

Cocultivation of caprine arthritis encephalitis virus-infected macrophages with primary goat synovial cells

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Abstract: Primary goat synovial cells were developed to be cocultivated with caprine arthritis encephalitis virus (CAEV)-infected monocytes. Two CAEV-seropositive goats and one CAEV-seronegative goat were confirmed for viral particles in the blood using the PCR method. Thereafter, heparinized blood samples from the three goats were collected and isolated for monocytes, which were further cultivated to differentiate into macrophages. Cultivated macrophages of 7–10 days old were cocultivated with the primary goat synovial cells for 7 days. Microscopic examination was performed every 2 days for multinucleated syncytial formation, and immunofluorescence assay was applied using monoclonal antibody against CAEV on day 7 to detect viral particles. Results showed that the primary goat synovial cells cultivated with CAEV-infected macrophages from the two seropositive goats had multinucleated syncytial formation with positive immunofluorescence, while the cocultured macrophages from the seronegative goats showed normal appearance with negative immunofluorescence. The multinucleated syncytial cells were prepared for transmission electron microscope examination and the results indicated that CAEV particles were clearly identified. In conclusion, our developed primary goat synovial cells and the cultivating system were proven to be an appropriate isolation assay for CAEV, which was the first report of cultivation and isolation of CAEV in goats in Thailand.

Key words: Caprine arthritis encephalitis virus, cocultivation, goat, macrophage

1. Introduction

Caprine arthritis encephalitis virus (CAEV) causes an enzootic viral infectious disease, which has been noted as one of the major causes of economic losses in the small ruminant industry worldwide. CAEV is a lentivirus that persistently infects goats and sheep. The principal manifestations are encephalitis and interstitial pneumonia in young animals, whereas arthritis and mastitis predominate in adult goats (1,2). Although most infected animals do not show any clinical signs, major losses from these infected animals are due to retarded growth and suboptimal production (2). The major transmission route of CAEV is vertical transmission by ingestion of colostrum or milk from infected dams to their offspring, in which CAEV infects white blood cells, especially mononuclear cells contaminating the colostrum and milk (3,4). Besides this common route, direct or indirect contact of infected discharges from the infected animals could be an efficient

horizontal transmission route of CAE infection (4). The specific diagnostic procedures deal with blood antibody detection in goats using enzyme-linked immunosorbent assay (ELISA), agar gel immunodiffusion (AGID), and antigenic or viral detection using polymerase chain reaction (PCR) and viral culture (3,5–8).

Viral cultivation was discussed as a definitive diagnosis of CAEV infection. The multinucleated syncytial formation as the cytopathic effect (CPE) is pathological changes of cultivated cells, which is a result of successful viral infection (6,9). This definitive diagnosis is laborious work, because it is time-consuming with a slow growth rate of virus in the cultivating system and naturally low numbers of viral-infected cells (10,11). In addition, cultivating conditions need highly specific cells and viral cultivation is indicated as an expensive diagnostic procedure.

In Thailand, some serological surveys on CAEV infection have been conducted. However, there is no

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report or evidence of CAEV cultivation and isolation from the infected goats (12,13). Primary goat synovial cells are discussed as specific and appropriate for CAEV cultivation (8,9,14–16). With this knowledge, study of the acceptability of primary fibroblastic cell culture of goat synovial cells to multiply CAEV is important and necessary for further CAEV isolation and for establishment of an in vitro model for the study of CAEV properties. Therefore, the objective of the study was to determine cocultivation of CAEV-infected macrophages with primary goat synovial cells collected from both seropositive and seronegative dairy goats.

2. Materials and methods

2.1. Goat samples

The study was conducted during March–June 2014. Two seropositive goats (aged 3 years) and one seronegative goat (aged 3 years) from a small-holder dairy goat farm in Ratchaburi Province were used. All three studied goats were clinically healthy. The goats were reared under the typical rearing conditions of Thailand, described as a small farm setting of approximately less than 40 goats per farm. Goats were kept in confined houses with a small free yard area. Perennial grasses and *Leucaena* plants were common feed and were offered to the goats by a cut-and-carry system. Rice straws were also used as a feed source during the drought season of the year. Commercial concentrate containing 14%–16% crude protein, supplemented with soybean husk, was normally fed as a source of concentrate. Amount of concentrates arbitrarily fed by farmers practically varied by milk yield and days in milk. Goats were milked once a day by hand. Natural breeding by bucks within the farms was commonly practiced. Kids remained in contact with their mothers until weaning at 4 to 5 months of age, depending on the kid’s weight.

