

Full Length Research Paper

Cloning and phylogenetic analysis of bovine leukemia virus *gag* gene in Iranian isolate

Hassan Momtaz

Department of Microbiology, Faculty of Veterinary Medicine, Islamic Azad University of Shahrekord branch, Shahrekord, Iran. P. O. Box.166. E-mail: hamomtaz@yahoo.com. Tel: 0098 381 3361083.

Accepted 22 December, 2009

Bovine leukemia virus (BLV) is a member of the family Retroviridae, genus *Deltaretrovirus* that has three important gene including *gag*, *pol* and *env*. This virus causes B-cell lymphocytosis and lymphosarcoma in cows. In the first step PCR product of *gag* gene of BLV isolated in different regions of Iran and BLV-FLK strain was cloned into a pTZ57R/T vector, then insert were digested by *Bgl*II and *Xho*I restriction enzymes and cloned into pET-28(a) as an expression vector. Analysis of the partial bovine leukemia virus (BLV) virus *gag* gene sequences obtained from insert and was carried out. The Iranian BLV *gag* sequence was compared to five other corresponding sequences of BLV isolated in different countries. Nucleotide analyzing of the sequences were shown a variation of 1 - 8.7% and constructing phylogenetic tree revealed two clusters in it. First cluster included New York, Maryland, Japan and Iranian sample, second cluster included Australian and Argentina strains. Iranian isolates were significantly identical to European and American isolates.

Key words: Bovine leukemia virus, p24 protein, pTZ57R/T vector, pET-28(a) vector, phylogenetic analysis.

INTRODUCTION

Like other complex retroviruses the bovine leukemia virus (BLV) genome contains the *gag*, *pol* and *env* structural genes and regulatory genes (Sagata et al., 1985). Most of the structural protein of BLV are immunogenic but the naturally infected animals develop antibodies to *env* encoded glycoproteins gp51 and gp30 as well as to *gag* encoded proteins p24 and p15 (Bicka et al., 2001; Deshayes et al., 1980).

Since the presence of antibodies to BLV is a constant and early feature of BLV infection, serological examination of cattle sera is the best method for detection of infected animals. Most commonly used serological tests are the agar gel immuno diffusion (AGID) and the enzyme linked immuno sorbent assay (ELISA). While in the ELISA the non specific reactions are difficult to distinguish from specific ones, western blot analysis allows precise resolution of the two reactions. So far limited studies have been performed to confirm the usefulness of the immunoblotting assay in the routine serological

detection of BLV antibodies (Bicka et al., 2001; Kittelberger et al., 1999; Simard and Richardson, 2000). Recently, the recombinant viral proteins have been found to be widely applicable in immunoassays for detection of specific antibodies. In particular, the use of the recombinant proteins synthesized in *Escherichia coli* has been well documented in retroviral serology (Bicka et al., 2001; De Giuseppe et al., 2004; Van den Heuvel et al., 2003, 2005).

In this study, the *gag* gene of BLV which encodes protein p24 from Iranian isolated virus was cloned in *E. coli* and finally phylogenetic analysis of the gene.

MATERIALS AND METHODS

Sample, plasmids and bacterial strains

The extracted DNA from buffy coat of one of the BLV infected cows which had previously shown positive molecular and serological results based on ELISA and PCR was selected to be cloned (Hemmatzadeh et al., 2008; Momtaz and Hemmatzadeh, 2003).

Plasmid pTZ57R/T (Ins T/A clone PCR Cloning kit, Fermentas) and *E. coli* strain JM107 (Fermentas) were used for initial cloning, sequencing and maintenance of DNA fragment. The required antibiotics were added to LB media according to the reference recommendation (Sambrook and Russell, 2001).

Abbreviations: BLV; Bovine leukemia virus, EBL; Enzootic bovine leucosis, gag; Group specific antigen, pol; Polymerase, env; Envelope, FLK; Fetal lamb kidney.

