plate were simultaneously infected with the recombinant baculovirus at an MOI of 10. Plates were incubated at 27 °C. At 24 hours postinfection and at subsequent 12-h intervals up until 84 h, the infected Sf9 cells were harvested. An equal amount of sample was used for 12% SDS-PAGE analysis to assess the level of Gn expression.

Purification of recombinant CCHFV Gn

All extraction and purification procedures were carried out at 4°C. The purification of the recombinant CCHFV Gn protein was based on the His-tag protocol under native conditions using commercial reagents (QIAGEN, Catalog no. 30210). Harvested cells were washed three times with cold PBS, resuspended in cold PBS, and lysed by three freeze-thaw cycles in liquid nitrogen. The lysate was centrifuged at 12000×g for 20 min at 4 °C to pellet the cell debris. Four ml of supernatant was mixed with 200 µl of

Fig. 2 Diagram of the recombinant transfer vector pFastBac HT B-Gn. Coding sequence of CCHFV Gn519-803 between the conserved cleavage sites RRLL and RKLL was selected, and the codons were optimized for expression in Sf9 cells (GenBank accession number KR921975). Two restriction sites (BamHI and XhoI) were inserted at the 5' and 3' ends of the construct, as indicated in red



Ni-NTA resin, incubated at 4 °C for 2 hours with continual shaking, poured into a QIAGEN column, and left to settle under gravitational force. The Ni-NTA resin was washed with 800 µl of washing buffer (50 mM NaH₂PO₄, 300 mM NaCl, and 20 mM imidazole, pH 8). Bound protein was eluted with 100 µl of elution buffer (50 mM NaH₂PO₄, 300 mM NaCl and 250 mM imidazole, pH 8). The eluate was dialyzed against PBS (pH 7.4). The purified protein was analyzed by SDS -PAGE and Western blot using an anti-His antibody (Sigma) to identify the recombinant protein.

Immunization

Female BALB/c mice (6 per group; aged 5-6 weeks) were injected subcutaneously with Sf9 cell lysate containing recombinant Gn protein. The lysates were prepared from Sf9 cells infected with recombinant baculovirus by three rounds of freeze-thawing followed by centrifugation at 200×*g* for 15 min. Mice were divided into three groups and immunized as follows: group 1, 50 µl of the clarified extract containing recombinant Gn protein mixed with an equal volume of complete Freund's adjuvant; group 2, 50 µl of clarified extract of uninfected Sf9 cells with an equal volume of adjuvant as a mock control; group 3, 100 µl of PBS as a negative control. Mice were boosted twice at 2-week intervals with the same preparation except that incomplete Freund's adjuvant was used for the second immunization. All animals were maintained under standard animal housing conditions with access to food and water at all times. Animals were bled 10 days after last immunization, and sera were prepared by centrifugation at 3000×*g* for 10 minutes and stored at -20°C for further analysis. At necropsy, samples of spleen and lymph nodes were collected for cytokine analysis.

Indirect enzyme-linked immunosorbent assay with mouse sera

Each well of a 96-well plate (Greiner) was coated overnight at 4°C with purified recombinant CCHFV Gn protein diluted in NaHCO₃ coating buffer (pH 9.4). Recombinant influenza virus HA antigen that was produced in a recombinant baculovirus–insect cell system [28] and uninfected Sf9 cells (mock) were used as negative controls. Plates were blocked for 15 min at room temperature (RT) with PBS containing 0.05% Tween-20 and 3% bovine serum albumin (BSA). After washing three times with 0.05% Tween-20 in PBS, 100 µl of mouse serum, diluted 1:200 in dilution solution (PBS containing 0.05% Tween and 1% BSA), was added and incubated at RT for 1 h. All subsequent washing steps were carried out three times as indicated above. After washing, plates were incubated with anti-mouse IgG-HRP-conjugated antibody (Sigma), diluted

1:5000 in dilution solution at RT for 1 h. After washing, 100 µl of substrate buffer containing 0.1 mg of 3,3',5,5'-tetramethylbenzidine (TMB) (Sigma) per ml was added, and the plates were incubated in the dark for 25 min. The reaction was stopped by the addition of 2 M H₂SO₄ and the optical density (OD) was measured at a wavelength of 450 nm using a microplate reader (Anthos 2020, Anthos Labtech, Austria).

Cytokine assay

Spleens from immunized mice were disaggregated into pieces by mechanical disruption. Red blood cells in the spleen preparations were removed by treatment with lysis buffer (0.15 M NH₄Cl, 1 mM KHCO₃ and 0.1 mM Na₂EDTA, pH 7.2). After washing twice in RPMI 1640 medium (Sigma) containing 5% FCS, cells were resuspended in RPMI 1640 medium enriched with 2 mM L-glutamine, 100 U of penicillin per ml, 100 µg of streptomycin per ml and 10% FCS. For proliferation assays 2 × 10⁵ cells/well were cultured in 96-well plates in the presence of 10 µg of CCHFV Gn protein, 10 µg of Sf9-Mock, or 5 µg of concanavalin A per ml. Cells were maintained at 37 °C in 5% CO₂. Supernatants were collected at 72 h post-stimulation. Secretion of IFN-γ and IL-10 were assessed using an ELISA kit (eBioscience, Inc, USA) according to the manufacturer's instructions. All tests were performed in triplicate.

