Enzootic Bovine Leukosis (EBL) in formalin-fixed and paraffin-embedded tissues (FFPPE)

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**บทคัดย่อ**

โรค Enzootic bovine leukosis (EBL) ทำให้เกิดผลกระทบต่อระบบภูมิคุ้มกันของสัตว์ และเกิดความเสี่ยงในการสูญ
ภูมิคุ้มกันจากการทุจริต โรคนี้อาจเกิดการสูญเสียทางเศรษฐกิจที่สำคัญกว่าการอุบัติการณ์ เซ็นทำให้ปริมาณภูมิคุ้มกันลดลง เสีย
การไวรัสจาก Organism ที่ติดเชื้อจากสัตว์ หรือจับได้ต้องส่งกลไกที่ติดเชื้อดังกล่าวเข้าสู่ระบบชมพู
ในการรักษา ซึ่งทั้งเกิดกับการสูญเสียโอกาสในการแจ้งขึ้นในระบบภูมิคุ้มกัน แต่จากการสังเกตุขึ้น จะทำให้เกิดการ persistent, asymptomatic และymphosarcoma ซึ่งสื่อถึงเป็นการที่ส่งผลกระทบต่อสัตว์ที่ได้รับเชื้อมาก
สุด ดังนั้นเพื่อวินิจฉัยว่า bovine lymphosarcoma ที่พบใน FFPPE มีสาเหตุจาก EBL หรือไม่จำเป็นต้องผลิต primary
antibody ต่อ protein ที่สำคัญของไวรัสในการเตรียม antigen คือ glycoprotein และ capsid protein เพื่อใช้ศึกษา
ทาง immunohistochemistry ของ lymphosarcoma โดยย้าย gene ของ glycoprotein ของไวรัสที่ติด signal protein
และ transmembrane region ออก ในขณะที่ full-length gene ของ capsid protein p24 เพื่อจำหน่ายและแสดงออก
โดยมี histidine tag ที่ปลาย N terminal region จากนั้น recombinant ตั้ง 2 ชนิดมาในเครื่องมือใช้ SDS-PAGE และ
Western blot พบว่า recombinant protein ตั้ง 2 ชนิด มี molecular weight ที่ถูกต้อง นอกจากนี้จากการทำ in situ
hybridization สามารถ hybridize proviral DNA ที่ nuclease, genomic RNA ที่ cytoplasm และที่ plasma
membrane ซึ่งทำให้ว่า rabbit IgGs antilymphoprotein สามารถตรวจหา antigen ได้ที่ plasma membrane เป็นส่วน
ใหญ่ ขณะที่ rabbit IgGs anticapsid protein ตรวจหา antigen ได้ที่ cytoplasm เป็นส่วนใหญ่ นอกจากนี้ยังพบว่า
อัตราการกระจายของไวรัสมีมากที่สุดที่มีมัน ต่อมน้ำเหลือง และ อวัยวะที่ cell นี้จะกระจายไปตามลำดับ จา
การศึกษาในครั้งนี้สามารถผลิต primary antibody เพื่อวินิจฉัย lymphosarcoma ที่เกิดจากไวรัส EBL ใน FFPPE
รวมถึงสร้างความเข้าใจเกี่ยวกับกลไกการเกิดโรคมากยิ่งขึ้น

**คำสำคัญ:** Enzootic bovine leukosis, gp51, p24, immunohistochemistry, Formalin-fixed and paraffin-embedded tissues (FFPPE)

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Production of Polyclonal Antibody from Recombinant Glycoprotein 51 (rgp51) and Capsid protein 24 (p24) for Diagnosis of Enzootic Bovine Leukosis (EBL) in Formalin-fixed and paraffin-embedded tissues (FFPPE)