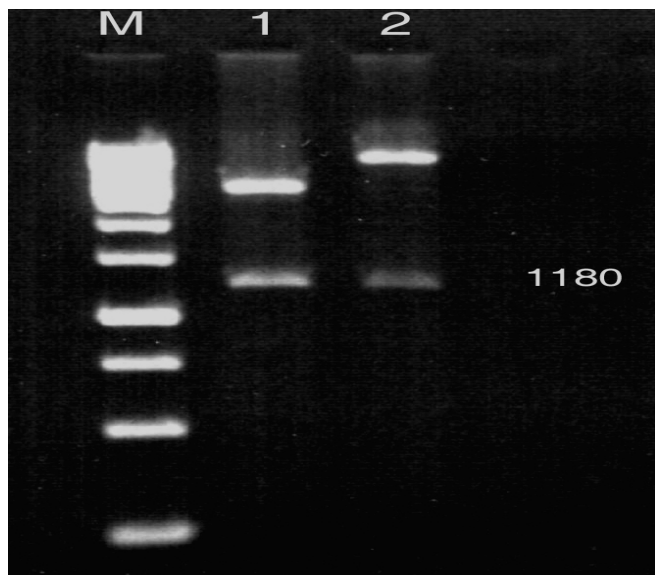


Figure 1. Restriction enzyme analysis of recombinant pTZ57R/T and pET-28(a) plasmids (Line M, Molecular weight marker 1kb DNA ladder; Line 1, digestion of recombinant pTZ57R/T plasmid; Line 2, digestion of recombinant pET-28(a) plasmid).

Primers design

Primers were designed according to the published sequence for gag gene of BLV (accession number: M 10987.1) (Rice et al., 1985). The forward primer, gag F: 5-GGC AGA TCI TGG GAA ATT CCC CCT CCT ATA-3 contain BglI site. Reverse primer, gag R: 5-CCG CTG GAG TAG TTT TTT GAT TTG AGG GTT GG-3 contain recognition site for XhoI. The restriction enzyme sites (underlined) were added to the primers for subsequent cloning procedure.

Gene amplification of gag (encoding the p24 protein)

PCR was performed in a 50 µl total volume containing 1 µg of template DNA, 1.5 µM of each primer, 1.5 mM MgCl₂, 150 µM dNTP, 1x PCR buffer and 1.5 unit of Taq DNA polymerase (Sigma). The following conditions were used for amplification: initial denaturation at 95°C for 4 min, followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 50 s. The program followed by a final extension at 72°C for 6 min. 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.

Cloning of gag gene

The PCR product was digested with *Bgl*I and *Xho*I and ligated to pTZ57R/T and pET-28(a), which were digested by the same restriction enzymes, using T4 DNA ligase (*Invitrogen*) at 14°C overnight. *E. coli* JM107 competent cells were prepared by calcium chloride method and were used for transformation of pTZ57R/T-p24 and pET-28(a)-p24 vectors, respectively. The transformed bacteria were selected by screening the colonies on LB media containing antibiotic. The suspected colony was further analyzed by restriction enzyme digestion and PCR (Sambrook and Russell, 2001).

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: M10987.1, NC001414.1, K02120.1, D00647.1, AF257515.1) were aligned separately using the Clustal X 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 BLV gag gene was compared with the corresponding sequences from other regions of the world. An unrooted dendrogramme was constructed using the Njplot software and statistical support for the dendrogramme was obtained by bootstrapping using 1000 replicates.

RESULTS

The purified PCR product (1180bp fragment) was cloned in pTZ57R/T vector and digestion with *Bgl*I and *Xho*I enzymes. Figure 1 shows recombinant plasmids after digestion.

The recombinant plasmid (pTZ57R/T-p24) was sequenced by specific primers and Sanger sequencing method (Macrogen, Korea). The sequencing result was confirmed by comparing with databases and using basic local alignment search tool (BLAST) software.

The result of sequencing of the gag fragment using Clustal W software was aligned with some of the Registered sequences in Genbank such as the sequences of this gene in Japan, Australia, USA and Argentina. After comparing differences and similarities applying Njplot software, the phylogenetic tree was drawn which is shown in Figure 2 and the diagram is demonstrated in Table 1.

DISCUSSION

Recognition and study of retrovirus infections are critical in different aspects. The occurrence of mutations consequently genotypic and phenotypic diversity is frequent in retroviruses because of their natural characteristics which are resulted from reverse transcription from their genomes. This feature makes the diagnostic value of most of the experimental tests uncertain. There are lots of articles that notice the special figures and numbers as sensitivity and special quality in diagnostic tests. The repeat of these tests by other researchers usually has had different results and it is reported that the reason can be found in genetic diversity of these viruses (Bicka et al., 2001; Bunger et al., 1994; Grover and Guillemain, 1992).

Anyway, one of the main goals of this research which was tracing of the coding gene of p24 protein of BLV in the infected samples to this virus, achieved for the first time in Iran and the presence of the corresponded gene was confirmed with the help of sequencing of the fragment.

