

Construction of a recombinant vector containing the respiratory syncytial virus (RSV) G subunit gene as a preliminary vaccine construct

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Abstract

Introduction: RSV is a single-stranded RNA virus with a nearly 15 kb genome containing 10 genes that encode 11 proteins. It has long been a leading cause of severe respiratory illness in children and the elderly. The G and F proteins are the most essential: G mediates attachment to host cells and stimulates immune responses, while both proteins directly contribute to the virus's pathogenicity.

Objective: The aim of this study was to develop a potential antigenic fragment based on a conserved region of the RSV- G gene, which was selected due to its key role in virus attachment to host cells and triggering an immune response.

Materials and Methods: For this purpose, five positive control RSV clinical samples were collected, and after RNA extraction, cDNA synthesis, and amplification of the central conserved region of the G gene using a nested PCR reaction, the resulting product was cloned into the pcDNA3.1+Hygro expression vector. Then, the transformation of Escherichia coli strain BL21 with the recombinant plasmid was performed, and after extraction, it was confirmed by enzymatic digestion and sequencing.

Results: All molecular steps, also confirming their proper quality, cloning, and transformation were completed successfully.

Conclusion: The results showed that the central conserved region of the G gene was cloned into the target vector without unwanted mutations, and the next phases of vaccine design can proceed. This study is a preliminary step towards the development of an RSV candidate vaccine.

Keywords: G gene, pcDNA3.1+Hygro, potential antigenic fragment , RSV, Vaccine

1. Introduction

For the first time, respiratory syncytial virus (RSV) was obtained from chimpanzees with respiratory sickness in 1955 [1]. RSV is identified as one of the important viral agents of severe lower respiratory tract diseases like pneumonia and bronchiolitis, especially in children and elderly people, and it consistently causes high rates of hospitalization in these populations [2]. The RSV genome is approximately 15.2 kilobases long. It consists of a single-stranded RNA containing ten genes encoding eleven viral proteins, among which two major proteins, G and F, are critically involved in virus entry into the host and contain immune interactions [3-5]. In the viral infection cycle, two proteins, F and G, play a vital role in causing infection in the host cell, as glycoprotein G mediates the stick of the virus to the receptors of the exterior surface in the host cell, and the F protein is involved in the fusion of the viral membrane and the host cell [6]. Also, protein G, and especially its Central Conserved Region (CCR), is an important target of antibodies neutralization and has high potential for use in vaccine design [7]. Despite the widespread circulation of RSV, no approved vaccine with high success has been introduced to prevent this virus in children [8]. Past efforts to progress vaccines have confronted challenges, for example, inducing insufficient immune responses or even exacerbating the disease [9]. In recent years, new technologies, including subunit vaccines and nucleic acid-based vaccines, have been proposed as promising approaches to overcome these challenges. In this regard, the G gene, and especially its conserved central domain, is considered as an one of the strategic targets in the design and manufacture of new vaccines due to its immunogenic potential and essential role in virus entry into the host cell [8]. As well, eukaryotic expression vectors, for instance pcDNA3.1+Hygro, provide a proper platform for the progress of nucleic acid-based vaccines due to their high potential for effective cloning and expression of target genes in eukaryotic cells [10]. Therefore, the aim of the current project was to clone the preserved central region of the RSV G gene into the pcDNA3.1+Hygro expression vector. This research is a preliminary step towards developing an effective subunit vaccine candidate against RSV and can pave the way for further studies in the field of immunogenicity and efficacy of this approach.

2. Materials and Methods

2.1. Sample collection: In order to obtain the target gene sequence required for cloning, positive control samples of respiratory syncytial virus were needed, for this purpose four nasopharyngeal swab samples from patients with RSV confirmed cases by molecular PCR method (two samples from Sari Health Center and two samples from Mofid Children's Hospital, Tehran) and one supernatant sample of cultured virus (Faculty of Health, University of Tehran) were collected. The RSV-positive status of the clinical samples had been confirmed previously by molecular PCR assays at the corresponding healthcare centers before being transferred for this study. Furthermore,

the identity of the RSV genetic fragment analyzed in this study was confirmed by amplification of the RSV G gene using nested-PCR, followed by real-time PCR amplification of the CCR region and Sanger sequencing of the cloned fragment. All samples were stored at -80°C immediately after collection to prevent degradation of viral RNA and to be used in the subsequent steps of RNA extraction and cDNA synthesis. The project's next steps were carried out at the Research Virology Laboratory of the University of Mazandaran, Babolsar.

