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Original Article

Designing a non-virulent HIV-1 strain: potential implications for vaccine and experimental research

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Abstract

BACKGROUND: Culturing and working with wild type HIV virions may produce contamination and infection. The main objective of this study was to design a non-infective HIV-1 strain that can be used safely in research laboratories for various research topics on HIV life cycle and pathogenesis.

METHODS: Non-infective HIV-1 strain (mzNL4-3) was designed by deleting a 2 Kb sequence of HIV-1 (NL4-3 strain) genome that codes reverse transcriptase (RT) and integrase (IN) enzymes.

RESULTS: The deletion removed 95% of RT and 34% of IN peptide sequences and abolished the functions of these enzymes totally. This deletion didn't produce any other alteration in HIV genome, not only in mRNA level but also in transcription or translation levels. We named this strain, mutated z NL4-3 (mzNL4-3).

CONCLUSIONS: Our mutated HIV-1 virions can be produced by transforming any mammalian cell line with mzNL4-3 vector (pmzNL4-3) but these virions can not replicate in any competent target cell. Hence, it can be used for many of the HIV-1 researches in a level 2 lab. mzNL4-3 has also the major antigen markers of HIV-1, NY5 and LAV strains, which are the most common strains of HIV-1. Therefore, mzNL4-3 can also be considered as a choice for HIV-1 vaccine investigation. Probably mzNL4-3 could be used for lab research and vaccine investigation.

KEYWORDS: HIV-1, mzNL4-3, non-infectious, mutant, safe research, vaccine.

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Human immunodeficiency virus (HIV) is RNA virus and a member of retroviruses family. HIV can transcribe its RNA genome to DNA, and then integrate it to the host cell genome using reverse transcriptase (RT) and integrase (IN) enzymes [1]. Infection with HIV causes acquired immunodeficiency syndrome (AIDS) in human, which is a chronic immune deficiency illness with severe decrease in CD4 cells count [1]. CD4 cells are the main target cells for HIV virions and depletion of CD4 cells during HIV infection causes chronic immune deficiency, which results in death from opportunist infections and tumors. Working with wild type HIV virions during research procedures may impose serious infection danger. Levels 3 & 4 labs are needed for research on the wild type HIV and there are many problems to set up these labs. Using a non-infective HIV strain for research procedures could be a solution for this problem and also a choice for vaccine investigation.

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During assembly process of HIV virions, they package two copies of their full length viral mRNA [2-5]. After recognition, virions adherence and fusion of the envelope to the target cells membrane, the inner capsid pushes into the cytoplasm [6]. Intermediating some host cytoplasmic factors uncoats the inner capsid [7-9] and releases the pre-integrating complex (PIC) to the cytoplasm of target cells. The PIC is made of contents of inner capsid and host cells factors [6]. The HIV RNA genome, RT and IN enzymes are the major parts of the PIC [6]. Mammalian cells transcription machinery can not read the RNA genome of HIV, thus HIV needs to convert its genome to dsDNA [6]. The role of RT, which exists in PIC as hetero-dimmer form, is to synthesize the genomic dsDNA [6,10]. The role of IN is to form two dimmers. Each dimmer interacts to one end of the provirus dsDNA and then two dimmers bind together to form a tetramer. The IN tetramer takes two ends of provirus close together. This tetramer binds to the target cell chromosomal DNA and takes two ends of virus DNA close to the cellular DNA and triggers the insertion or integration of provirus to the cellular DNA [6,11]. The transcription and translation machinery of the cell can recognize and read the integrated provirus and begin to produce the viral mRNAs and proteins.

Researches have demonstrated that, mutations in the RT or IN sequences of pol gene can block the replication of HIV virions in target cells. Only a point mutation on these sequences is sufficient to abolish these functions of enzymes. Hence, the large deletion in these enzymes can totally block virions replication [6,12-15]. This non-reversible large deletion mutation deletes 2 Kb of HIV-1 genome in the polymerase (pol) gene that codes the pol part of gag-pol polyprotein. The mutation deletes nucleotide number 2620 to 4551 of the HIV genome. The sequences that are codes of the RT and IN enzymes are in pol gene and they are adjacent together. There is no overlapping in the RT and IN sequences with other sequences in pol gene (figure 1).

