www.roavs.com EISSN: 2223-0343
RESEARCH OPINIONS IN ANIMAL & VETERINARY SCIENCES

Molecular detection of Bovine Leukemia Virus (BLV) in the frozen semen samples of bulls used for artificial insemination in Iran

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Abstract

Bovine Leukemia Virus *(*BLV*)* is a causative agent of bovine leukosis responsible for lymphoproliferative disorders in cattle. The aim of this study was to detect the BLV in the frozen semen samples of Iranian bulls used for artificial insemination using PCR. A total of 45 frozen Semen samples were obtained from 45 bulls used for artificial insemination centres. DNA was extracted and 385 bp fragment of the gag gene of the virus was visualized in a 1% agarose gel electrophoresis. Viral DNA was detected in nine of 45 (20%) frozen semen samples of bull's that were used for artificial insemination. The results of this study demonstrated the high prevalence of BLV DNA in frozen semen samples of bulls. According to this finding it is clear that testing, segregation and culling programs can be effective in the control and prevent the bovine leukosis disease caused by BLV. Furthermore, examination of cattle semen samples that are used for artificial insemination is necessary to prevent the spread of BLV in this region.

Keywords: Artificial insemination; *Bovine leukemia virus*; bulls; PCR, semen

To cite this article: Khamesipour F, A Doosti, AK Shahraki and M Goodarzi, 2013. Molecular detection of Bovine Leukemia Virus *(*BLV*)* in the frozen semen samples of bulls used for artificial insemination in Iran. Res. Opin. Anim. Vet. Sci., 3(11), 412-416.

Introduction

In Iran, not withstanding adopted labours, the prevention and eradication of viral infections in farm animals have continually been a problem. The latent infection by retroviruses such as Bovine Leukemia Virus *(*BLV*)* confuses early diagnosis of disease make happen via the viruses (Jafari and Asadpour, 2010; Sharifzadeh et al., 2011).

BLV induces tenacious lymphocytosis in cattle and some other animals. It has a single-stranded RNA genome which encodes a DNA intermediate that is inserted into the host DNA. The virus is mostly transmitted horizontally via direct contact to biological fluids (blood, semen, milk and saliva) polluted by infected lymphocytes (Lorin et al., 2007). All the same viral antigens and proviral DNA have been recognized in semen, milk and colostrums. Typical transmission via these secretions has not been demonstrated (Romero et al., 1983; Choi et al., 2002). There are three pathological forms of infection including: asymptomatic course, lymphosarcomas and persistent leucocytosis (PL). These three forms can follow each other, or happen without previous other symptoms (Burny et al., 1985; Mirsky et al., 1996). Signs of BLV infections cause severe economic losses for milk producers. These signs include tumours in lymphoid tissues, decreased milk production, enlarged lymph nodes, loss of appetite, weight loss, fever, rear limb weakness or paralysis, gastrointestinal obstructions

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protruding eyeballs and increased blood lymphocytes counts (Motton and Buehring, 2003; Ott et al., 2003).

Because BLV infection affects directly the immune system, its power on herd health and economy could be more extensive than direct loss from death of a single individual following lymphomas (Trainin and Brenner, 2005; Hanuš et al., 2007; Janu et al., 2007). Cellular damages could force cells to shed free DNA into body fluids. Many studies have demonstrated tumour or viral specific sequences of DNA or RNA recovered from the plasma or seminal fluid of patients by several diseases, a finding that could be helpful in molecular diagnosis and prognosis (Poon et al., 1993; Carsten et al., 2002; Amanda et al., 2003).