Seropositivity and seronegativity were determined by ELISA (IDEXX CAEV/MVV Total Ab Test, IDEXX Laboratories, Inc., Westbrook, ME, USA) test results. Furthermore, the three goats were finally confirmed for CAEV appearance in their blood by PCR technique according to a method described previously (17). The

primers were designed corresponding to the most highly conserved sequences in the gag regions from available small ruminant lentivirus genomes in public databases to produce a 1327-bp amplicon. Four primers for the first round and two primers for the second round of PCR are demonstrated in the Table.

2.2. Preparation of primary fibroblastic cell culture of goat synovial cell

The primary goat synovial cells were prepared using the previous protocol (8). Briefly, the synovial tissues from one healthy goat that was proved to be seronegative against CAEV infection were harvested from the slaughter house (8). The tissues were digested by collagenase type 1 (GIBCO BRL) in RPMI 1640 (GIBCO BRL) supplemented with 3% fetal calf serum at 37 °C for 1 h. The digested tissues were centrifuged at 1500 rpm and 4 °C for 5 min. The supernatant was discarded, and the sediment was washed by PBS. The cells were later kept in culture medium consisting of RPMI 1640 supplemented with glutamine (2.05 mM), penicillin (100 U/mL), streptomycin (100 µg/mL), sodium pyruvate (2 mM), 2-mercaptoethanol (50 µM), and 10% FCS at 37 °C and 5% CO₂. The culture medium was changed twice a week, and the passages of the cells were stored at –80 °C when the cell growth was complete. Therefore, the primary goat synovial cells used in this study were preserved by the mixture of cultivating medium supplemented with fetal calf serum and dimethyl sulfoxide, and were already kept at –80 °C for approximately 5 years (13). When the present study was conducted, the preserved primary synovial cells were tested for CAEV using PCR, and the result was absolutely negative.

2.3. Mononuclear cell (monocyte) isolation and cultivation

Twenty milliliters of heparinized blood from the studied goats was diluted with PBS in an equal volume of blood. The blood solution was gently poured into Ficoll-Histopaque 1.077 gradient using the proportion of blood solution and Ficoll-Histopaque of 4:1. The suspension was centrifuged at 2000 rpm at 5 °C for 20 min. The mononuclear cells were separated as an obvious thin white layer above the red

Table. List of primers used in the study (5’ to 3’).

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

blood cell layer. Cells were washed by PBS and centrifuged at 2000 rpm at 5 °C for 20 min. Thereafter, the isolated mononuclear cells were transferred into 2 cultivating flasks; one flask was prepared for immunofluorescence assay and the other was for transmission electron microscopic examination. Approximately 4×10^6 cell/mL of isolated mononuclear cells per flask were resuspended in the medium for their differentiation to macrophages at 37 °C and 5% CO₂ for 7 days. The medium contained RPMI 1640 supplemented with L-glutamine (2 mM), gentamicin (50 µg/ml.), HEPES buffer (10 mM), 2-mercaptoethanol (50 µM), and 10% FCS. The medium was changed and nonadherent cells were removed every other day.

2.4. Cocultivation of CAEV with primary goat synovial cells

Macrophages from 2 seropositive goats and 1 seronegative goat were trypsinized at 37 °C for 5 min and then PBS was added to stop the reaction. The culture flask was then flushed with cultivating medium to remove the detached macrophages and the suspension was centrifuged at 2000 rpm and 4 °C for 5 min. The supernatant was discarded, and the sediment was transferred with cultivating medium to the primary goat synovial cells cultivating system for detection of multinucleated syncytial formation as a CPE. The pathological change of cultivated cells resulted from successful viral infection, which was determined twice a week for 2 weeks.

2.5. Immunofluorescence assay for detecting viral particles

At 7 days after cocultivation, the cocultivated cells from seropositive and seronegative goats were washed with PBS and adhesive cells were detached with trypsin at 37 °C for 5 min. The reaction was stopped by adding PBS; the solution was flushed to remove nonadhesive cells with cultivating medium; and it was centrifuged at 2000 rpm and 4 °C for 5 min. The supernatants were discarded and the sediments were dried on glass slides before fixing with ether for 15 min. Thereafter, the glass slides were washed with tap water and dried at room temperature. For immunofluorescence assay, the mouse anti-CAEV monoclonal antibody (CD Creative Diagnostics), a primary monoclonal antibody that can detect the p28 core polypeptide epitope of the virus at a dilution of 1:100, was applied to the slides to detect the viral particles by confocal immunofluorescence microscope.