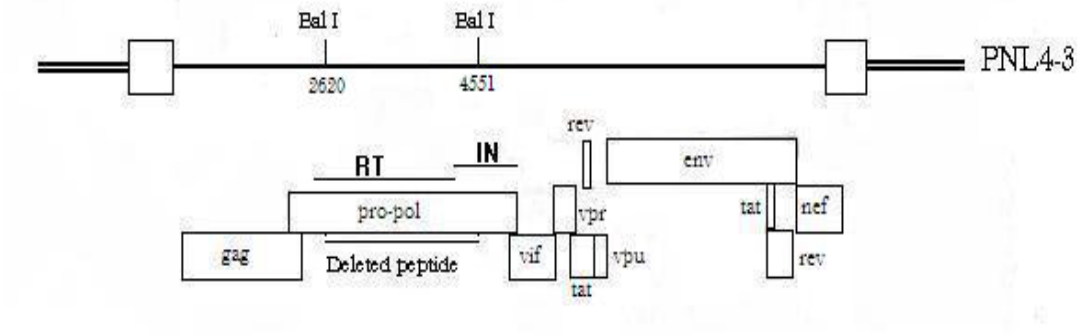

Figure 1. There is no overlapping between the deleted sequence of mzNL4-3 in RT and IN sequences from nucleotide 2620 to 4551 (restriction sites of Bal I restriction enzyme) and the other HIV provirus sequences. For more details about the HIV-1 mRNAs, splicing sites, translation sites and the mRNA processing sites of HIV mRNAs see the reference number 6.

The deletion mutation deletes 1610 nucleotides of RT sequence at its 3´ (the whole RT sequence was 1680 nucleotides). In fact, 96% of the sequence that codes the RT enzyme is deleted and the remained 4% of RT sequence is at its 5` end. This deletion abolishes the total function of RT enzymes [12-14]. In addition, the deletion mutation also deletes 320 nucleotides of IN sequence at the 5` end of IN sequence. In fact, 34% of IN amino acids has been deleted from amino terminal of IN enzyme peptide (97 out of 288 amino acids). HHCC domain is a zinc finger structure of IN and is needed for interaction of IN with the newly synthesized HIV provirus ends and this interaction is needed for integration of HIV provirus to the

cellular genome [6,15]. Following this deletion, the total HHCC domain (zinc finger) of IN enzyme and a small part of the central catalytic core of this enzyme would be deleted. This deletion would disrupt the function of IN [15]. In these mutated virions the PIC is not functional any more because of the absence of RT and IN enzymes and this PIC can not synthesize the provirus and integrate it into the cellular DNA [15]. After the translation of virion protein, the assembling of virion particles is the main step. The newly budded virions are immature and the activation of viral protease, triggers the formation of mature HIV-1 virion particles [6,16]. Some covalent interactions between parts of viral gag polyproteins and the viral RNA genome are needed for assembling the newly virion particles under the plasma membrane surface. E/DLS (encapsidation/dimerization linkage site) structure in the 5` end of RNA genome is a secondary RNA structure, which is needed for the recognition of the RNA genome by gag. The nucleoprotein NC of gag is responsible for formation of this structure (NC is a nucleic acid chaperon) [6,17-19]. Existence of these factors is needed for budding newly virion particle from the surface of host cell and no one of these factors is affected by the deletion mutation. Previous studies showed that deleting *RT* and *IN* sequences would not block virion production. It seems that mRNA level and transcriptional and translational products are intact in these mutants so that they could produce new virions [14,15]. Preceding research has demonstrated that the 7 Kb genome lengths are sufficient for correctly assembling and budding the HIV virions from host cell surface. Thus, the 7 Kb genome length of mutated Z NL4-3 (mzNL4-3) could be sufficient for the budding of virions [20].