Immunoassays with human sera

To date, there are no reports on the immunoreactivity of baculovirus -expressed recombinant CCHFV Gn protein with human sera from naturally infected CCHFV patients. Five human sera that were previously confirmed to be CCHFV IgG positive by the Iranian Centre for Disease Control (CDC) were processed for Western blot analysis. The Sf9 cells expressing CCHFV Gn were lysed in sample buffer, separated by SDS-PAGE, and transferred electrophoretically to nitrocellulose membranes, which were blocked with 5% BSA dissolved in 10 mM Tris-HCl (pH 7.2), 150 mM NaCl, and 0.05% Tween-20 for 1 h at RT. Human sera were added as primary antibody (diluted 1/100 in blocking solution) and incubated for 2 hours at RT. After washing three times with PBS containing 0.05% Tween-20 for up to 10 min, the membranes were incubated with alkaline-phosphatase-conjugated mouse anti-human IgG (Sigma) for 1 h at room temperature (diluted 1/8000 in blocking solution). The membrane was washed thoroughly as described previously, and antigen-antibody complexes were visualized with DAB chromogenic substrate (3,3-diaminobenzidine tetrahydrochloride). Healthy human sera from individuals with no history of CCHF disease and a

mouse anti-V5 antibody (Life Technologies) were used as negative and positive controls, respectively.

Statistical analysis

The mean values of mouse antibody levels and cellular response (secretion of interferon gamma and IL-10) were compared using two-way analysis of variance (ANOVA) and Tukey's post-hoc tests using SPSS statistical software (version 16, Scientific Package for Social Sciences, Chicago, IL, USA). A *P*-value less than 0.05 was considered significant.

Results

Generation and expression of recombinant baculovirus from CCHFV Gn

The sequences of the original and codon-optimized CCHFV Gn genes were aligned (Fig. 1). A recombinant plasmid harboring the CCHFV envelope Gn protein was constructed and named pFastBac HT B-Gn (Fig. 2). The correctness of pFastBac HT B-Gn was confirmed by DNA sequencing. DH10Bac cells were transformed, and colonies harboring the recombinant bacmid were selected by blue and white screening. The isolated recombinant bacmid DNA was introduced into cells by transfection using Cellfectin. After the infected cells stopped dividing and showed enlargement with pathological changes, the supernatant of Sf9 cells was collected and named P1. Virus amplification was repeated in fresh Sf9 cells to obtain high-titer baculovirus stocks.

To examine the expression of the CCHFV envelope Gn protein in Sf9 cells, the cells were infected with recombinant baculoviruses. Analysis by SDS-PAGE using a 12% polyacrylamide gel and Coomassie blue staining revealed a recombinant protein with the expected apparent molecular weight of 37-kDa (Fig. 3A) [27]. Western blot analysis with monoclonal anti-V5 antibody demonstrated binding to the 37 kDa recombinant protein (Fig. 3B). No expression of any immunoreactivity protein was detected in mock preparations.

In this experiment, SDS-PAGE and Western blot analysis showed that expression of CCHFV Gn protein could be detected as soon as 24 h postinfection. The peak level of expression of Gn protein was determined at 36–48 h postinfection based on the intensity of the protein band on an SDS-PAGE gel. By 72 h postinfection, no visible bands were observed (Fig. 4).

The recombinant CCHFV Gn protein was purified by affinity chromatography using Ni-NTA resin. The retained recombinant Gn was eluted from the resin with 250 mM

imidazole (Fig. 5A) and confirmed to be recombinant CCHFV Gn protein by Western blot (Fig. 5B). The concentration of purified protein was determined using a NanoDrop spectrophotometer that was set to measure the absorbance at 280 nm.

Immunogenicity of recombinant CCHFV Gn protein in mice

There have been no studies of the immune response to baculovirus-expressed recombinant CCHFV Gn protein in mice. In order to examine the immunogenicity of the recombinant CCHFV Gn obtained from the insect cells in mice, we immunized BALB/c mice with rGn baculovirus-infected Sf9 cell lysates containing recombinant CCHFV Gn protein, with uninfected Sf9 cells, or with PBS. Serological analysis showed that none of the mock- or PBS-immunized mice contained antibodies against CCHFV Gn, as determined by ELISA (Fig. 6). In contrast, the results show that recombinant CCHFV Gn protein with Freund's adjuvant elicited a strong immune response, as demonstrated by the production of antigen-specific antibodies ($p < 0.001$).

We proceeded to explore whether the *in vitro*-generated CCHFV Gn could serve as an immunogen *in vivo*. Splenocytes from all groups of immunized mice were assayed for their IFN-gamma and IL-10 cytokines levels in the supernatants after antigenic stimulation (Fig. 7). The mitogen concanavalin A was used as positive control. Mice immunized with recombinant CCHFV Gn protein demonstrated higher levels of IFN-gamma and IL-10 when compared with those that received the mock preparation or PBS, and this difference statistically was significant ($p < 0.001$).

Western blot analysis with human serum

Finally, we examined whether the *in vitro*-generated CCHFV Gn could react with human sera from naturally infected CCHFV patients. The results are shown in Fig. 8. All five patient sera reacted with the recombinant CCHFV Gn protein in Western blot analysis, as seen by detection of a protein with the expected size of 37 kDa (Fig. 8), while no band was observed in uninfected Sf9 cells with CCHFV patient sera.