2.6. Examination by transmission electron microscope (TEM)

The cocultivated cells from both seropositive and seronegative groups were prepared for electron microscopic examination by prefixing with 2.5% glutaraldehyde at 4 °C for 2 h. The cells were scraped and pelleted in a microcentrifuge tube. The pellets were transferred to vials and washed with glutaraldehyde 2.5% in 0.1 M phosphate

buffer, pH 7.0–7.4, three times and were postfixed with 1% osmium tetroxide for 1 h. The postfixed cells were rinsed with distilled water for 30 min, and this step was repeated 3 times. The samples were dehydrated with 8 steps of acetone series method for 1 day and then were infiltrated with a mixture of acetone and Spurr's resin with different proportions, which were 2:1, 1:1, and 1:2 for 3 h at each step. Samples were infiltrated and embedded with pure Spurr's resin 3 times for 3 h before polymerization was performed at 70 °C for 8 h in a vacuum chamber. Ultrathin sections were cut and applied on a supportive grid before staining the sections with uranyl acetate for 30 min and with lead citrate for 30 min. All the prepared samples were examined by TEM (Hitachi HT7700, Hitachi High-Technologies Corporation, Tokyo, Japan) at the Scientific Equipment and Research Division of the Kasetsart University Research and Development Institute.

3. Results

The primary goat synovial cells in this study were previously prepared and were kept at –80 °C. These stored primary synovial cells were successfully recultured, as demonstrated by a monolayer of fibroblastic cells with spindle-shaped appearance as shown in Figure 1. These cells could actually be cultivated and multiplied for more than nine passages while maintaining their viability. Isolation and cultivation of monocytes for differentiation into macrophages was characterized by the presence of adherent cells and cytoplasmic processes of activated macrophages, as shown in Figure 2. After cocultivating the primary goat synovial cells with the macrophages differentiated from monocytes collected from seropositive goats, the multinucleated syncytial formation of the highly vacuolated synovial cells was considerably noted, whereas multinucleated syncytial formation was not observed in the cocultivation with the macrophages differentiated

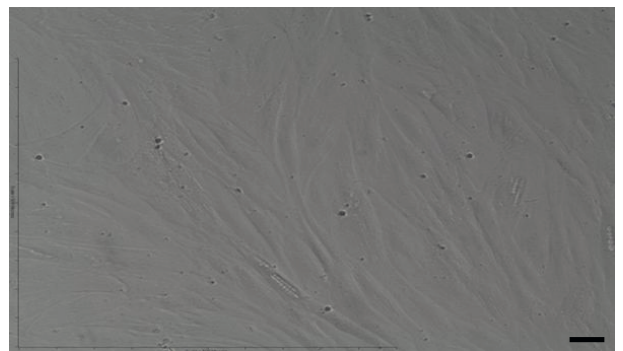


Figure 1. A primary fibroblastic cell of a goat synovial cell on days 7–10 of the cultivating period showed a spindle-shaped cell characteristic of fibroblastic cells (bar = 10 µm, 400× magnification).

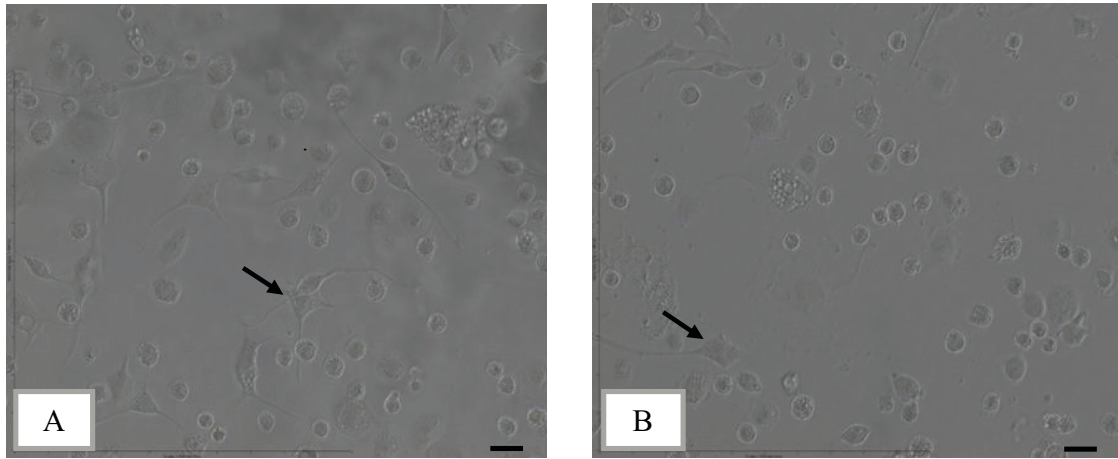


Figure 2. Macrophage cultivation on days 7–10 indicated adherent cells and cytoplasmic processes of activated macrophages (arrow) harvested from 2 seropositive goats (A: the first goat and B: the second goat; bar = 10 μ m, 400 \times magnification).

