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Caprine arthritis encephalitis virus detection in blood by loop-mediated isothermal amplification (LAMP) assay targeting the proviral gag region

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ABSTRACT

Caprine arthritis encephalitis virus (CAEV), of the genus *Lentivirus* of the Retroviridae family, causes persistent disease, which is characterized by polyarthritis and mastitis in adult goats and progressive paresis (leukoencephalomyelitis) in kids. A loop-mediated isothermal amplification (LAMP) assay was developed for the detection of CAEV in blood samples. Species-specific primers amplifying the *gag* gene region in the provirus were used for the detection of CAEV. The LAMP assay result was obtained 30 min after incubation on a constant temperature at 63 °C in a heat block. Resulting amplicons were visualized by addition of SYBR green dye after the reaction and checked by agarose gel electrophoresis. The sensitivity of LAMP assay was evaluated by comparing the result with the nested polymerase chain reaction. Based on the experiments, the result of the assay indicated a rapid and sensitive test for the detection of CAEV.

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1. Introduction

Caprine arthritis encephalitis virus (CAEV) belongs to the *Lentivirus*, a genus of the Retroviridae family (CFSPH, 2007; L'Homme et al., 2011; Ali Al Ahmad et al., 2012; ICTV, 2012), which consist of non-oncogenic viruses that produce multi-organ diseases characterized by long incubation period and persistent infection. This virus is able to cause persistent infection due to its ability to integrate into host cell and invade the host's immunity by infecting immune cells specially the macrophage and T cells (Clements and Zink, 1996). Lentiviruses also mutate and replicate at high rate making it difficult to diagnose and eradicate. Other viruses that are classified under *Lentivirus* are HIV that causes AIDS and maedi-visna virus in sheep (APHIS, 2008; Clements and Zink, 1996; Miller et al., 2000; Pisoni et al., 2007; Ramirez et al., 2012). In small ruminants, the common clinical signs of CAEV infection are polyarthritis and mastitis in adult goats and leukoencephalitis or progressive paresis among kids. Pneumonia can also be observed in infected animals (APHIS, 2008; CFSPH, 2007; Clements and Zink, 1996; OIE, 2008; Smith and Sherman, 2009).

The CAEV infection is subclinical in nature, and if the affected animal shows clinical sign of infection, usually only 1 of the mentioned signs dominate. The virus is transmitted vertically through consumption of CAEV-infected colostrum and milk, while horizontal transmission by direct contact with the affected animal is also possible (APHIS, 2008; CFSPH, 2007; Herrmann-Hoesing, 2010; OIE, 2008; Reddy et al., 1993). Recent studies suggest that this virus can be isolated and possibly be transmitted through body fluids (Ali Al Ahmad et al., 2008; Ali Al Ahmad et al., 2012). This virus can cause great economic loss (Leitner et al., 2008) among goat raisers and farmers since the animal affected would have a decrease in milk production in addition to increase in culling due to persistent arthritis (CFSPH, 2007; APHIS, 2008). The CAEV as well is a barrier to the exportation of goats from countries where it is endemic (CFSPH, 2007).

In the Philippines, CAEV is classified by the Department of Agriculture as an emerging disease of goats (DA-AO 17, 2004). A study conducted by Lluz et al. (2011) reported CAEV in Eastern Visayas using competitive enzyme-linked immunosorbent assay (cELISA), while Gonzales et al. (2013) detected CAEV among goats in different farms in Region III using nested polymerase chain reaction (nested-PCR). It is indeed alarming since the goat industry is 1 of the fastest growing agricultural sectors in the Philippines. According to the Philippine Council for Agriculture, Forestry, and Natural Resources Research Development (PCAARRD), the goat inventory is 3.9 million heads as of 2011 (PCAARRD, 2011). However, the health and growth of this industry are being threatened by the emergence of CAEV.

The CAEV screening and detection of CAEV are done mainly through antibody tests such as radioimmunoprecipitation assay, agar gel immmunodiffusion assay (AGID), enzyme-linked immunosorbent assay (ELISA), and virus isolation (CFSPH 2007; Herrmann-Hoesing, 2010; Reddy et al., 1993). The OIE-approved tests for CAEV in goats for international trade are AGID test and ELISA (Herrmann-Hoesing, 2010; Office International des Epizootes, 2008). The above-mentioned tests have been proven to be useful in the detection and controlling the spread of the virus, but these have their limitations and

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drawbacks. Virus isolation, for example, is time consuming and even costly, while the serologic based tests has a problem in sensitivity because of the presence of maternal antibody (Reddy et al., 1993; CFSPH, 2007) and strain differences (Herrmann-Hoesing, 2010).

