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A Recombinase Polymerase Amplification Lateral Flow Dipstick for Field Diagnosis of Bovine Leukemia Virus Infection and its Effectiveness Compared to iiPCR and ELISA

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Abstract

For better control and eradicate enzootic bovine leukosis (EBL) in Taiwan, a more sensitive but also convenient method for detecting proviral bovine leukemia virus (BLV) DNA is required. The retrovirus BLV establishes a persistent infection that can result in reduced milk production and reduced survival rates, causing substantial economic losses in the dairy industry. BLV replicates by integrating its proviral DNA into the host genome; therefore, the detection of proviral DNA is recommended for identifying BLV carriers to help establish BLV-free herds. The integration of recombinase polymerase amplification (RPA) and lateral flow dipstick (LFD) in this study for on-site BLV detection. The optimal amplification condition for the RPA was 30 min at 37°C and followed by 5 min of LFD at room temperature. The sensitivity of this assay of 400 pg of total DNA and 10 copies of plasmid DNA. The method showed no cross-reaction with other tested viruses, including bovine foamy virus, bovine immunodeficiency virus, and caprine arthritis-encephalitis virus. For the detection of BLV field samples, the RPA-LFD was parallel tested with serological enzyme-linked immunosorbent assay (ELISA) and hydrolysis probe insulated isothermal PCR (iiPCR). The RPA-LFD assay exhibited a better sensitivity, with 83.5% of the 200 samples collected in Taiwan testing positive. A significant difference in the positive rates was found between the iiPCR and RPA-LFD methods, indicating that the RPA-LFD method for detecting BLV nucleic acid is sensitive at a lower limit. This RPA-LFD protocol can serve as an alternative tool to ELISA for the preliminary screening of BLV for its simplicity and portability, and is suitable for both laboratory and field application.

Keywords: Bovine leukemia virus (BLV), Recombinase polymerase amplification (RPA), Lateral flow dipstick (LFD)

Introduction

Bovine leukemia virus (BLV), classified in the genus *Deltaretrovirus*, family *Retroviridae*, is in close relationship with human T cell leukemia virus types 1 and 2 (HTLV-1 and HTLV-2) [1]. It is the causative etiologic agent responsible for enzootic bovine leukosis (EBL) [2]. The EBL is the most common neoplastic disease of cattle worldwide, though most of BLV infected cattle remain clinically asymptom at the aleukemic (AL) stage. However, about one-third of such infections may manifest as persistent lymphocytosis (PL; an increasing number of B lymphocytes) and around 5-10% may be diagnosed as B cell lymphoma [3]. BLV infections have been reported throughout the world, including countries in South America, Asia, Middle East, and Eastern Europe. It is also seriously prevalent in Taiwan, with a seropositive rate of approximately 96.10% [4]. BLV affects both the beef and dairy industries, with the direct economic losses resulting from the disease including death; decreases in milk yield, fertility, and life span; and condemnation at slaughter [5,6]. The economic losses resulting from the effects imposed by BLV infection on bovine breeding selection and export restrictions are also immense [7]. Uncontrolled BLV infection causes the extent of BLV spreading in herds, and leads to production and economic losses. Screening for BLV antibodies is the primary method for detecting BLV infection in cattle. There is currently no effective vaccine or therapy for BLV. The eradication of BLV relies on the precise diagnosis of the infection.

The BLV genome contains Gag, Pol, and Env structural and enzymatic protein genes. Other regulatory genes contained in the genome include the *tax*, *rex*, *R3*, and *G4* genes [8]. Two identical long terminal repeats (LTRs) include a U3 region, a long R region, and a U5 region in the BLV genome. Efficient transcriptional promoter activity exerted by LTRs in cells infected with BLV [8]. Once infected, the host cell genome integrates BLV into itself as a provirus [9]. In its period of latency, the expression of BLV is blocked at the transcriptional level [10,11] and BLV antibodies remain undetected [12]. Infected cattle retain at least one copy of the full-length BLV proviral genome throughout the course of the disease [13].