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Abstract

Enzootic bovine leukosis (EBL) can cause detrimental effect to host immune system. Decline of host immunoglobulins makes it vulnerable to infection and failure in producing protective antibody from vaccination. EBL also can cause significant economic loss to farmers such as decreasing in milk yield, cost of treatment, high premature culling rate and loss of international trade. EBL can be classified into 3 categories: persistent lymphocytosis, asymptomatic and lymphosarcoma, the most importance aspect of EBL. To detect BLV in bovine lymphosarcoma found in FFPPE, we produce rabbit anti serum against glycoprotein and capsid protein of bovine leukemia virus (BLV) for immunohistochemical study of lymphosarcoma. The importance proteins for antigens preparation, a gene encodes glycoprotein of BLV is amplified without signal peptide and transmembrane region; while, a gene almost full-length encoding capsid protein p24 is amplified. The recombinant protein with N terminal histidine tag is expressed and purified. SDS-PAGE and Western blot are brought to verify presence of the proteins. Furthermore, in situ hybridization is performed to identify proviral DNA associated EBL. Rabbit IgGs antibody raised against both recombinant proteins are purified and used for immunohistochemistry. Both recombinant proteins are successfully expressed with correct expected molecular weight. For in situ hybridization technique, proviral DNA of BLV is hybridized mainly in nuclease, genomic RNA in cytoplasm and plasma membrane. Interestingly, purified rabbit IgGs-anti glycoprotein is intensely recognized this antigen predominantly in plasma membrane; in contrast, IgGs-anti capsid protein is mainly localized antigen in cytoplasm. Importantly, distribution of the virus is found in spleen more than the other organs of neoplastic involvement. The data from this study demonstrates that both rabbit antibodies can be used for diagnosis of EBL in FFPPE and it generates better understanding about this pathogen.

Key words: Enzootic bovine leukemia, gp51, p24, immunohistochemistry, formalin-fixed and paraffin-embedded tissues (FFPPE)

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Introduction

Enzootic bovine leukemia (EBL) can cause significant economic loss to farmers from direct impact such as reduce milk production (Otta et al., 2003), production loss, reduced slaughter value, a high premature culling rate and increase replacement cost (Pelzer, 1997). The indirect impact comes from treatment cost and losses of international market share (Brenner et al., 1989; Chi et al., 2002). Besides the economic impact, EBL has an effect to host immunity. From the previous study, it was found that mastitis is related to EBL infection (Yoshikawa et al., 1997) and the function of neutrophil in mammary gland of natural infected cows is impaired (Della Libera et al., 2015) Furthermore, several studies have been reported the immunoglobulins produced from affected animals are impaired and it makes the infected hosts are vulnerable to infection and primary antibody response to vaccination is failure (Trainin et al., 1996).

EBL caused by Oncogenic lymphotropic virus belongs to Family Retroviridae, Genus Deltaretrovirus. In this Family reverse transcriptase plays a crucial role in viral infection. After infection the target cells are mostly found in B-lymphocytes (Johnson et al., 1987) and other cellular types such as T-lymphocytes, macrophage, granulocytes (Panei et al., 2013) and dendritic cells (Szczotka et al., 2012). The host immune system directly responses against the viral envelope (env) glycoprotein which is important for the virus to recognize target receptors and enter into host cells and this glycoprotein is the main target for neutralizing antibody. Moreover, the env gene of Bovine leukosis virus (BLV) encodes surface unit (SU), the polyprotein precursor (gpr72) which is cleaved into gp51 and gp30 transmembrane glycoproteins (Llames et al., 2001). Like gp51, p24 is immunogenic, and it is the main capsid protein which is encoded by gag gene, involved in formation of viral capsid. This precursor capsid protein is 45 kDa protein, finally cleaved to p15, p24 and p 12 (Schwartz and Levy, 1994).

After infection, hosts are life-long infected, usually they are asymptomatic, however 30-70% of infected animal can develop persistent lymphocytosis and less than 10% can develop lymphosarcoma (Johson and Kaneene., 1991a). The clinical pictures of affected animals can be seen like emaciation, disorder in several organs such as respiratory, circulatory, digestive, reproductive and nervous sign depending on organs involved by neoplasm. Therefore, it is necessary to make a definite diagnosis for neoplasm commonly found in histopathology slides of affected cattle. In general the most common neoplasm seen in cattle can be divided into 2 types: enzootic bovine leukosis (EBL), caused by bovine
leukemia virus (BLV), and the other type is sporadic bovine leukosis (SBL) which is not related to BLV infection. From histopathological study, both of them are morphologically indistinguishable. The objective of this study is to produce rabbit polyclonal antibodies against BLV gp51 and p24 to be used for immunohistochemistry application in cattle found death from neoplasm.