With the advent of molecular diagnostics, PCR-based assay for the detection of CAEV is becoming more common (Herrmann-Hoesing, 2010) since the rapidity of the assay aid in detection of infected goats even before antibody is detected using ELISA (Brinkhof et al., 2008; CFSPH, 2007). Recently, a molecular technique called loop-mediated isothermal amplification (LAMP) was developed by Notomi et al. in 2000. This technique uses 4–6 primers that amplify 6–8 regions of the target gene. Amplification and detection of the target gene can be completed in a single step, by subjecting the mixture of sample, primers, buffers, and DNA polymerase at a constant temperature between 60 °C to 65 °C. With this number of primers that works simultaneously, the DNA can be amplified 10^9–10^{10} in 15–60 min with high efficiency and specificity (Notomi et al., 2000; Parida et al., 2006; Parida et al., 2008); hence, it is an alternative technique that requires short time and inexpensive machine (Le Roux et al., 2009; Mitarai et al., 2011; Song et al., 2012; Thekisoe et al., 2009). Recently, LAMP for the detection of CAEV has been developed targeting the p25 gene region of the CAEV provirus (Huang et al, 2012).

Considering the current animal health status and possible economic impact of the CAEV in the goat industry in the Philippines, an effective method is needed in the detection and screening of CAEV among goats. The purpose of this study was to develop a simple and rapid test that could detect the presence of CAEV among affected animals. Specifically, this study was conducted to evaluate the sensitivity and specificity of the LAMP assay. The gag region of the caprine arthritis encephalitis (CAE) was used to amplify the virus. This gene is well conserved among small ruminant lentivirus (SRLV) and an ideal target for molecular diagnosis (L'Homme et al., 2011). The method that could be generated in this study would be useful in the early diagnosis and screening of animals harboring CAEV.

2. Materials and methods

2.1. Blood collection and DNA extraction

Blood samples were collected from different farms with reported cases of CAE as confirmed through cELISA and from goats showing clinical signs of infection such as arthritis, pneumonia, and mastitis. About 5 mL of blood was collected from the jugular vein of the animals using vacutainer tubes with EDTA. The samples were centrifuged at 4000× rpm for 5 min to separate the buffy coat layer. A total of 500 μL of the buffy coat was recovered and transferred to 1.5 mL microcentrifuge tubes and was added with 700 μL of cell lysis buffer. It was mixed by pipetting and was centrifuged at 14000 rpm for a minute. The supernatant was discarded, and the same procedure was repeated 2 times to remove the cell debris in the pellet. Using the recovered cell pellet, genomic DNA was extracted using Wizard Genomic DNA purification kit (Promega, Madison, WI, USA) following the manufacturer's instructions. The extracted DNA was stored at 4 °C until used.

2.2. Primer design for LAMP

The primers used for CAEV detection were designed targeting the gag gene, a relatively well-conserved region of the SRLV, which makes it an ideal target region for primer design (Abelson and Schoborg, 2003; L'Homme et al., 2011). The gag gene also encodes structural proteins and enzymes that could be detected by antibody tests (Miller et al., 2000). Six primers comprising 2 outer (F3 and B3), 2 inner (FIP and BIP), and 2 loop (F-loop and B-loop) were generated using the Primer Explorer V4 (http://primerexplorer.jp/elamp4.0.0/index.html; Eiken Chemical Co., Tokyo, Japan). The details of the primers used are given in Table 1. These primers were designed based on the sequence of the CAEV that was detected in the Philippines using nested-PCR. The primers were synthesized by AITbiotech Pte Ltd., No. 01–08 The Rutherford, Science Park 1, Singapore.

Table 1
Primer sequences used (5′-3').