Methods

Plasmid : pNL4-3 which is a recombinant proviral clone and contains DNA from HIV-1 NY5 and LAV strains (the most common strains of HIV-1), in pUC18 vector (the vector of NL4-3) was used in this project. In the recombinant strain NL4-3, gag-pol polyproteins sequence is from NY5 and the virion surface protein ENV sequence is from LAV strain. This vector after transformation to mammalian cell lines can code and trigger the production of the wild type HIV-1 virions (figure 2). Five µg of this plasmid was obtained through the NIH and then transformed into the $CaCl_2$ treated competent E. coli DH5alfa cells [21]. Transformed bacteria were then detected using ampicillin positive LB agar culture. To provide high amounts, plasmids were then miniprepped.

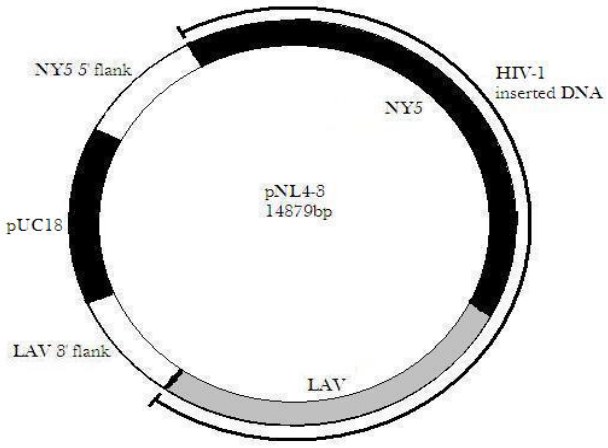

Figure 2. pNL4-3 is a recombinant proviral clone that contains DNA from HIV-1 NY5 and LAV. NY5 integrated proviral DNA from a SmaI site about 1.5 Kb in the 5` flanking site at 5.8 Kb plus LAV integrated proviral DNA from the EcoRI site extending to 1.5 Kb into 3` flanking cellular DNA was blunt end cloned into pUC-18 after removing fragment of pUC-18 that contained the pUC polylinker site.

Digestion and Ligation: To prepare deletion mutation, the MLS I (Bal I) restriction enzyme has been applied (rapid ligation kit of Fermentas). The Bal I restriction enzyme can recognise and cleave TGG↓CCA sequences. This restriction enzyme has two restriction sites on the pNL4-3 vector and can cleave the nucleic acids number 2620 and 4551 of this vector. The digestion reaction was done on 100 ng of pNL4-3 DNA using 10 U of Bal I (2 µL of 5 U/µL glycerol solution). Digestion reaction was performed for 5 hours at 37^{oC} in a 20 µL volume reaction using 2 µl of 10× buffer (10mM tris-HCl, pH 8.5 at 37^{oC}, 10 nM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA). The digestion result was fractionated on 0.7% agarose. The 12 Kb band (pmzNL4-3) was cut out of the gel and DNA was extracted using silica powder method. The extracted DNA was eluted in 40 µl deionised water. Ligation reaction was performed using rapid ligation kit (Fermentas) in a 50 µl reaction volume containing 10 µl (18.2 ng) mzNL4-3 plasmid, 1 µl T4 DNA ligase and 5µl of 10× ligation buffer. The reaction was incubated for 1 hour at 37^{oC}. Ten µl of ligation reaction (4 ng pmzNL4-3 plasmid) was used for transformation of $CaCl_2$ treated competent DH5alfa E. coil cells. Transformed bacteria were detected using ampicillin positive LB agar culture and then miniprepped and stored at -70^{oC} in 10% glycerol LB stock solution.

Confirming Digestion: To confirm that the 2Kb sequence is not present in pmzNL4-3 plasmid colon, DNA was extracted from 10 colonies of pmzNL4-3 transformed bacteria by miniprepped method. The confirming digestion reaction was done on the 200 ng of 10 pmzNL4-3 plasmid colonies. 130 ng of the pNL4-3 was also used as positive control. The digestion reaction was done by 10 U of Bal I in a 20 µl volume containing 2 µl reaction buffer (10 mM tris-HCl, pH 8.5 at 37^{oC}, 10 nM $MgCl_2$, 100 mM KCl, 0.1 mg/ml BSA). Digestion reactions were incubated for 1 hour at 37^{oC} before fractionation on 0.7% agarose.

