Cloning, expression, purification and immunoreactivity analysis of gag derived protein p17 from HIV-1 CRF35 in fusion with thioredoxin from human subjects

Mohammad Askari[1], Fazel Gorjipour[2], Zohreh Sharifi[3], Mohammad Morad Farajollahi[1*]

[1]*Department of Medical Biotechnology, Faculty of Allied Medical Sciences, Tehran University of Medical Sciences, P.O. Box 1449614535, Tehran, I.R. Iran* [2]*HIV Molecular Research Laboratory, Division of Virology, Department of Pathobiology, School of Public Health and Health Research Institute, Tehran University of Medical Sciences, P.O. Box 19395-5731, Tehran, I.R. Iran* [3]*Blood Transfusion Research Center, Institute for Research and Education in Transfusion Medicine, P.O. Box 14665-1157, Tehran, I.R. Iran*

Abstract

So far, recombinant antigens of HIV-1, the etiologic cause of Acquired Immunodeficiency Syndrome (AIDS), have been widely used for the diagnosis and vaccine development. P17 or the matrix protein formed by the proteolytic cleavage of gag is strongly antigenic and is as conserved and immunogenic as p24. In some cases, antibodies to p17 are more prevalent than antibodies to p24 and the decline in the level of p17 antibodies is an earlier prognostic marker for disease progression than decline in the level of antibodies to p24. The aim of this study was to clone and express the gag derived p17 protein in soluble form in *E. coli* and then assess the immunoreactivity of produced recombinant p17. DNA sequence encoding p17 matrix protein was cloned from PTZ-gag53-IR vector that has the complete gag polyprotein sequence. The T7-promoter-based expression system used in this study was TOPO directional cloning strategy that expressed p17 matrix protein in fusion with thioredoxin in *E. Coli*. Purification of produced recombinant protein has been done using Ni-NTA nickel chelating agarose beads. Sequencing result of the cloned sequence showed that it belongs to CRF35_AD subtype of HIV-1 which is highly prevalent in Iran and Afghanistan. The immunoreactivity of produced recombinant p17 to sera from infected individuals showed 93.8% sensitivity and 100% specificity.

Keywords: HIV-1 CRF35_AD; Cloning; Recombinant protein; p17 antigen; Fusion protein

Correspondence to: **Mohammad Morad Farajollahi, Ph.D.**
Tel: +98 912 2169076; Fax: +98 21 88622533
E-mail: mfarajol@tums.ac.ir

INTRODUCTION

There are two types of HIV known as; HIV1 and HIV2. Human immunodeficiency virus type 1 (HIV-1) is the main cause of HIV disease, the end stage of it is the acquired immunodeficiency syndrome (AIDS). In Iran, nearly all of case reports of the HIV infections are due to HIV-1 (Sarrami-Forooshani *et al.*, 2006). This virus population is characterized by extensive genetic variability that gives rise to many groups and subtypes. At present, HIV-1 classified into three phylogenetically distinct groups consists of Major (M), Outlier (O), and non-major, non-outlier (N). Among these groups, group M are the most prevalent and the main cause of worldwide epidemics. This group, further subdivided into subtypes A-D, F-H, J and K, sub-subtypes consists of unique recombinant forms (CFUs) and 43 circulating recombinant forms (CRFs). Subtypes A, B and C and circulatory recombinant forms of CRF35_AD, A1D and AD of HIV-1 has been shown to be the preva-

lent subtypes among Iranian populations. Among these subtypes, the CRF35_AD is one of the highly prevalent forms of HIV-1 in Iran and Afghanistan that should be more investigated (Soheilli *et al*., 2009).

Gag polyprotein from HIV-1 is one of the most conserved viral proteins. Gag derived proteins consisting Capsid (CA) or p24 or Nucleocapsid (NC), and Matrix (MA) or p17 that are synthesized by proteolytic cleavage of gag polyprotein, are the essential components of the HIV-1 virion and therefore have the great importance as potential targets of therapeutic and vaccine studies (Naderi *et al*., 2006; Ono and Freed, 1999; Cannon *et al*., 1997; Schulte *et al*., 1988). p24 (CA) is the most immunogenic protein of the virus and appears in the blood at the early stages of the disease. This protein is an integral component of most diagnostic kits and is used as a prognostic marker for HIV infections (Urnovitz *et al*., 1997; Schulte, Meurer *et al*., 1988). P17 or matrix protein is strongly antigenic as p24 and recently, various articles reported that p17 antibody levels are better serological marker of disease progression than p24 antibody levels (Metha *et al*., 1990; Lange *et al*., 1987). It has been reported that detection of antibodies to p17 along with p24 antigen detection could shorten the window period in the diagnosis of HIV infection (Hashida *et al*., 1996).