LAMP assay	
F3	ATGAAGAGGCGGAAAGGT
B3	GTTTGTGAGCCATATGCC
FIP	CCTACTCCCATAATTTGATCCACTGGGAGAAGGAATAATCCACCA
BIP	GCACAGGGCTAACATGGATCAAGACTGCTCTTAAGGCTGTT
F-loop	TTAATCCTCCCCCCTGCCG
B-loop	AGGCAGACATGCCTGGG
Nested-PCR assay	
P1/F1	CAA GCA GCA GGA GGG AGA AGC
P2/R1	TCC TAC CCC CAT AAT TTG ATC CAC
P3/F2	GTT CCA GCA ACT GCA AAC AGT AGC AAT G
P4/R2	ACC TTT CTG CTT CTT CAT TTA ATT TCC C

2.3. Optimization of the LAMP assay

Initially, the CAEV LAMP reaction was performed in a total volume of 12.5 μL containing 4.5 μL RNAse free water, 3.0 uL of 5 mol/L Betaine (Sigma, Munich, Germany), 1.5 uL of 10× Thermopol Buffer (New England Biolabs, Ipswich, MA, USA), which consist of 20 mmol/L Tris-HCl, 10 mmol/L $(NH_4)_2SO_4$, 10 mmol/L KCl, 2 mmol/L $MgSO_4$, and 0.1% Triton X, 0.2 mmol/L each dNTPs (Intron, Seongnam, South Korea), 0.75 μL of primer mix consist of 3.846 pmol each of outer primers, 30.769 pmol each of inner primers, and 15.38 pmol each of loop primers, 0.75 of 8000 U/mL Bst Polymerase (New England Biolabs) and 1.0 μL of DNA template. The amplification was performed at 61 °C, 62 °C, 63 °C, 64 °C, and 65 °C in a heat block. After determining the optimal temperature for the assay, LAMP was also performed at different incubation time for 15, 30, and 45 min. During the optimization, a DNA from a goat positive for CAEV confirmed through cELISA and sterile double distilled water (DDW) was used as a positive and negative control, respectively.

2.4. Analysis of LAMP product

For the analysis of LAMP product, different methods suggested by researchers were used. A positive result of the amplification can be determined by direct observation of a color change in the LAMP reaction after addition of 0.5 uL of 10× SYBR Green dye (Invitrogen, Carlsbad, CA, USA) into the tube after incubation. A color change from orange to green would indicate samples positive for CAEV, while the negative sample would remain orange. The result of the assay could also be analyzed by gel electrophoresis. A characteristic of multiple ladder-like band patterns in gel under UV illuminator could be observed among CAEV-positive samples.

2.5. Detection of CAE-V by LAMP and nested-PCR assay

A total of 15 randomly selected blood samples from different farms were extracted using commercial DNA extraction kit. The extracted DNA from the samples was subjected to LAMP and nested-PCR for the detection of CAEV. The LAMP reaction was performed following the protocol used during optimization. The nested-PCR was carried out in volumes of 12.5 μL containing 2.0 μL of 5× polymerase chain reaction (PCR) buffer, 1.4 μL of 25 mmol/L MgCl2, 0.5 μL of 2.5 mmol/L each dNTP, 0.2 μL of each of the 10.0 μmol/L forward and reverse primers, 0.05 μL of Taq polymerase (Promega), 3.0 μL of the genomic DNA, and sterile DDW. The primers used for the nested-PCR (Table 1) were based on the study of Konishi et al. (2004) and Gonzales et al. (2013). The cycling conditions were initial denaturation at 94 °C for 3 min

followed by 40 cycles of denaturation at 94 °C for 45 seconds, annealing at 55 °C for 1 min and extension at 72 °C for 45 seconds and a final extension for 7 min. For the second PCR, forward and reverse inner primers and 3.0 μL of the first PCR product were used. The volume and concentration of the reagents and the PCR condition preceded following the PCR protocols for the first set of primers. The PCR amplification products were electrophoresed on 2% agarose gel stained with ethidium bromide in 1× tris acetate EDTA buffer. Following incubation, 3.0 uL of the amplified PCR product was added with 2.0 uL of 2 × loading dye (Invitrogen). The resulting bands were visualized using UV transilluminator after gel electrophoresis. The expected amplicon sizes for the first and second PCR were 256 and 185 bp, respectively.

2.6. Specificity of the LAMP assay

The LAMP assay was evaluated for its sensitivity by checking its cross reactivity to other virus, specifically the porcine epidemic diarrhea (PED) virus of the Coronaviridae and the foot and mouth disease (FMD) virus of the Picornaviridae that were isolated from commercial vaccines. The LAMP reaction was carried out following the above-mentioned protocol. DDW was also used as a negative control.