Domestic cattle are the usual hosts for BLV. The most important target cell of the virus is generally the B lymphocyte. Though, it can too establish in T lymphocytes, macrophages, monocytes, granulocytes, mammary epithelial cells and in the cells of many organs and tissues (Heeney et al., 1992; Buehring et al., 1994). Extensive research has shown that semen from BLV-infected artificial insemination stud bull does not pose a risk of infection to progeny or even to the dam. However, the potential of transmission may exist when heifers are bred by infected bulls. But from a pragmatic point of view this can be alleviated by testing of bulls for BLV antibodies, whenever semen is collected (Kaja and Olson, 1982; Monke, 1986; Choi et al., 2002; Dus Santos et al., 2007; Santose et al., 2007; Sharifzadeh et al., 2011).

Numerous indirect and direct methods have been used for detection of BLV-infected carriers including haematological and syncytial tests, Agar Gel Immunodiffusion (AGID), Western Blot, Enzyme-linked Immunosorbent Assay (ELISA), and Polymerase Chain Reaction (PCR) (Simard et al., 2000; Martin et al., 2001). The method presently working for BLV-infection diagnosis is the AGID technique, due to its ease of use, rapidity and simplicity in obtaining results. Else, AGID has low sensitivity and failsto detect antibodies in the early stages of the infection (Eaves et al., 1994). Amplification techniques, such as PCR has been described as another method, which directly detects the presence of proviral DNA in BLV infected cattle with low, transient or absent antibody titers (Naif et al., 1992; Klintevall et al., 1994). The aim of this study was to detect the *Bovine Leukemia virus* in the semen samples of bulls that used for artificial insemination in Iran by using PCR technique.

Materials and Methods

Sampling and DNA isolation

The study was carried out between December, 2012 and January, 2013. A total of 45 frozen semen samples, from 45 bulls, used for artificial insemination, were aseptically obtained from Iranian bulls (From semen bank at artificial insemination centre, in Iran), randomly selected from different farms. Frozen semen samples were sent to the Biotechnology Research Centre of Islamic Azad University of Shahrekord Branch in a cold cabinet. Each of the specimen was stored at -20°C for further use. Genomic DNA was extracted from specimens using DNA extraction kit (Bioneer, Daejeon, Korea) according to the manufacturer's protocol. The total DNA was measured at 260 nm optical density according to the method described by Sambrook and Russell (2001). The extracted DNA of each semen sample was kept frozen at -20°C until its use.

Gene amplification

To detect BLV DNA, nested PCR was performed via the DNAs extracted from semen samples. Two sets of oligonucleotide primer pairs were used for amplification. The external primers specific to the BLV region was used as described by Wang et al (2002). The sequence of primers for first round was BLV-F1: 5-ATGGGAAATTCCCCCTCCTAT-3 and BLV-R1: 5-GTTTTTTGATTTGAGGGTTGG-3 (Wang et al., 2002). The second amplification round was performed to amplify a 385 base pair (bp) fragment, using a pair of inner primers of the gag region in table 1 (BLV- F2: 5-AACACTACGACTTGCAATCC-3 and BLV-R2: 5-GTTCCTTAGGACTCCGTCG-3) in the BLV genome (Gene Bank accession number K02120). The FLK-BLV DNA served as a positive control for amplification (FLK-BLV stands for fetal lamb kidney cells infected with *bovine leukemia virus*).

Two set of nested-PCR program was carried out in 25 µL total reaction volumes, each containing 100 ng of template DNA, 0.2 pM of each primer, 2.5 µL of 10X PCR buffer, 1.5 Mm $MgCl_2$, 200 Mm dNTPs and 1 unit of *Taq* DNA polymerase (Fermentas, Germany). The amplification reaction consisted of 5 min of pre-denaturing at 94°C, followed by 30 cycles of 1 min denaturation at 94°C, 1 min annealing at 55°C and 1 min extension at 72°C and then by a final extension at 72°C for 5 min. Two µL amplicon from the first round was used as a template in a Gradient Palm Cycler (Corbett Research, Australia). The second round PCR was performed with inner oligonucleotide primers for 25 cycles with the same concentration of reagents and temperature conditions.