Discussion

CCHF is the most prevalent tick-borne disease in the world [29]. Despite serological evidence of infection in Iran reported in 1975, CCHFV has recently received much more attention as a potential threat to public health by health

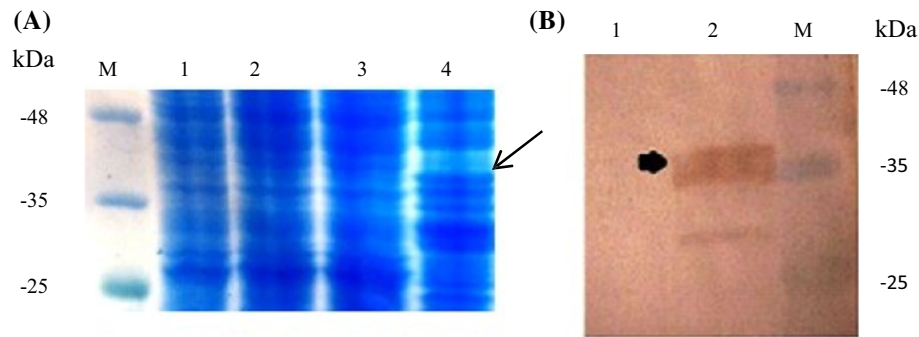


Fig. 3 SDS-PAGE and Western blot analysis of CCHFV Gn expression using recombinant baculovirus in Sf9 cells. (A) SDS-PAGE analysis of Sf9 cell lysates. Infected or uninfected Sf9 cells were cultured and analyzed using a 12% polyacrylamide gel. M, protein marker; lanes 1-3, Sf9-Mock; lane 4, Sf9-Gn. (B) Western

blot analysis of Sf9 cell lysates. Infected or uninfected Sf9 cells were cultured, homogenized, and analyzed by immunoblotting using anti-V5 tag mAb. Lane 1, uninfected Sf9 cell lysates (Sf9-Mock); lane 2, baculovirus-infected Sf9 cell lysates (Sf9-Gn); M, protein marker. The arrows indicate the location of the Gn protein

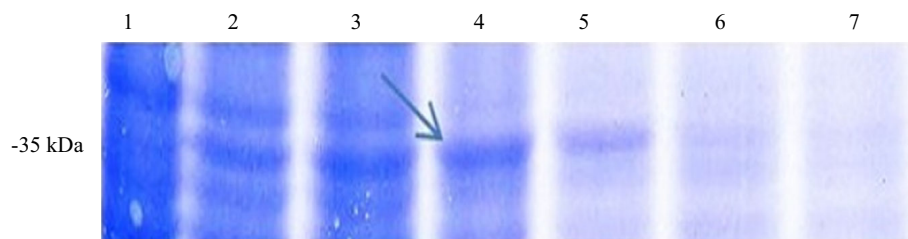


Fig. 4 Time course analysis of recombinant CCHFV Gn expression. Sf9 cells were infected with recombinant baculovirus expressing codon-optimized CCHFV Gn at an MOI of 10 and incubated at 27°C for 24-84 h. Equal volumes of culture supernatant were used for

analysis by 12% SDS-PAGE. Lane 1, uninfected Sf9 cells (Sf9-Mock); lanes 2-7, baculovirus-infected Sf9 cell lysates (Sf9-Gn) at 24, 36, 48, 60, 74 and 84 h postinfection, respectively

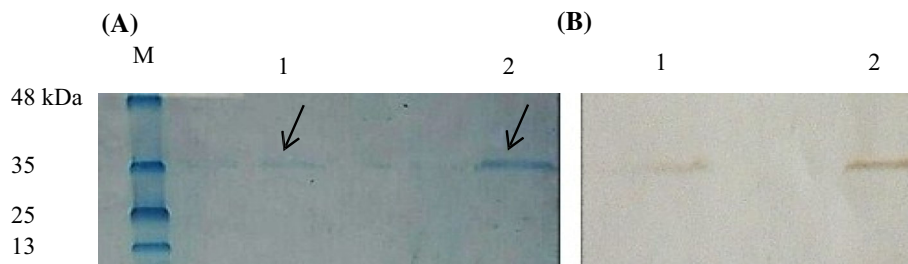


Fig. 5 Purification of histidine-tagged recombinant CCHF Gn protein (A) SDS-PAGE of purified CCHFV Gn protein by affinity chromatography. Lanes 1 and 2, fractions of purified CCHFV Gn

protein eluted with 250 mM imidazole; M, protein marker. (B) Western blot analysis of the purified recombinant protein using anti-His antibody

authorities since an outbreak in 1999 [11, 30]. The risk of CCHFV to the community is highlighted by a report of a nosocomial outbreak in Mashhad in northwestern Iran, which was found to be associated with inadequate self-care procedures of a physician during the handling of CCHF patients [31].

The development of a safe recombinant vaccine against CCHF disease in endemic regions is urgently needed. Despite its potential threat to public health, unfortunately, there is no licensed vaccine or specific treatment available against CCHFV. Because of its high level of pathogenicity and infectivity and the lack of a vaccine, working with

CCHFV requires BSL-4 laboratories, which are restricted to a few specific centers, making it difficult for antigen production using live-virus systems [32–35]. Fortunately, these challenges can be overcome by recent progress in bioinformatics tools and DNA cloning technology, which have opened new strategies and facilitated the production of recombinant antigens from highly pathogenic agents such as CCHFV [36, 37].

