

Detection of Enzootic Bovine Leukosis in Cattle using Nested Polymerase Chain Reaction Assay

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Abstract

Enzootic bovine leukosis (EBL) is caused by bovine leukemia virus (BLV) infection. BLV was detected in cattle using nested polymerase chain reaction (PCR) assay and were identified BLV infected cattle farms in five selected provinces in the Philippines. A total of 300 cattle blood samples were used. BLV Proviral DNA was extracted and amplified using nested PCR assay targeting the BLV long terminal repeat (LTR). Results showed that 11 samples (3.67%) of the 300 cattle blood samples used were positive for BLV infection. This study is considered first report of cattle EBL in the Philippines.

Keywords: BLV, cattle, EBL, nested PCR, Philippines

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บทคัดย่อ

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Enzootic bovine leukosis (EBL) เกิดจากการติดเชื้อไวรัส bovine leukemia (BLV) ในการศึกษาครั้งนี้ตรวจ BLV ในโคด้วยวิธี nested polymerase chain reaction (PCR) ในพลาสมาโคที่ติดเชื้อ BLV จากห้าจังหวัดของประเทศฟิลิปปินส์ ทำการสกัด proviral ดีเอ็นเอของเชื้อ BLV จากตัวอย่างเลือดโคจำนวน 300 ตัวอย่าง และทำการเพิ่มจำนวนด้วยวิธี nested PCR โดยกำหนดเป้าหมายไว้ที่ BLV long terminal repeat (LTR) ผลการศึกษาพบว่าพบตัวอย่างที่ให้ผลเป็นบวกจำนวน 11 จากเลือดโค 300 ตัวอย่าง (ร้อยละ 3.67) ซึ่งการศึกษานี้ถือเป็นการรายงานครั้งแรกของการตรวจพบการติดเชื้อ EBL ในโคของประเทศฟิลิปปินส์

คำสำคัญ: BLV โค EBL nested PCR ประเทศฟิลิปปินส์

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Introduction

Enzootic bovine leukosis (EBL) is caused by bovine leukemia virus (BLV), which is a retrovirus of the Family Retroviridae (Khan, 2010). B-lymphocytes of cattle and water buffaloes are affected causing the development of lymphomas in the peripheral lymph nodes and other organs (Ambroski et al., 1989; Murtaugh et al., 1991; Pollari et al., 1992). Approximately 30% of infected cattle develop persistent lymphocytosis while usually less than 5% develop lymphoma (Pollari et al., 1992). BLV is related genetically and functionally to human T-lymphotropic viruses 1 and 2. Its major target cells are the B lymphocytes. The virus particle is spherical and about 80-100 nm in diameter. It consists principally of single-stranded RNA, some surface glycoproteins and several enzymes, including reverse transcriptase (Venables and Lucas, 2004). During retroviral replication, the single-stranded viral genomic RNA is transcribed to double-stranded DNA molecules called provirus or proviral DNA (Klintevall et al., 1994). Proviral DNA integrates into the nuclear DNA of the host cell and establishes infection that persists for the life of the animal.

The provirus is flanked by two identical long terminal repeat sequences (LTRs) and contains the open reading frames corresponding to gag, prt, pol and env. Several orfs coding for Tax, Rex, R3 and G4 are present in the X region between env and the 3'LTR (Sagata et al., 1984^{a,b}). The genomic RNA transcript initiates and terminates in the 5' and 3' LTRs,

respectively (Gillet et al., 2007). LTR is the control center for gene expression and is also involved in retro-transcription and integration. The LTR structure has three parts (U3, R, U5) in both extremes of the proviral DNA (Fig 1). The 3' LTR acts in transcription termination and polyadenylation, although it has exactly the same sequence arrangement as the 5' LTR, the latter has dominant control as promoter. If the 5' LTR is disrupted, the 3' LTR can act as a promoter (Arellano et al., 2006).