3. Results

In the present study, we evaluated the LAMP assay, which is considered as the simplest nucleic acid amplification method developed by Notomi et al. (2000). The LAMP method is capable of amplifying DNA under isothermal condition with high specificity, efficiency, and speed. This assay is a simple diagnostic tool in which the reaction can take place in a single tube without the use of complicated and expensive laboratory equipments.

3.1. Optimization of LAMP assay

Using the DNA from a CAEV-positive goat as confirmed by cELISA, 3 pairs of primers and reagents for the LAMP assay are mixed in a single tube and incubated in a heat block at an isothermal temperature between 61 °C and 65 °C for 15 up to 45 min.

As shown in Fig. 1A, the LAMP assay for CAEV could be performed at any temperatures from 61 °C to 65 °C, and the result of the assay could be obtained as early as 30 min after incubation as observed in Fig. 1B. The samples positive for CAEV had a color change from orange to green when SYBR Green dye was added on the reaction, while a characteristic of multiple ladder-like bands pattern was seen after gel electrophoresis. The experiment was repeated more than 3 times to check the repeatability of the protocol as well as the efficiency of the technique used (data not shown).

3.2. Detection of CAEV by LAMP and nested-PCR assay

The CAEV was detected in the samples using 2 different molecular techniques. The results of the detection of CAEV and nested-PCR are presented on Table 2. Using LAMP assay for the detection CAEV in the 15 samples, 5 were detected positive for CAEV after addition on SYBR green (Fig. 2A) and upon gel electrophoresis (Fig. 2B). In nested-PCR, 4 samples were positive for CAEV, which is seen as single bright band on the gel at the level of 185 bp (Fig. 2C). The negative control and samples were not observed to show color change and characteristics band pattern on gel electrophoresis. In this study, the use LAMP assay for the detection of CAEV had a comparable result with nested-PCR.

3.3. Specificity of the LAMP assay

The specificity of the LAMP assay was also evaluated by subjecting PED and FMD virus to the optimized LAMP condition for CAEV. Following the optimized LAMP protocol for CAEV, there was no positive reaction observed from PED and FMD virus after addition of SYBR green dye (Fig. 3A) and gel electrophoresis (Fig. 3B). A color change that would indicate a positive reaction was not observed from PED and FMD. Also, multiple bands pattern was not seen on the agarose gel on transilluminator.

4. Discussion

The CAE, a disease in goats, has caused a damage that has a big impact in the goat industry. Animals affected shows decrease in productivity. At the moment, there is no vaccine developed for this particular disease, and the only way to control the spread of the virus is through culling and sacrificing the animal (CFSPH, 2007). The CAEV causes a persistent disease in goats, which is characterized by encephalomalacia, pneumonia, arthritis, and mastitis. Encephalomalacia is usually seen among kids, while arthritis is more common in

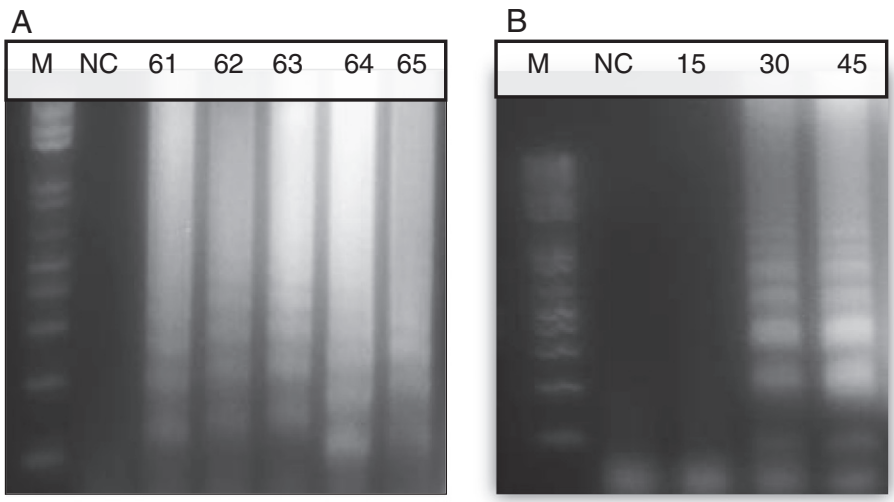

Fig. 1. (A) Result of the LAMP assay subjected to different temperatures. Lane 1, DNA marker; lane 2, negative control; and lanes 3–7, result of LAMP assay at different incubation temperature (in ºC). (B) LAMP assay subjected at different incubation time. Lane 1, DNA marker; Lane 2, negative control; lanes 3–5, LAMP assay at different incubation time (in minutes).