In previous studies we isolated a clone of HIV-1 CRF35_AD gag gene and expressed the p24 coding region in *E. coli* successfully. In the current study we expressed p17 coding region of the same isolated gag sequence in *E. coli*. P24 antigen can be used for production and purification of antibodies for the detection of p24 antibodies, meanwhile p17 can be used for double antigen sandwich enzyme immunoassay (DAGS-EIA). Taking the advantage of antibodies against p24 protein for p24 antigen detection and sensitive antibody detection using naturally folded p17 antigen, fourth generation sensitive EIA kits can be developed for serodiagnosis of HIV-1 at low costs. This cheap and sensitive enzyme immune assay serodiagnostics are highly needed for national screening and control programs for HIV-1 epidemic (Hashida *et al*., 1996 a; Hashida *et al*., 1996 b; Hashida *et al*., 1998; Ishikawa *et al*., 1998; Tomala *et al*., 2010).

MATERIALS AND METHODS

Serum samples: HIV-1 positive serum samples were kindly provided by virology lab of the Iranian Blood Transfusion Organization (IBTO). All samples were confirmed to be HIV positive or negative by western blotting and PCR.

Viral RNA isolation and cDNA synthesis: We used viral RNA MiniKit (Qiagen, USA) to isolate viral RNA from HIV positive serum samples according to the manufacturers guide. cDNA synthesis was done under following conditions: 1 µg of isolated viral RNA were added to RT reaction mixture containing 40 units of MuLV RT enzyme, 100 pmol of random hexamer, 4 µl of 5x RT buffer, 20 units of Thermo Scientific RiboLock RNAse inhibitor, and DNTP mix 10 mM each (all from Fermentas, Lithuania) adjusted to the final volume of 20 µl. The mixture was then incubated for 10 min at 25°C followed by 60 min at 37°C. The reaction was terminated by heating at 70°C for 10 min.

Nested PCR and cloning into T-vector: To isolate gag gene coding sequence we used a consensus nested primer pairs (Table 1). We used pfu DNA polymerase (Stratagen, USA) for amplification of target sequence according to the recommendations of the manufacturer. The PCR product was A-tailed using Taq DNA polymerase (Fermentas, Lithuania) and purified using PCR Cleanup Kit (Roche, Germany). Purified PCR product was then cloned into PTZ57R (T) vector using Instaclone TA-cloning kit (Fermentas, Lithuania). All procedures were done according to the instruction manuals provided by kit manufacturers. Insertion of Gag sequence was confirmed by sequencing. The resulting recombinant vector bearing gag coding sequence from HIV-1 CRF35_AD was designated PTZ-gag53-IR and sequence was submitted to NCBI GenBank (NCBI accession No. HQ233645).

Vector and p17 coding region: The primer sets Fp17 (CACCATGGGTGCGAGAGCGTCA) and Rp17 (TTC-CTCTATTTTATCTAGAGCTTC) were designed for amplification of p17 coding sequence from PTZ-gag53-IR. CACC sequence at the start of forward primer gained from topo cloning instruction for the insertion of blunt end PCR product into TOPO cloning vector in the right direction. DNA encoding p17 was PCR amplified by pfu DNA polymerase (Stratagen, USA) and inserted into the pET102D/TOPO vector (Invitrogen, USA) according to the manufactures instruction and transformed into BL21 (DE3) *E. coli* strain.

Expression of rp17: *E. coli* BL21 (DE3) transformed with constructed expression vector was cultured

overnight at 37°C in LB medium containing 100 ng/µl ampicillin. 500 µl of overnight culture was added to 50 µl of LB medium and was grown in shaker incubator (150 rpm) in 37°C until the logarithmic phase was reached (at OD_{600} of 0.5-0.6). At this point *E. coli* BL21 (DE3) cells transformed with constructed expression vector were induced by IPTG at a final concentration of 0.1 mM for 4 h in 37°C. After 4 h cell culture were centrifuged at 5000 RPM (Eppendorf 5804) for 10 min. Culture pellet was harvested and used for subsequent analysis using western blot and SDS-PAGE.