Materials and methods

Ethics statement

The animal in this experiment was conducted in accordance with the guiding principles for the care and use of research animals, and the protocol was approved by the Committee on the Ethics of Animal Experiments by National Institute of Animal Health, Thailand (Permit number EA-002/57(B)).

Production of full length of env (gp72) and p24 capsid gene

Fetal Lamb Kidney Cells Infected with bovine leukemia virus (FLK-BLV) was kindly provided by NIAH Japan, keep in -80°C. According to the complete genome sequence accession number NC_001414 (acc. No. AF033818), it was used to design primers for construction of env and p24 full length sequence. After cloning into pGEM T Easy vector (Promega, United States) according to manufacturer’s instruction, briefly, primers forward envgPrT2F and reward primer envgPrT2R are designed (Table 1). The reaction was performed in 25 ul total volume, which contains 1x PCR buffer, minus Mg, 0.2 mM dNTP mixture, 1.5 mM MgCl₂ 0.2 uM of forward and reward primers final concentration and 5 ul of template DNA, 1 unit of Platinum® Tag DNA polymerase (Invitrogen®). Amplification reaction was carried out in a thermocycler (BioRad®) in the following cycle: the initial incubation at 94°C 2 min, follow by repeat 35 cycle: denaturation 94°C 30s, annealing 55°C 30s and extension at 72°C 2 min. The last cycle was run at 72°C 7 min. Furthermore, p24 capsid gene was constructed by forward primer p24F and reward primer p24R (Table 1) containing BamH1 and HindIII restriction site, respectively. The PCR reaction was performed in 25 ul total volume, which contains 1x PCR buffer, minus Mg, 0.2 mM dNTP mixture, 1.5 mM MgCl₂ 0.2 uM of forward and reward primers final concentration and 5 ul of template DNA, 1 unit of Platinum® Tag DNA polymerase (Invitrogen®). Amplification reaction was carried out in a thermocycler (BioRad®) in the following cycle: the initial incubation at 94°C 2 min, followed by repeat 35 cycle: denaturation 94°C 30s, annealing 55°C 30s and extension at 72°C 1 min. The last cycle was run at 72°C 7 min. Finally, they were designate pGEM T
Easy_Gpr72 and pGEM T Easy_rp24, respectively. For pGEM T Easy_Gpr72, it will be used as template for amplification glycoprotein 51 gene without signal peptide and transmembrane region (designate gp51/TM-).

Identification of positive clone

Escherichia coli (strain JEM 109, Promec@) transformed with pGEM T Easy_Gpr72 and pGEM T Easy_p24. They were analyzed by selective agar containing 100 ug/ml ampicillin 80 ug/ml, X-Gal and 0.05 mM IPTG. White colonies were picked and grown overnight in 1 ml of LB broth (Sigma®) containing 50 ug/ml final concentration of ampicillin. Plasmid was extracted by using alkali lysis miniprep, in brief, 1ml culture medium was centrifuged at 3500 rpm for 2 min at 4°C, discard supernatant and remove all LB broth as much as possible. After adding 200 ul solution 1(50 mM glucose, 25mM Tris-HCL, pH 8, 10 mM EDTA, pH8), the pellet was suspended by vortexing. Then, 200 ul of solution 2(1% SDS, 0.2 N NaOH) was added and mixed by inverting tubes and wait for 5 min. Solution 3 (3M NaOAc) 200 ul was added and then mixed by inverting tubes and wait for 5 min on ice. Centrifuge 15,000 rpm 5 min at 4°C and collect supernatant to new tubes. Absolute ethanol 600 ul was added and waited for 5 min then centrifuge 15,000 rpm 15 min at 4°C. Pellet was washed by 70% Ethanol and sterile water was added. Finally, extracted plasmid was subjected analyzed by PCR or restriction enzyme digestion.