2.2. Viral RNA extraction: Viral RNA was carried out from clinical nasopharyngeal swab specimens and viral culture supernatants using a commercial silica column-based Viral RNA extraction kit (KiaGene Fanavar Aria, Iran). Briefly, 150 µL of each sample was subjected to lysis in the presence of carrier RNA and proteinase K, followed by incubation at 58 °C to facilitate viral particle disruption. Ethanol was then added to promote RNA binding, and the mixture was transferred to spin columns where nucleic acids were selectively retained on the membrane. After sequential washing steps to remove proteins and other contaminants, viral RNA was eluted using 50 µL of the provided elution buffer. To minimize RNA degradation, the purified RNA was immediately processed for cDNA synthesis.

2.3. cDNA synthesis: Complementary DNA was synthesized using a heat-stable reverse transcription kit (cDNA-Ultra, Pars Toos, Iran), which is suitable for transcripts with low abundance.

2.4. Quantitative and qualitative examination of RNA and cDNA: In order to examine the quantity and quality of extracted RNA and native cDNA, the absorbance at two wavelengths of 260 and 280 nm was read by a spectrophotometer.

2.5. Positive control polymerase chain reaction (PCR) to confirm cDNA synthesis: To verify successful cDNA synthesis and assess the performance of the PCR system, a positive control amplification targeting the beta-2 microglobulin (B2M) gene was performed. The PCR reaction was prepared in a final volume of ten microliters, including five microliters of PCR master mix, two microliters of cDNA template, zero point five microliter of B2M-specific primers, and two point five microliters nuclease-free water.

Amplification was initiated with an initial denaturation step at 95 °C for 5 minutes to ensure total strand separation. This was followed by 35 thermal cycles consisting of denaturation at 94 °C for 30 seconds, primer annealing at 57 °C for 30 s, and extension at 72 °C for 30 s. A final extension phase was carried out at 72 °C for 5 min to allow complete synthesis of amplified products. The resulting PCR product was used as a quality control to confirm the integrity of the synthesized cDNA and the suitability of the reaction conditions.

2.6. Agarose gel electrophoresis: PCR products were analyzed on 1.2% agarose gel prepared in

TAE 1x buffer and stained with Safe Stain. A 100 bp DNA ladder was used as a molecular weight marker, and the amplified fragments were examined to confirm the expected product size.

Target region	Primers Name	Sequence (5'–3')	Product size (bp)
RSV- G-gene	Forward-G	5' TCGGCAAACCACAAAGTCACAC 3'	507
	Reverse-G	5' TTTCTGTGGGCTTGGTGGTAG 3'	
RSV-G-CCR	CCR-F	5' GCCACCATGACCACAGTCAAGACCAAGAAC 3'	105
	CCR-R	5' GGCACAAAGCTGAACACTTCA 3'	

2.7. Primer design and ordering: After confirming cDNA synthesis, for amplification of the G subunit gene and its central conserved region, specific primers were designed using Gene Runner software. The sequence of the target gene was extracted from the NCBI database. In the design of the primers, parameters such as melting temperature, Percentage of guanine and cytosine bases, primer length, secondary structures, and specificity of the primer to the target gene were examined. To optimize sequences for cloning and increased expression in eukaryotic systems, the 5' end of the forward primer CCR, the HindIII cleavage site, the initial part of the Kuzak sequence (GCCACC), and the start codon (ATG) were included, and the 5' end of the reverse primer was the XhoI cleavage site. The primers designed for synthesis were sent to Sinaclone Iran Company and produced by this company. The primers used included external and internal primers, the information of which is shown in Table 1.

Table 1. Information regarding the designed primers

2.8. Nested-PCR Optimization: In order to achieve the optimal annealing temperature of the outer and inner primers of the Nested-PCR reaction, a temperature gradient was established for the outer and inner PCR products.

2.9. First round of PCR for G gene amplification and product examination by agarose gel

(External PCR): Amplification of the RSV G gene was initially performed using an outer PCR reaction. A total of 20 μ L reaction mixture was prepared containing PCR pfu master mix (Kiagene Fanavar Aria, Iran), cDNA template, forward and reverse outer primers, along with nuclease-free water.