Confirming PCR: Deletion was confirmed using PCR method. This was to make sure that the mutated plasmid colon was not contaminated with original pNL4-3 plasmid. Primers that were used in this step were specific for two sites inside the deleted sequence (table 1).

Table 1. Primers used for confirming the deletion mutation, were specific for two sites inside the deleted sequence. This amplified product is between nucleotide number 2697 and 4455.

Specific site	Primer sequence
2697S	5'-tcaggcctgaaaatccataca
4455A	5'-tatccactggctacatgaact

The PCR program was 1 cycle of 94^{oC} for 2 minutes followed by 30 PCR cycles of 94^{oC} for 45 seconds, 54^{oC} for 45 seconds and 72^{oC} for 2 minutes and one cycle of 72^{oC} for 5 minutes. The PCR was performed in a reaction mixture containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 125 ng of each primer and 1 U DNA polymerase (AmpliTaq; final volume, 25 µl). The PCR products were then fractionated on the 0.7% agarose gel.

Results

Fractionating of digestion reaction demonstrated two bands in the size of 12 Kb and 2 Kb. The 12 Kb band was the pmzNL4-3 and the 2 Kb band was the removed RT and IN sequence of the pol gene. Figure 3 shows that the 2 kb DNA was removed from the pNL4-3 vector.

The 12 kb band was cut out of the gel and the pmzNL4-3 DNA was extracted from the gel. Then, this plasmid was transformed into the $CaCl_2$ treated competent E. coli cells. Transformed bacteria were then detected using ampicillin containing LB agar culture. Plasmids were amplified and miniprepped in order to provide high amount of the plasmid. The extracted plasmid from this clone was checked for deletion mutation using digestion reaction and PCR. The deletion was confirmed by another Bal I digestion reaction. Then, digestion result was loaded on 0.7% agarose gel. The result is shown in figure 4.

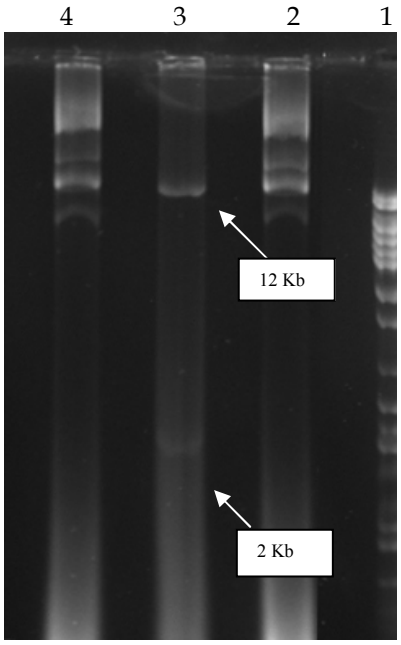

Figure 3. Digestion of pNL4-3 with Bal I restriction enzyme produced a 12 Kb (pmzNL4-3) band and a 2 Kb which is part of RT and IN sequence of pol gene (lane3). Lanes 2 and 4 are undigested pNL4-3 control plasmids and lane 1 is 14 Kb DNA size marker. The 12 Kb (pmzNL4-3) and the 2 Kb bands are marked by arrows.

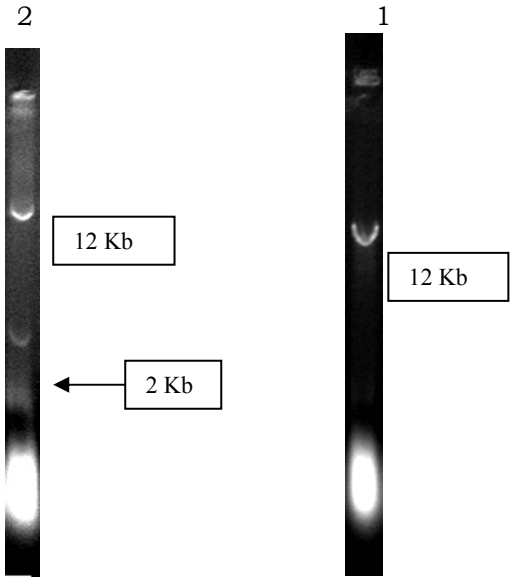

Figure 4. The digestion reaction results of pmzNL4-3 and pNL4-3 are fractionated on the 0.7% agarose gel. The lane 1 is the digestion result of pmzNL4-3 and the lane 2 is the digestion result of pNL4-3. A 2 kb band (which is marked with arrow) is seen in the pNL4-3 digestion reaction (lane 2) but this band was not seen in pmzNL4-3 digestion reaction (lane 1). The 12 Kb band is seen in both lanes, which is the linear DNA of pmzNL4-3.