Expression and purification of Recombinant protein gp51 without signal peptide and transmembrane region (rgp51/TM-) and Recombinant protein p24 (rp24)

The gene encoding target sequence gp51/TM- was amplified by using primers forward and reward (Table1) designed according to sequence accession number NC_001414 containing restriction enzyme BamH1 and HindIII, respectively. The PCR reaction was performed in 25 ul total volume, which contains 1x PCR buffer, minus Mg , 0.2 mM dNTP mixture, 1.5 mM MgCl2 0.2 uM of forward and reward primers final concentration and 5 ul of template DNA, 1 unit of Platinum® Tag DNA polymerase (Invitrogen®). Amplification reaction was carried out in a thermocycler (BioRad®) in the following cycle: the initial incubation at 94°C 2 min, followed by repeat 35 cycle: denaturation 94°C 30s, annealing 55°C 30s and extension at 72°C 1.5 min. The last cycle was run at 72°C 7 min. The amplicons was cloned into pGEM T Easy vector and positive cloned was identified the same protocol as mentioned above. The pGEM T
Easy vector harboring rgp51/TM- and rp24 was subcloned into pQE 30 expression vector (addgene®, united states) and expressed as histidine-tag protein in E. coli. A fresh 5 ml overnight culture from single colonies of the E. coli/BL21(DE3) (invitrogen®, united states) transformed with pQE 30_rgp51/TM- and pQE 30_rp24 plasmid was grown in 500 ml LB broth containing 50 ug/ml of ampicillin at 37°C with shaking at 180 rpm for 3 hr. The expression was induced by using 1 mM Isopropyl-thiogalactoside (IPTG) followed by expression at 20°C with shaking at 90 rpm for 16 h. The E. coli cultures were centrifuged at 3,000 rpm 30 min at 4°C, and the cell pellets were resuspended in lysis buffer (100 mM NaH2PO4, 10 mM Tris Cl pH 8.0) containing 8 M Urea, 50 mg/ml lysozyme, protease inhibitor (amresco®). The E. coli cell suspensions were chilled on ice for about 1 hr and sonicated for 5 min. The sonicated cell cultures were immediately centrifuge at 10,000 rpm for 30 min at 4°C. To purify the recombinant protein, the supernatant was bound to Ni-NTA agarose beads (QIAGEN®) according to the manufacturer’s instruction. The recombinant protein bound to beads was washed three times with washing buffer (100 mM NaH2PO4, 10 mM Tris Cl pH 6.0) and eluted with elution buffer (100 mM NaH2PO4, 10 mM Tris Cl pH 5.0). The concentration of protein was determined by using a DC protein assay kit (Biorad®), applying bovine serum albumin (BSA) as standard. The specific band of the expressed was determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970).
SDS-PAGE and Western blotting.

The expressed recombinant proteins were verified by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) stained with Coomassie blue, and the antigenicity was confirmed by Western blot analysis. To identify antigenicity of the recombinant proteins, the proteins concentration was determined using a BCA protein assay kit (Pierce Biotechnology, Rockford, Illinois, USA), and stored at -80°C until use. Proteins in the extract were size-separated by electrophoresis in 12% SDS-PAGE and then electroblotted onto a nitrocellulose membrane. The membrane was blocked with 0.05% Tween 20 in PBS (PBST) plus 5% skim milk and probed with specific primary antibodies against BLV purchased from ID.Vet® (France). After washing with PBST, a secondary antibody, a horseradish peroxidase (HRP)-conjugated anti-bovine IgG antibody (Bethyl, Montgomery, Alabama, USA) was applied. The substrate solution (5 ug DAB, 5 ml of 1x PBS and 3 ul of 30% hydrogen peroxidase) was added to the membrane and the specific band corresponding to the molecular weight of the protein was detected.

Production of anti-rgp51/TM- and rp24 sera and IgG purification.

Two New Zealand White Rabbits (2 kg.) supplied by National Laboratory Animal Center ([http://www.nlac.mahidol.ac.th/nlacen/](http://www.nlac.mahidol.ac.th/nlacen/)) were immunized subcutaneous (s.c.) with 150 ug of the purified rgp51/TM- and rp24 emulsified in 100ul of Freud’s complete adjuvant (Sigma, St. Louis, Missouri, USA). Three boosters were given s.c. using 150 ug of the same antigens emulsified in Freund’s incomplete adjuvant (Sigma) at 14-day intervals. Sera were collected before inoculation and after the last booster and checked for specific antibodies by iELISA. Furthermore, total IgGs were purified from rabbit sera through a chromatography columns according to the manufacturer’s instruction (Bio-Rad Laboratories, Foster, California, USA). The fractions containing IgG were run on SDS-PAGE to test for the purity and concentration. An iELISA was performed to test the binding activity of the IgGs.