The same outer PCR process as the positive control PCR process for cDNA synthesis was used, except that the annealing temperature in this process was 58°C.

The specificity and size of the amplified products were examined by electrophoresis 1.2 percent agarose gel.

2.10. Second round of nested-PCR using real-time PCR conditions for amplification of the RSV G gene CCR region (Internal PCR): Quantitative real-time PCR was employed to amplify the central conserved region (CCR) of the RSV G gene, generating a 105 bp amplicon. Reactions were performed using CCR-specific primers and a SYBR Green I-based master mix containing Hot-start Taq DNA polymerase (Pars Toos, Iran). Each reaction was gathered in a final volume of 20 μ L, including master mix, cDNA template, primers, ROX reference dye, and nuclease-free water.

Real-time PCR cycling conditions consisted of an initial denaturation at 94 °C for 5 min, followed by 35 cycles of denaturation at 94 °C for 30 s, primer annealing at 54 °C for 30 s, and extension at 72 °C for 30 s. A final extension step was carried out at 72 °C for 5 min. Fluorescence data were collected during each amplification cycle, and the resulting amplification curves were used to confirm specific amplification of the CCR fragment and to generate products suitable for subsequent cloning procedures.

2.11. Preparation of pcDNA3.1+Hygro vector: In this study, pcDNA3.1+Hygro vector (Invitrogen/Thermo Fisher, received from Pasteur Institute, Tehran) was used as a cloning and gene expression platform. This vector, with a size of approximately 5.6 kilobases, has a strong cytomegalovirus promoter that allows high gene expression in mammalian cells. Also, this vector contains hygromycin B and ampicillin resistance genes, and its applications include expression of recombinant proteins, design of subunit vaccines, transient or stable transfection, and production of secreted or membrane proteins (9).

2.12. Cloning of the CCR of the G gene in the pcDNA3.1+Hygro vector: The amplified central conserved region (CCR) of the RSV G gene was cloned into the pcDNA3.1+Hygro expression vector using restriction enzyme-mediated ligation. Both the purified nested PCR product and the plasmid vector (10 μ L each) were independently digested with HindIII (1 μ L) and XhoI (1 μ L) in the presence of Tango buffer (2 μ L), with sterile water added to complete a final reaction volume of 20 μ L.

Enzymatic digestion was carried out at 37 °C for 2 h, then enzyme inactivation at 80 °C for 20 min. Subsequently, the digested insert and vector were ligated at an optimized 3:1 molar ratio (insert to vector). The ligation reaction mixture consisted of 1 μ L vector DNA, 3 μ L insert DNA, 2 μ L ligase buffer, 2 μ L T4 DNA ligase, and 12 μ L distilled water, resulting in a total volume of 20 μ L. The ligation was incubated at 37 °C for 20 min and then heat-inactivated at 80 °C for 10 min. The ligation product was used directly for bacterial transformation.

2.13. Preparation of competent cells and transformation: Chemically competent *Escherichia coli* BL21 cells were prepared using a calcium chloride-based protocol. This bacterial strain was used because it produced competent cells to high efficiency for transformation in previous studies and is a popular host for recombinant proteins. A single bacterial colony was cultured in 10 mL of LB broth and incubated for 16 h at 37 °C. A portion of 50 µL from the overnight culture was transferred into 5 mL of fresh LB medium and incubated with shaking at 37 °C until the optical density reached $OD_{600} = 0.5\text{--}0.6$. Cells were harvested by centrifugation at 9000 rpm for 3 min, washed repeatedly, and resuspended in cold 0.1 M $CaCl_2$. Following incubation on ice for 20 min, the cells were finally resuspended in 300 µL of 0.1 M $CaCl_2$ and aliquoted into sterile microtubes. To the transformation, 10 µL and 20 µL of the ligation product were added to competent cells in two separate vials, and one vial was used as a negative control. The mixtures were incubated on ice at 0 °C for 30 min, were treated with heat shock at 42 °C for 90 s, and immediately returned to ice for 2 min. After recovery in 900 µL of LB medium for 1 h at 37 °C, transformed cells were plated on LB agar supplemented with ampicillin and incubated overnight at 37 °C.

2.14. Plasmid DNA extraction: To extract plasmid DNA, the base degradation method was used, similar to the protocol described by Rasani et al [11].

