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Original article

Diagnosis of Caprine Arthritis Encephalitis Virus infection in dairy goats by ELISA, PCR and Viral Culture

S. Panneum^{1,2,3,4}, T. Rukkwamsuk^{1,2,3,4}¹ Faculty of Veterinary Medicine, Kasetsart University, Kamphaeng Saen, Nakhon Pathom 73140, Thailand² Center for Advanced Studies for Agriculture and Food, Kasetsart University Institute for Advanced Studies, Kasetsart University, Bangkok 10900, Thailand³ Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand⁴ Center of Excellence on Agricultural Biotechnology: (AG_BIO/PERDO-CHE), Bangkok 10900, Thailand

Abstract

For preventive and control strategies of Caprine Arthritis Encephalitis Virus (CAEV) infection in dairy goats, performance of the available diagnostic tests was described as one of the most important and necessary aspects. The study aimed at evaluating the diagnostic test performance, including PCR, ELISA and viral culture, for CAEV infection in dairy goats in Thailand. Blood samples of 29 dairy goats from five low- to medium-prevalence herds and one very low-prevalence herd were collected for PCR and ELISA methods. The performance of these two diagnostic methods was evaluated by comparing with cytopathic effects (CPE) in the co-cultivation of CAEV and primary synovial cells. Results indicated that sensitivity, specificity were, respectively, 69.6%, 100%, for PCR; and 95.7%, 83.3% for ELISA. The PCR assay tended to have lower sensitivity and higher specificity than ELISA. When multiple tests were applied, parallel testing provided sensitivity and specificity of 98.7% and 83.3%, while series testing showed sensitivity and specificity of 66.6% and 100% respectively. These results indicated that combination of ELISA and PCR provided some advantages and possibly offered optimal methods to detect CAEV-infected goats. Kappa value of the agreement between PCR and ELISA test was 0.34, indicating fair agreement. Regarding the possibility of antigenic variation between CAEV strains used in both PCR and ELISA assays, the actual circulating CAEV strain should be reviewed in order to develop and enhance the diagnostic tests using the CAE viral antigens derived from specific local strains of Thailand.

Key words: caprine arthritis encephalitis virus, dairy goat, ELISA, PCR, viral culture

Introduction

Caprine arthritis encephalitis virus (CAEV) is classified as small ruminant lentivirus (SRLVs), which cause a persistent, slowly progressing and debilitating disease in goats. In dairy goats, the revenue is negatively influenced by decreased milk production and milk quality, reduced birth rate, lowered body weight gain, reduced conception rate or even loss from early culling of infected goats, particularly in the more intensive farms (Peterhans et al. 2004, Reina et al. 2009, Brinkhof et al. 2010). These obvious evidences emphasize and indicate the importance of prevention and control strategies against CAEV. For the prevention and control measures, identification of infected animals, particularly in the early infection stage, is essential for determination of infection rates which would be the initial action in any eradication schemes (Peterhans et al. 2004, Reina et al. 2009). Based on clinical signs that frequently appear late and are not specific to infection, laboratory-based diagnosis for early detection, comprising antibody and/or viral nucleic acid detection should be appropriately applied. To our knowledge, efforts to find the gold standard for CAEV or SRLV diagnosis have not yet been successful. Agar gel immunodiffusion (AGID) and, more recently, enzyme-linked immunosorbent assay (ELISA) are internationally prescribed tests (OIE 2004, Reina et al. 2009). These serological tests show a limited sensitivity but excellent specificity, regarding the relatively long time between infection and seroconversion period. In addition, the possibility of intermittent antibody production leading to false negative results may be due to antigenic variation between the viral strains used in the test and the actual circulating strains (Grego et al. 2005). Thus, the detection of viral nucleic acid via molecular biological methods, like polymerase chain reaction (PCR) or viral cultivation and immunochemistry assay, may be of particular value in early detection of infection and for non- or slow-responders. For the test performance, PCR has lower sensitivity than many ELISA tests because of SRLV strain variation. High rate of mutation during viral replication causes misdiagnosis from inappropriately designed probes to local circulating strain. Low viral load *in vivo* is another possible reason for low sensitivity value for PCR. Immunohistochemical methods, such as immunofluorescence assay (IFA), using specific antibody against SRLV protein, was claimed as unsuitable regular screening test due to high technical skill requirements and high operation costs. However, it has been often applied as a standard reference test regarding to the high specificity (Reina et al. 2009, Brinkhof et al. 2010). Combination of the tests can provide a higher effectiveness