*In situ* hybridization to confirm Bovine lymphosarcoma associated with BLV infection

444 biotinylated in situ hybridization probe to detect BLV infection in formalin-fixed paraffin embed tissue (FFPPE) was previously described by Nawaporn (2014). Briefly, the plasmid pGEM T Easy_gpr71
was used as a template for nested PCR (Fechner et al., 1996). The hybridization probe was produced from the second PCR by mixed dNTP and Biotin-dUTP (Jena Bioscience, Germany). For specimens preparation for in situ hybridization (ISH), the paraffin embedding tissue was cut in 3-5 μM and put on plus slide/superfrost®. Warm slide at 60°C for 30 min and deparaffinized and rehydration by xylene (5 min, 2 times), absolute ethanol(5 min, 3 times), 95% ethanol(5 min, 2 times) and DEPC-H₂O (5 min). Proteinase K (Dako, ready to use) was applied for 20 min and incubate at room temperature. Then the slides were rinsed in DEPC-H₂O (3 times, 5 minute). Next, peroxidase was got rid of by treating slides with 0.3% H₂O₂ in methanol for 20 min at room temperature. Rinse slides in DEPC-H₂O (3 times, 5 min), then dry slides. For prehybridization step, apply 100 ul of hybridization buffer with probe 500 ng/slide and incubate at 95°C for 10 min. For hybridization step, incubate slides at 42°C 16 hours. And for post hybridization, remove coverslip by soaking in TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20). The slides were subjected for stringency wash in Saline Sodium Citrate buffer, SCC (3M NaCl in 0.3M sodium citrate, pH 7.0) in decreasing concentration in at 42°C as followed, 2X SSC, 1X SSC , 5 min and finally in 0.5X SSC, 30 min.

Then incubate slides shortly in TBST for 5 min and blocking in 3% normal horse serum in TBST for 10 min. For detection step, streptavidin-HRP (Dako®) was applied for 3-4 drops to cover the specimens and incubate for 15 min, wash slides with TBST(5 min, 3 times) and tap off the excess TBST. Then, apply 3-4 drops of biotin (Dako®) and incubate for 15 min. Wash slides with TBST (5 min, 3 times), then repeat streptavidin-HRP and wash. Finally, DAB chromogen (3,3'-Diaminobenzidine) was added and incubated at room temperature for 5 min. The slides were counter stained with hematoxylin.

Sequencing

The E. coli positive clones, pQE 30_rgp51/TM- and pQE 30_rp24 were subjected to 5 ml. culture and plasmids were extracted (Promega®) according to manufacturer’s instruction, and the plasmids were sent to local company to sequence (WardMedic, Thailand) to confirm in frame of histidine tag. Then the amino acid sequence of both proteins was predicted (http://web.expasy.org/translate/) and aligned with reference sequence MEGA v.4 (Tamura et al., 2007).
Development of immunohistochemistry to detect EBL in FFPPE

Paraffin-embedding blocks (the cases of SBL and EBL were confirmed by PCR, kindly provided by NIAH, Japan and a case gives positive result to bovine viral diarrhea virus) were cut 3 μM and placed on positive charge slides. Then the slides were put in an incubator for 30 min. The slides were deparaffinized in xylene (2 times x 5 min) and start rehydration in 100% ethanol for 3 min and descending concentrations of ethanol (90% 80%, 70%, 50%) and immerse sections in distilled water. From this point on, be careful not allow the slide to dry out throughout the entire process, following treating slides with 0.3% H₂O₂ in methanol for 20 min at room temperature. Proceeding, heat induces epitopes retrieval for 5 min at 100°C in streamer while immersed in citrate buffer solution (10mM Citric Acid, 0.05% tween 20, pH 6.0). Afterwards allow sections to cool off for 30 min. Then equilibrate slides in Phosphate buffer solution (137 mM NaCl; 2.7 mM KCl; 10 mM Na₂HPO₄; 1.8 mM KH₂PO₄, pH 7.4 ) once. Non specific binding was blocked by 1% normal bovine serum. Purified Rabbit IgGs antibody raised against rgp51 and rp24 1 in 100 dilution were used to incubate overnight at 4°C together with positive and negative control (preimmunized rabbit-anti serum in replacement of primary antibody was used in slides of positive control and PBS is used to replaced primary antibody in order to observed the immunoreactivity of polymer-anti rabbit antibody, secondary antibody to bovine immunoglobulins). Then the slides were rinsed in phosphate buffer solution three times, five min each. Polymer-anti rabbit antibody horseradish peroxidase conjugate was added and incubated at room temperature for 10 min. Repeat washing step. Finally AEC (3-amino-9-ethylcarbazole) substrate chromogen was applied and incubated in room temperature for 2 min. The reaction was stopped in distilled water before counterstain with hematoxylin, and covered the slides with mounting medium.