Analysis of PCR products

The amplified products were detected in 1% agarose gel electrophoresis. The electrode buffer was TBE (Tris-base 10.8 g 89 mM Boric acid 5.5 g 2mM EDTA (pH 8.0) 4 ml of 0.5 M EDTA (pH 8.0) which combine all components in sufficient H_2O and Aliquots 10 µL of PCR products were applied to the gel.

Constant voltage of 80 V for 30 min was used for products separation. After electrophoresis, images were obtained in UVIdoc gel documentation systems (Uvidoc, UK).

Results

Nested-PCR specimens producing a band of expected size (385 bp) were considered positive (Fig. 1). BLV viral DNA was detected in 9 samples out of 45 (20%) frozen bull's semen samples. The positive control (FLK-BLV DNA) showed the expected amplification product specific for BLV (385 bp). The size of the bands matched the positive control.

The results showed a high incidence of BLV DNA in frozen bull's semen samples that were used for artificial insemination in Iran. This finding suggests that control and eradication programs for BLV infection are necessary in Iranian cattle. The results of the prevalence of BLV in frozen semen samples of cattle are shown in Table 1.

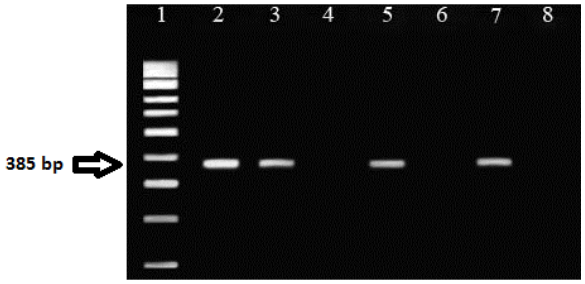

Fig. 1: Ethidium bromide-stained agarose gel electrophoresis of PCR products (385 bp) for detection of BLV **DNA in frozen semen samples after PCR amplification.** Agarose gel electrophoresis for identification of BLV DNA in cattle semen samples. lane 1: 100 bp DNA ladder (Fermentas, Germany); Lanes 2: positive control; lanes 3, 5 and 7: positive samples (385 bp); lanes 4 and 6: negative samples, 8: Negative control.

Table 1: The results of the prevalence of BLV **in frozen semen samples of bulls**

Animal	Specimens type	Total number	Positive	Negative
Bull's	frozen semen	45	9 (20%)	36 (80%)

Discussion

BLV is the causative agent of bovine leucosis and an extremely fatal form of cancer in cattle (Motton and Buehring, 2003). The virus is transmitted through white blood cells (WBC) and very small amount of blood can transfer the virus (Poon et al., 1993). Nasal secretions, semen and saliva and bronchoalveolar lavage fluid, have been evaluated for the presence of provirus and their skill to transmit BLV (Camargos et al., 2002).

Previous studies by numerous investigators suggested that semen from BLV-infected bulls was non infectious for recipient cows (Monke, 1986). Recently, polymerase chain reaction (PCR) for the detection of BLV proviral DNA has been described (Naif et al., 1992; Klintevall et al., 1994; Czarnik et al., 2002; Menendez-Arias, 2002; Amills et al., 2004). Numerous routine management practices such as ear tagging, dehorning, giving injections without changing needles and performing multiple rectal examinations with the same obstetrical sleeve can transmit BLV. The most popular transmission of BLV is horizontal. Close contact among BLV negative and BLV positive cattle has been thought to be a risk factor (Wang et al., 2002; Kohara et al., 2006). Though, semen contaminated with viral agents may cause an enormous spread of sure diseases, since it could infect many farms, areas, or even countries in a short period of time (Van Rijn et al., 2004).

The economic losses associated with BLV included reduced production, increased veterinary costs and increased culling rate of infected adults. Extra significant losses are practiced via purebred herd with the loss of domestic and foreign seed stock sales may establish a significant economic burden. Various countries, particularly those in the European Economic Community do not accept cattle semen, or embryos from positive individuals or herds (Santose et al., 2007).

