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Construction of a Prokaryotic Expression Vector harboring Two HIV-1 Accessory Genes

Original Article

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ABSTRACT

Background and objectives: HIV-1 Nef and Vpr antigens have been described as suitable candidates for therapeutic HIV vaccine development. The aim of this study was to generate *Nef-Vpr* fusion gene construct and to clone the construct into pET-23a (+), a prokaryotic expression vector.

Methods: HIV-1 *Nef* and *Vpr* genes were PCR-amplified from the *pNL4-3* plasmid using specific primers and Pfu DNA polymerase. Results of PCR amplification were visualized by electrophoresis on 0.8% agarose gel. At first, the amplified Nef fragment was cloned into *Nhe*I and *Bam*HI restriction sites of pET-23a expression vector. Next, cloning of *Vpr* gene was performed into *Bam*HI and *Hind*III restriction sites of the pET-23a-Nef vector. Finally, purity of the recombinant pET-23-*Nef-Vpr* construct was determined by NanoDrop spectrophotometry.

Results: PCR amplification of *Nef* and *Vpr* genes was confirmed by detection of 620 bp and 291 bp bands, respectively. Cloning of the *Nef-Vpr* construct into the vector was confirmed by detection of a 911 bp fragment following enzymatic digestion with *Nhe*I and *Hind*III and sequencing.

Conclusion: The successful construction of recombinant fusion plasmid encoding a chimeric *Nef-Vpr* gene was performed in a prokaryotic expression vector for development of HIV-1 recombinant protein vaccine in near future.

Keywords: HIV-1, Nef, Vpr , Cloning.

INTRODUCTION

Acquired immune deficiency syndrome (AIDS) is a disease caused by infection with the human immunodeficiency virus (HIV) and still remains one of the leading causes of death worldwide. The virus belongs to the genus Lentivirus within the family of Retroviridae and can be transmitted through various body fluids or secretions, including blood, semen, milk, *etc*. (1). Since the start of the HIV epidemic, 75 million people have been infected with the virus, nearly 32 million of whom have died from the disease. According to the WHO, 38 million people were living with HIV in 2019 (2).

The HIV virus is classified into two types based on the characteristics and differences in viral antigens: HIV-1 and HIV-2. The virus genome consists of two RNA strands encapsulated in the envelope that encode nine viral genes (*gag, pol, env, tat, rev, nef, vif, vpr, vpu*). The genes *gag, pol* and *env* are involved in the production of structural proteins (matrix, capsid, nucleocapsid and p6), viral enzymes (protease, reverse transcriptase and integrase) and envelope proteins (Gp120 and Gp41). The remaining six genes encode regulatory proteins (Tat and Rev) and accessory proteins (Vif, Vpu / Vpx, Vpr, and Nef) (3).

Vpr is a relatively small protein containing 96 amino acids that is present in all HIV types (4). Despite its small size, the presence of this protein is essential for the optimal progression of the early stages of the virus life cycle in newly infected cells. The general functions of this protein include induction of cell cycle arrest (G2/M stage) and apoptosis, regulation of reverse transcription, insertion of viral DNA into macrophage nuclei and immune cells, facilitation of virus infection and regulation of transcription of viral and host genes (7, 8). Moreover, functional impairment in Vpr reduces the rate of disease progression in HIV patients (7). Nef is a 27-32 kDa protein, highly expressed in the early stages of the infection process that negatively affect the presence of CD4 and HLA molecules on the surface of HIV-infected cells, thereby slowing down the immune response, especially by CTL (8). This protein also promotes infectivity and pathogenesis, particularly in HIV-1. In addition to the various functions of this protein in infected cells, Nef can be released into the extracellular space in the form of virion or exosome and act as a good measure of viral protein expression in treated individuals (9).

Despite the benefits of antiviral drug combinations or highly active antiretroviral therapy, these drugs do not cure the disease and are associated with numerous side effects. This emphasizes the urgent need for developing novel or complementary therapies such as the development of new vaccines and antiviral drugs (10). The use of recombinant proteins allows the targeting of immune responses focused against particular antigens. In general, recombinant protein vaccines are safe, easy to produce in large quantities and free from potential concerns raised by traditional vaccines based on purified macromolecules (11). There are a variety of expression systems with different advantages, allowing the production of large quantities of proteins depending on the required characteristics. Multiple approaches utilizing viral and DNA vectors have shown promise in the development of an effective vaccine against HIV. The most straightforward method to produce large amounts of recombinant protein suitable for a vaccine is to clone the gene into a prokaryotic expression vector and produce the protein in *Escherichia coli* (12). Given the crucial role of HIV-1 accessory proteins Nef and Vpr in infectivity, viral proliferation and disease progression, we aimed to design a Nef-Vpr fusion construct and clone the construct into a prokaryotic vector to produce a potential immunogenic recombinant protein in future.