There are different techniques for the diagnosis of BLV infection. The agar-gel immunodiffusion (AGID) test and ELISA are both recommended by OIE [14] and serve as routine diagnosis methods for BLV infection. Antibodies to the main viral proteins such as gp51 and p24 were used for serological methods [15,16]. Hemmagglutination (IHA), radioimmunoassays, indirect fluorescent antibody test, and the

syncytium-inhibition test for antibody detection have also been used for the diagnosis of BLV infection. The removal of positive animals by these serological tests significantly reduced the number of BLV-infected animals. But the complete eradication BLV infection on farms cannot be done because they are not sensible for the early infection of BLV [17-19]. More specifically, it takes weeks to months for an animal to seroconvert, even as the animal continues shedding virus and covertly infecting other cattle within the herd.

Single PCR, nested PCR, real-time PCR, in situ-PCR, and PCR-ELISA tests have been developed for the direct detection of BLV proviral nucleic acid [15,16,20-25]. Isothermal nucleic amplification techniques were also developed, including the loop-mediated isothermal amplification (LAMP) method and iiPCR method, and used for the rapid diagnosis of BLV infection [26-28]. Both assays can amplify specific DNA sequences without the requirement for thermal cycling. However, a more simple, fast, and cost-effective technique is still needed for eradication programs and epidemiological studies. In iiPCR, 3 different temperature zones (denaturation, annealing, and extension) of PCR were achieved by natural liquid convection in a capillary tube sequentially [29-31]. Primers and probes bind to sequences at the annealing step is not done at a fixed temperature in iiPCR, allowing sequences with minor mismatches [32]. iiPCR were used in companion animals, livestock animals, and aquaculture animals for various bacterial and viral pathogens detection. In the field of food safety and health care, iiPCR has demonstrated a comparable result to that of the reference nested PCR, real-time PCR, and/or virus isolation method [33-35]. The evaluations of clinical sensitivity were higher than that of the reference real-time PCR methods in several cases [34]. Also, the detection of target sequences with notable variations, it was reported that clinical specificity could be maintained through the careful design of the primer and probe [36,37].

The recombinase polymerase amplification technique is another rapid and specific DNA amplification approach under isothermal temperature conditions. It does not require the use of expensive and sophisticated thermal cyclers and amplifies DNA within a single temperature range (37-42˚C) [38]. Unlike the LAMP method, which *Bst* DNA polymerase synthesizes various sizes of concatenated inverted repeats of DNA amplicons with 4-6 primers [39]. The RPA method aggregates two oligonucleotide primers to scan for a homologous target sequence in a DNA template uses phage-derived recombinase *UvsX* and its cofactor *UvsY*. When the specific homologous sequence is identified, the *Staphylococcus aureus*-derived DNA polymerase (*Sau* DNA polymerase) leads strand invasion and strand displacement *via* to generate double-stranded DNA (dsDNA) amplification [40-42]. Multiple techniques can be used for RPA amplification product detection, including agarose gel electrophoresis (AGE) [38] and real-time fluorescent detection [43], or be visualized by LFD assay [44,45].

The aim of this study was to develop a rapid and efficient diagnostic method combining RPA and LFD for BLV infection. The provirus pol-segment DNA of BLV in white blood cells were targeted by RPA-LFD assay. The diagnostic results of blood samples collected from accredited cattle flocks after exposure to natural infection, and samples from infected cattle herds were compared with those of serological ELISA and iiPCR tests. The molecular specificity and sensitivity of the RPA-LFD assay were also evaluated.

Materials and Methods

Animal and blood samples

The cattle blood samples were collected from 3 flocks of various breeds (147 heads of Holstein-Friesian, 35 heads of Taiwan Yellow Cattle, and 18 heads of Wagyu) from different regions of Taiwan. All three cattle herds were previously identified as BLV-positive or negative herds by the presence or absence of anti-BLV antibody by ELISA as described below. Both seronegative and seropositive cattle were sampled. The EDTA-treated blood was collected from the cattle by jugular venipuncture.