of diagnosis with minimized false negative results from limitation of serological tests. The combined test can eventually minimize the false positive results from non-infected, but maternally positive kids. In Thailand, results from serological survey reported by several studies (Ratanapob 2010, Chanlad and Prasitphon 2010, Lin 2011, Parchariyanon 2012) revealed that herd and individual prevalence were 11.5-37.5% and 5.5-21%, respectively. Prevention and control strategies for CAEV infection in goats in Thailand depended on the specific rearing system, management condition and disease prevalence (Peterhans et al. 2004, Reina et al. 2009). In this circumstance, the most efficient strategy that should be developed to reach the CAEV-free herd status may be the test and culling measure; additionally, the effective measures of hygiene and management to control the viral transmission within and between herds are recommended in Thailand. The diagnostic tests for detection of infected animals should be proven for their performance before applying them as the suitable method in the prevention and control strategy. In this study, the performance of available diagnostic tests, including commercial indirect ELISA, nested PCR and viral culture for diagnosis of CAEV infection in goats were evaluated.

Materials and Methods

Animals

Twenty nine adult dairy goats aged between 2 and 8 years old from 6 farms reared in Ratchburi and Bangkok province were used. Six goats from one very low seroprevalence (<10%) farm that had been seronegative against CAEV infection by indirect ELISA for 3 consecutive times in one year were treated as true negative goats. The other 23 goats were from 5 low-to-medium seroprevalence farms, have been tested for CAEV infection by indirect ELISA for 2 to 3 times in one year, in which the clinical infected goats had been presented in these farms during the study period. All goats were reared under the typical rearing condition of Thailand, that was a small farm settling of approximately less than 40 goats per farm and have been kept in a confined house with some free yard area. The goats were offered commercial concentrates; and the roughage sources were perennial grass, leucaena and rice straws. Amount of concentrates fed by farmers usually varied by milk yield and days of milking. Goats were milked once a day by hand milking method. Natural breeding was usually done by bucks of their own farms. Kids were allowed to live together with their

Table 1. List of primers for nested PCR.

Primer name	Sequence	Location	PCR	Amplicon size
CAEV F0	AACTGAAACTTCGGGGACGCCTG	304-326	First round	1191 bp
CAEV R0	GTTATCTCGTCCTAATACTTCTACTGG	2092-2118		
CAEV F1	AAGGTAAGTGACTCTGCTGTACGC	334-357		
CAEV R1	TTTTTCTCCTTCTACTATTCCYCC	2000-2024		
CAEV F2	TGGTGAGTCTAGATAGAGACATGG	513-536	Second round	1327 bp
CAEV R2	GGACGGCACACACGTAKCCC	1820-1840		

does until weaned at 4-5 months of age depending on kid's weight.

Sample collection and analyses

Blood samples from all goats were collected from jugular vein using aseptic technique for PCR, ELISA and viral co-cultivation with primary synovial cells. For PCR, 1 mL of blood was collected in EDTA tube (micro-EDTA K2, FUSHINO®, Qualify Group Co., Ltd. Bangkok, Thailand), and for ELISA, 4 mL of blood was collected in serum activating blood tube (Vacuette®, Greiner-one, Pennsylvania, USA). For monocyte isolation and viral co-cultivation with primary goat synovial cells, 12 mL of blood was collected in lithium-heparinized tube (IMPROVACUTER®, Improve Medical Instrument Co., Ltd, Guangzhou, China).