2.15. Verification of cloning and extraction of plasmid DNA: To verify the success of cloning and DNA extraction, the recombinant plasmid was digested with *Xho*I and *Mlu*I enzymes, and the resulting products were examined on a 1% agarose gel.

2.16. Sequencing of the extracted recombinant plasmid genome: To ensure the accuracy of cloning and the absence of unwanted mutations in the PCR reaction, the extracted product was sent to Nedaye Fan, Iran Company, and sequenced by Gene Ray, China Company. Sequencing was performed by Gene Ray Company using the Sanger sequencing method and on an ABI 3730XI capillary sequencer.

3. Results

3.1. Quantitative and qualitative examination of RNA and cDNA: The A260/A280 absorbance ratio of all extracted RNA and synthesized cDNA samples ranged between 1.8 and 2, confirming their acceptable purity.

3.2. Confirmation of cDNA synthesis with positive control PCR: PCR amplification using the B2M positive control primer produced the expected amplicon in all cDNA samples, confirming successful cDNA synthesis. Based on the electrophoretic results, one clinical sample obtained from Mofid Hospital, Tehran, was selected for the subsequent cloning experiments. The proper quality of the synthesized cDNA was also confirmed with a spectrophotometer as in the previous step.

3.3. Optimization of the external polymerase chain reaction: The optimal annealing temperature for amplification of the RSV G gene was determined to be 57°C, which produced the expected specific PCR product with the best amplification efficiency.

3.4. Agarose gel electrophoresis: The desired band was observed in the range of 507 bp using a gel dock device to confirm the accuracy of the PCR product size obtained during primer design and to obtain the optimal annealing temperature (Figure 1).

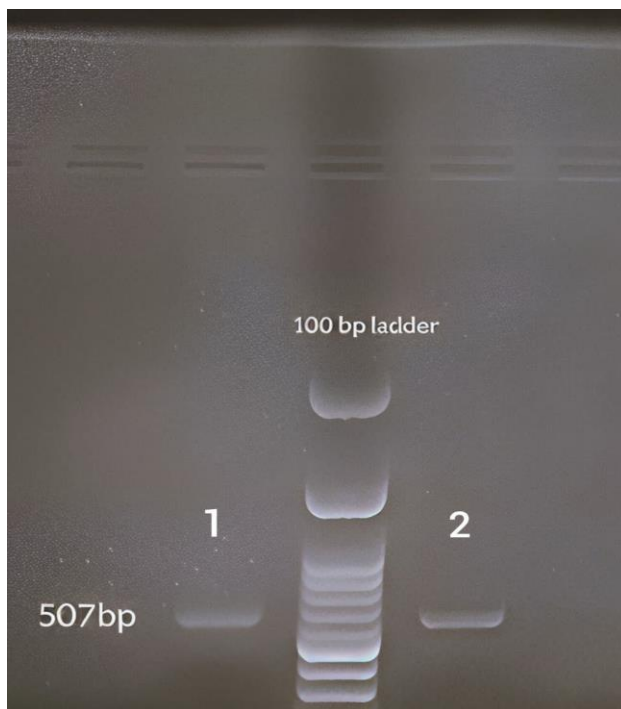
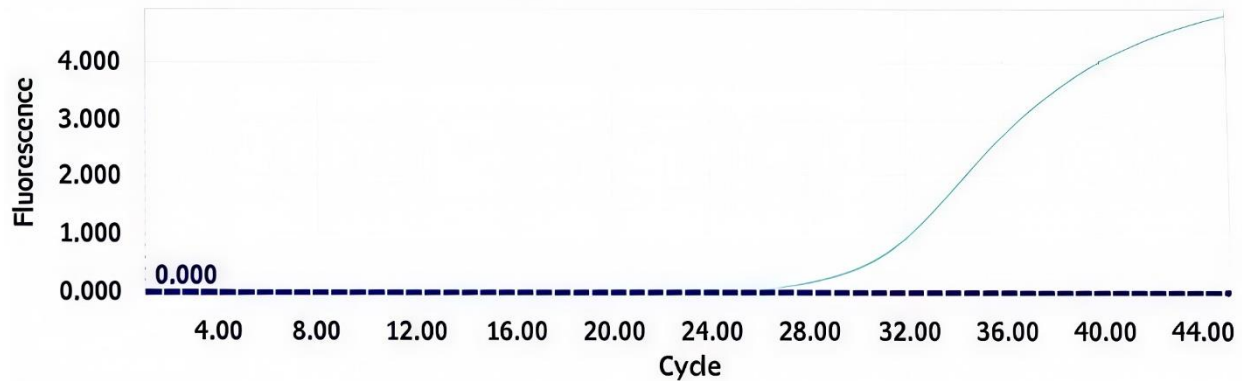