from monocytes collected from the seronegative goat. This result demonstrated that the seropositive goats carried CAEV in their monocytes. The presence of the viral particle in the cocultivated cells was confirmed by the positive result of immunofluorescence assay, while the negative control showed normal appearance and negative immunofluorescence as presented in Figures 3 and 4, respectively. The examination of CAEV in cocultivated goat synovial cells by TEM is presented in Figure 5. The cluster of mature virions and budding-like virions is marked, which indicated that co-cultivating cells allowed the CAEV to complete its replication cycle.

4. Discussion

The viability of these primary synovial cells that were preserved at -80°C for approximately 5 years was tested by recultivation from storage. The monolayer of goat synovial cells adhering to the bottom of the flask was detected and the cells reached full confluence after 7–10 days of cultivation, as described in our previous study (13). In order to cocultivate CAEV with synovial cells, the synovial cells in the culture system should align as monolayer cells. This step therefore limits the use of stored synovial cells. However, our developed primary goat synovial cells were easy to recover after such a long period of storage. This might be an advantage for further study to produce continuous cell lines of synovial cells, which might be useful for future research.

In cocultivating primary goat synovial cells with macrophages, it was noted in our study that subcultivation was not necessary within 7 days since cocultivation. This is according to the CPE formation that could be observed within 7 days of coculture without any further subcultivation. The characteristic of CPE of infected goat synovial cells in this study was mainly

syncytial formation and multinucleated giant cells, while a lysed lesion was rarely found. This was classified as the persistent and nonlytic type, which was a characteristic of CAEV infecting goat synovial cells; otherwise, the lysed lesion was predominantly the CPE of the maedi-visna virus (MVV), another closely related genetic ovine lentivirus, infecting the goat synovial cells (5,9,18,19). Macrophages would play a major role in the cultivating system because viral expression is linked to the ability of monocyte differentiation into mature macrophages, which are important sites and target cells for viral replication. Meanwhile, the monocyte was the primary cell carrying provirus infectivity in the blood and that virus became productive when the monocyte was differentiated into a macrophage (14–16,19). The primary goat synovial cell could be used as a diagnostic tool for CAEV and MVV infection by differentiating cytopathic phenotypes of caprine and ovine lentivirus (9,11,20).

A concrete confirmation of virus present in primary goat synovial cells was performed by immunofluorescence technique. Although our study did not prove the CAEV presenting in the macrophages by both immunofluorescence and TEM technique, we could hypothesize that CAEV-infected macrophages, even in small proportions, could easily replicate and multiply in the specific and appropriate cultivating system as clearly observed in this study. The appearances of positive fluorescence in infected cells by the reaction of specific monoclonal antibody to the CAEV capsid antigen p28 were noted and proven as a suitable cultivating system for CAEV multiplication. Both intracytoplasmic and intranuclear fluorescence were detected, which was described previously (1,9,21,22), while the goat synovial cells that were cocultivated with macrophages from the seronegative goat did not express fluorescence, which