BLV infection causes significant animal health problems like reduced milk production and poor reproductive efficiency and can also be a major cause of economic loss worldwide due to high morbidity and condemnation at slaughter (Venables and Lucas, 2004; Yavru et al., 2007). It is listed in the World Organization for Animal Health as a disease of importance to international trade (Kobayashi et al., 2010; OIE, 2000). EBL is widely distributed in the world, but with distinct regional differences in prevalence (Venables and Lucas, 2004).

In the Philippines, BLV infection is classified as an exotic disease based on the classification of the OIE listings of diseases which mean that the disease is not present in the country (BAI, 2004). Mingala et al. (2009) pioneered the surveillance study of BLV infection in the country with 27.6% prevalence of the disease in water buffaloes. As of today, no study of BLV infection has been reported in cattle in the Philippines. According to the DA-BAI Administrative Order No. 12 series of 2004, it is an exotic disease in the country. However, it is said to be already present and suspected in the country (Mingala et al., 2009).

Several studies had been made in many countries that detected the disease at molecular level through DNA based technique, but in the Philippines, there is only one study on the detection of the disease through PCR because of the late introduction of PCR technology in the country.

The study will be of great help in detecting EBL in cattle in the Philippines. Currently, the Philippine production for livestock especially in cattle and water buffalo is increasing. Therefore, spread of BLV infection can be prevented and controlled by means of diagnosing through detection by nested PCR. The objective of the study was to detect BLV infection in cattle using nested polymerase chain reaction assay.

Materials and Methods

Cattle blood sample collection: A total of 300 cattle blood samples which were previously collected from selected farms in five provinces in the Philippines were used. One hundred fifty three blood samples from Laguna, 42 from Pangasinan, 12 from La Union, 21 from Bohol and 72 from Cebu were collected. Blood samples were from native and crosses of native, Brahman, Holstein, Jersey and Sahiwal breeds. Five ml of blood were collected from each animal via the jugular vein. Blood samples were placed in sterile vacutainer tube with anti-coagulant, labelled accordingly and stored at 4°C until arrival at the laboratory.

Genomic DNA extraction and nested-PCR: Blood samples were centrifuged at 10,000 rpm for ten minutes. Three hundred microliter buffy coat was collected and transferred to a microcentrifuge tube for DNA extraction. Each tube was labelled according to the sample number. Genomic DNA extraction was done using the Wizard Genomic DNA Purification Kit (Promega, USA) following the manufacturer's instruction.

All samples were tested by conventional PCR for the presence of β -actin gene (housekeeping gene) to assure that genomic DNA was extracted from all the samples. PCR was carried out in a total volume of 20 μ l containing 13.3 μ l DDW, 2.0 μ l 10x PCR buffer (Takara, Japan), 1.6 μ l dNTPs (Takara, Japan), 0.5 μ l each of ten pmol forward (F - CGC ACC ACC GGC ATC GTG AT) and reverse (R -TCC AGG GCC ACG TAG CAG AG) primers (Tajima et al., 1998), 0.1 μ l of rTaq polymerase (Takara, Japan) and 2.0 μ l of DNA template. PCR condition was as follows: an initial denaturation at 94°C for five min followed by 35 cycles each consisting of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec. It was followed by final extension at 72°C for 5 min.

BLV-LTR of the proviral DNA (Fig. 1) was amplified by nested PCR in a 20 μ l reaction volume, containing 12.3 μ l DDW, 2.0 μ l 10x PCR buffer (Takara, Japan), 1.6 μ l dNTPs (Takara, Japan), 0.5 μ l each of 10 pmol of the external primers (Tajima et al., 1998), forward (LTR 1-TGT ATG AAA GAT CAT GCC GAC) and reverse (LTR533- AAT TGT TTG CCG

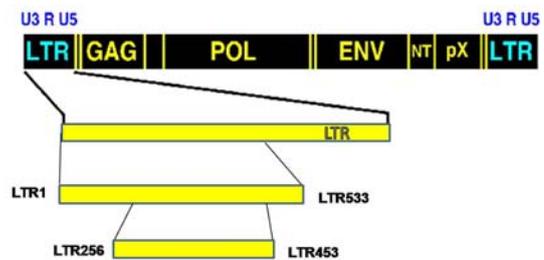


Figure 1 Diagram of the location of nested primers used from the long terminal repeat (LTR) of BLV proviral gene.