Figure1. Results of 1.2% agarose gel electrophoresis of the external PCR product of the gene that makes the RSV-G protein. cDNA was amplified with the 507 base pair primer at the optimal temperature of 57 ° C. The positive control samples for RSV were amplified within the 507 base pair range of the 100 base pair gene ruler. Both bands observed on the gel (1 and 2) correspond to the sample received from Mofid Hospital in Tehran.

3.5. Optimization of the second round of nested-PCR using real-time PCR conditions: The optimal annealing temperature for amplification of the RSV G gene CCR fragment was determined to be 54°C, which produced the expected specific amplification and was selected for the subsequent real-time nested PCR analysis.

3.6. Results of RT-qPCR of the G gene CCR region: Real-time PCR analysis demonstrated successful amplification of the CCR fragment. As shown in Figure 2, the amplification curve exhibited a consistent increase in fluorescence signal, and the melting curve showed a single distinct peak, confirming the specificity of the amplified product and the absence of detectable non-specific amplification or primer-dimer formation.

A) Amplification curve



B) Melting curve

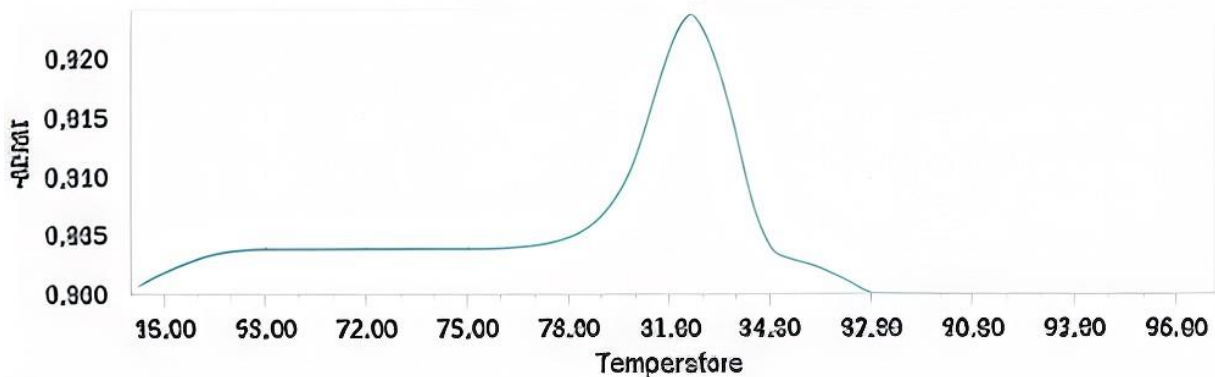


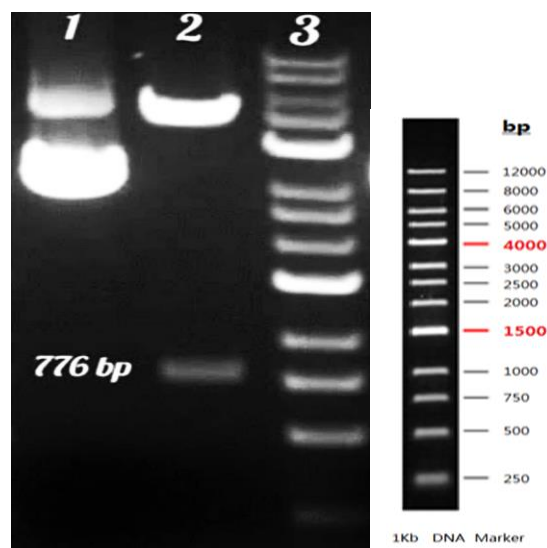
Figure 2. Real-time nested PCR analysis of the RSV G gene central conserved region (CCR). A) Amplification curve B) Melting curve of Real-time PCR reaction

3.7. Results of cloning of the CCR of the G gene in the pcDNA3.1+Hygro vector: The CCR fragment of the RSV G gene was successfully cloned into the pcDNA3.1+Hygro expression vector. The recombinant plasmid containing the target insert was obtained and further analyzed for confirmation of the cloning process.