Rapid, definitive and accurate diagnosis of BLV is very important for a positive result of eradication programmes. The detection of the BLV in bull's semen is important and PCR is a sensitive method for this purpose. The majority of the PCR assay is based on sensitivity and highly quantitative of his technique for detection of viral DNA such as BLV against other diagnostic methods (Sherman et al., 1992).

The BLV provirus has been demonstrated by PCR in cattle (Naif et al., 1990; Murtaugh et al., 1991; Sherman et al., 1992). In the study of Sharifzadeh et al. (2011), BLV DNA was detected in 36 of the 172 (20.93%) semen samples of bulls used for artificial insemination in Iran (Sharifzadeh et al., 2011). Jafari and Asadpour (2010) also detected BLV provirus in seminal plasma of bulls.

Mohammadi et al. (2011) reported that 29.9% BLV seropositivity was detected among the sampled cattle population. Previous study on slaughterhouse prevalence of BLV in Holstein cattle reported cattle seroprevalence rates of 22.3% (Tooloei et al., 2009).

The results of this study showed that the frozen semen samples served as a reservoir of BLV in Iran. These reservoirs increase the risk of the potential spread

of disease to other animals and this deserves special attention.

Conclusions

In conclusion, the results presented high incidence of BLV infection in cattle semen samples and control and eradication programs for prevention and reduction of economic loses of BLV infection are necessary. Moreover, the results of the present study suggested that PCR is a highly sensitive and specific diagnostic test for identification and differentiation of BLV and regular screening of this virus in all regions is essential to prevent the spread of the disease.

Acknowledgements

The authors would like to express their deep sense of gratitude and sincere thanks to the staff of the Biotechnology Research Center of Islamic Azad University of Shahrekord Branch in Iran.

References

Amanda, L., Dunne, F.M., Kelly, M., Gordon Baker, H.W., Garland, S., Clarke, G.N., Mijch, A. and Crowe, SM. 2003. Analysis of HIV-1 viral load in seminal plasma samples. *Journal of Clinical Virology*, 26: 239-245.

Amills, M., Norimine, J., Olmstead, C.A. and Lewin, H.A. 2004. Cytokine mRNA expression in B cells from bovine leukemia virus-infected cattle with persistent lymphocytosis. *Cytokine*, 28: 25-28.

Buehring, G., Kramme, P. and Schultz, R. 1994. Evidence for bovine leukemia virus in mammary epithelial cells of infected cows. *Laboratory Investigation*, 71: 359-365.

Burny, A., Bruck, C., Cleuter, Y., Couez, D., Deschamps, J., Gregoire, D., Ghysdael, J., Kettmann, R., Mammerickx, M., and Marbaix, G. 1985. Bovine leukemia virus and enzootic bovine leukosis. *Onderstepoort Journal of Veterinary Research*, 52: 133-144.

Camargos, M.F., Stancek, D., Rocha, M.A., Lessa, L.M., Reis, J.K. and Leite, R.C. 2002. Partial sequencing of env gene of bovine leukaemia virus from Brazilian samples and phylogenetic analysis. *Journal of veterinary medicine. B, Infectious diseases and veterinary public health*, 49: 325-331.

Carsten, G., Markus, M., Bernd, S. and Kurt, M. 2002. DNA alterations in body fluids as molecular tumor markers for urological malignancies. *European Urology*, 41: 668-676.

Choi, K., Monke, D. and Stott, J. 2002. Absence of bovine leukosis virus in semen of seropositive bulls. *Journal of Veterinary Diagnostic Investigation*, 14: 403-406.

Czarnik, U., Kamin'ski, S., Rulka, J. and Walawski, K. 2002. Modified procedure for PCR detection of proviral DNA of bovine leukaemia virus. *Bulletin of the Veterinary Institute in Pulawy*, 44: 143-147.