MATERIALS AND METHODS

PCR amplification of the Nef and Vpr accessory genes

Complete sequences of HIV-1 *Nef* and *Vpr* genes were obtained from the HIV-1 vector *pNL4-3* available at the NCBI website (accession no. AF324493). Specific primers comprising the restriction site of relevant restriction enzymes were designed using the SnapGene software. Table 1 shows the sequence of the primers used for amplification of *Nef* and *Vpr* genes.

To obtain the genes, PCR amplification was performed using the *pNL4-3* vector and pfu DNA polymerase. The final reaction solution (50 µl) contained 5 µl buffer (10X), 2 µl Dntp (2mM), 1 µl of each primer (10 pmol), 0.5 µl

pfu DNA polymerase (2.5 U/µl), 1 µl *pNL4.3* (100 ng) and 39 µl H_2O. H_2O was used as negative control. Cycling conditions were carried out as shown in Table 2. PCR products were subjected to agarose gel electrophoresis for 40 min at 90V.

At first, the amplified Nef fragment was cloned into *Nhe*I and *Bam*HI restriction sites of pET-23a (+) expression vector (Novagen, Merck, Germany). For this purpose, 10 µl of each template (the vector and gene) were digested in a final reaction solution of 50 µl containing 1.5 µl *Nhe*I (FastDigest, Thermo Scientific), 1.5 µl *Bam*HI (FastDigest, Thermo Scientific), 5 µl buffer (10X) and 32 µl deionized water for one hour. The digested pET23a vector and *Nef* were first run on 0.8% agarose gel and then purified using the GEL/PCR Purification Mini Kit (FAVORGEN Biotech Co., Taiwan). The digested *Nef* gene was ligated into the linearized pET23a vector at the ratio of 1:7 (vector: gene) using T4 DNA ligase (Thermo Scientific, USA) and 10X T4 DNA ligase buffer for 16 hours at 4°C. Next, the ligation mixture was used to transform competent *E. coli* DH5α cells by heat shock. The pET23a vector harboring the *Nef* gene was extracted from recombinant clones using FavorPrep Plasmid DNA Extraction Mini Kit (FAVORGEN Biotech Co., Taiwan) and verified by restriction enzyme digestion.

Table 1- Sequences of the primers used for amplification of HIV-1 *Nef* and *Vpr* genes

Primer	Sequence 5`-3`
Forward-Nef	TAGCTAGCATGGGTGGCAAGTGGTC
Reverse-Nef	AGGGATCCGCAGTTCTTGAAGTAC
Forward-Vpr	AAGGATCCATGGAACAAGCCCCAG
Reverse-Vpr	AGAAGCTTGGATCTACTGGCTCC

Table 2- PCR program for amplification of *Nef* and *Vpr* genes

Stage	Temperature	Time	Cycle(s)
Initial denaturation	95 °C	5 minutes	1
Denaturation	95 °C	30 seconds	30
Annealing	63 °C	45 seconds	
Extension	72 °C	60 seconds	
Final extension	72 °C	5 minutes	1