Total DNA template preparation

Total DNA was extracted from the EDTA-anticoagulated blood using a QIAamp DNA Mini Kit (Qiagen, Courtaboeuf, France) according to the manufacturer's instructions. The concentration and quality of DNA were determined using a NanoDrop 1000 Spectrophotometer (Thermo Fisher Scientific Inc., Waltham, MA, USA) at 260 and 280 nm, and then adjusted to 50 ng/μL by DNase-free water. The quantified DNA was stored at -20˚C until RPA was performed. One microliter of the total DNA template was used in each optimized RPA-LFD reaction.

Recombinase polymerase amplification primer and probe design

An series of primer screening assay of candidate primers and select a preferred primer pair is recommended. A series of suitable candidate primers were designed using Primer 3 (http://primer3.ut.ee/) to target the conserved regions in the BLV *pol* gene (2132-4669 nt, GenBank accession number: NC_001414.1) according to the appendix in the TwistAmp® reaction kit manual. Following the TwistAmp® reaction kit manual for primer screening, the forward primer BLV-01F (5′-TAGACGAACCCCACCTTCCCATGACTCAGGC-3′, nt 2180-2210) and the reverse primer BLV-02R with a 5′-biotin label (5′-biotin-AGTTTATCAGAGCCCTTGGGTGTTTCTCCGC-3′, nt 2315-2345) for the detection of the BLV *pol* gene (nt 2180-2345, 166 bp) used the TwistAmp® Basic kit (TwistDx, Cambridge, UK). For the integration of RPA with LFD assay, two nfo DNA probes (BLV-01Probe and BLV-02Probe) used were designed from the sequence between the BLV-01F and BLV-02R primers. The probes were consisted with a 5′-FAM antigenic label at the 30 nt upstream oligonucleotide stretch, and were connected *via* a Tetrahydrofuran (THF) spacer adjacent with a 15 nt downstream and a 3′ C3-spacer (polymerase extension blocking group) at its end. The designed nfo probes were tested using a TwistAmp® nfo kit (TwistDx, UK) for their compatibility with the forward BLV-01F and reverse BLV-02R primers in amplifying the BLV gene (nt 2132-4669). The BLV-01Probe (5′-FAM-CGAGCCCTCTGGACTCACAATCAGATTAAC-THF-TCCTACCAATTCTAA-C3-spacer-3′) demonstrated the best target amplification (166 bp). All the primers and probes were synthesized by Integrated DNA Technologies (Coralville, IA, USA).

Recombinant plasmid construction

The BLV *pol* gene sequences located between the nt positions 2132 and 4669 of the BLV proviral genome (GenBank accession number: NC_001414.1) were synthesized using pUCIDT-AMP vector provided by Integrated DNA Technologies (IDT) (Coralville, IA, USA). According to the manufacturer's protocol for plasmid resuspension,

the recombinant plasmid was centrifuged and re-suspended in 80 µL of IDTE (10 mM Tris, 0.1 mM EDTA) buffer (pH 7.5-8.0) to reach an approximate concentration of 50 ng/µL as a stock concentration and then stored at -20˚C until use. One microliter of the template was used in each optimized RPA reaction.

RPA conditions and optimization

For primer screening, 50 µL of RPA was performed using a TwistAmp® Basic kit (TwistDx, Cambridge, UK) with the manufacturer's protocol. A mixture of 1 ng of recombinant plasmid DNA, 0.42 µM of each RPA primer, 14 mM magnesium acetate, 1x of rehydration buffer, and DNase-free water was added to the dry enzyme pellet and thoroughly mixed. Magnesium acetate was pipetted into the tube lids. The initiation of the RPA mechanism at 37˚C for 60 min was by centrifugation of the magnesium acetate into the tubes. The RPA amplification products were visualized by 3% agarose gel electrophoresis (AGE). For LF probe (BLV-01Probe or BLV-02Probe) screening, RPA was performed in a 50 µL volume using a TwistAmp® nfo kit (TwistDx, Cambridge, UK). The BLV-01F and BLV-02R primers were used in the RPA assay by labelling 5′ biotin residue at the reverse BLV-02R primer. Both primers were tested for the compatibility with RPA LF probe which was designed according to the TwistDX guidelines. The 0.42 µM of each RPA primer (BLV-01F and BLV-02R) and 0.12 µM of an LF probe (BLV-01Probe or BLV-02Probe) with 1x rehydration buffer and DNase-free water were firstly mixed, and then added to the dry pellet of the TwistAmp® nfo kit (TwistDx, Cambridge, UK). Following the addition of recombinant plasmid DNA (1 ng) and 14 mM of magnesium acetate into the tube lids. The initiation of the RPA mechanism was by centrifugation. The reactions were visualized by 3% AGE.

