رهیافت های نوین در علوم سلولی و مولکولی
JNACMS
دوره ۳ شماره ۱ بهار ۱۴۰۴
Journal homepage: https://sanad.iau.ir/journal/nacms

کلونینگ و آنالیز فیلوژنتیکی ژن gag ویروس نقص ایمنی گاوی در جدایه‌های ایرانی

الهه تاج بخش*

۱. گروه زیست شناسی، واحد شهرکرد، دانشگاه آزاد اسلامی، شهرکرد، ایران.

چکیده

ویروس نقص ایمنی گاوی عضوی از خانواده رتروویریده، جنس لنتی ویروس، دارای سه ژن مهم به نام‌های *gag*، *pol* و *env* است محصول PCR ژن *gag* ویروس نقص ایمنی گاوی جدا شده از مناطق مختلف ایران، در ناقل pTZ57R/T کلون شد؛ سپس قطعات الحاقی توسط آنزیم‌های محدودکننده BamHI و XhoI هضم و سپس در ناقل بیانی pGEX-4T-3 کلون شدند. تجزیه و تحلیل توالی‌های ژن *gag* ویروس نقص ایمنی گاوی به دست آمده از قطعه الحاقی انجام شد. توالی *gag*ویروس نقص ایمنی گاوی ایرانی با ۵ توالی متناظر دیگر از BIV جدا شده در کشورهای مختلف مقایسه شد. در نهایت، مشخص شد که تجزیه و تحلیل نوکلئوتیدی توالی‌ها، تنوعی بین ۰/۴ تا ۱۰/۶ درصد را نشان می‌دهد و رسم درخت فیلوژنتیکی دو خوشه را در آن آشکار کرد.

اطلاعات مقاله

تاریخچه مقاله:
دریافت:۱۴۰۴/۰۵/۰۳
پذیرش:۱۴۰۴/۰۵/۲۹
چاپ: بهار ۱۴۰۴

DOI:
doi.org/10.82415/NACMS.2025.1212999

کلمات کلیدی:
آنالیز فیلوژنتیکی، ویروس نقص ایمنی گاوی، ژن *gag* ، وکتور pTZ57R/T، وکتور pGEX-4T-3

* نویسنده مسئول: *Email*
ee_tajbakshh@yahoo.com

Introduction

Bovine immunodeficiency virus (BIV) is a lentivirus causing lymphadenopathy, lymphocytosis, central nervous system lesions, progressive weakness and emaciation (1-3). Studies have shown that BIV resembles human immunodeficiency virus (HIV) type1 and other lenntiviruses, e.g., equine infectious anemia virus and feline immunodeficiency virus, in its structural, genetic, antigenetic and biological properties (4-6).

Basically all lentiviruses infect monocyte/macrophage cells. Moreover, feline immunodeficiency virus (FIV), simian immunodeficiency virus (SIV) and human immunodeficiency virus (HIV) infect T cells and consequently, are mainly associated with clinical signs of immunodeficiency in the infected hosts (7-10).

Bovine immunodeficiency virus (BIV) is an enveloped virus 120–130 nm in diameter. The bilayer viral envelope, which containing the viral surface (SU) gp100 and transmembrane (TM) gp45 proteins, surrounds conical-shaped capsid (CA) and nucleocapsid (NC) structures that protect the BIV genome. The genome is composed of a capped and polyadenylated diploid RNA 8482 nucleotides in length that is closely associated with viral proteins p7 and p13 (11).

The lentivirus genome offers a complex structure including several regulatory/accessory genes that encode proteins, some of which involved in the regulation of virus gene expression (12).

The BIV proviral DNA is 8960 nucleotides long and resembles other retroviruses with the typical 5'–3' *gag*, *pol* and *env* gene arrangement. Besides, the major structural genes of BIV (*gag*, *pol* and *env*) encode polyprotein precursors to generate structural (Gag and Env) and viral enzyme (reverse transcriptase, integrase and protease) proteins. Furthermore, the *gag* (for group antigen-associated gene) gene, located downstream from the 5’ LTR, encodes the Gag (Gag Pr53) precursor protein. It also encodes a portion of the Gag-Pol (Pr170) precursor (13-15). In addition, the BIV genome has been cloned, and

the complete nucleotide sequence has been determined (5). Among the structural properties predicted by the nucleotide and amino acid sequences of BIV is, however, the core protein encoded by the *gag* gene.