CCHFV encodes two structural surface glycoproteins, Gn and Gc, which are involved in the initiation of the virus life cycle and also in eliciting host immune system responses and neutralizing antibodies. Humoral immune

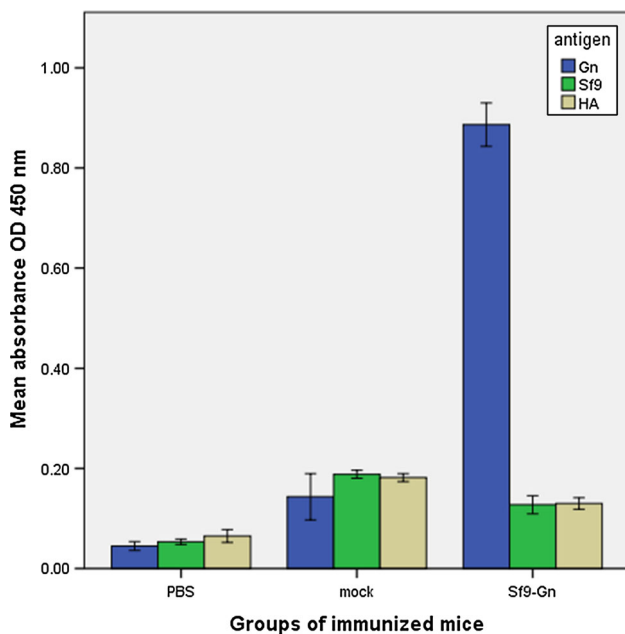


Fig. 6 Reactivity of mouse sera to recombinant CCHFV Gn protein antigen by indirect ELISA post-immunization. Female BALB/c mice were immunized subcutaneously three times at two-week intervals with Sf9-Gn/adjuvant. Control groups consisted of mock/adjuvant and PBS. Ten days after the last immunization, mice were bled, and immune responses to the recombinant CCHFV Gn protein were measured by ELISA. Data are expressed as the mean of at least three independent experiments. Sf9- Mock, uninfected Sf9 cells; Sf9-Gn, baculovirus-infected Sf9 cell lysates; Sf9-HA, baculovirus-infected Sf9 cell lysates. The significance of differences was determined using an ANOVA test. *, $p < 0.001$

responses have been proposed to be crucial for the recovery of the patients infected with CCHFV [38]. This conclusion is partially supported by the observation that passive transfer of human convalescent sera to acutely CCHFV-infected patients provided some protection. In a study conducted by Kubar *et al.*, administration of hyperimmune globulin to CCHFV patients resulted in recovery of 13 out of 15 patients (86.6%) [39]. On the other hand, it has been shown that in fatal cases of CCHF, no IgG response against CCHFV was produced [39–41]. In animal model studies, it has been shown that monoclonal antibodies (MAbs) against Gn were able to protect animals from lethal CCHFV infection [14]. Altogether, these results suggest that CCHFV glycoproteins may have potential clinical applications and could be used as vaccine candidates. Indeed, the only vaccine candidate for CCHFV based on a recombinant expression system, modified vaccinia Ankara (MVA), has demonstrated the ability to provide protection against infection when using the CCHFV glycoprotein as an antigen [42]. A similar system using nucleoprotein was not protective [43].

Eukaryotic glycoproteins expressed in prokaryotic systems are not suitable for immunoassays as a consequence

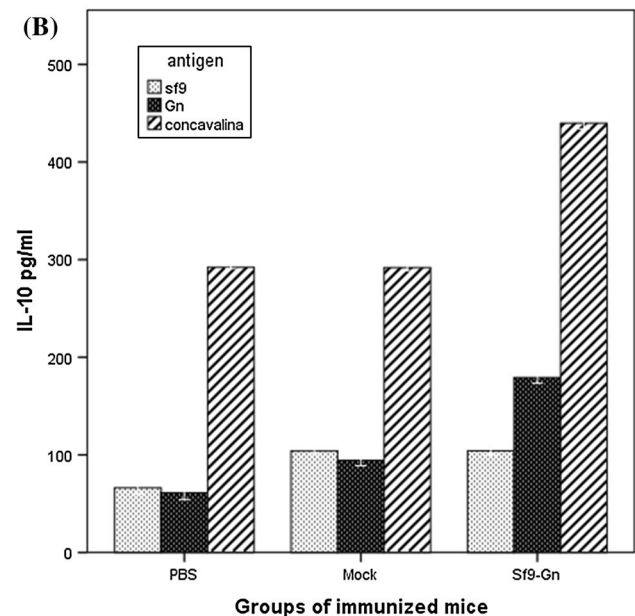
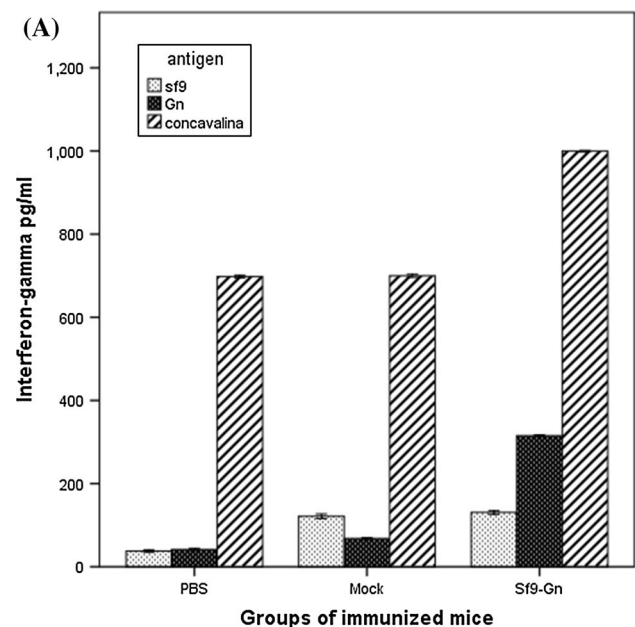


Fig. 7 *In vitro* production of IFN-gamma and IL-10 from splenocytes of immunized mice. 2×10^5 cells recovered from the splenocytes of three groups of immunized mice and single cell preparations were plated in triplicate in 96-well microtiter plates and stimulated *in vitro* with Gn (10 μ g/ml), Sf9-Mock (10 μ g/ml) or concanavalin A (5 μ g/ml) as a positive control. Seventy-two hours later, supernatants were harvested, and IFN-gamma and IL-10 were assayed by ELISA. Sf9-Mock, uninfected Sf9 cells; Sf9-Gn, baculovirus-infected Sf9 cell lysates. Data are expressed as the mean of at least three independent experiments. The significance of differences was determined using an ANOVA test. *, $p < 0.001$

of the inability of the bacteria to glycosylate and properly fold eukaryotic proteins. Yeast species such as *Saccharomyces cerevisiae* and *Pichia pastoris* can be used as alternative hosts for the expression of eukaryotic proteins.