Purification of recombinant protein: Recombinant protein purification was done using Ni^{2+}-NTA resin column that employed an in ProBond purification system (Invitrogen, USA) according to the manufacturer's guide. Purity of the purified protein was assessed using electrophoresis on the 15% polyacrylamide gel and Commassie blue staining. The concentration of purified protein was assessed by Micro BCA protein assay kit (PIERCE, USA).

Enzyme immunoassay for immunoreactivity of rp17: 100 µl of 40 µg/ml rp17 in PBS (ph: 7.3) was used for coating ELISA microplates (Nunc, Denmark). After overnight incubation at 4°C the microplate was slap dried and then 250 µl blocking reagent (PBS ph: 7.3 containing 1% BSA and 1% Tween 20) was added to each well. Then 3 washes with 350 µl of wash buffer (0.1% Tween 20 in PBS) were applied. At this point 200 µl of diluted serum samples (1:250 in PBS) were added and incubated for 30 min and washed subsequently with 350 µl of wash buffer 2 (1% Tris 0.05 M containing 0.1% Tween20) 3 times. Mouse antihuman-ALP conjugate was then added and incubated at room temperature for 30 min. 3 times washing were applied and finally 100 µl of PNPP was added to each well. After 30 min OD_{450} was determined using ELISA reader (Biochrome Ltd., UK). For determining the immunoreactivity of produced rp17, ELISA test with 40 serum samples from HIV-1 infected and 40 control serums was carried out.

RESULTS

PCR ampliConstruction of p17 prokaryotic expression vector: The blunt end DNA encoding p17 protein was PCR amplified as described above (Fig. 1) and then directionally inserted into PET102D/TOPO vec-

tor according to manufacturer's instructions. The constructed vector was sequenced by the forward and reverse primers used for the amplification of p17 coding region and sequencing result of cloned sequence showed that it belongs to p17 coding sequence of CRF35_AD subtype of HIV-1 virus which is highly prevalent in Iran and Afghanistan. Computational ORF analysis showed that p17 coding region was inserted in-frame next to N-terminus HP-thioredoxin fusion and C-terminus V5 epitope and (6X) His-tag.

Expression of p17 recombinant protein: In this study rp17 was expressed in fusion with (6X) His-tag and HP-thioredoxin that increased the molecular weight of the product from 17kDA to near 29kDA. In SDS-PAGE analysis, after induction, a distinct band of approximately 29 kDa was seen. Meanwhile, there was no expression of rp17 fusion protein in transformed BL21 (DE3) without IPTG induction and BL21 (DE3) alone. SDS-PAGE analysis and subsequent Coommassie blue staining of soluble fraction and cell debris pellet was done and nearly all of the expressed recombinant protein was found in the soluble fraction, suggesting that the His-tagged p17-thio protein was mainly in soluble form (Figs. 2A and 2B).

Purification and identification of p17 recombinant protein: Concentration of the purified recombinant protein as measured by Micro BCA protein assay kit (PIERCE, USA) was seen to be 150 ng/µl. For the identification and confirmation of produced rp17, western blotting with anti-His Tag and HIV+ serum of
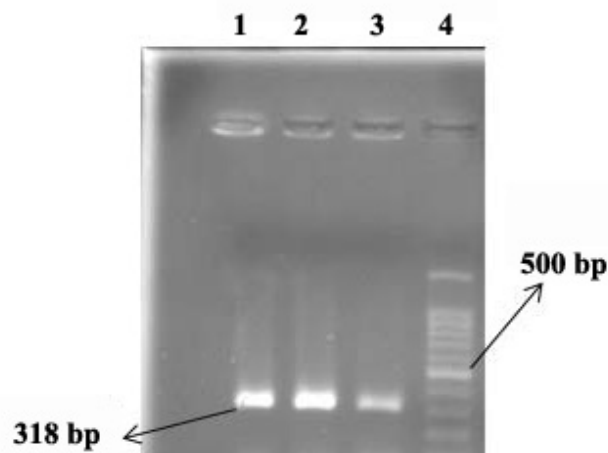