3.8. Transformation and propagation of the recombinant plasmid in E. coli BL21 :Successful transformation of E. coli BL21 with the recombinant plasmid was confirmed by bacterial growth on selective LB agar containing ampicillin. In contrast, no growth was observed in the negative control, confirming the successful propagation of the recombinant construct.

3.9. Confirmation of plasmid DNA extraction and cloning confirmation: As shown in Figure 3, to verify plasmid DNA extraction and cloning, the recombinant vector—both intact and digested with the restriction enzymes XhoI and MluI—was loaded onto a 1% agarose gel; the formation of a 776-bp product, which encompasses the 105-bp target region (consistent with the plasmid map in Figure 5), confirmed the success of the cloning.

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266

267 **Figure 3. Electrophoresis results to confirm plasmid DNA extraction and cloning. Number 1**
 268 **recombinant plasmid DNA extracted in the range of 5625 bp of one kilobase gene line, number 2**
 269 **plasmid digested with two enzymes XhoI and MluI, and number 3 one kilobase ladder.**

270

271 **3.10. Confirmation of cloning by sequencing of extracted recombinant plasmid DNA:**

272 According to Figure 4, the results of sequencing of extracted recombinant plasmid DNA confirmed
 273 the accuracy of cloning and the absence of unwanted mutations in the PCR reaction. The sequence
 274 of the 105 bp product obtained from the second round of nested-PCR performed under real-time
 275 PCR conditions for amplification of the central conserved region of the RSV G gene is shown
 276 below.

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1  GACGGATCGG GAGATCTCCC GATCCCCTAT GGTGCACTCT CAGTACAATC TGCTCTGATG
61  CCGCATAGTT AAGCCAGTAT CTGCTCCCTG CTGTGTGTGT GGAGGTCGCT GAGTAGTGCG
121 CGAGCAAAAT TTAAGCTACA ACAAGGCAAG GCTTGACCGA CAATTGCATG AAGAATCTGC
181 TTAGGGTTAG GCGTTTTGCG CTGCTTCGCG ATGTACGGGC CAGATATACG CGTTGACATT
241 GATTATTGAC TAGTTATTAA TAGTAATCAA TTACGGGGTC ATTAGTTCAT AGCCCATATA
301 TGGAGTTCGG CGTTACATAA CTTACGGTAA ATGGCCCGCC TGGCTGACCG CCCAACGACC
361 CCCGCCCATT GACGTCAATA ATGACGTATG TTCCCATAGT AACGCCAATA GGGACTTTCC
421 ATTGACGTCA ATGGGTGGAG TATTTACGGT AAAGTGCCCA CTTGGCAGTA CATCAAGTGT
481 ATCATATGCC AAGTACGCCC CTTATTGACG TCAATGACGG TAAATGGCCC GCCTGGCATT
541 ATGCCCAGTA CATGACCTTA TGGGACTTTC CTACTTGGCA GTACATCTAC GTATTAGTCA
601 TCGCTATTAC CATGGTGAIG CGTTTTTGCG AGTACATCAA TGGGCGTGGA TAGCGGTTTG
661 ACTCACGGGG ATTTCCAAGT CTCCACCCCA TTGACGTCAA TGGGAGTTTG TTTTGGCACC
721 AAAATCAACG GGACTTTCCA AAATGTCGTA ACAAATCCGC CCCATTGACG CAAATGGGCG
781 GTAGGCGTGT ACGGTGGGAG GTCTATATAA GCAGAGCTCT CTGGCTAAGT AGAGAACCCA
841 CTGCTTACTG GCTTATCGAA ATTAATACGA CTCACTATAG GGAGACCCAA GCTGGCTAGC
901 GTTAAACTT AAGCTTGCCA CCATGACCAC AGTCAAGACC AAGAATTTG AAGTGTTCAA
961 CTTTGTACCT TGCAGCATAT GCAGCAACAC TTCAACAAGT GAAACACGGC TCGAGTCTAG
1021 AGGGCCCCGT TAAACCCGCT GATCAGCCTC GACTGTGCTT TCTAGTTGCC AGCCAICTGT
1081 TGTTTGCCCC TCCCCGTGCG CTTCCCTTGC CCTGGAAAGG GCCACTCCCA CTGTCCTTTC
1141 CTAATAAAAT GAGGAAATTG CATCGCATTG TCTGAGTAGG TGTCATTCTA TTCTGGGGGG
1201 TGGGGTGGGG CAGGACAGCA AGGGGGAGGA TTGGGAAGAC AATAGCAGGC ATGCTGGGGA
1261 TCGGTTGGGC TCTATGGCTT CTGAGGCGGA AAGAACCAGC TGGGGCTCTA GGGGGTATCC

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Figure4. Image of a portion of the plasmid genome sequencing result containing the CCR fragment. The fragment in question is highlighted in a different color.