The Gag precursor of BIV has been shown to have a molecular mass of 53KDa, and by analogy to HIV cleavage products, p17, p26 and p15, which are the matrix, capsid and nucleocapsid proteins, respectively (16). The *gag* gene product is also an important viral antigen that induces a strong immune response in infected cattle. Recently, a purified recombinant BIV Gag protein was used in an immunoblot assay to detect BIV antibodies in field bovine serum samples.

In this study, the *gag* gene of BIV from Iranian isolated viruses was cloned in *E.coli* and finally phylogenetic analysis of the gag gene was determined.

Matherial and Methods

1- Sample, plasmids and bacterial strains: The extracted DNA from buffy coat of five BIV infected cows isolated from shahrekord and isfahan, Iran, and showing positive molecular results based on PCR were selected to be cloned. Besides plasmids pTZ57R/T (Ins T/A clone PCR Cloning kit, Fermentas) and pGEX-4T-3 (Pharmacia) and *E.coli* strain JM107 (Fermentas) were used for cloning, sequencing and maintenance of DNA fragment. The required antibiotics were added to LB media according to the reference recommendation (14).

2-Primers design: Perimers were designed according to the published sequence for proviral genome of BIV (accession number: M32690.1). BIV-gag-F: 5'-GGATCCGAGGCCAGAGCTGATAAGGAA-3'contain *BamH*I site. Reverse primer, BIV-gag-R: 5'-CTCGAGATCCCACTACCCTACATGCT-3' contain recognition site for *Xho*I. The restriction enzyme sites (underlined) were also added to the primers for subsequent cloning procedure.

3- Gene amplification of *gag* : PCR was performed in a 50µl total volume containing 1µg of template DNA, 1 µM of each primer, 2 mM Mgcl2 , 200µM dNTP, 1x PCR buffer and 2 unit of Taq DNA polymerase (Roche applied science). In addition, the following conditions were provided for amplification: initial denaturation at 95ċ for 5 min, followed by 33

cycles of denaturation at 94ċ for 1 min, annealing at 59ċ for 60s and extension at 72ċ for 50s. The program followed by a final extension at 72ċ for 10 min. Further, the PCR product was analyzed by electrophoresis in 1% agarose gel in 1X TBE buffer and visualized by ethidium bromide staining on UV transilluminator. The PCR product was purified by High pure PCR product purification kit (Roche applied science) according to the manufacturer recommendation.

4- Cloning of *gag* gene: The PCR product was digested with *BamH*I and *Xho*I and ligated to pTZ57R/T and pGEX-4T-3, digested by the same restriction enzymes, using T4 DNA ligase (Invitrogen) at 14ċ overnight. *E.coli* JM107 competent cells were also prepared by calcium chloride method and were used for transformation of pTZ57R/T-*gag* and pGEX-4T-3-*gag* vectors, respectively. In addition, the transformed bacteria were selected by screening the colonies on LB media containing antibiotic. The suspected colony was further analyzed by sequencing and PCR (14).

5- Sequence analysis: The nucleotide sequences were edited using Edit View v.1.0.1 (Applied Bioscience, Australia) and the 5 sequences registered in GenBank (accession numbers: NC001413-USA , L64972-USA , EF661980-IRAN, EF661988-IRAN , AY426724-India) were aligned separately using the Clustal W v1.81 in order to obtain a consensus sequence. Subsequently, the sequences were analyzed using the BioEdit package v.7.0.4.1 to compare the nucleotide sequences.

The nucleotide sequence of the Iranian BIV *gag* gene was then compared with the corresponding sequences from other regions of the world. An unrooted dendrogramme was constructed using the Njplot software. Fainally, statistical support for the dendrogramme was obtained by bootstrapping using 1000 replicates.