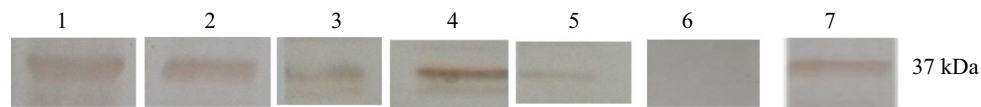


Fig. 8 Recognition of recombinant CCHFV Gn protein by human patient serum. Western blot analysis was carried out using serum samples from known CCHFV convalescent patients. Lanes 1-5, serum

samples from five known CCHFV patients; lane 6, serum sample negative for antibody against CCHFV; lane 7, antiV5- tag monoclonal antibody as a positive control

Yeasts are able to glycosylate the target proteins, but it has been shown that the resulting N- and O-linked oligosaccharide structures are significantly different from those of their mammalian counterparts [44]. Hypermannosylation (addition of a large number of mannose residues to the core oligosaccharide) is an additional disadvantage of using yeast because it hinders the proper folding and therefore the activity of the protein [45–48]. The baculovirus system is widely used for eukaryotic protein expression in insect cells as a compromise between bacterial and mammalian expression. The baculovirus expression system allows overproduction of antigens and also facilitates the formation of disulfide bonds, which are essential for the proper folding of the CCHFV Gn protein [49–52].

To our knowledge, there are no reports about non-optimized Gn expression in insect cells, but some studies have shown that high-level expression of recombinant proteins is affected by factors such as the different codon preferences in different expression systems, and codon optimization can significantly increase the expression level [53–56]. This is the first report on the cloning and expression of CCHFV Gn glycoprotein in a baculovirus expression system. This study was prompted by the consideration that baculovirus is a suitable expression system for glycoproteins. The results obtained in this study provide further evidence in support of using a baculovirus as a heterologous expression system.

In the first part of this work, we cloned CCHFV Gn into a baculovirus by using the pFastBac HT B vector. As observed by SDS-PAGE, the CCHFV Gn produced in Sf9 cells generated a protein with the expected molecular weight for the glycosylated form of Gn (37 kDa) as reported by Sanchez *et al.* [15]. To determine when the level of recombinant protein reaches maximum levels, a time course analysis of Gn expression was conducted. The results demonstrated that 36–48 hours postinfection is the best time for harvesting to obtain the highest amount of recombinant protein. Further delays in harvesting may result in a loss of recombinant antigen, which may be influenced by factors such as proteolysis. Since the polyhedrin promoter is a very late promoter [22] and expression from this promoter reaches high levels after 48–72 hours, it can be concluded that infection of Sf9 cells with recombinant baculovirus expressing CCHFV Gn results in cell death, but the reason for this is unknown.

Production and affinity purification of the recombinant protein resulted in a low yield of CCHFV Gn. This could be due, at least in part, to CCHFV glycoproteins having specific properties, including having undergone complex post-translational modifications [29]. On the other hand, the CCHFV Gn glycoprotein has several cysteine amino acids in its structure, suggesting the presence of complex internal disulfide bonds [14]. Another cause may be the possible instability of the recombinant protein, although further work would be required to assess protein stability.

Western blot analysis demonstrated that the baculovirus-produced CCHFV Gn glycoprotein was recognized by sera from CCHFV patients. This reactivity of CCHFV Gn by patient sera indicates that it could be used in diagnostic assays or in the production of monoclonal antibody applications. Previous studies using baculovirus expression systems for expression of proteins of other pathogens have shown that these proteins were expressed in the correct conformation and could be used as potential serodiagnostic targets for monitoring the host humoral immune response to infection and/or vaccination [24, 49, 57, 58]. Additionally, an antigen-specific response to the recombinant CCHFV Gn protein was detected in the sera of immunized mice after two boosts with the recombinant protein, demonstrating that it was capable of inducing a significant humoral response in mice.

Unfortunately, despite the significant medical impact of CCHF disease and its ability to cause nosocomial infection, the pathogenicity of CCHF is not very well understood. Although specific cytotoxic T-lymphocytes (CTLs) are critical in the recovery from infection and the clearance of viruses and the Th2 immune response against viral infection is also protective, this scenario in CCHF may be complicated. Clinical studies have shown that there are significant positive correlations between the viral load, IFN-gamma, and IL-10 cytokines. [59]. Moreover, studies on the effect of virus infection on cytokine release *in vitro* have demonstrated an increase in the release of tumor necrosis factor (TNF)- α , interleukin (IL)-6, and IL-10 in the supernatant of infected cells [59]. IFN-gamma is a pro-inflammatory cytokine that is critical for innate and adaptive immunity against viral infections and is produced by natural killer (NK) cells and activated memory CTL cells [60]. IL-10 is a regulatory cytokine that inhibits cellular immunity by downregulating the cytokine IL-12 [61]. In