GTC TCT C), 0.1 μ l of rTaq polymerase (Takara, Japan) and 3.0 μ l of DNA template. This first PCR included an initial denaturation at 94°C for 30 sec, 40 cycles of denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec and extension at 72°C for 30 sec, and a final extension at 72°C for 10 min. DDW was used as negative control.

The second PCR was carried out in a total reaction volume of 20 μ l which contained 13.3 μ l DDW, 2.0 μ l 10x PCR buffer (Takara, Japan), 1.6 μ l dNTPs (Takara, Japan), 0.5 μ l each of 10 pmol of the internal primers (Tajima et al., 1998): forward LTR256-GAG CTC TCT TGC TCC CGA GAC and reverse LTR453- GAA ACA AAC GCG GGT GCA AGC CAG, 0.1 μ l of rTaq polymerase (Takara, Japan) and 2.0 μ l of the first PCR products. PCR condition was the same as the first PCR except for the annealing temperature at 60°C. The amplified PCR products were visualized on 2% agarose gel. The results were observed under an ultraviolet light.

Results

A total of 300 cattle blood samples from selected cattle farms in five provinces in the Philippines were used. All 300 (100%) of the total cattle blood samples were confirmed to contain DNA as they exhibited bands at the expected 227 bp for the β -actin genes after PCR (Fig 2). This also ensures that DNA from all the samples had been successfully extracted.

On the other hand, the PCR products of the first BLV-LTR PCR amplification did not exhibit a desired 533 bp using the external primers. One reason is that the product of the first amplification for the nested PCR assay contained only very few number of target genes specific for the external primers. The second PCR amplification for BLV-LTR internal

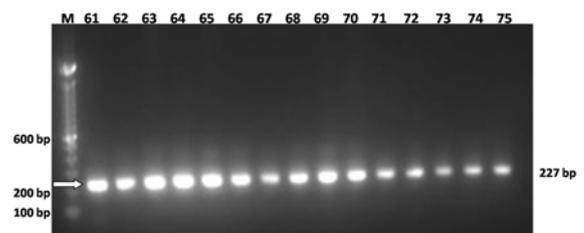


Figure 2 Representative figure exhibiting the amplification of β -actin gene used as the housekeeping gene. M, represent the 100 bp ladder. White arrow shows the β -actin bands (227 bp).

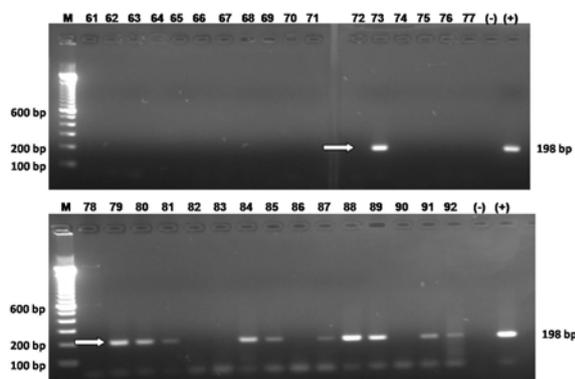


Figure 3 Nested PCR results for BLV-LTR 2nd amplification (samples no. 61-92). M, represents the 100 bp ladder. White arrows show positive results (198 bp). (+) positive and (-) negative controls.

primers resulted to 11 (3.67%) positive samples of the total 300 cattle blood samples. Interestingly, out of 153 cattle blood samples from the two farms in Laguna, all 11 (7.19%) positive samples came from only one farm (Fig 3).

