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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.

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

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