For BLV diagnosis of the field samples, RPA was performed in a 50 µL volume using a TwistAmp® nfo kit (TwistDx, Cambridge, UK). One µL of the field genomic DNA sample, 0.42 µM of each RPA primer (BLV-01F and BLV-02R primers), 0.12 µM of BLV-01 Probe, 14 mM magnesium acetate, 1x rehydration buffer, and DNase-free water were first mixed and added to the dry enzyme pellet. Magnesium acetate was then pipetted into the tube lids following by centrifugation of the magnesium acetate into the tubes to initiate the RPA. The reaction was performed at 37˚C for 30 min.

Lateral flow dipstick (LFD) assay

The RPA amplification products detection was observed as a positive test line using the LFD strip (Milenia Biotec, Giessen, Germany). To perfume the RPA amplicon detection by LFD, 10 µL of RPA product were added to 120 µL of assay buffer (1x phosphate buffered saline with 0.1% Tween 20) in a new tube. Then the LFD strip was dipped into the mixture. The entire LFD assay was performed at room temperature for 5 min to visualize the test result.

Serological BLV diagnostic tests

Clotted blood samples by centrifugation at 1200x g for 20 min, the sera were collected and stored at -20˚C until needed. The presence or absence of antibodies to BLV was analyzed using a commercially available ELISA kit, specifically, the IDEXX Leukosis Serum Screening Ab Test whole virus ELISA from IDEXX Laboratories Inc (Netherlands). The test was carried out following the instructions of the manufacturer.

The hydrolysis probe-iiPCR BLV diagnostic tests

A portable iiPCR device (POCKIT™) was used for on-site BLV detection. The test was carried out according to the manufacturer's instructions. The singleplex BLV iiPCR (POCKIT™ BLV Detection Kit) targeted the *env* gene of BLV. The lyophilized reagent was hydrolyzed with 50 µL of premix buffer B then mixed with 5 µL of DNA extract. The final mixture were transferred to an R-tube (GeneReach Biotech, Taiwan) then loaded into a POCKIT™ device. The POCKIT™ device used a built-in algorithm to consider samples generated signals and detected by the 550 nm and 520 nm channels. The qualitative results showed on the display screen within 1 hour.

Molecular specificity of RPA-LFD

The molecular specificity of the RPA-LFD assay using the RPA BLV-01F and BLV-02R primers and BLV-01Probe was evaluated. A cross-reaction tests using 100 ng of DNA or cDNA templates extracted from two other animal viruses, namely, bovine foamy virus (BFV) and bovine immunodeficiency virus (BIV), under the determined optimal conditions for BLV detection. The virus specimens were provided by the Animal Health Research Institute, Council of Agriculture, Executive Yuan (Taiwan). The resulting products were analyzed with LFD and 3% AGE.

Evaluation of molecular sensitivity of RPA using LFD and AGE

Five-fold serial dilutions (50 ng, 10 ng, 2 ng, 400 pg, and 80 pg) of the total DNA extracted from a BLV-infected cow, and 10-fold serial dilutions (108, 107, 106, 105, 104, 103, 102, and 10 copies) of the plasmid DNA at were used as templates for RPA. The comparison result of RPA products detected by LFD and AGE was performed.

Reliability and positive rate comparison of RPA-LFD, iiPCR, and serological ELISA

The screening of samples from cattle with unknown BLV status was conducted using the RPA-LFD assay, iiPCR kit, and a serological ELISA kit as follows: (1) the RPA-LFD test and the hydrolysis probe-iiPCR test were conducted according to procedures previously described for BLV diagnosis of field samples using the same genomic DNA. (2) The serological ELISA test was conducted on serum samples using the IDEXX Leukosis Serum Screening Ab Test whole virus ELISA from IDEXX Laboratories Inc (Netherlands) following the instructions of the manufacturer. The results for McNemar's test of agreement were also calculated using the coefficient, with 0 indicating no agreement beyond chance and 1.0 indicating perfect agreement between the RPA-LFD and ELISA tests.