The PCR method was used as a second way for checking the purity of pmzNL4-3 clone. The PCR was performed using a pair of primers that were specific for two sites in the deleted sequence in Pol gene of pNL4-3. Using this method a pure clone of pmzNL4-3 was selected as the source of virion producing vector. The confirming results of the deletion mutation as well as the purity of pmzNL4-3 clone are shown in figure 5.

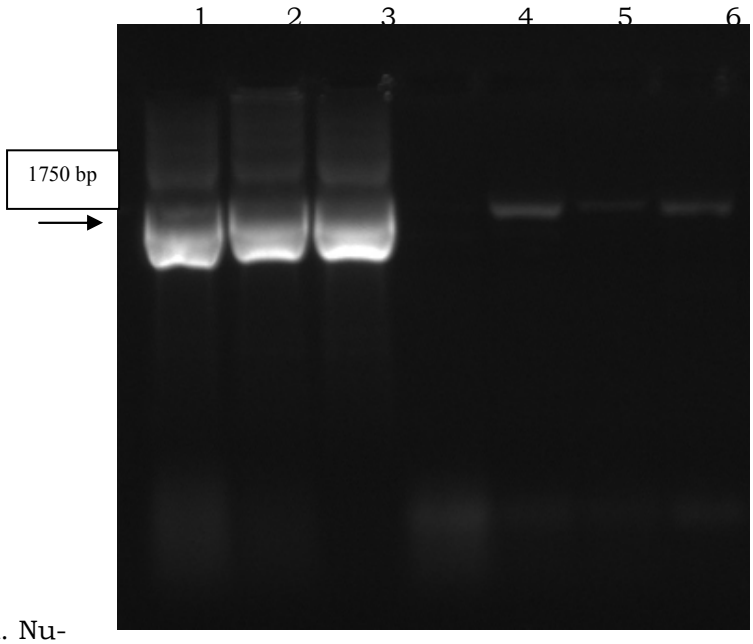

Figure 5. Confirming the deletion by PCR. Nucleotide 2697 to 4455 of the pNL4-3 plasmid was amplified. This part should not be present in pmzNL4-3. The absence of the 1750 PCR product in the pmzNL4-3 PCR confirms the deletion mutation. After fractionating the PCR products on agarose gel, the 1750 bp band (which is marked with arrow) was seen in the PCR products of pNL4-3 (the lanes 1 to 3) but this band was not seen in the PCR products of pmzNL4-3 (lanes 4-6).