CCHF disease, studies have shown that increased levels of IL-10 may result in increased viremia, as CCHF patients with fatal outcomes produce higher serum levels of IFN-gamma and IL-10 cytokines than patients who survive [59]. This may be due to the L protein of CCHFV containing an ovarian tumor domain (OTU), which acts in the evasion of innate immunity [8, 62]. The results of this study support these findings, as the cytokine assay indicated that the levels of Th1-type cytokines (IFN-gamma) and Th2-type cytokines (IL-10) produced in mice immunized with recombinant Gn were significantly higher than in the groups given Sf9 mock preparations or PBS. This demonstrates that the full-length Gn protein contains multiple epitopes, which in this study stimulated intensive cellular immunoreactivity. The high levels of IFN-gamma in mice administered Sf9-derived Gn protein could potentially be attributed to substantial activation of NK and memory CTL cells. Unfortunately, due to the lack of BSL-4, facilities virus challenge studies in immunized mice to investigate protective efficacy were not performed in the present study.

In conclusion, a CCHFV Gn recombinant baculovirus was successfully constructed and expressed in insect cells. The expressed Gn protein had a molecular weight that was in line with expectations. Compared to the control groups, CCHFV Gn was able to elicit a significant humoral and cellular immune response and, importantly, reacted with CCHFV patient sera. Together, this implies that baculovirus could be a favorable platform for CCHFV glycoprotein antigen expression. It offers the unique advantages of the baculovirus, especially the relative ease of manipulation, sufficient antigen expression, and safety.

Acknowledgements We thank Dr. Dorostkar for kindly providing human CCHF-positive sera. We are grateful to all co-workers who support us in conducting this project. The views expressed are those of the authors and do not necessarily reflect those of the employing institutes.

Compliance with ethical standards

Funding This study was funded by Health Research Institute, Infectious and Tropical Diseases Research Center, Ahvaz Jundishapur University of Medical Sciences, Ahvaz, Iran (PhD Grant No. 91119).

Conflict of interest The authors declare that they have no conflict of interest

Ethical approval Animal experiments described in this paper were conducted according to NIH guidelines for the care and use of laboratory animals and were approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (Approval No. 2015-4-13). All procedures performed in studies involving human samples were approved by the ethics committee of Ahvaz Jundishapur University of Medical Sciences (Approval No. 2015-4-13) and were in accordance with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards.

Informed consent Studies using human samples were retrospective, and therefore, informed consent from the patients for their use in this study was not obtained.

References

- Sanchez A, Vincent M, Erickson B, Nichol S (2006) Crimean-Congo hemorrhagic fever virus glycoprotein precursor is cleaved by Furin-like and SKI-1 proteases to generate a novel 38-kilodalton glycoprotein. *J Virol* 80:514–525
- Ergonul O, Tuncbilek S, Baykam N, Celikbas A, Dokuzoguz B (2006) Evaluation of Serum Levels of Interleukin (IL)-6, IL-10, and Tumor Necrosis Factor- α in Patients with Crimean-Congo Hemorrhagic Fever. *J Infect Dis* 193:941–944
- Dash A, Bhatia R, Sunyoto T, Mourya D (2013) Emerging and re-emerging arboviral diseases in Southeast Asia. *J Vector Borne Dis* 50:77–84
- Gray J, Dautel H, Estrada-Peña A, Kahl O, Lindgren E (2009) Effects of climate change on ticks and tick-borne diseases in Europe. *Interdiscip Persp Infect Dis*
- Atkinson B, Chamberlain J, Logue CH, Cook N, Bruce C et al (2012) Development of a real-time RT-PCR assay for the detection of Crimean-Congo hemorrhagic fever virus. *Vector-Borne Zoonotic Dis* 12:786–793
- Izadi S, Holakouie-Naieni K, Majdzadeh SR, Chinikar S, Nadim A et al (2006) Seroprevalence of Crimean-Congo hemorrhagic fever in Sistan-va-Baluchestan province of Iran. *Jpn J Infect Dis* 59:326
- Duh D, Nichol S, Khristova M, Saksida A, Hafner-Bratkovic I et al (2008) The complete genome sequence of a Crimean-Congo Hemorrhagic Fever virus isolated from an endemic region in Kosovo. *Virol J* 5:7
- Bergeron E, Vincent M, Nichol S (2007) Crimean Congo hemorrhagic fever virus glycoprotein processing by the endoprotease SKI-1/S1P is critical for virus infectivity. *J Virol*
- Sargianou M, Panos G, Tsatsaris A, Gogos C, Papa A (2013) Crimean-Congo hemorrhagic fever: seroprevalence and risk factors among humans in Achaia, western Greece. *Int J Infect Dis* 17:e1160–e1165
- Keshkar-Jahromi M, Sajadi MM, Ansari H, Mardani M, Holakouie-Naieni K (2013) Crimean-Congo hemorrhagic fever in Iran. *Antiviral Res* 100:20–28
- Sharifi-Mood B, Metanat M, Ghorbani-Vaghei A, Fayyaz-Jahani F, Akrami E (2009) The outcome of patients with Crimean-Congo hemorrhagic fever in Zahedan, southeast of Iran: a comparative study. *Arch Iran Med* 12:151–153
- Papa A, Mirazimi A, Koksai I, Estrada-Pena A, Feldmann H (2014) Recent advances in research on Crimean-Congo hemorrhagic fever. *J Clin Virol* 64:137–143
- Ahmed AA, McFalls JM, Hoffmann C, Filone CM, Stewart SM et al (2005) Presence of broadly reactive and group-specific neutralizing epitopes on newly described isolates of Crimean-Congo hemorrhagic fever virus. *J Gene Virol* 86:3327–3336
- Bertolotti-Ciarlet A, Smith J, Strecker K, Paragas J, Altamura LA et al (2005) Cellular localization and antigenic characterization of Crimean-Congo hemorrhagic fever virus glycoproteins. *J Virol* 79:6152–6161
- Sanchez AJ, Vincent MJ, Nichol ST (2002) Characterization of the glycoproteins of Crimean-Congo hemorrhagic fever virus. *J Virol* 76:7263–7275
- Altamura LA, Bertolotti-Ciarlet A, Teigler J, Paragas J, Schmaljohn CS et al (2007) Identification of a novel C-terminal cleavage of Crimean-Congo hemorrhagic fever virus PreGN that leads to generation of an NSM protein. *J Virol* 81:6632–6642