Figure 1. PCR amplification of HIV p17 coding sequence. Lane 1 and 2, the amplified product of HIV p17 by taq DNA polymerase (318 bp); lane 3, the amplified product of HIV p17 by pfu DNA polymerase; lane 4, DNA marker 100.
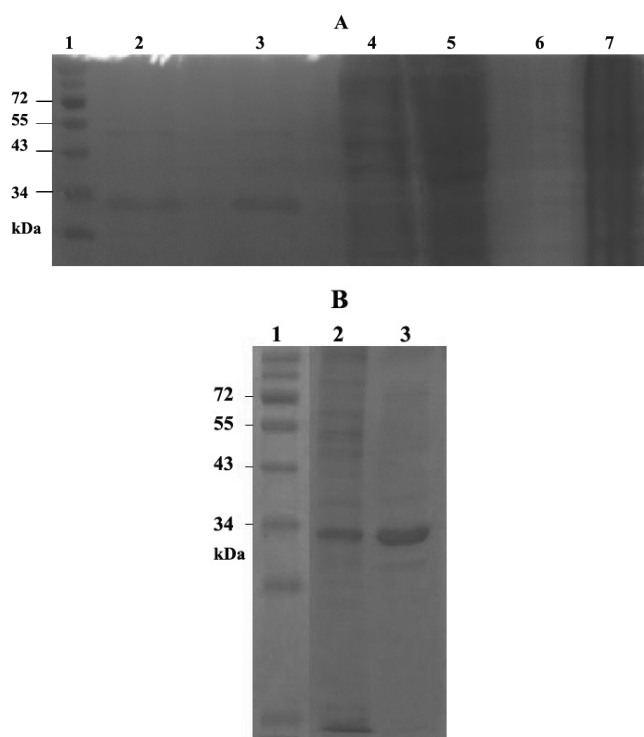
Figure 2. A: the SDS-PAGE analysis of recombinant thioredoxin-p17 fusion protein. Lane 1, protein molecular marker; lane 2, Supernatant of BL21 (DE3) crude cell lysate harboring recombinant plasmid after induction with IPTG (about 29 kDa) after centrifugation; lane 3. Recombinant p17 protein purified by the Ni^{2+}-NTA agarose affinity gel; lane 4, *E. coli* BL21 (DE3) Cell lysate harboring recombinant plasmid before induction with IPTG; lane 5, host cell *E. coli* BL21 (DE3) (as negative control). Control; lane 6, empty; lane 7, cell extract of *E. coli* with recombinant plasmid (pellets). B: The SDS-PAGE analysis of the solubility of the recombinant thioredoxin-p17 fusion protein. Lane 1, protein molecular marker; lane 2, supernatant; lane 3, purified HIS-p17-thio.

HIV infected individuals were carried out. Clear bands corresponding to 29 kDa molecular weight were observed (Figs. 3A and 3B).

Immunoreactivity analysis of p17 recombinant protein: Results showed that produced rp17 specifically reacted with HIV-1 infected serum samples (positive for 37 out of 40 HIV-1 infected serums) and did not react with any HIV-1 negative control serums. The samples were provided by virology lab of the Iranian Blood Transfusion Organization (IBTO) and all of them were confirmed to be HIV positive or negative by commercially available kits for western blotting and PCR.

Statistical analysis was performed by ROC analysis and the cut-off (CO) value set to 0.715 according to the Youden's Index (CO is equal to the point where sensitivity+specificity-1 reaches the highest amount).

The immunoreactivity of produced recombinant p17 to sera from infected individuals by ROC analysis showed 93.8% sensitivity and 100% specificity.