Polymerase chain reaction (PCR)

Nested PCR assay was processed at Molecular Diagnostic Laboratory, Veterinary Diagnostic Unit, Faculty of Veterinary Medicine, Kasetsart University. The DNA was extracted from whole EDTA blood using E.Z.N.A.® Blood DNA Mini Kit (Omega Bio-tek, Inc., Georgia, USA) following the manufacturer's instructions. PCR condition and amplification were done according to description by L'Homme et al. (2011), in which primers were designed corresponding to the most highly conserved sequences in the gag regions from available SRLV genomes in public data bases to produce a 1327 bp amplicon. Four primers for the first round and two primers for the second round PCR are demonstrated in Table 1.

The general conditions for PCR were described as follows: approximately 0.5-1 µg of total DNA; 1X PCR buffer; 200 µM of each dATP, dCTP, dGTP and dTTP; 250 nM of each primer, 3 mM MgCl₂; and 1U Platinum Taq DNA polymerase as final reaction volume of 50 µL. Activation of Platinum Taq and initial denaturation was done at 94°C for 2 min followed by

35 cycles at 94°C for 1 min, 55°C for 1 min and 72°C for 2 min. Nested amplifications were carried out under the same conditions as the first round of PCR with 1 µL from PCR reaction 1. Amplicons were detected using 1% agarose gel electrophoresis and visualized with ethidium bromide staining.

Enzyme-linked immunosorbent assay (ELISA)

Serum samples were tested for the CAEV antibodies by IDEXX CAEV/MVV Total Ab Test® (IDEXX Laboratories, Inc., Maine, USA) following the instruction of manufacturer. The CAEV infection was based on the antibody detection of an immunogenic peptide (p28 protein) of a transmembrane protein (TM, ENV gene) and of the recombinant p28 protein, which enters into the composition of the viral capsid (GaG gene).

Co-cultivation of Caprine Arthritis Encephalitis Virus Infected Macrophage with Primary Goat Synovial Cells

Heparinized blood samples from all goats were isolated for monocytes using Ficoll-Histopaque – 1.077 (Sigma-Aldrich Co., Singapore). The isolated monocytes were further cultivated to differentiate into macrophages in the medium at 37°C, 5% CO₂ for 7 days. The medium contained RPMI1640 supplemented with L-glutamine (2 mM), gentamicin (50 µg/ml), Hepes buffer (10 mM), 2-mercaptoethanol (50 µM) and 10% FCS. Seven to ten days-old cultivated macrophages were co-cultivated with the primary goat synovial cells for 7 days. Microscopic examination was performed every 2 days for multinucleated syncytial formation defined as cytopathic effect (CPE) which indicated the successful CAEV infection. IFA was applied using the mouse anti CAEV monoclonal antibody (CD Creative Diagnostics®, New York, USA) at dilution 1:100 on day 7 to detect viral particles in the co-cultivated primary goat synovial cells. The presence of both CPE and positive fluorescence test were reported as positive result.

Table 2. Cross tabulation of PCR and ELISA testing results compared to CPE as a reference method for detection of CAEV infection in 29 goats.

		CPE	
		positive	negative
PCR	positive	16	0
	negative	7	6
ELISA	positive	22	1
	negative	1	5

Table 3. Results of combination of PCR, ELISA testing and CPE formation in detection of CAEV infection in 29 goats.

PCR	ELISA	CPE	
		positive	negative
Positive	positive	15	0
Positive	negative	1	0
Negative	positive	7	1
Negative	negative	0	5
		23	6

Table 4. Sensitivity and specificity of PCR and ELISA testing compared to CPE formation as a reference method for detection of CAEV infection in 29 goats.

Test	CPE	
	sensitivity	specificity
PCR	69.6 (47.1, 86.8) ¹	100.0 (54.1, 100.0)
ELISA	95.7 (78.1, 99.9)	83.3 (35.9, 99.6)
Parallel	98.7	83.3
Series	66.6	100

¹ 95% confidence interval.