Results

DNA amplification: The proviral DNA of BIV virus from buffy coat of 5 BIV infected cows in Iran was prepared and used as template for amplification and cloning of the *gag* gene. The amplified fragment had the expected size of 754bp comparing to 1kb DNA ladder (Fermentas) (Figure1)

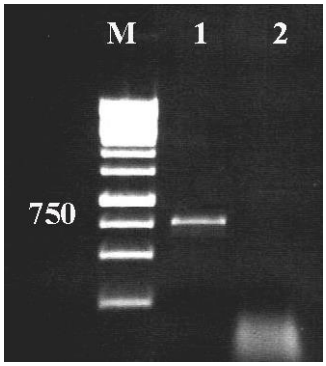

Figure 1- *gag* gene amplification by PCR (Lane M, Molecular weight marker 1 kb DNA ladder; Lane 1: amplified gag gene in sample; Lane 2, Negative control)

PCR product cloning: The purified PCR products were cloned in pTZ57R/T and pGEX-4T-3 vectors and digestion with *BamH*I and *Xho*I enzymes. Figure 2 shows recombinant plasmids after digestion.

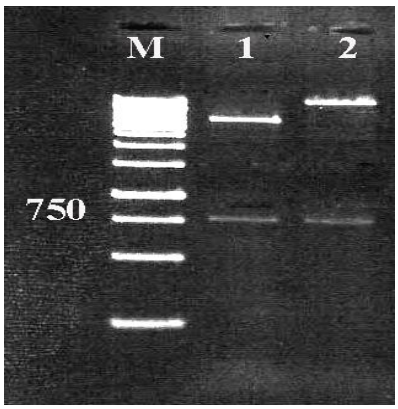

Figure 2- Restriction enzyme analysis of recombinant pTZ57R/T and pGEX-4T-3 plasmids (Lane M, Molecular weight marker 1kb DNA ladder; Lane 1, digestion of recombinant pTZ57-R/T plasmid; Lane 2, digestion of recombinant pGEX-4T-3 plasmid)

The recombinant plasmids (pTZ57R/T-*gag* and pGEX-4T-3-*gag*) were sequenced by specific primers and Sanger sequencing method (Macrogen, Korea). The sequencing result was confirmed through being compared with databases and also using basic local alignment search tool (BLAST) software. The result of sequencing of the *gag* fragment using ClustalX (1.81) software was aligned with some of the registered sequences in Genebank such as the sequences of this gene in India and USA. After comparing differences and similarities by using Njplot software, the phylogenetic tree was drawn which is shown in figure 3.

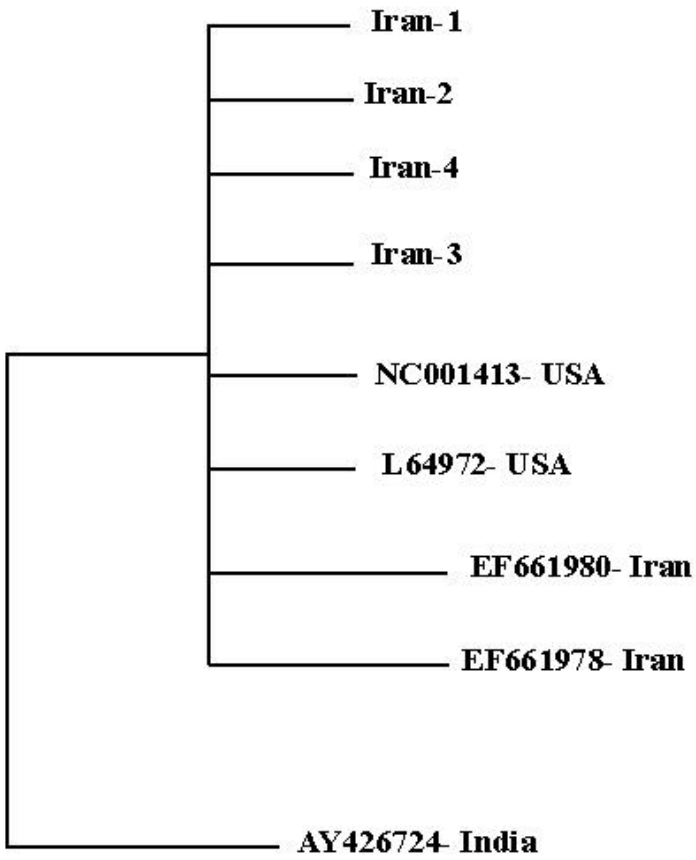

Figure 3- Phylogenetic tree of the sequence of the BIV virus *gag* gene in Iranian isolates and in other countries

Discussion

One of the main goals of this research, achieved for the first time in Iran, was trace the *gag* gene of BIV in the infected samples of this virus, presence of the corresponded gene was also confirmed with the help of sequencing of the fragment.