Results

Determination of the optimal RPA-LFD conditions

The optimal RPA amplification temperature was determined using 103 copies of plasmid DNA as templates at 37, 38, or 39˚C, respectively. The amplification results determined by AGE did not show significant differences for amplification at 37, 38, or 39˚C (data not shown). Thus, given the testing results and the manufacturer's instructions, 37˚C was selected as the assay temperature. For RPA reactions at 37˚C, the quantities of amplification products for 30, 45, and 60 min were similar, but higher than those at 10 and 20 min by

using 102 copies of plasmid DNA as templates. In fact, the amplification products were barely observable at 10 min and were faint at 20 min when visualized by AGE Figure 1. The incubation time for further use in the field was selected as 30 min for efficiency. The RPA amplification products using LF strips for detection were detectable after 10 min of incubation time, while a clear positive test band was observed at 30 min (data not shown).

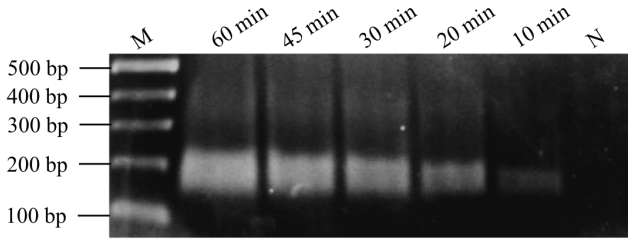

Figure 1: Optimization of RPA reaction temperature using 102 copies of BLV *pol* gene recombinant plasmid DNA. Lanes M and N are the molecular marker and negative control (DNase-free water), respectively.

Molecular specificity of RPA-LFD

For the evaluation of specificity for RPA BLV-01F, BLV-02R primers and BLV-01Probe, reactions were performed with the DNA from other vertebrate animal viruses under the optimal conditions. No cross-reactions were found for testing BFV, BIV, and caprine arthritis-encephalitis virus (CAEV) and detected by AGE (Figure 2A) and LFD (Figure 2B), suggesting that the primers and the probe were specific for BLV *pol* gene detection. The assay gave positive results for its expected amplification product (186-bp) and 2 red-purple color detection lines. No template control (NTC) showed only primer/probe residuals and no false-positive results as determined by AGE, and showed only a red-purple color control line (Figure 2B).

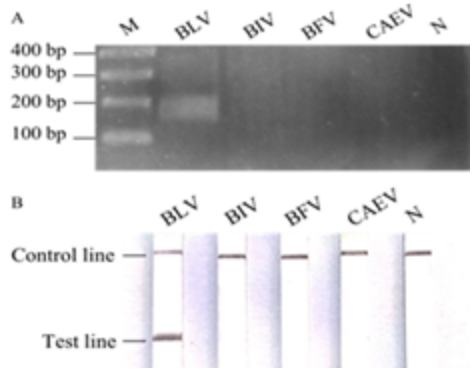

Figure 2: Molecular specificity of RPA-AGE and RPA-LFD carried out using total DNA of animals naturally infected with Bovine immunodeficiency virus (BIV), Bovine foamy virus (BFV), and Caprine arthritis-encephalitis virus (CAEV) as templates. (A) Results visualized by AGE. (B) Results visualized by LFD. N: negative control (DNase-free water).