With respect to this point that primers applied for identification of the gag gene in this study involve the main part

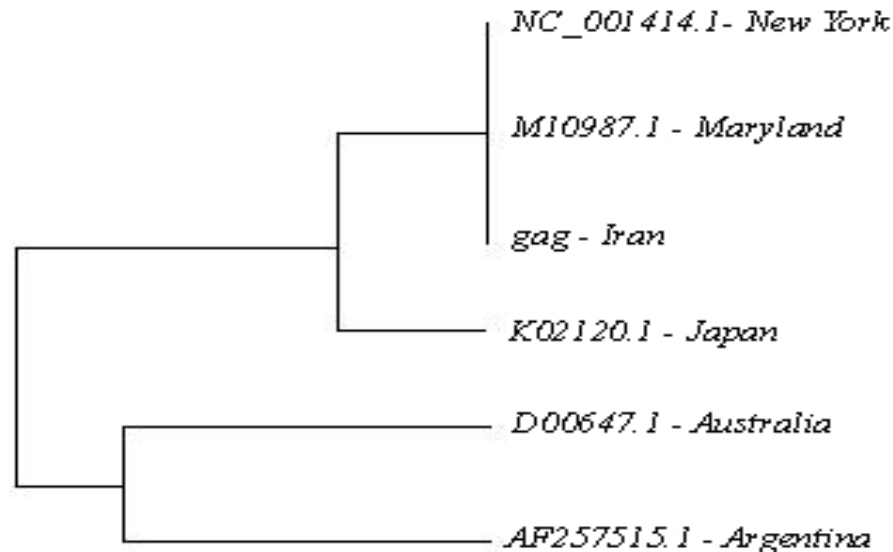


Figure 2. Phylogenetic tree of the sequence of the BLV *gag* gene in Iran and in other five countries.

Table 1. The results of comparison the sequence of the BLV *gag* gene in Iran with other countries (Sequence Identity Matrix)

Seq	M10987.1 Maryland	NC001414.1 New York	Gag Iran	K02120.1 Japan	D00647.1 Australia	AF257515.1 Argentina
M10987.1 Maryland	ID	1	0.990	0.950	0.923	0.935
NC001414.1 New York	1	ID	0.990	0.950	0.9123	0.935
gag Iran	0.990	0.990	ID	0.950	0.913	0.926
K02120.1 Japan	0.950	0.950	0.950	ID	0.912	0.920
D00647.1 Australia	0.923	0.923	0.913	0.912	ID	0.913
AF257515.1 Argentina	0.935	0.923	0.926	0.920	0.913	ID

of encoding frame of the gene thus, from the beginning the primers were designed for cloning and gene expression of *gag* in the way that the amplified fragment could be able to be cloned in different vectors such as cloning and expressing vectors.

The second goal of this study was cloning of the mentioned gene in each of the cloning vector (pTZ57R/T vector) and expressing vector (pET-28(a)). 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 Genbank indicates the success in cloning the gene into the related vector. Such vectors have the capacity to be proliferated in the competent bacterial cells, to be digested because of several sites for restriction enzymes, to be extracted and to be inserted in the expressing vectors.

Many researchers show that p24 and gp51 proteins which are the products of the *gag* and *env* genes of BLV are strong antigens and usually the first serological responses will be against these antigens. As the gp51 antigen is a very suitable candidate for manufacturing the

recombinant vaccines because of its glycoprotein structure and positioning in the membrane of virus. So, many researchers have started to design diagnostic methods based on tracing of antibody for p24 antigen and currently several institutions have designed and supplied ELISA and immunoblot methods specific to p24 (Altaner et al., 1991; Kono et al., 1986).

With respect to the remarkable frequency of infection to BLV in Iran and the necessity of controlling it through vaccination with recombinant vaccines of gp51, manufacturing and applying the recombinant p24 protein are vital goals in recognition and distinction between infection and responses caused by vaccine. As the amplified fragment by PCR involves all the domains of p24 and be placed in the expressing frame based on first designs of primers and has successfully been cloned in the expressing vector of pET-28(a) so, the expression of this gene and the preparing recombinant protein will be applied in near future for designing Dot-ELISA kit for detection of antibodies against p24 antigen of bovine leukemia virus in infected and vaccinated cows.

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 1 - 8.7% genetic diversity in the fragment. Most of the agreements were related to the known sequences in the gag gene in USA (M10987, NC001414) and most of differences were related to strain of this virus in Australia (D00647.1) (Table 1)

The phylogenetic tree of compared sequences was drawn using Clustal W and Njplot softwares. As it shown in Figure 2 the Iranian isolate is set in the branch of American sequences and there is significant difference between them and the separated strain in Australia and Argentina.