17. Cifuentes-Muñoz N, Salazar-Quiroz N, Tischler ND (2014) Hantavirus Gn and Gc envelope glycoproteins: key structural units for virus cell entry and virus assembly. *Viruses* 6:1801–1822
18. Shi X, Goli J, Clark G, Brauburger K, Elliott RM (2009) Functional analysis of the Bunyamwera orthobunyavirus Gc glycoprotein. *J Gene Virol* 90:2483–2492
19. Metz SW, Pijlman GP (2011) Arbovirus vaccines; opportunities for the baculovirus-insect cell expression system. *J Invertebr Pathol* 107:S16–S30
20. Faburay B, Lebedev M, McVey DS, Wilson W, Morozov I et al (2014) A glycoprotein subunit vaccine elicits a strong Rift Valley fever virus neutralizing antibody response in sheep. *Vector-Borne Zoonotic Dis* 14:746–756
21. Mandell RB, Flick R (2011) Virus-like particle-based vaccines for Rift Valley fever virus. *J Bioterr Biodef* S1:008. doi:10.4172/2157-2526.S1-008
22. Patterson RM, Selkirk JK, Merrick BA (1995) Baculovirus and insect cell gene expression: review of baculovirus biotechnology. *Environ Health Perspect* 103:756–759
23. Aucoin MG, Mena JA, Kamen AA (2010) Bioprocessing of baculovirus vectors: a review. *Curr Gene Ther* 10:174–186
24. Hitchman RB, Possee RD, King LA (2009) Baculovirus expression systems for recombinant protein production in insect cells. *Recent Pat Biotechnol* 3:46–54
25. Y-c Hu (2005) Baculovirus as a highly efficient expression vector in insect and mammalian cells. *Acta Pharmacol Sin* 26:405–416
26. Rahpeyma M, Fotouhi F, Makvandi M, Ghadiri A, Samarraf-Zadeh A (2015) Crimean-Congo hemorrhagic fever virus Gn bioinformatic analysis and construction of a recombinant bacmid in order to express Gn by baculovirus expression system. *Jundishapur J Microbiol* 8(11):e25502. doi:10.5812/jjm.25502
27. Sambrook J, Russell DW (2006) Preparation and transformation of competent *E. coli* using calcium chloride. *Cold Spring Harbor Protocols* 2006: pdb. prot3932
28. Yousefi A, Fotouhi F, Hosseinzadeh S, Kheiri M, Farahmand B et al (2012) Expression of antigenic determinants of the haemagglutinin large subunit of novel influenza virus in insect cells. *Folia Biologica* 58:151
29. Vincent MJ, Sanchez AJ, Erickson BR, Basak A, Chretien M et al (2003) Crimean-Congo hemorrhagic fever virus glycoprotein proteolytic processing by subtilase SKI-1. *J Virol* 77:8640–8649
30. Keshkar-Jahromi M, Kuhn JH, Christova I, Bradfute SB, Jahrling PB et al (2011) Crimean-Congo hemorrhagic fever: Current and future prospects of vaccines and therapies. *Antiviral Res* 90:85–92
31. Naderi H, Sheybani F, Bojdi A, Khosravi N, Mostafavi I (2013) Fatal nosocomial spread of Crimean-Congo hemorrhagic fever with very short incubation period. *Am J Trop Med Hygiene* 88:469–471
32. Borio L, Inglesby T, Peters CJ et al (2002) Hemorrhagic fever viruses as biological weapons: Medical and public health management. *JAMA* 287:2391–2405
33. Connolly-Andersen A-M, Douagi I, Kraus AA, Mirazimi A (2009) Crimean Congo hemorrhagic fever virus infects human monocyte-derived dendritic cells. *Virology* 390:157–162
34. Saijo M, Tang Q, Shimayi B, Han L, Zhang Y et al (2005) Recombinant nucleoprotein-based serological diagnosis of Crimean-Congo hemorrhagic fever virus infections. *J Med Virol* 75:295–299
35. Andersson I, Lundkvist Å, Haller O, Mirazimi A (2006) Type I interferon inhibits Crimean-Congo hemorrhagic fever virus in human target cells. *J Med Virol* 78:216–222
36. Pop M, Salzberg SL (2008) Bioinformatics challenges of new sequencing technology. *Trends Genet* 24:142–149
37. Muyrers JP, Zhang Y, Stewart AF (2001) Techniques: recombinogenic engineering—new options for cloning and manipulating DNA. *Trends Biochem Sci* 26:325–331
38. Dreshaj S, Ahmeti S, Ramadani N, Dreshaj G, Humolli I et al (2016) Current situation of Crimean-Congo hemorrhagic fever in Southeastern Europe and neighboring countries: a public health risk for the European Union? *Travel Med Infect Dis* 14:81–91
39. Kubar A, Haciomeroglu M, Ozkul A, Bagriacik U, Akinci E et al (2011) Prompt administration of Crimean-Congo hemorrhagic fever (CCHF) virus hyperimmunoglobulin in patients diagnosed with CCHF and viral load monitorization by reverse transcriptase-PCR. *Jpn J Infect Dis* 64:439–443
40. Ergonul O (2012) Crimean-Congo hemorrhagic fever virus: new outbreaks, new discoveries. *Curr Opin Virol* 2:215–220
41. Zhu Z, Dimitrov AS, Chakraborti S, Dimitrova D, Xiao X et al (2006) Development of human monoclonal antibodies against diseases caused by emerging and biodefense-related viruses
42. Buttigieg KR, Dowall SD, Findlay-Wilson S, Miloszezewska A, Rayner E et al (2014) A novel vaccine against Crimean-Congo haemorrhagic fever protects 100% of animals against lethal challenge in a mouse model. *PLoS One* 9:e91516
43. Dowall S, Buttigieg K, Findlay-Wilson S, Rayner E, Pearson G et al (2015) A Crimean-Congo Haemorrhagic Fever (CCHF) viral vaccine expressing nucleoprotein is immunogenic but fails to confer protection against lethal disease. *Human Vaccin Immunotherap*
44. Brooks SA (2004) Appropriate glycosylation of recombinant proteins for human use. *Mol Biotechnol* 28:241–255
45. Rai M, Padh H (2001) Expression systems for production of heterologous proteins. *Curr Sci Bangal* 80:1121–1128
46. Fuerst TR, Niles EG, Studier FW, Moss B (1986) Eukaryotic transient-expression system based on recombinant vaccinia virus that synthesizes bacteriophage T7 RNA polymerase. *Proceed Natl Acad Sci* 83:8122–8126
47. Macauley-Patrick S, Fazenda ML, McNeil B, Harvey LM (2005) Heterologous protein production using the *Pichia pastoris* expression system. *Yeast* 22:249–270
48. Hannig G, Makrides SC (1998) Strategies for optimizing heterologous protein expression in *Escherichia coli*. *Trends Biotechnol* 16:54–60
49. Vialard J, Lalumiere M, Vernet T, Briedis D, Alkhatib G et al (1990) Synthesis of the membrane fusion and hemagglutinin proteins of measles virus, using a novel baculovirus vector containing the beta-galactosidase gene. *J Virol* 64:37–50
50. Abe T, Takahashi H, Hamazaki H, Miyano-Kurosaki N, Matsuura Y et al (2003) Baculovirus induces an innate immune response and confers protection from lethal influenza virus infection in mice. *J Immunol* 171:1133–1139
51. Kajigaya S, FuJII H, Field A, Anderson S, Rosenfeld S et al (1991) Self-assembled B19 parvovirus capsids, produced in a baculovirus system, are antigenically and immunogenically similar to native virions. *Proceed Natl Acad Sci* 88:4646–4650
52. Fotouhi F, Soleimanjahi H, Roostae MH, Behzadian F (2008) Enhancement of protective humoral immune responses against Herpes simplex virus-2 in DNA-immunized guinea-pigs using protein boosting. *FEMS Immunol Med Microbiol* 54:18–26
53. Qian B, Shen H, Xiong J, Chen L, Zhang L et al (2006) Expression and purification of the synthetic preS1 gene of Hepatitis B Virus with preferred *Escherichia coli* codon preference. *Protein expression and purification* 48:74–80
54. Xu Z, Zhong Z, Huang L, Peng L, Wang F et al (2006) High-level production of bioactive human beta-defensin-4 in *Escherichia coli* by soluble fusion expression. *Appl Microbiol Biotechnol* 72:471–479
55. Peng L, Xu Z, Fang X, Wang F, Yang S et al (2004) Preferential codons enhancing the expression level of human beta-defensin-2 in recombinant *Escherichia coli*. *Protein Pept Lett* 11:339–344