Comparative molecular sensitivity of the RPA-AGE and the RPA-LFD assays

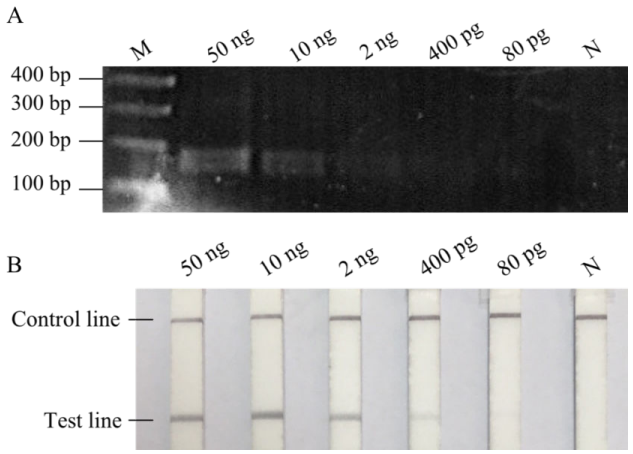

Figure 3: Molecular sensitivity test results of RPA using 5-fold serially diluted total DNA extracted from BLV-infected goat as templates. (A) Results visualized by AGE. (B) Results visualized by LFD for the same RPA amplicons. 50 ng, 10 pg, 2 ng, 400 pg, and 80 pg: dilution series of total DNA prepared from CAEV-infected goat; N: no-template control (DNase-free water).

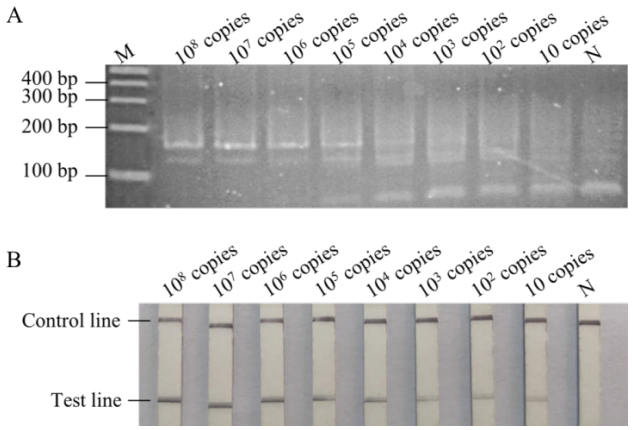

Figure 4: Molecular sensitivity test results of RPA using 10-fold serially diluted plasmid DNA as templates. (A) Results visualized by AGE. (B) Results visualized by LFD for the same RPA products. Lanes M and N: molecular marker and negative control (DNase-free water), respectively.

Using 5-fold serial dilutions of the total DNA extract for testing the sensitivity of the RPA-LFD assay, a detection limit of 400 pg of cattle total DNA was found (Figure 3B). This was 25 times greater than the detection limit of the RPA-AGE assay, which was 10 ng (Figure 3A). When the recombinant plasmids were used as templates, the RPA-LFD assay showed a detection limit with a positive signal at 10 copies (Figure 4B), but the RPA-AGE only yielded a faint band at 100 copies (Figure 4A). The results indicated that for the detection of RPA product, using LFD had a higher sensitivity than the use of AGE. More

positive signals being interpreted by the LFD when it was not clear or difficult to interpreted by AGE.

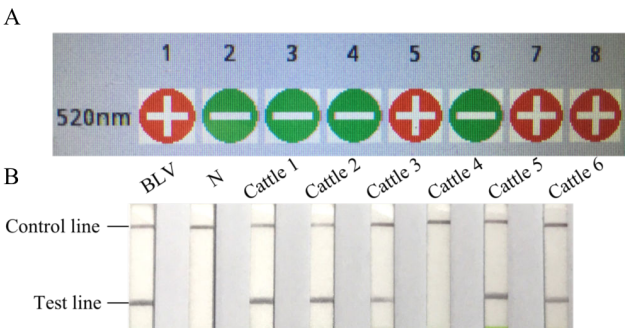

Figure 5: Comparison of iiPCR results from BLV-seropositive cattle with results for RPA-LFD using pol primers. (A) Results visualized by iiPCR. (B) Results visualized by RPA-LFD. N: negative control (DNase-free water).

Reliability and positive rate comparison of RPA-LFD, iiPCR, and serological ELISA

We used field samples to evaluate the statistical sensitivity and specificity of the RPA-LFD assay, in parallel with the hydrolysis probe-iiPCR and a serological ELISA assay. A comparison of the respective results of the iiPCR, the ELISA, and RPA-LFD assays is shown in Table 1.

RPA-LFD	BLV status diagnosed by other assays			
	ELISA		iiPCR	
	Positive	Negative	Positive	Negative
Positive	162	5	120	47
Negative	0	33		33
McNemar's test	P=0.06		P<0.01	

Table 1: BLV diagnostic performance of the RPA-LFD assay, compared with ELISA and iiPCR assays.