Dus Santos, M.J., Trono, K., Lager, I. and Wigdorovitz, A. 2007. Development of a PCR to diagnose BLV genome in frozen semen samples. *Veterinary Microbiology*, 119: 10-18.

Eaves, F.W., Mohillo, J., Dimmock, C. and Eaves, L. 1994. A field evaluation of the polymerase chain reaction procedure for the detection of bovine leukaemia virus proviral DNA in cattle. *Veterinary Microbiology*, 39: 313-321.

Hanuš, O., Frelich, J., Janu, L., Macek, A., Zajíčková, I., Genčurová, V. and Jedelská, R. 2007. Impact of different milk yields of cows on milk quality in Bohemian Spotted cattle. *Acta Veterinaria Brno*, 76: 563-571.

Heeney, J., Valli, P., Jacobs, R. and Valli, V. 1992. Evidence for bovine leukemia virus infection of peripheral blood monocytes and limited antigen expression in bovine lymphoid tissue. *Laboratory Investigation*, 66: 608-617.

Jafari, R. and Asadpour, R. 2010. Bovine leukemia provirus: evidence of presence of part of gag gene in seminal plasma of naturally infected bulls. *Veterinary Research Forum*, 1: 44-49.

Janu, L., Hanus, O., Frelich, J., Macek, A., Zajíčková, I., Genčurová, V. and Jedelská, R. 2007. Influences of different milk yields of Holstein cows on milk quality indicators in the Czech Republic. *Acta Veterinaria Brno*, 76: 553-561.

Kaja, R.W. and Olson, C. 1982. Non-infectivity of semen from bulls infected with bovine leukosis virus. *Theriogenology*, 18: 107-112.

Klintevall, K., Ballagi-Pordany, A., Naslund, K. and Belak, S. 1994. Bovine leukemia virus: rapid detection of proviral DNA by nested PCR in blood and organs of experimentally infected calves. *Veterinary Microbiology*, 42: 191-204.

Kohara, J., Konnai, S. and Onuma, M. 2006. Experimental transmission of Bovine leukaemia virus in cattle via rectal palpation. *Japanese Journal of Veterinary Research*, 54: 25-30.

Lorin, A., Lins, L., Stroobant, V., Brasseur, R. and Charloteaux, B. 2007. Determination of the minimal fusion peptide of bovine leukemia virus gp30. *Biochemical and Biophysical Research Communications*, 355: 649-653.

Martin, D., Arjona, A., Soto, I., Barquero, N., Viana, M. and Gómez-Lucía, E. 2001. Comparative study of PCR as a direct assay and ELISA and AGID as indirect assays for the detection of Bovine Leukaemia Virus. *Journal of Veterinary Medicine Series B*, 48: 97-106.

Menendez-Arias, L. 2002. Molecular basis of fidelity of DNA synthesis and nucleotide specificity of

retroviral reverse transcriptases. *Progress in Nucleic Acid Research and Molecular Biology*, 71: 91-147.

Mirsky, M.L., Olmstead, C.A., Da, Y. and Lewin, H.A. 1996. The prevalence of proviral bovine leukemia virus in peripheral blood mononuclear cells at two subclinical stages of infection. *Journal of Virology*, 70: 2178-2183.

Mohammadi, V., Atyabi, N., Nikbakht Brujeni, G.H., Lotfollahzadeh, S. and Mostafavi, E. 2011. Seroprevalence of bovine leukemia virus in some dairy farms in Iran. *Global Veterinaria*, 7: 305-309.

Monke, D. 1986. Noninfectivity of semen from bulls infected with bovine leukosis virus. *Journal of the American Veterinary Medical Association*, 188: 823-826.

Motton, D.D. and Buehring, G.C. 2003. Bovine leukemia virus alters growth properties and casein synthesis in mammary epithelial cells. *Journal of Dairy Science*, 86: 2826- 2838.