Table 2
Result of the detection of CAEV by LAMP and nested-PCR assays.

Result	LAMP assay	nested-PCR
Positive	5	4
Negative	10	11

adults. This virus in goats is believed to be transmitted directly by ingestion of colostrum and milk contaminated with the virus, while direct contact with the infected animal also considered a way of acquiring the disease. In a study by Villoria et al. (2013), CAEV was detected in air and water samples collected from the pen of infected animals. A PCR assay was used as a detection tool in the mentioned study.

Different methods have been developed in the detection of the CAEV. Serologic assays and virus isolation are the main methods in detection of the virus. The PCR has been widely used by different research institutions and became a routine diagnostic tool in laboratories.

The purpose of this study was to develop a rapid, sensitive, and specific tool that could detect CAEV. In this study, we optimized LAMP assay for the detection of CAEV. The optimized protocol amplified CAEV at an isothermal condition of 63 °C for 30 min. Five out of 15 samples were positive for CAEV using LAMP assay, while only 4 samples were detected positive for the virus using the nested-PCR. When other viruses were subjected to the optimized LAMP assay protocol for CAEV, no positive reaction for LAMP was observed.

In a study by Brinkhof et al. (2008), it was mentioned that the PCR, though a powerful molecular tool, has its own limitations. In the detection of CAEV using PCR, 2 difficulties was mentioned by Cruz et al. (2009), and these are strain variation and virus load of the animal. The PCR assay requires certain level of virus in the host to be detected using blood sample (Ali Al Ahmad et al., 2008; Cruz et al., 2009), while genomic heterogeneity among CAEV could explain why primers based from published articles sometimes does not work when used (Gjerset et al., 2006; Gomez-Lucia et al., 2013; Kaba et al., 2009).

Since the introduction of LAMP assay in 2000, this technique became widely used in different fields including detection of different diseases both virus and bacteria such as PED virus (Ren and Li, 2011), Japanese encephalitis virus (Parida et al., 2006), *Mycobacterium* spp. (Iwamoto et al., 2003), and *Brucella* spp. (Song et al., 2012). In the previous studies that used LAMP assay, its efficiency to detect disease causing microorganisms was frequently evaluated by comparing its results to the standard detection methods and to the commonly used molecular technique such as PCR. The LAMP assay amplified these disease causing microorganisms with high sensitivity and specificity. The simplicity and rapidity of the assay were also superior compared to other molecular method such as PCR. The same result was observed in this study.

The assay's sensitivity and specificity can be attributed to the presence of primers that recognizes specific regions of the target sequence (Fukuda et al., 2006; Notomi et al., 2000; Parida et al., 2008). The activity of the *Bst* polymerase, which is active at a high temperature, also prevents non-specific priming (Negamine et al., 2002; Parida et al., 2008) making the LAMP assay specific. Rapidity of the assay is a result of 4 to 6 primers that works simultaneously to amplify a specific sequence (Notomi et al., 2000; Parida et al., 2008) at an isothermal temperature. Amplification at an isothermal condition provides high amplification efficiency, wherein the target DNA is being amplified at a very high rate in 15–60 min (Parida et al., 2008). The LAMP assay generates a large amount of amplification by-product, which is the magnesium pyrophosphate that can be seen as white turbidity (Mori et al., 2001), which correlates with the amount of DNA being synthesized (Notomi et al., 2000; Parida et al., 2008). In the presence of fluorescent intercalating dye such as calcein, it combines with magnesium ion, which produces fluorescence under a UV lamp (Parida et al., 2006) or a color change from orange to green that can be observed by the naked eye (Peng et al., 2011). Similarly, when the DNA with template of the pathogen is present, the LAMP primers recognize the nucleic sequences and cause multiple stem loop DNA structures with several inverted repeats of the target region. These repeats are visualized as ladder-like multiple bands on agarose gel analysis or gel electrophoresis (Notomi et al., 2000).

The LAMP reaction can be performed by incubating the reagents, specific primers, and sample in a single tube. Simple equipment such as water bath or a heat block can be used for this molecular technique for the detection of diseases in particular. The property of the assay wherein the results can be analyzed by the naked eye makes it a good diagnostic tool that could be used outside the laboratory or field.