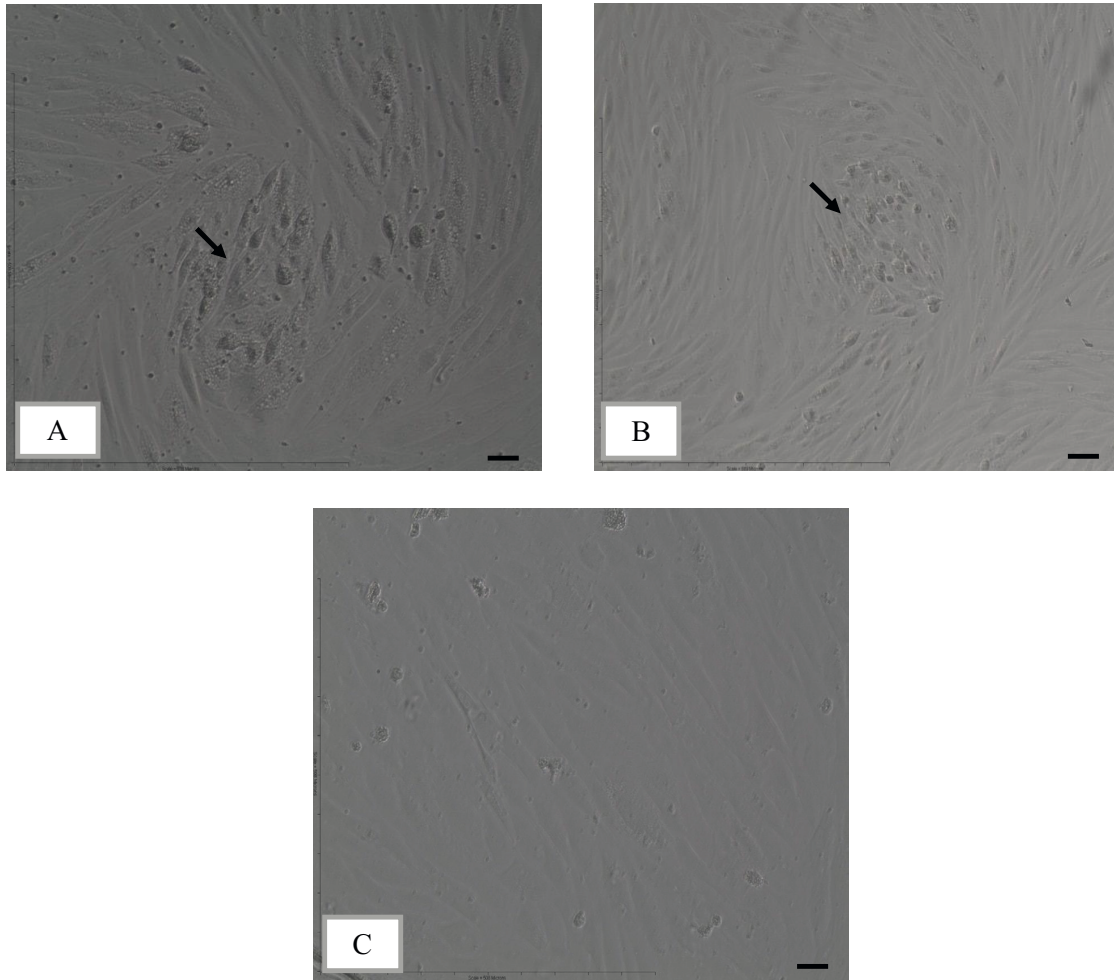


Figure 3. Cytopathic effect (CPE) from 2 seropositive goats (A: the first goat and B: the second one), presenting multinucleated giant cells and syncytial cells that resulted from the fusion of infected cells (arrow). The CPE of these viral infections is usually found as multifoci in the goat synovial cells while Figure 3C shows no CPE present from the seronegative goat (bar = 10 μ m, 400 \times magnification).

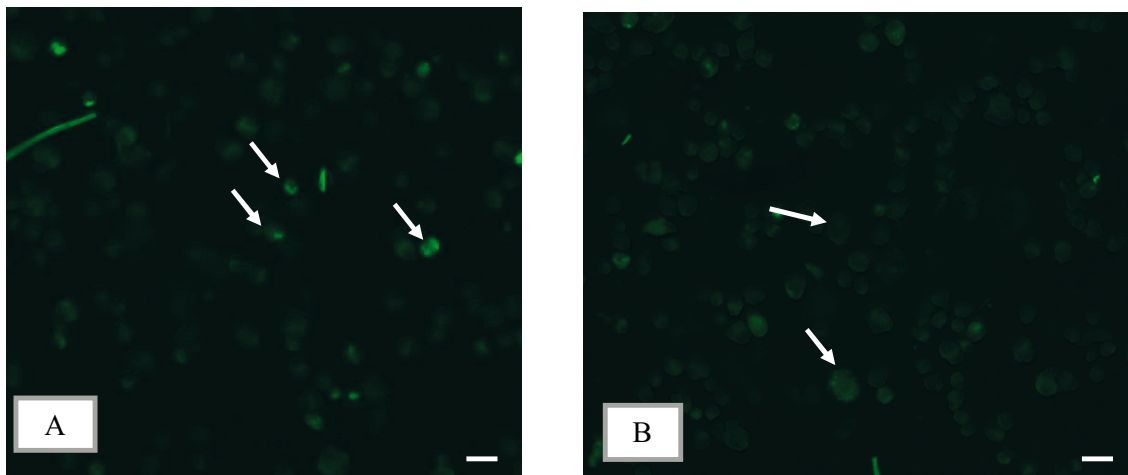


Figure 4. Immunofluorescence assay indicated the CAEV antigen in goat synovial cells by retained green fluorescence (A) in the cytoplasm and nucleus (arrow), while uninfected cells were not stained (B) (bar = 10 μ m, 400 \times magnification).