Statistical analyses

All seropositive and seronegative goats were tested and confirmed by the presence of CPE, which was the result of co-cultivation and supplemented with fluorescence assay regarding as the gold standard. Cross tabulation of PCR versus CPE and ELISA versus CPE results were reported as descriptive statistics. Evaluation of diagnostic parameters (sensitivity and specificity) for PCR and ELISA compared with CPE as the gold standard method, was performed using MEDCALC® easy-to-use statistical software (MedCalc Software bvba, Belgium), and the effect of parallel and series testing of both PCR and ELISA on sensitivity and specificity were calculated according to the formula reported previously (Cebul et al. 1982). In addition, kappa statistics was applied to determine the agreement of the test results from PCR and ELISA using Win Epi Working in Epidemiology (Universidad de Zaragoza, Zaragoza, Spain).

Results

Based on the origin of the goats and the confirmation of the infection status using CPE plus IFA, 23 goats were found as truly positive and 6 goats as truly negative. When considering co-cultivation as the reference method for indicating infection status in dairy goats, PCR provided 100% of positive result (16/16) as infected goats, and ELISA provided 85.7% (22/23). However, PCR gave only 46.2% of negative result (6/13) as healthy goats, and ELISA presented 83.3% (5/6) (Table 2). According to the CPE plus IFA, PCR provided 69.6% of sensitivity and 100% of specificity, and ELISA gave 95.7% of sensitivity and 83.3% of specificity. Results revealed that when both PCR and ELISA were positive, 65.2% (15/23) of goats were found to be infected. In addition, when both tests were negative, 83.3% of goats were found to be healthy (Table 3). If the situation of a gold standard was not available, multiple testing of both parallel and

series testing were employed to figure out the best performance for detecting CAEV infection in goats (Table 4). Parallel testing was conducted as two or more tests at the same time, and the sensitivity was calculated by counting positive results in either of the tests, and specificity was the true negative result from both tests. For the series testing, diagnostic tests were sequentially performed. The animals positive to an initial test were tested again by the other test. Therefore, only animals that were positive to both tests were considered to be infected. Application of the parallel testing for detecting the disease can increase the sensitivity (98.7%), hence false negative result were found to be lower, while application of series testing could increase the specificity (100%) and, thereby reducing the false positive results. While, in this circumstance which the gold standard was not available, kappa statistics values were calculated and described as an agreement between tests. Kappa value of the agreement between PCR and ELISA test was 0.34, indicating fair agreement.

Discussion

Attempts to find a gold standard for CAEV diagnosis in goats have been unsuccessful (Reina et al. 2009, Brinkhof et al. 2010). In this study, co-cultivation of CAEV infected macrophages with primary goat synovial cells under practical cultivating system was developed by our laboratory (Panneum et al. 2017). And, detection of viral antigen in the co-cultured cells by IFA was employed as a reference method. It is noted that viral co-cultivation, resulting in CPE formation, may not be effective in an early diagnosis. This is due to laborious methods that may be hampered by the non-permissiveness of cells to particular viral strains and generally poor growth characteristics of these viruses (Thormar 2005, Brinkhof et al. 2010). However, viral co-cultivation is efficiently suitable for the definitive diagnostic purpose (Clavijo and Thorsen 1996), especially when supplementary test such as RIA or IFA is applied together. This method is often used as the gold standard test for diagnosis (Archambault et al. 1988, de Andres et al. 2005).

When CPE formation combined with an IFA were used as the reference method, it was highly likely that the animals were infected or diseased when PCR was positive. While, when PCR was negative, approximately a half of these negative results were really uninfected or healthy animals. This low negative PCR results in healthy goats might be affected by the low sensitivity of the PCR test which might be due to the viral heterogeneity leading to mismatch in the primer

binding region. In this study, it was most likely that the infected or healthy goats could be differentiated using ELISA because of its high sensitivity and specificity. This finding suggested that in any disease circumstance, ELISA has a possibility of around 95% to detect the truly infected goats and has a possibility of around 83% to indicate the healthy goats. This result depended on the sensitivity that might be caused by the fluctuation of antibody response after infection as well as on the disease prevalence (de Andres et al. 2005, Thrusfield 2007, Brinkhof et al. 2008)