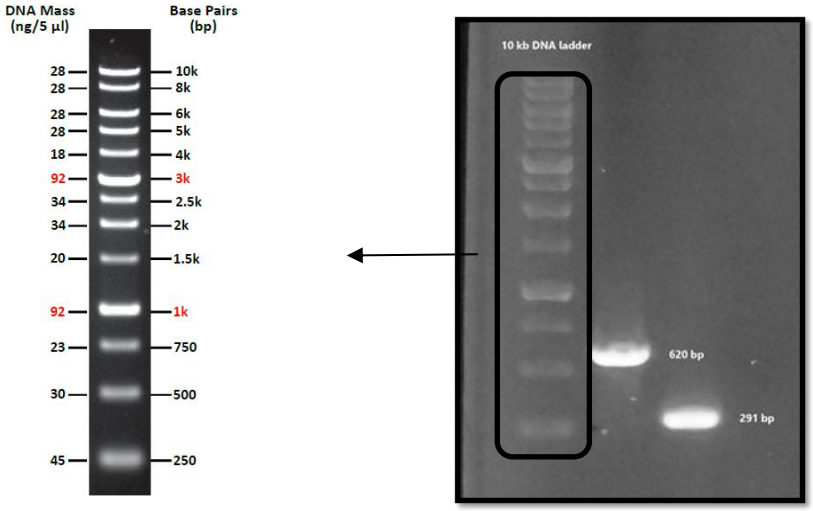

Figure 1- Detection of ~ 620 bp and ~ 291 bp fragments confirmed successful amplification of *Nef* and *Vpr* genes, respectively

PCR-amplified Vpr fragment was cloned into *Bam*HI and *Hind*III restriction sites of the pET23a-*Nef* plasmid as explained for *Nef*.

The digested *Vpr* and pET23a-*Nef* were first run on 0.8% agarose gel and then purified using the kit.

Next, the ligation mixture was used to transform competent *E. coli* DH5α. Plasmid DNA containing pET23a-*Nef-Vpr* was extracted from recombinant clones using the kit. The concentration and purity of the pET23a-*Nef-Vpr* were evaluated using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA). Successful cloning of the *Nef-Vpr* construct into pET23a vector was verified by restriction enzyme digestion and sequencing. Bacteria stocks were prepared in 0.8% glycerol solution and stored at -70 ^{o}C for future use.

RESULTS

PCR amplification of the Nef and Vpr genes

Amplification of HIV-1 *Nef* and *Vpr* genes were carried out using specific primers. Detection of 620 bp and 291 bp fragments in agarose gel electrophoresis confirmed successful amplification of *Nef* and *Vpr* genes, respectively (Figure 1).

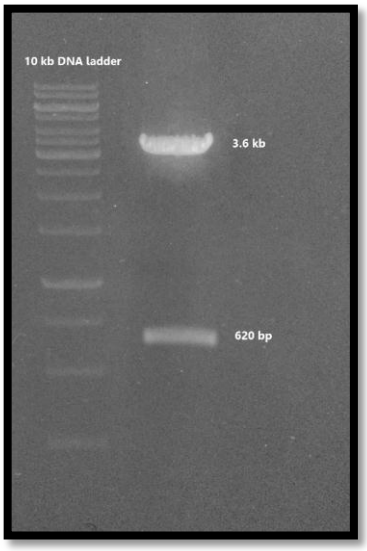

Figure 2- Detection of ~ 620 bp fragment after enzymatic digestion with *Nhe*I and *Bam*HI enzymes confirmed cloning of *Nef* gene

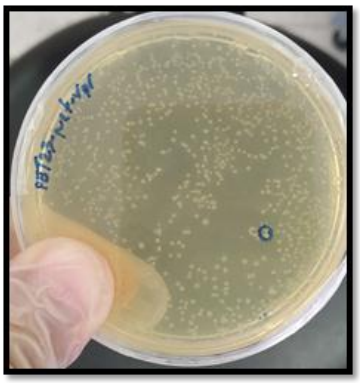

Figure 3- Transformation of pET23-*Nef-Vpr* into the competent DH5α cells.

The PCR-amplified Nef fragment was cloned into *Nhe*I and *Bam*HI restriction sites of pET23a expression vector. Successful cloning of *Nef* gene was confirmed by enzymatic digestion of the plasmid with *Nhe*I and *Bam*HI enzymes (Figure 2).

The amplified Vpr fragment was cloned into *Bam*HI and *Hin*dIII restriction sites of the pET23a-Nef plasmid. The recombinant clones after transformation was shown in Figure 3. Successful cloning of the *Nef-Vpr* fusion was then confirmed by enzymatic digestion of the plasmid using *Nhe*I and *Hin*dIII enzymes (Figure 4).