Discussion

One of the methods in confirming the success of DNA extraction is the exhibition of housekeeping gene (i.e. β actin gene) by PCR. β -actin gene exists and is transcribed in most cell types as components of cytoskeleton and as mediators of internal cell motility (Lanetty et al., 2008; Zilli et al., 1988). B-actin is typically selected as a housekeeping gene because of its responsibility for the key biological pathways, ubiquitous expression and its presence in all genomic DNA (Pearce et al., 2007). Over the past years, β -actin has become the most common internal control gene in gene expression and other related molecular studies. It is not only an adequate housekeeping gene but also the best performing and most stable gene because it determines the overall quality of the DNA (Lanetty et al., 2008; Ferreira et al., 2010).

The nested-PCR is usually used to reduce the presence of undesirable bands due to contaminations and to increase the specificity of amplifying the target gene. Usually, the first PCR product would not exhibit bands after electrophoresis particularly in cases of low DNA yield of the target gene. The gene load is very low that it only produced invisible bands. The presence of the expected second PCR amplicon is usually viewed as confirmation of the presence of the target gene in a sample (EPA, 2004). According to Rimstad and Hyllseth (1990) the second amplification will surely form a band because it is already specific to a sequence found within the DNA of the initial amplification during nested PCR.

In connection to the detected BLV infection despite no case in the past, the possible reason particular in the farm where the samples came from is that stocks were previously imported from Australia and New Zealand. These two countries are known to have BLV infection (Hillink, 1988; Ambroski et al., 1989; Eaves et al., 1994; Teekayuwat et al., 2000; Lew et al., 2004; AQIS, 2009; Erectus, 2011). Cattle blood

samples from Pangasinan, La Union, Bohol and Cebu were all negative for BLV infection.

Amplification and detection of BLV proviral DNA genes by the double or nested PCR provides a sensitive means of direct diagnosis of BLV infection. Most studies of PCR assays are based on single PCR using only one pair of primers. However, studies on BLV and other viruses proved that sensitivity of nested PCR, using two pairs of primers, was considerably higher than that of the single PCR (Klintevall et al., 1994).

Mingala et al. (2009) detected BLV proviral DNA in riverine and swamp type water buffaloes in the Philippines using the BLV- LTR primers designed by Tajima et al. (1998). Their study resulted to 75 positive results out of 272 (27.6%) blood samples collected from various parts of the Philippines, mainly in Luzon. In Canada, Jacobs et al. (1992) detected BLV provirus in cattle having lymphoma using nested PCR. The result of the nested PCR was 16 out of 27 (59.3%) cattle with lymphoma were positive for BLV provirus. They also concluded that nested PCR is very specific for detecting BLV provirus. In the US, Kuckleburg et al. (2003) also detected BLV provirus using nested PCR and showed that a very significant number of samples were positive to BLV infection in blood and milk samples collected. The study accomplished that nested PCR might not be designed to replace the traditional antibody assays. However, it can provide as a useful supplement for the early detection and confirmation of EBL since delayed seroconversion is also possible in the event of early BLV infection (Wu et al., 1989). To the best of our knowledge, this is the first report of EBL detection in cattle in the Philippines using nested-PCR.

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References

- Amborski, G.E., Lo, J.L. and Seger, C.L. 1989. Serological detection of multiple retroviral infections in cattle: Bovine leukemia virus, bovine syncytial virus and bovine visna virus. *Vet Microbiol.* 3: 247-253.
- Arellano, E.R., Soriano, V., Alcami, J., Holguin, A. 2006. New findings on transcription regulation across different HIV-1 subtypes. *AIDS Rev.* 8: 9-16.
- Australian Quarantine and Inspection Services. 2009. *Enzootic Bovine Leucosis*. [Online]. Available: <http://www.daff.gov.au/animal-plant-health/pests-diseases-weeds/animal/eb1>. Accessed November 8, 2011.
- Bureau of Animal Industry. 2004. Amendment to DA-BAI Administrative Order No. 12 series of 2004