56. Lu X, Wang J, Jin X, Zhu J (2015) High-level expression of a novel liver-targeting fusion interferon with preferred *Escherichia coli* codon preference and its anti-hepatitis B virus activity in vivo. *BMC Biotechnol* 15:1
57. Treanor JJ, Schiff GM, Hayden FG, Brady RC, Hay CM et al (2007) Safety and immunogenicity of a baculovirus-expressed hemagglutinin influenza vaccine: a randomized controlled trial. *JAMA* 297:1577–1582
58. Van Oers MM (2006) Vaccines for viral and parasitic diseases produced with baculovirus vectors. *Adv Virus Res* 68:193–253
59. Saksida A, Duh D, Wraber B, Dedushaj I, Ahmeti S et al (2010) Interacting roles of immune mechanisms and viral load in the pathogenesis of Crimean-Congo hemorrhagic fever. *ClinVacc Immunol* 17:1086–1093
60. Schoenborn JR, Wilson CB (2007) Regulation of Interferon- γ During Innate and Adaptive Immune Responses. *Adv Immunol: Academic Press* 96:41–101
61. Watford WT, Moriguchi M, Morinobu A, O'Shea JJ (2003) The biology of IL-12: coordinating innate and adaptive immune responses. *Cytokine Growth Factor Rev* 14:361–368
62. Frias-Staheli N, Giannakopoulos NV, Kikkert M, Taylor SL, Bridgen A et al (2007) Ovarian tumor domain-containing viral proteases evade ubiquitin-and ISG15-dependent innate immune responses. *Cell Host Microbe* 2:404–416