We are evident of not much diversity in the sequences of BLV 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 refer to the European countries and Canada, so the perceived genetic similarities in this research can justify this claim. In the other side, transportation of livestock between Far-East Countries (Australia and Japan) or South America (Argentina) and Iran basically does not have historical background. Thus, placing of Japanese, Australian and Argentinean strains in other branches of phylogenetic tree is indicating more differences in the sequence of this virus between Iran and the mentioned countries.

ACKNOWLEDGEMENTS

We thank Dr. A. Sharifzadeh, Dr. M Rohani, Dr. S. Nejat and Dr. M. Momeni for their cooperation. This work was supported by Grant No. 35510 from the Islamic Azad University of Shahr-e-kord Branch in Iran.

REFERENCES

- Altaner C, Ban J, Altanerova V, Janik V (1991). Protective vaccination against bovine leukemia virus infection by means of cell-derived vaccine. *Vaccine* 9: 889–895.
- Bicka L, Kuzmak J, Kozaczynska B, Plucienniczak A, Skorupska A (2001). Expression of bovine leukemia virus protein p24 in *Escherichia coli* and its use in the immunoblotting assay. *Acta Biochim. Pol.* 48: 227-232.
- Bunger I, Khalaf H, Cripe C, Rimpler M (1994). Detection of antibodies against bovine leukaemia virus in milk and serum samples by immunoblotting. *Dtsch. Tierarztl. Wochenschr.* 101: 402-405.

- De Giuseppe A, Feliziani F, Rutili D, De Mia GM (2004). Expression of the bovine leukemia virus envelope glycoprotein (gp51) by recombinant baculovirus and its use in an enzyme linked immuno sorbent assay. *Clin. Diagn. Lab. Immunol.* 11: 147 – 151.
- Deshayes L, Levy D, Parodi AL, Levy JP (1980). Spontaneous immune response of bovine leukemia virus infected cattle against five different viral proteins. *Int. J. Can.* 25: 503-508.
- Grover YP, Guillemain B (1992). An immunoblotting procedure for detection of antibodies against bovine leukemia virus in cattle. *J. Vet. Med. B.* 39: 48-52.
- Hemmatzadeh F, Raza Tofighi E, Keyvanfar K, Monadi A, Rohani M, Momtaz H., Rahmani F (2008). Investigation of env gene of bovine leukaemia virus in infected cows. *Ind. Vet. J.* 85: 20-22.
- Kittelberger R, Reichel MP, Meynell RM (1999). Detection of antibodies against the core protein p24 BLV in cattle. *J. Virol. Methods* 77: 109-114.
- Kono Y, Arai K, Sentsui H, Matsukawa S, Itohara S (1986). Protection against bovine leukemia virus infection in sheep by active and passive immunization. *Nippon Juigaku Zasshi.* 48: 117–125.
- Momtaz H, Hemmatzadeh F (2003). A serological survey of BLV on cattle in Chaharmahal and Bakhtiary province of Iran. *Iran. J. Vet. Res.* 4: 37-44.
- Rice NR, Stephens RM, Burny A, Gilden RV (1985). The gag and pol genes of bovine leukemia virus: Nucleotide sequence analysis. *Virology* 142: 357-377.
- Sagata N, Yasuaga T, Tsuzuku-Kawamura J, Ohish K, Ogawa Y, Ikawa Y (1985). Complete nucleotide sequence of the genome of bovine leukemia virus: Its evolutionary relationship to other retroviruses. *Proc. Natl. Acad. Sci. U.S.A.* 82: 677-681.
- Sambrook J, Russell DW (2001). *Molecular cloning :a laboratory manual.* (3th edn). Cold Spring Harbor, New York.
- Simard C, Richardson S, Dixon P, Belanger C, Maxwell P (2000). ELISA for the diagnosis of bovine leukosis: comparison with AGID test approved by the Canadian food Inspection Agency. *Can. J. Vet. Res.* 64: 101-106.
- Van den Heuvel MJ, Jefferson BJ, Jacobs RM (2005). Purified bovine plasma blocking factor decreases bovine leukemia virus p24 expression while increasing protein synthesis and transcriptional activity of peripheral blood mononuclear cells in short-term culture. *Can. J. Vet. Res.* 69: 186-192.
- Van den Heuvel MJ, Portetelle D, Jefferson B, Jacobs RM (2003). Adaptation of a sandwich enzyme linked immuno sorbent assay to determine the concentration of bovine leukemia virus p24 and optimal conditions for p24 expression in short-term cultures of peripheral blood mononuclear cells. *J. Virol. Methods* 111: 61-67.