Of the 200 cattle sampled and tested with ELISA, iiPCR, and RPA-LFD, 162 (81.0%) heads were identified as positive by both the ELISA and RPA-LFD assays, and 120 (60.0%) heads were identified as positive by both the iiPCR and RPA-LFD assays. Of the sample tested with ELISA, iiPCR, and RPA-LFD, 38 (19.0%) and 33 (16.5%) heads were identified as negative by ELISA and RPA-LFD, respectively, with five out of the 38 (2.5%) seronegative cattle being RPA-LFD positive; 80 (40.0%) and 33 (16.5%) heads were identified as negative by iiPCR and RPA-LFD, respectively, with 47 out of the 80 (23.5%) iiPCR negative cattle (Figure 5A) being RPA-LFD positive (Fig. 5B). The RPA-LFD assay thus exhibited a higher BLV-positive rate (83.5%) than did the ELISA assay (81.0%) (P<0.1), and it also had a significantly higher BLV-positive rate (83.5%) than did the iiPCR assay (60.0%) (P<0.01). A strong agreement (McNemar's test coefficient was 0.91) between the ELISA and RPA-LFD assays were found. But only medium level of agreement (McNemar's test coefficient was 0.45) was estimated between the iiPCR and RPA-LFD assays.

Discussion

Previous studies with respect to conventional PCR methods have detected BLV based on the *env* and the LTR genes [16,46], and a real-time PCR method was developed by Rola-Łuszczak et al. [23]. The LAMP assay for the diagnosis of BLV infection was developed by Komiyama et al. as an isothermal nucleic acid amplification method [26]. In this study, we used the *pol* gene as the BLV diagnostic target region, since the *pol* gene has been found to be the most conserved region between strains [47]. In fact, other researchers have also chosen the *pol* gene as a detection target [21,47-49].

BLV exhibits less genetic variation among strains as compared with most other retroviruses, and the genomes of viruses isolated from multiple countries around the world share approximately 97% of their nucleotide sequences in common [50]. Thus, consistent with this sequence fidelity, the reverse transcriptase enzyme of BLV is less error prone than that of other retroviruses. Recent phylogenetic analysis of BLV using *env* genes showed that this virus could be classified into 8 or more genotypes from multiple geographical locations [51,52]. Within seven [53] and twenty-two [20] geographically different strains, only small differences, mainly point mutations were found. A study conducted by Taiwan's Council of Agriculture included a total of 2,333 cattle sera from 77 dairy herds in 16 prefectures of Taiwan, which were collected in 2009. The results showed that the seropositive rates were 68.58% (1600/2333) and 63.78% (1488/2333) for ELISA and AGID, respectively. The rates of herds with seropositive cattle were 96.10% (74/77). The major BLV isolates prevalent throughout the world were found to belong to genotype 1, while only a few isolates at the US and Japanese were classified as genotype 3 [54].

The developed RPA method showed high specificity and sensitivity. With a sensitivity limit of 10 copies, it could be used for the detection of provirus in field samples suspected to be infected with BLV. In another study, a real-time PCR assay showed a detection limit of one copy, which was more sensitive than a nested PCR assay [23]. Previous research reported that nested PCR had the detection limitation of less than eight provirus copies in a background of two million negative lymphocytes [55]. In the post-seroconversion phase of BLV infection, low viral loads in the sample often cause possible false-negative results. The developed RPA-LFD and formerly established real-time PCR and nested PCR provirus detection methods could minimize the problem. Moreover, the current developed RPA-LFD method is more suitable for use in the less well-equipped field.