- on the Classification of Animal Diseases. [Online]. Available: <http://spsissuances.da.gov.ph/attachments/article/695/DA-BAI%20Administrative%20Order%20No.%2017%20Series%20of%202004.pdf>. Accessed June 13, 2011.
- Eaves, F.W., Molloy, J.B., Dimmock, C.K. and Eaves, L.E. 1994. A field evaluation of polymerase chain reaction procedure for the detection of bovine leukemia virus proviral DNA in cattle. *Vet Microbiol.* 39: 313-321.
- Environmental Protection Agency-US. 2004. Quality assurance / quality control guidance for the laboratories performing PCR analyses on environmental samples. [Online]. Available: <http://nepis.epa.gov/Exe/ZyPDF.cgi?Dockey=P100993V.PDF>. Accessed October 10, 2011.
- Erectus, Q. 2011. Diseases in Australia: Cattle and Dairy Herds. [Online]. Available: <http://www.instituteforsocialsurvivalism.org>. Accessed November 8, 2011.
- Ferreira, E.C., Gontijo, C.M., Cruz, I., Melo, M.N. and Silva, A.M. 2010. Alternative PCR protocol using a single primer set for assessing DNA quality in several tissues from a large variety of mammalian species living in areas endemic for leishmaniasis. *Mem Inst Oswaldo Cruz.* 105: 895-898.
- Gillet, N., Florins, A., Boxus, M., Burteau, C., Nigro, A., Vandermeers, F., Balon, H., Bouzar, A.B., Defoiche, J., Burny, A., Reichert, M., Kettmann, R. and Willems, L. 2007. Mechanisms of leukemogenesis induced by bovine leukemia virus: prospects for novel anti-retroviral therapies in human. *Retrovirology* 4: 18.
- Hillink, F. 1988. Bovine leukemia virus infection in herds in New Zealand. *Surveillance.* 5: 4-5.
- Jacobs, M., Song, Z., Poon, H., Herney, J.L., Taylor, J.A., Jefferson, B., Vernau, W. and Valli, V.E.O. 1992. Proviral detection and serology in bovine leukemia virus-exposed normal cattle and cattle with lymphoma. *Can J Vet Res.* 56: 339-348.
- Khan, C.M. (ed). 2010. *The Merck Veterinary Manual*. 10th ed. Whitehouse Station, New Jersey, USA. Merck & Co., Inc. 671-674.
- Klintevall, K., Pordany, A.B., 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. *Vet Microbiol.* 42: 191-204.
- Kobayashi, S., Tsutsui, T., Yamamoto, T., Hayama, Y., Kameyama, K., Konishi, M. and Murakami, K. 2010. Risk factors associated with within-herd transmission of bovine leukemia virus on dairy farm in Japan. *BMC Vet Res.* 6: 1-6.
- Kuckleburg, C.J., Chase, C.C., Nelson, E.A., Marmac, A.E., Dammer, M.A. and Hemmings, J.C. 2003. Detection of bovine leukemia virus in blood and milk by nested and real-time polymerase chain reaction. *J Vet Diagn Invest.* 15: 72-76.
- Lanetty, M.R., Philips, W.S., Dove, S., Guldborg, O.H. and Weis, V.M. 2008. Analytical approach for selecting normalizing genes from a cDNA microarray platform to be used in q-RT-PCR assays: a cnidarians case study. *J Biochem Biophys Methods.* 70: 985-991.
- Lew, A.E., Bock, R.E., Molly, J.B., Minchin, C.M., Robinson, S.J. and Steer, P. 2004. Sensitive and specific detection of proviral BLV by 5' Taq nuclease PCR using a 3' minor groove binder fluorescent probe. *J Virol Methods.* 115: 167-175.