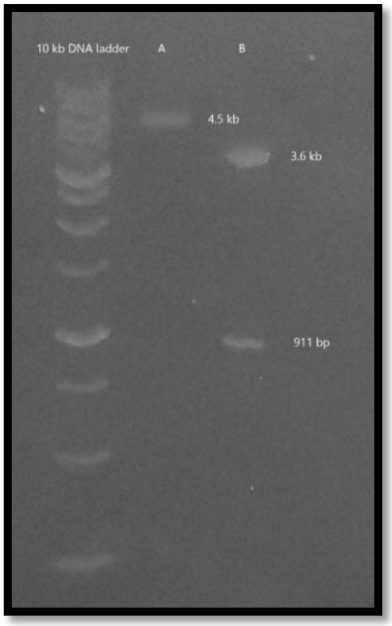

Figure 4- Successful cloning of *Nef-Vpr* fusion was confirmed by detection of ~911 bp band after enzymatic digestion of the pET23a-*Nef-Vpr* recombinant plasmid with *Nhe*I and *Hin*dIII enzymes. A. Undigested pET23a-*Nef-Vpr* (~ 4.5 kb); B. Digested plasmid (3.6 kb) and Nef-Vpr fragment (~911 bp).

DISCUSSION

HIV/AIDS remains a global health concern with over 39 million HIV/AIDS-related deaths to date. The recent advancements in antiviral drug combinations or highly active antiretroviral therapy and increased access to this therapy, have led to 51% reduction in HIV mortality and 17% reduction in incidence rates (13). However, the issue of finding complementary or alternative treatment and prevention strategies still persists.

Among numerous HIV genes examined for generation of immunogenic recombinant DNA and proteins, the highly-conserved HIV-1 accessory protein Nef has been widely regarded as a highly immunogenic candidate for development of therapeutic vaccines (14–17). This is mainly attributed to the main role of Nef in HIV pathogenesis via downregulation of cell-surface expression of multiple membrane-associated proteins, enhancement of virion infectivity, stimulation of viral replication, interference of cellular signal transduction, and manipulation of the apoptosis mechanism (14). On the other hand, Vpr has been known as a crucial component of HIV pathogenicity and progression. It is particularly involved in virus spread by promoting replication in dividing and non-dividing cells (18). It has been also suggested that targeting Vpr as an early-expressing HIV-1 protein could decrease HIV infection and $CD4^+$-T-cell dysfunction (19). Both *Nef* and *Vpr* are highly-conserved HIV genes that have been long considered as promising targets for development of therapeutic strategies against HIV infection (20).

Despite its limitations, restriction digestion- and ligation-based cloning is still widely used to generate DNA constructs for a variety of molecular biology applications. Faster growth rate, higher plasmid yield and higher transformation efficiency are among the advantages of *E. coli* compared to other hosts, such as *Saccharomyces cerevisiae* (21). DH5α

is one of the most popular *E. coli* strains used for maintenance and amplification of plasmid DNAs. These cells are suitable for stable production of recombinant plasmid DNA due to the recA and endA deletions, thereby preventing heterologous recombination to ensure a higher insert stability and endonuclease digestion of the plasmid during the isolation procedure (22).

Originally derived from pBR322, the pET system vectors controlled by the T7lac promoter, are a suitable choice for inducible heterologous expression of genes with high protein yields (23). According to Novagen, this series of vectors are the most robust vectors for cloning/ sub-cloning and overexpression of recombinant proteins in *E. coli* (24). There are various cloning strategies in order to generate protein expression constructs for structural and functional applications. In the present study, we used type II restriction endonucleases that cleave DNA outside of the recognition site, creating unique sticky ends that enable directional and seamless cloning of the gene of interest. Our PCR-amplified genes were inserted into the multiple cloning site of the pET23a vector, downstream of a T7 promoter for IPTG- inducible transcription by the T7 RNA polymerase. The pET vectors have been widely used for designing HIV proteins expression constructs (16,25–27). In the present study, we successfully constructed a recombinant fusion plasmid encoding a chimeric *Nef-Vpr* gene in pET23a expression vector for generation of a recombinant fusion protein in near future.

CONCLUSION

Generating recombinant vectors for efficient expression of HIV-1 antigens is a pivotal part of developing therapeutic and preventive vaccines. Given the higher effectiveness of multi-antigen HIV-1 targeted therapeutic strategies, in this study, we designed and constructed a recombinant prokaryotic vector harboring HIV-1 accessory genes *Nef* and *Vpr*. The expression of the recombinant *Nef-Vpr* fusion construct will be investigated in the next studies.

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CONFLICT OF INTEREST

The authors declare that there is no conflict of interest regarding publication of this study.

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