Current ELISA tests are based on the BLV gp51 protein, and mutations in this envelope protein have been found to influence detection rates [53]. It was reported by Fechner et al. [56] that some BLV variants in infected cattle could be failed by antibody detection. But Licursi et al. [57] and Asfaw et al. [58] did not observe a relationship between the serological status and the BLV genotypes of infected animals because of different detection targets of the two types of assays. It is not appropriate to directly compare the sensitivity and specificity of the BLV RPA-LFD assay and the ELISA assay [59]. The results showed no significant differences in the BLV-positive rate between the ELISA and RPA-LFD assays in this study. The RPA-LFD assay had just a slightly higher positive rate than the ELISA assay. Moreover, the five samples tested negative by the serological ELISA tests but were positive according to the RPA-LFD assay, they were tested positive by the serological ELISA test after 6 months. It is possible that these cows were in the early infection stage, and that there is not enough of detectable antibody been produced yet.

Although, iiPCR and RPA-LFD assays both detect BLV proviral DNA as a target. Low concordance was found between the iiPCR and RPA-LFD methods in this study. One possible reason for this low concordance is the target *env* gene used by iiPCR, which was previously shown to have a lower sensitivity and positive rate in comparison with using the *pol* gene and LTR as target regions [23]. The other possible reason was that the RPA can theoretically detect even a single template DNA copy. The annealing step is not done at a fixed optimal temperature in iiPCR. It is said to allowing primers and probes to be able to tolerant of minor mismatches in sequences. However, the test's amplification efficiency might also be compromised under such non-optimal conditions. Therefore, the low proportion of lymphocytes carrying the provirus in the peripheral blood might cause the failure of iiPCR in detecting BLV-positive cattle. When using a published real-time PCR method for the quantitative analysis of proviral copies [23], less than 102 copies of the *env* gene were found in the 47 discordant samples. Therefore, the detectability of BLV provirus DNA is directly relevant to the copy number [49,60]. Overall, the results indicated in this study showed that the new RPA-LFD method is a suitable diagnostic tool for early stage BLV infection in terms of both sensitivity and specificity.

Taiwan has been known as a BLV epidemic area since the first survey of BLV infections was conducted in 1987. In 1987, animals in nineteen counties all tested positive for BLV infection using a serological AGID method, with a positive rate of 17.31% in dairy cattle and 0.84% in dairy goats. In 2001, a survey of dairy cattle in seven counties using a serological AGID method revealed a BLV-positive rate of 38.84% in Taiwan. More recently, a 2011 survey using a serological ELISA method showed positive rates of 68.58% among all tested animals and 96.10% among tested farm animals [54]. Currently, ELISA is being used by agricultural authorities for the assessment of BLV suspect animals [14]. The positive rates of BLV infection in dairy cattle have increased widely and rapidly for the past two decades. Therefore, the problem of BLV infection has drawn substantial attention from the livestock industry and agricultural authorities in Taiwan. A national BLV control and eradication program will be executed beginning in 2019. For use in addition to the standard serological ELISA method used in the OIE reference laboratory in Taiwan, a novel isothermal RPA-LFD method for BLV provirus nucleic acid detection was developed for routine quick on-farm diagnosis in this study. A simple, rapid, and cost-effective RPA-LFD procedure presented for detecting BLV proviral DNA. The total assay time for RPA amplification combined with LFD detection is approximately 35 min. The assay time needed by RPA-LFD is only a quarter of RPA-AGE, one third of iiPCR, and one sixth of ELISA method used for the detection of BLV. The key advantages of this method are the high sensitivity and specificity, the short RPA amplification and LFD detection time. This procedure requires only a simple heating block for incubation, which can be powered by mobile batteries. Thus, using the method for the diagnosis of BLV infections is much more possible in less well-equipped laboratories or even in the field. To the best of our knowledge, this is the first study of the development of a sensitive and specific RPA-LFD method for detecting BLV infection.

Conclusion

Previous research indicated that BLV can be transmitted horizontally by blood-sucking vectors [1]. In the early stage of infection, a short period of viremia is observed in cattle, and in this condition the horizontal transmission is possible without a vector [61]. To detect the pathogen infection, PCR and ELISA are generally used, but entire procedure for using conventional PCR and ELISA takes about 3-6 h, and special instruments are required. Therefore, it is limited for the use of ELISA to eradicate cattle in the early stage of BLV infection on-farm. The developed RPA-LFD assay in this study will provide a new method for on-site detection of BLV-infected cattle. This should give a more effective tool for BLV eradication program for the diagnosis of BLV infection.

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