- Mingala, C.N., Konnai, S., Cruz, L.C., Onuma, M. and Ohashi, K. 2009. Comparative molecule-immunological analysis of swamp- and riverine-type water buffaloes responses. *Cytokine.* 46: 273-282.
- 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. *J Virol Methods.* 33: 73-85.
- Office Internationale Des Epizooties. 2000. *OIE Manual of Standards for Diagnostics Tests and Vaccines*. 4th ed. Paris, France. OIE.
- Pearce, M., Jurinke, C. and Oeth, P. 2007. "Reference gene selection for gene expression studies." [Online]. Available: http://www.google.com.ph/url?sa=t&rct=j&q=reference%20gene%20selection%20for%20gene%20expression%20studies.&source=web&cd=2&sqi=2&ved=0CE0QFjAB&url=http%3A%2F%2Fwww.sequenom.com%2Ffiles%2Fgenetic-analysis-files%2Fqge-application-pdfs%2Fwhite_paper&ei=CbMgUJO2FY6kiQe134Ao&usg=AFQjCNHa9EU_8z8LV_Qa1EAVHfiyZ0Ha3Q&cad=rja. Accessed November 8, 2011.
- Pollari, F.L., Wangsuphachat, V.L., Digiacamò, R.F. and Fevermann, J. 1992. Effects of bovine leukosis virus infection on production and reproduction in dairy cattle. *Can J Vet Res.* 56: 289-295.
- Rimstad, E. and Hyllseth, B. 1990. Double PCR and magnetic separation of DNA used in identification of infectious pancreatic necrosis virus, a dsRNA virus. *J Virol Methods.* 34: 446-455.
- Sagata, N., Yasunaga, T., Ohishi, K., Tsuzuku-Kawamura, J., Onuma, M. and Ikawa, Y. 1984a. Comparison of the entire genomes of bovine leukemia virus and human T-cell leukemia virus and characterization of their unidentified open reading frames. *EMBO J.* 3: 3231-3237.
- Sagata, N., Yasunaga, T., Ogawa, Y., Tsuzuku-Kawamura, J. and Ikawa, Y. 1984b. Bovine leukemia virus: unique structural features of its long terminal repeats and its evolutionary relationship to human T-cell leukemia virus. *Proc Natl Acad Sci USA.* 81: 4741-4745.
- Tajima, S., Ikawa, Y. and Aida, Y. 1998. Complete bovine leukemia virus (BLV) provirus is conserved in BLV-infected cattle throughout the course of B-cell lymphosarcoma development. *J Virol.* 113: 55-56.
- Teekayuwat, T., Pfeiffer, D.U. and Hayes, D.P. 2000. Spatial clustering of bovine leukemia virus infection in New Zealand. In: *Proceedings of the 9th Symposium of the International Society for Veterinary Epidemiology and Economics*. Breckenridge, Colorado, USA, p. 526.
- Venables, C. and Lucas M.H. 2004. *Enzootic bovine leukosis*. In: *Bovine Medicine. Diseases and Husbandry of Cattle*. 2nd ed. A.H. Andrews,

- R.W. Blowey, H. Boyd and R.G. Eddy. Ames, Iowa, USA. Blackwell Publishing Co. 693-699.
- Wu, M.C., Shanks, R.D. and Lewin, H.A. 1989. Milk and fat production in dairy cattle influenced by advanced subclinical bovine leukemia virus infection. *Proc Natl Acad Sci USA*. 86: 993-996.
- Yavru, S., Kale, M., Ata, A., Yapkic, O., Bulut, O. and Gulay, M.S. 2007. Effects of subclinical bovine leukemia virus infection on fertility of holstein cows and heifers. *Medycyna Wet*. 63: 667-669.
- Zilli, L.D., Tuyns, A.S. and Paterson, B.M. 1988. A 40-base pair sequence in the 3' end of the β actin gene regulates β actin mRNA transcription during myogenesis. *Proc Natl Acad Sci USA*. 85: 1389-1393.