DISCUSSION

Rp17 is one of the viral proteins which have been widely used as a part of serodiagnostic kits for the diagnosis of HIV-1 infection. Many reports are around use of rp17 for sensitive diagnosis of antibodies raised against HIV-1 after infection (Ishikawa, Hashinaka *et al.*, 1998; Lange, De Wolf *et al.*, 1987; Metha, Rupprecht *et al.*, 1990). In a report by Hashida *et al*, they used detection of antibodies against this antigen along with p24 antigen detection for early and highly sensitive diagnosis of HIV-1 infection with immune complex transfer EIA (Hashida, Hashinaka *et al.*, 1996; Hashida, Hashinaka *et al.*, 1996; Ono and Freed 1999). In another report the sensitivity of antibody detection using recombinant p17 antigen was estimated to be 41% in asymptomatic carriers, 30% in patients with ARC (AIDS-related-complex) and 14% in patients with AIDS (Gupta *et al.*, 2001). Currently, p17 is one of the components of several commercially available kits for the serodiagnosis of HIV-1 infection. In the current study, for production of recombinant p17 protein we used the sequence from isolate IBTO-1 sequence cloned in a vector PTZ-gag53-IR (NCBI accession No. HQ233645) and cloned p17 coding sequence in a thioredoxin-fusion expression vector for T7 promoter controlled expression in *E. coli*. N-terminus thioredoxin fusion will promote protein folding, expression and solubility as proved by others (Sun *et al.*, 2011; Tomala, Lavrentieva *et al.*, 2010; Wang *et al.*, 2010). Furthermore, increased solubility will decrease the formation of inclusion body and will ease the purification of recombinant proteins. P17 was successfully expressed in *E. coli* strain BL21 (DE3) and western blotting and Coomassie blue staining confirmed the proper expression. Single-step purification of rp17 was successfully done using nickel affinity column and the purity of the product was estimated to be more than 95% by SDS-PAGE and subsequent staining by Coomassie blue. Immunoreactivity of rp17 was comparable to those achieved during similar studies by Gupta *et al*. (2001) which showed that rp17 produced in the current experiment is a qualified antigen for serodiagnosis of HIV-1 infection. Low cost of producing biologicals in bacteria for diagnostic and therapeutic purposes was also consistent with our study.
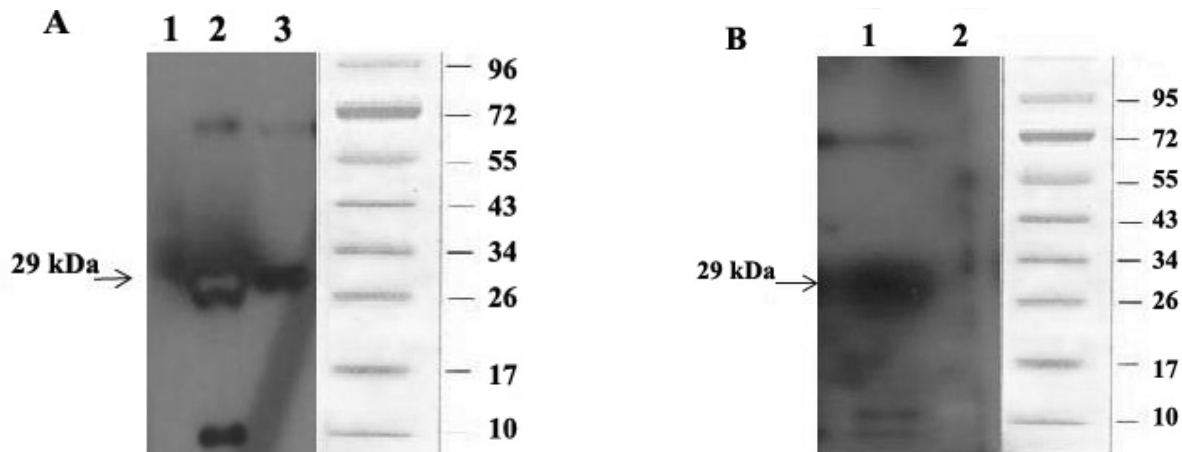
Figure 3. A, Western blotting analysis of the recombinant p17 protein with mouse anti-HIS tag. Lane 1, host cell without recombinant plasmid (control negative); lane 2, cell lysate harboring recombinant plasmid after induction; lane 3, purified recombinant p17. B, Western blotting analysis of the recombinant p17 protein with HIV infected sera. Lane 1, purified recombinant p17; Lane 2, host cell without recombinant plasmid (control negative).

CONCLUSIONS

Highly expressed rp17 in this experiment can provide a cheap and highly qualified candidate for use in the diagnostic kits for the serodiagnosis of HIV-1 infection. Accompanied with p24 antigen detection, detection of antibodies using this recombinant antigen can provide a platform for early and sensitive screening and detection of HIV-1 infection in national screening programs. Recombinant antigens are also a part of subunit vaccines. Although we have not studied the potency of this antigen for the induction of immune response, but it is expected that this can be a potential source of recombinant antigen for further studies.

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