In this study, a relatively high specificity of PCR was expected (Reddy et al. 1993, Rimstad et al. 1993, Wagter et al. 1998, Celer et al. 2000, Extramiana et al. 2002). This indicated a suitable performance used as a confirmatory test. The PCR method probably gave zero false negatives and had the possibility for diagnosis of CAEV infection by detecting proviral sequences when antibody is absent in circumstances of early infection prior to seroconversion. On the other hand, PCR assay is very useful to prove viral infection in seropositive kids which received maternal antibody from their dams (de Andres et al. 2005, Brinkhof et al. 2008). When considering the test performance of ELISA, we found satisfactory results as also described by de Andres et al. (2005), that the sensitivity values ranged from 92 to 100%. In this study, ELISA specificity was calculated to be lower than the previous study by Zaroni et al. (1994), in which they found the specificity of the developed ELISA based on highly purified whole virus antigen as 99.3% when compared with an established ELISA based on recombinant GAG-GST fusion protein. The possible explanation was that this study was performed in a limited number of animals, especially when considering only 6 truly negative animals. However, the present findings indicated a suitable performance for screening test of antibody response to CAEV infection, which was the international prescribed test announced by OIE (2004). However, about 4% of false negative and 17% of false positive from ELISA testing were found in this study. It could be speculated that antibody titers may have fluctuated after infection and intermittent antibody responses such as slow seroconversion responders could occur, as previously reported (de Andres et al. 2005). In addition, the viral strain used in an ELISA method might fail to detect specific antibodies against particular field strains (Reina et al. 2009).

To improve sensitivity and specificity, parallel and series testing by PCR and ELISA were conducted. Results were reported in this study that increasing of sensitivity by parallel testing and increasing of specificity by series testing were expected. This finding indicated an advantage of combination of serology and

PCR, because PCR positive results had been obtained in some seronegative goats. These combined tests might be optimal for detecting infected goats when the eradication scheme was implemented, especially for detecting the infection in high genetic value animals (de Andres et al. 2005, Reina et al. 2009). Although slightly increasing the sensitivity of the combined tests when compared with the ELISA alone was observed in a parallel testing, it might not demonstrate any advantages. The additional value from parallel testing, both detecting of antibody and viral antigen, was also reported by Brinkhof et al. (2010). In that study, the rapid eradication of an SRLV infection from a genetically valuable sheep flock were conducted using both antibody detection and real-time PCR at short intervals, and were applied in the intensive flock management. The authors claimed that eradication of the infection could be completed by two testings and culling rounds within a 3 months interval.

When an agreement between the two tests was considered, the kappa value was calculated and indicated a fair agreement between PCR and ELISA (Thrusfield 2007). Relative sensitivity of PCR result in this study was lower than expected and lower than other studies (Brodie et al. 1993, Reddy et al. 1993, Rimstad et al. 1994). It was probably due to viral heterogeneity and low viral loads, which might hamper the application of PCR, resulting in a limitation of this PCR test. The diagnostic value of PCR assay largely depends on the design of the oligonucleotides for priming and of the probes for detecting; therefore, false negative results in this study could be due to mismatch in the primer binding region as also hypothesized with low sensitivity (de Andres et al. 2005, Brinkhof et al. 2008). The phylogenetic study of various strains of circulating viruses and the selection of suitable primer sequences from a relatively conserved region should be emphasized in order to minimize the effects of strain variation and increasing the sensitivity (de Andres et al. 2005). It is therefore necessary and recommended to create an effective PCR assay before applying this diagnostic method for prevention and control strategy in Thailand.

In conclusion, PCR has been proven for its advantage to detect of infection in seronegative goats since it has 100% specificity and this could decrease false negatives when series testing was applied. This suggested that the combination of ELISA and PCR might be optimal for detecting CAEV-infected goats. However, the antigenic variation between the viral strain(s) used in both assays and the actually circulating strains needs to be concerned and evaluated. The development of ELISA and PCR containing antigens derived from specific local strains of epi-

demiological interest such as in Thailand should be performed to enhance the diagnosis performance (Grego et al. 2005).

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