Given that primers applied for identification of the *gag* gene in this study involved the main part of encoding frame of the gene, the primers were designed for cloning and gene expression of *gag* in the way that the amplified fragment could be cloned in different vectors such as cloning and expressing vectors.

Further the second goal of this study was to clone the above mentioned gene in each of the cloning vector (pTZ57R/T vector) and expressing vector (pGEX-4T-3). The cloning of this gene in the cloning vector after sequencing and comparing resulted sequences to other known sequences of the *gag* gene available in Genebank indicates the success in cloning the gene into the related vector. Besides such a vector have the capacity to be proliferated in the competent bacterial cells and to be digested due to several sites for restriction enzymes, and also to be extracted and to be inserted in the expressing vectors.

Morever the *gag* gene product is an important viral antigen that induces a strong immune response in infected cattle. Recently, a purified recombinant BIV Gag protein was used in an immunoblot assay to detect BIV antibodies in field bovine serum samples .The method proved to be sensitive and specific by comparing the results with PCR (14).

As the amplified fragment by PCR involves all the domains of *gag* and has the capacity be placed in the expressing frame based on first designs of primers and has successfully been cloned in the expressing vector of pGEX-4T-3 so, the expression of this gene and the preparing recombinant protein will be applied in near future for designing recombinant protein for detection of bovine immunodeficiency virus in Iran.

Besides, for sequencing of the *gag* gene and comparing its genetic diversity in the Iranian isolates with other available viruses in the world, we compared the known sequences of this gene in Genbank of NCBI. The results indicated that there was 0.4-10.6% genetic diversity in the fragment. Most of agreement was related to the known sequences in the *gag* gene in USA (NC001413) and most of differences was also related to strain of this virus in India (AY426724).

The phylogenetic tree of compared sequences was also drawn using ClustalX and Njplot softwares. Is clear from in figure 3, the Iranian isolate is set in the branch of American sequences and there is significant difference between them and the separated strain in India..

We are evident of not much diversity in the sequences of BIV in the other studies. Despite of little diversity in the sequences which are being studied, we can justify the genetic diversity of the

virus based on its geographical distribution. As the origins of many Iranian noble cattle's refer to the America, so the perceived genetic similarities in this research can justify this claim. Besides, transportation of livestock between Far-East Countries (Japan) and Iran basically does not have any noticeable historical background. Thus, placing of Indian strains in other branches of phylogenetic tree highlights differences in the sequence of this virus between Iran and the mentioned countries.

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Cloning and phylogenetic analysis of Bovine immunodeficiency virus *gag* gene in Iranian isolates

Elahe Tajbakhsh

Department of Biology, ShK.C., Islamic Azad University, Shahrekord, Iran

Corresponding author: ee_tajbakshh@yahoo.com

Abstract

Bovine immunodeficiency virus (BIV), a member of the family *Retroviridae*, genus *Lentivirus*, has three important genes namely *gag*, *pol* and *env*. In the first step, PCR product of *gag* gene of BIV, isolated in different regions of Iran, were cloned in to a pTZ57R/T vector; then insert were digested by *BamH*I and *Xho*I restriction enzymes and then cloned in to pGEX-4T-3 as an expression vector. Analysis of the partial bovine immunodeficiency virus (BIV) *gag* gene sequences obtained from insert and was run. The Iranian BIV *gag* sequence was compared to 5 other corresponding sequences of BIV isolated in different countries. In the end, It was revealed that nucleotide analysis of the sequences witnessed, a variation of 0.4-10.6% and constructing phylogenetic tree revealed two clusters in it.

Key words: Bovine immunodeficiency virus, *gag* gene, pTZ57R/T vector, pGEX-4T-3 vector, Phylogenetic analysis