Results

Table1. Primer sequence for amplification target gene

<table>
<thead>
<tr>
<th>Primers(5’-3’)</th>
<th>Position in BLV Genome sequence</th>
<th>PCR product</th>
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<tbody>
<tr>
<td>envgPr72F</td>
<td>4615..4635</td>
<td>1548 bp</td>
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<tr>
<td>Primer</td>
<td>Sequence</td>
<td>Range</td>
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<td>-------------</td>
<td>---------------------------------------------</td>
<td>----------</td>
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<td>GATAAAGCTTTTGGAAAAACCCAGCGGATGTACACC</td>
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</table>

Amplification of full length of env (Gpr72), gp51/TM without signal peptide and capsid gene encoding p24

A reference sequence of BLV is deposited in GenBank accession number NC_001414. cDNA encodes full length of envelope gene gpr72 is successfully amplified and the amplicons are in the expected calculation. The pGEM T Easy harboring full-length sequence of env gene is used as a template for amplification. The PCR products are in the calculate site (Picture 1 A) as well as a gene encodes p24 protein (Picture 1 B).

![Picture 1. A; PCR products are analyzed in 1% agarose gel stained with fluorescent dye (Novel Juice). Lane M is 1 kb DNA marker (Thermo scientific). Lane1-5 are 1051 PCR product, and 6-10 are 1548 PCR products. Lane neg. is negative control. B; successful amplification of 651 bp product is analyzed in 1% gel.](image_url)
agarose gel stained with fluorescent dye. Lane M is 100 bp ladder (H3 RTU (Ready-to-Use)), 1-5 are PCR product, and neg is negative control.

**SDS-PAGE and Western blotting analysis of rgp51/TM- and rp24**

SDS-PAGE analysis after purification of the his-tag rgp51/TM- and rp24 by Ni²⁺-NTA resin column. The eluted recombinant proteins show band corresponding to the expected molecular weight of rgp51/TM- (40kDa) and rp24 (21kDa). In order to confirm the present of both proteins, Western blot is performed by using positive and negative bovine serum against BLV.

![Picture 2](image)

Picture 2. Panel A: Expression and purification of rgp51/TM- in *E. coli*. A comassie blue-staining; M, Molecular protein size marker (See blue®, Invitrogen); Lane a is a purified fraction of the rgp51/TM-; Lane b is purified rp24. Panel B; the purify rgp51/TM- and rp24 was run on 12.5% SDS-PAGE together with molecular weight marker and transferred to PVDF membrane (BioRad®). Western blot to detect rgpgp51/TM- (a) and rp24 by bovine anti-BLV-IgG positive serum (pos) and negative serum(neg)

**In situ** hybridization of Bovine lymphosarcoma associated with BLV infection

In order to confirm bovine lymphosarcoma caused by BLV infection *in situ* hybridization is conducted. Many infected cells are detected. The reactivity is observed mostly in nuclease, cytoplasm and plasma membrane.
Positive labelling is observed in nuclease (a, black arrow) cytoplasm (d, arrow head) and plasma membrane (d, white arrow). Negative control (b). Positive control (c), neoplastic cells is characterized by irregular hyperchromatic nuclei of varying in size with small amount of cytoplasm.

**Predicted amino acid sequence alignment**

Amino acid sequence alignment of full-length sequence between BLV surface unit 60 (glycoprotein 51 with transmembrane 30) and mature peptide without transmembrane region (gp51/TM-) are performed (A). Additionally, full-length sequence of p24 capsid protein is also aligned with rp24 (B)
Picture 4. Matrix: BLOSUM 62, *Painwise sequence alignment* with 1000 of bootstraps. Reference sequence is NC_001414 and predicted rgp51/TM-. Green area is a signal peptide. Red arrow indicates the origin of mature peptide. Blue area is transmembrane region and cytoplasmic domain (A). Capsid protein p24 of reference sequence is compared with rp24 (B). – (dash) indicates delete amino acid sequence in the area of non agreement.