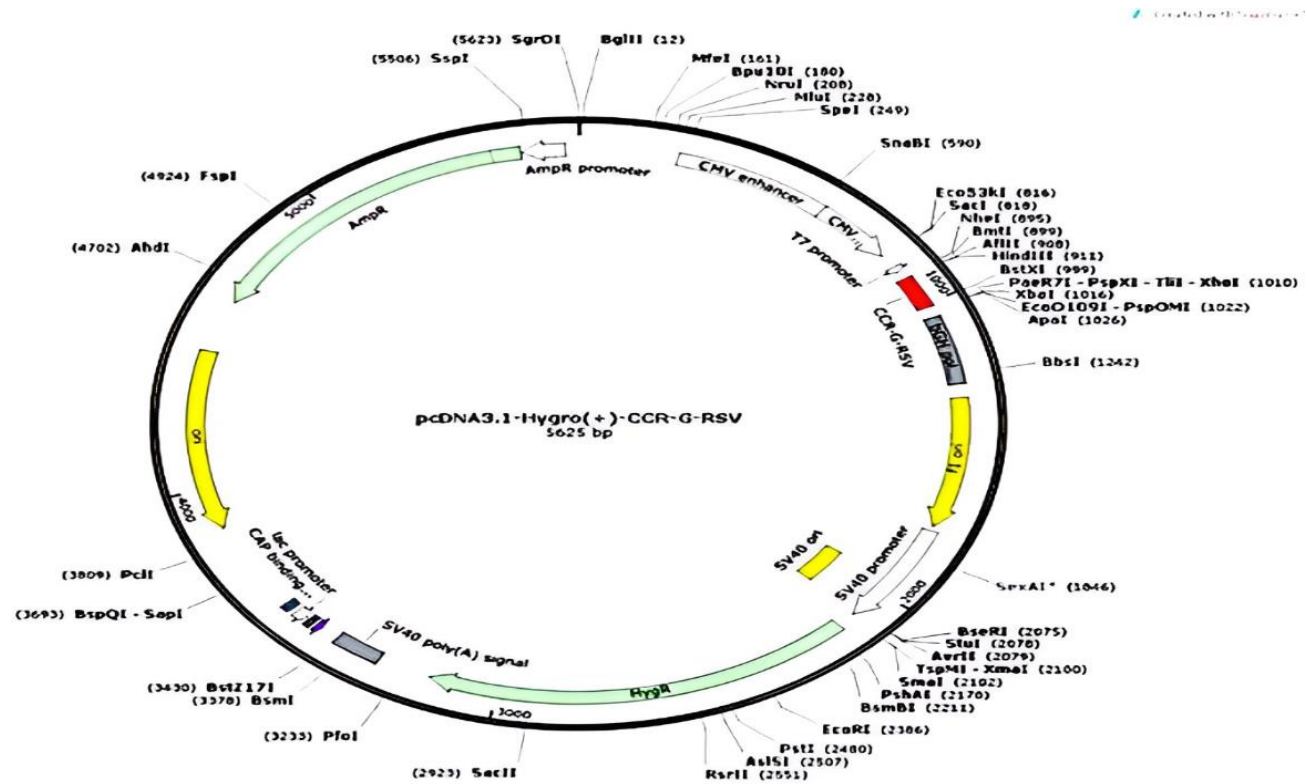


Figure 5. Schematic image of the recombinant vector pcDNA3.1+Hygro in which the CCR region of the respiratory syncytial virus G gene has been cloned into the aforementioned plasmid vector by enzymatic digestion with the enzymes HindIII and XhoI and ligation with the enzyme T4DNA Ligase.