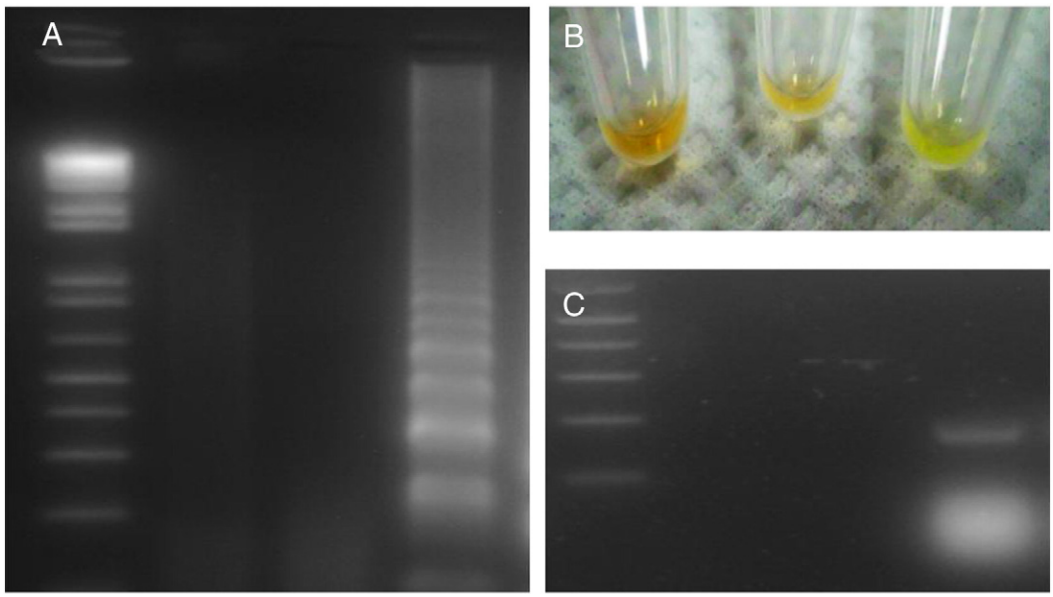

Fig. 2. (A) Result of the LAMP assay on gel electrophoresis and visual inspection by (B) addition of SYBR green dye. Five samples were positive for CAEV. (C) Result of the nested-PCR assay. Four samples were positive for CAEV (as indicated by arrows) using the 2 sets of primers.

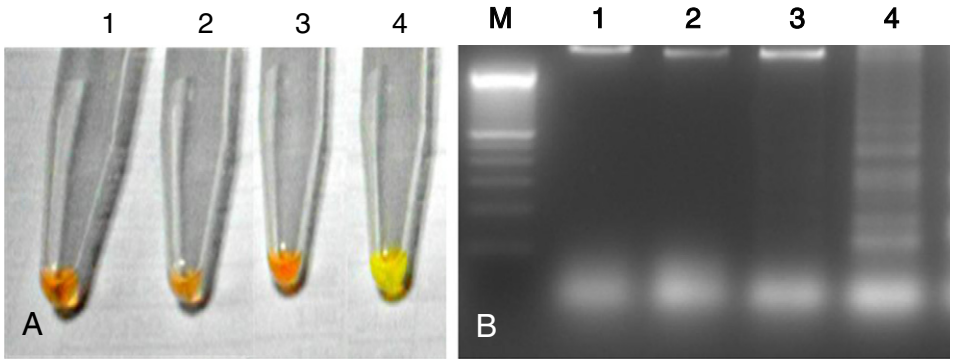

Fig. 3. Evaluation of specificity. (A) Visual inspection with SYBR green dye. Samples 1–3: DDW, PED, and FMD samples, while 4 is the CAEV sample. (B) Gel electrophoresis result, M – DNA ladder. Only sample 4 appeared positive after LAMP assay as indicated by the color change (A) and multiple ladder-like band (B).

Expensive and complicated equipment is not needed in performing this technique.

Using the LAMP assay protocol generated from this study, the goats in the Philippines can be rapidly screened with CAEV infection. By using this, animal health programs and monitoring for eradication and control of the spread of the virus could be possibly improved or generated. However, studies should also be conducted for further improvement of the method to enhance its applicability on field or on-site diagnosis of the disease.

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