**Immunohistochemistry to detect EBL in FFPPE**

Bovine lymphosarcoma from BLV infected and sporadic lymphosarcoma are utilized to validate rabbit-anti gp51 (A) and p24 (B)
Primary antibody from purified rabbit IgGs raised against glycoprotein and capsid protein of BLV is able to localized those antigens in infected cells as previously studied (Llames et al., 2001). However, our observed glycoprotein of the virus is detected more at plasma membrane of infected cells than in the cytoplasm. This finding might be because rabbit anti-gp51/TM antibody cannot detect signal peptide, but those studies use monoclonal antibody against anti precursor glycoprotein and this monoclonal antibody might detect in both in cytoplasm and plasma membrane of infected cells. These points are due to the fact
that monoclonal antibody might recognize epitopes at the signal peptide. Additionally, this finding suggests that duration for transferring precursor glycoprotein and cleaving signal peptide at endoplasmic reticulum for producing mature peptide is short (Gillet et al., 2007). Furthermore, the intensity of immunoreactivity of p24 is localized mostly in cytoplasm. This might be due to p24 is very conserve among BLV making it easier for anti-p24 antibody to bind virion which capsid protein will assembly in the late state of morphogenesis. Furthermore, expression of recombinant glycoprotein without transmembrane which is predicted to be high hydrophobicity is expressed in a high concentration; however, p24 is tended to form inclusion body. Additionally, recognition of recombinant protein by positive serum for both gp51 and p24 (Rodák et al., 1997) was used to study the antigenicity of BLV. This implies the antigenicity of both recombinant proteins will be able to develop for ELISA test kit in the future. For in situ hybridization to detect BLV in bovine lymphosarcoma, our study got the same result as the previous study (Okada et al., 1991). Interestingly immunohistochemistry for rabbit anti-gp51/TM- and p24 primary antibodies are able to recognized the antigen at paracortical area in spleen with the small number of immunoreactivity in lymph nodes. This is because white pulp contains large number of B and T lymphocytes which are the target cells of infection (Chiba et al., 1995). Other organs infiltrated by neoplastic cells have less numbers of infected cells. This finding supports transformation of neoplastic cells from protein inducing non apoptosis of cells by the viral infected cells, or neoplastic cells do not come from insertion of proviral DNA of the virus because immunoreactivity cannot be observed in all neoplastic cells. However, to extend this finding electron immunogold electron microscopy and confocal microscope technique should be brought to study in the future.

Conclusions

From this study, rabbit IgGs raised against glycoprotein and capsid protein are able to recognize the antigens in bovine lymphosarcoma in FFPPE and both antibodies can distinguish EBL from SBL. However, rabbit anti-glycoprotein to locate the antigen at plasma membrane of infected cells more than within the cytoplasm; in contrast, rabbit IgGs anti-p24 antibody recognize the antigen in the cytoplasm of infected cells better than in the plasma membrane. Generally immunohistochemistry is employed to study distribution of the pathogen associated disease or organs involvement in the disease in order to gain understanding in pathogenesis and pathogenicity or severity of the pathogen, BLV tends to increase the
numbers of virion in spleen and a small number is detected in peripheral lymph nodes and organs that neoplastic cells infiltrate.

Primary antibodies used in this study are able to differentiate between EBL and SBL. This means both of the primary antibodies could be utilized in diagnosis of lymphosarcoma associated with EBL. Knowledge from this study also emphasis the importance of lymphosarcoma cases because only small numbers of infected cells producing a high number of infected or mature virion. Therefore, only small number of infected cell can establish infection and moreover this data leads us to investigate long terminal repeat (LTRs) of the virus relating to genotypes of the virus presented in Thailand. Additionally primary antibodies produced in our institute will be able to use to produce lateral flow immunochromatography test as well as they will be assisted in immunoperoxidase diagnosis for cell culture infected with the virus in order to establish OIE reference laboratory center for EBL diagnosis in the future.

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