4. Discussion

RSV was first isolated in 1955 from chimpanzees with respiratory disease [12]. It is transmitted by respiratory droplets and direct interaction [13]. RSV is an extremely contagious seasonal virus that causes approximately 3 million hospitalizations and 120,000 deaths annually among children under 5 years of age [14]. Despite decades of research, RSV vaccine development remains challenging because of the young age of the target population, immune evasion by the virus, incomplete protection following natural infection, the lack of an ideal animal model, and the historical failure of the formalin-inactivated vaccine [15–18]. Currently, more than 30 RSV vaccine candidates based on recombinant, subunit, live attenuated, chimeric, and nucleic acid platforms are under development, several of which have reached phase III clinical [19]. Subunit vaccines aim to induce immunity more effectively than natural virus infection [15]. The RSV G protein induces neutralizing antibodies and is considered a promising candidate for nucleic acid vaccine [20]. Previous studies have demonstrated the potential of the RSV G protein as a nucleic

acid vaccine antigen. Li Xiaomao et al. reported that a plasmid DNA vaccine encoding the full-length G protein induced balanced humoral and cellular immune responses, generated neutralizing antibodies, and did not cause enhanced pulmonary inflammation following viral challenge [21]. Similarly, Saydi et al. showed that monoclonal antibodies against the G protein, particularly those targeting the central conserved region (CCR), were effective in preventing RSV infection [22]. Jones et al. demonstrated that the CCR contains highly conserved neutralizing epitopes capable of inducing cross-neutralizing antibodies against both RSV A and B subtypes [23]. Furthermore, Bergeron et al. reported that antibodies directed against the CCR blocked the interaction between the RSV G protein and the CX3CR1 receptor, thereby inhibiting viral attachment and infection [24]. Based on these findings, the CCR of the RSV G gene was selected as a preliminary vaccine construct and successfully cloned into the pcDNA3.1+Hygro expression vector. The CCR was selected because of its high sequence conservation between RSV A and B subtypes, with the aim of inducing cross-neutralizing immune responses. The pcDNA3.1+Hygro vector was chosen because of its high stability and efficient gene expression in eukaryotic [25,26]. Compared with viral vectors, plasmid vectors offer several advantages, including ease of production, a favorable safety profile, and the absence of pre-existing anti-vector immunity. Despite the successful construction and molecular validation of the recombinant plasmid, this study has some limitations. The in vitro transcription assay and evaluation of target gene expression were not performed because the primary objective of this study was the design, cloning, and molecular confirmation of the recombinant construct. Therefore, further studies are needed to evaluate its transcriptional activity, expression efficiency, immunogenicity, and protective efficacy in appropriate in vitro and in vivo models. In addition, although the CCR was selected because of its high conservation, future studies should investigate its immunogenicity alone and in combination with suitable molecular adjuvants to enhance both humoral and cellular immune responses.

5. Conclusion

In this study, the central conserved region (CCR) of the RSV G gene was successfully amplified by nested-PCR and cloned into the pcDNA3.1+Hygro expression vector as a preliminary DNA vaccine construct. The recombinant plasmid was successfully propagated in *E. coli* BL21, and accurate insertion of the target fragment was confirmed by sequencing. These findings demonstrate the successful molecular construction and validation of a recombinant plasmid containing the conserved CCR of the RSV G gene. The conserved nature of the CCR between RSV A and B subtypes makes this construct a promising candidate for further vaccine development. However, the present study was limited to molecular construction and confirmation of the recombinant plasmid. Therefore, additional studies are required to evaluate its transcriptional activity, protein expression, immunogenicity, and protective efficacy in appropriate in vitro and in vivo models before its potential as a DNA vaccine candidate can be determined.

Ethical Considerations

Compliance with ethical guidelines

All experimental procedures were conducted in accordance with the guidelines of the Bioethics Committee of the University of Mazandaran (Ethical approval code: IR.UMZ.REC.1403.110).

Data availability

Data will be available on editors request.

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Authors contributions

Supervision, writing – review & editing: Fatemeh roodbari; Investigation, Methodology, Writing – original draft: Amirhossein ashkeshi

Conflict of interest

The authors declared no conflict of interest.

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Declaration of generative AI and AI-assisted technologies in the writing process

During the preparation of this work, the authors used Grammarly for grammatical corrections and to improve readability, and IMGLARGER to enhance the quality of certain images. No generative AI was used to create the content. Following the use of these tools, the authors reviewed and—where necessary—edited the content, and they assume full responsibility for the material presented in this work.

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