Murtaugh, M.P., Lin, G.F., Haggard, D.L., Weber, A.F. and Meiske, J.C. 1991. Detection of bovine leukemia virus in cattle by the polymerase chain reaction. *Journal of Virological Methods*, 33: 73-85.

Naif, H.M., Brandon, R.B., Daniel, R.C.W. and Lavin, M.F. 1990. Bovine leukaemia proviral DNA detection in cattle using the polymerase chain reaction. *Veterinary Microbiology*, 25: 117-129.

Naif, H.M., Daniel, R.C., Cougle, W.G. and Lavin, M.F. 1992. Early detection of bovine leukemia virus by using an enzyme-linked assay for polymerase chain reaction-amplified proviral DNA in experimentally infected cattle. *Journal of Clinical Microbiology*, 30: 675-679.

Ott, S.L., Johnson, R. and Wells, S.J. 2003. Association between bovine-leukosis virus seroprevalence and herd-level productivity on US dairy farms. *Preventive Veterinary Medicine*, 61: 249-262.

Poon, H., Jimenez, E., Jacobs, R.M., Song, Z. and Jefferson, B. 1993. Detection of bovine leukemia virus RNA in serum using the polymerase chain reaction. *Journal of Virological Methods*, 41: 101-112.

Romero, C., Cruz, G. and Rowe, C. 1983. Transmission of bovine leukemia virus in milk. *Tropical Animal Health and Production*, 15: 215-218.

Sambrook, J. and Russell, D.W. 2001. Molecular cloning: A laboratory manual. 3rd Edition. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp: 58-152.

Santose, M.J.D., Trono, K., Lager, I. and Wigdorovitz, A. 2007. Development of a PCR to diagnose BLV genome in frozen semen samples. *Veterinary Microbiology*, 119: 10-18.

Sharifzadeh, A., Doosti, A. and Ghasemi Dehkordi, P. 2011. Molecular detection of Bovine leukemia virus (BLV) in the Semen Samples of Bulls. *World Journal of Zoology*, 6: 285- 290.

Sherman, M.P., Ehrlich, G.D., Ferrer, J.F., Sninsky, J.J., Zandomeni, R., Dock, N.L. and Poiesz, B. 1992. Amplification and analysis of specific DNA and RNA sequences of bovine leukemia virus from infected cows by polymerase chain reaction. *Journal of Clinical Microbiology*, 30: 185-191.

Simard, C., Richardson, S., Dixon, P., Bélanger, C. and Maxwell, P. 2000. Enzyme-linked immunosorbent assay for the diagnosis of bovine leukosis: comparison with the agar gel immunodiffusion test approved by the Canadian Food Inspection Agency. *Canadian Journal of Veterinary Research*, 64: 101-106.

Tooloei, M., Bazargani, T.T., Nikbakht Brujeni, G.R., Nikbakht-Brujeni, Z., Khaki Bokaei, S. and Ashrafi, I. 2009. An abattoir survey on prevalence of enzootic bovine leukosis virus infection and associated clinical, hematological and flow cytometric findings in Holestein cattle in Tehran. *Journal of Veterinary Research*, 64: 147-156.

Trainin, Z. and Brenner, J. 2005. The direct and indirect economic impacts of bovine leukemia virus infection on dairy cattle (Review). *Israel Journal of Veterinary Medicine*, 60: 94-105.

Van Rijn, P., Wellenberg, G., Hakze-van der Honing, R. Jacobs, L., Moonen, P.L. and Feitsma, H. 2004. Detection of economically important viruses in boar semen by quantitative Real Time PCR technology. *Journal of Virological Methods*, 120:160-161.

Wang, H., Norris, K.M. and Mansky, L.M. 2002. Analysis of bovine leukemia virus gag membrane targeting and late domain function. *Journal of Virology*, 76: 8485- 8493.