Discussion

Previous studies have prepared wide range of HIV mutants for investigating the role of each part of virion particle. Many of these mutants are non-infectious, weakly infectious or have new pathogenic specifications. RT and IN genes are two of the HIV genes that researchers altered them to investigate their functions during the virions replication [12-15]. In many cases, only a point mutation in the catalytic domain of these genes can abolish or highly reduce their functions. Hence, these genes are among the best choices for designing a non-infective HIV strain. The study of Zhu et al showed that Cys residues generally play crucial role in integrase protein structure and function. They introduced Cys-to-Ser substitutions at positions 56, 65, and 130 of HIV-1 integrase to determine their effects on integration activity and viral replication. When introduced into the NL4-3 molecular clone of HIV-1, mutant viruses encoding Cys mutations at positions 56 and 65 of integrase replicated similarly to the wild-type virus in CD4 (+)-T-cell lines, whereas the C130S-containing virus was non-infectious [22]. Nakamura et al examined the effect of mutations in the HHCC motif on viral infectivity. The results suggested that the replication of HIV-1 carrying point mutations in the HHCC motif was blocked at the step after adsorption/entry and prior to the initiation of reverse transcription, presumably at the uncoating step. Furthermore, electron microscopy revealed that the observed complete lack of viral infectivity caused by introducing an amino acid substitution into the HHCC motif is not always accompanied by apparent abnormal morphology or maturation of virus particles [15]. Diallo et al and Wei et al studied the ability of HIV constructs containing the M184V substitution in RT. The findings provided biological and biochemical evidence that M184V-containing viruses are impaired in replication fitness [23-25]. The study of Frankel et al on RT mutant has shown that, L74V-containing virions in their RT display diminished replication capacity and that this is associated with reduced levels of synthesis of early reverse-transcribed viral DNA molecules [26]. RT is a heterodimer of 66-kDa and 51-kDa subunits. The latter is formed by HIV PR-catalyzed cleavage of p66 during virion maturation, resulting in removal of the RNase H (RNH) domain of a p66 subunit. In order to study the apparent need for RT heterodimers in the context of the virion, Abram et al introduced a variety of mutations in the RT p51-RNH protease cleavage site of an infective HIV-1 molecular clone. Repeated passage of MT-2 cells exposed to mutant viruses led to the appearance of virus with improved replication capacity [27]. There is no previous study, which deleted the RT and IN sequences. The above-mentioned studies made some point mutations in the RT and IN genes. The results of these studies showed that point mutations are sufficient for disinfection of virions. This is because of the strong role of HIV-1 IN and RT proteins during the virions life cycle. Considering these results, it is highly possible that deletion of these two enzymes abolishes the virus replication.

A non-infective mutant of HIV that has the all abilities of wild type HIV for producing the virion proteins, assembling the virion particles, producing the mature virions, recognition of target cells, adhesion to target cells and uncoating the inner capsid can be used for lab researches with no lab contamination and infection danger. We have designed a mutation for HIV-1 genome, which does not abolish the above functions but makes the virions non-infectious. It is possible to work on this mutated non-infective HIV strain in a common level 2 lab. This deletion mutation was done in wild type recombinant HIV-1 NL4-3 strain to create a lab safe HIV strain (mzNL4-3). Structural health of mutated virions and effective production of virions in cell culture were two major advantages of this non-infective strain in addition to the safety. Therefore, this non-infective HIV strain could be used for the wide range of researches on HIV life cycle or even on the pathogenesis of HIV with safety. This mutated HIV strain can not support the researches that are going to be done on the re-

verse transcription and integration steps of HIV life cycle, because this strain does not have the RT and IN enzymes. The mzNL4-3 virions will be produced after transformation of any mammalian cell lines by pmzNL4-3, which is the mzNL4-3 vector. The mature mzNL4-3 virions would be the same as wild type virions. The major antigens of HIV-1 exist in mzNL4-3 and these mutated virions would have the same abilities for recognition and adhesion to target cells. After recognition of competent target cells, entry and uncoating the inner capsid, these non-infective virions can not replicate because of the defect in reverse transcription of genomic RNA and viral DNA integration into the host cell genome.

Safety of working on HIV is the main problem of research on this virus not only in Iran but also in most countries in the world. Therefore, mzNL4-3 is a great hope to solve this problem. The mzNL4-3 virions could be produced very easily by transforming any mammalian cell line with pmzNL4-3. Production of this clone was the first step for us and now we can work on the details of our second and third research phases. The second phase of our project is to produce the mzNL4-3 virions and test the infectivity of virions. The third phase of our work is to compare the specifications of mzNL4-3 and wild type NL4-3 virions. If the mzNL4-3 virions are produced correctly in the second and third phase, it is possible to use mzNL4-3 for vaccine design investigations. The genes of the major antigen markers of the two most common HIV strains, NY5 and LAV are present in mzNL4-3. The mzNL4-3 virions are non-infectious so they can be a choice for the recombinant vaccine investigation. McBurney et al [14] designed RT negative HIV-1 virions and showed that they are safe and could be used for clinical trial of HIV vaccine. Next step in our research plan is to investigate the potential of this clone for production of HIV recombinant vaccine.

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