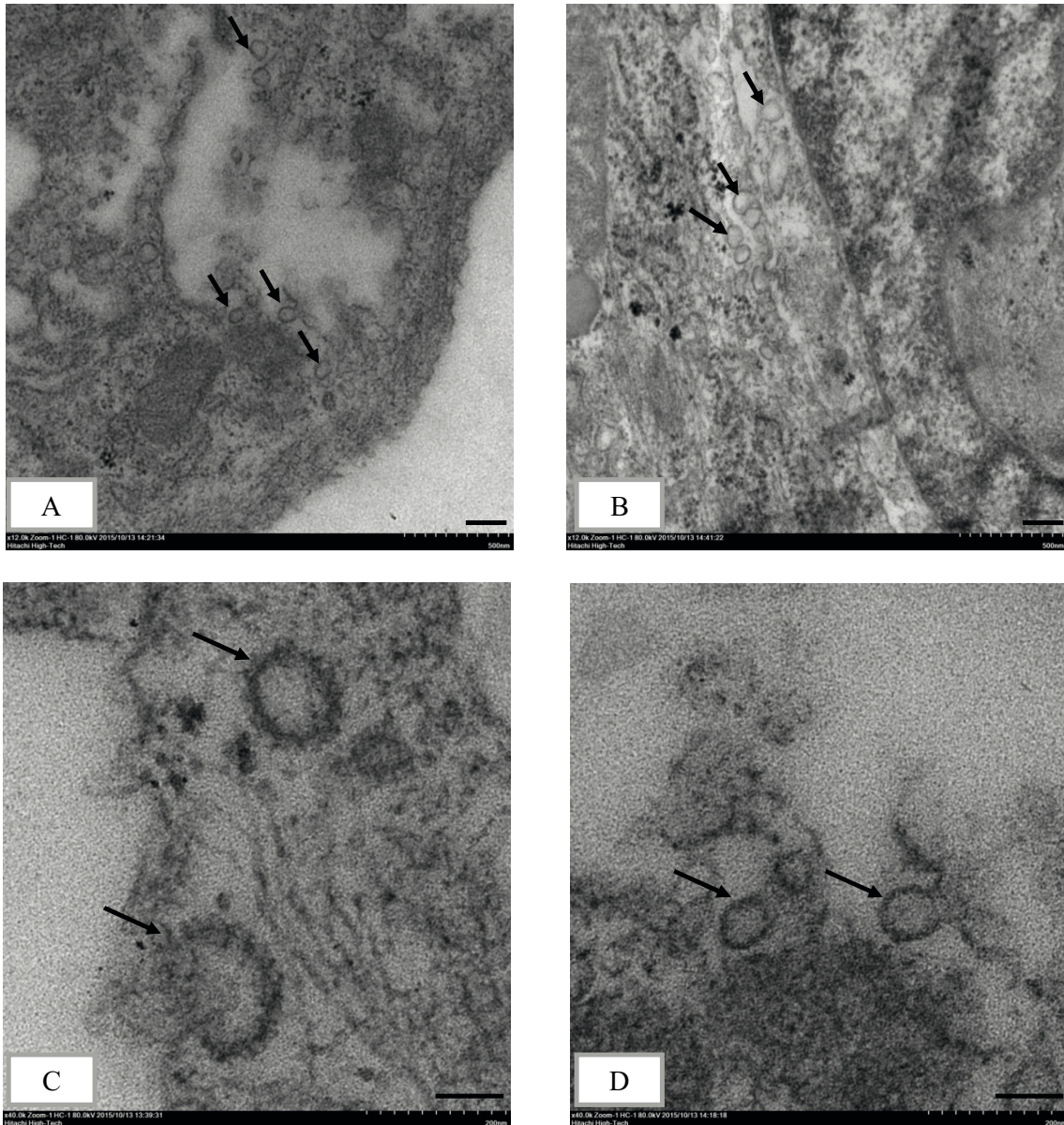


Figure 5. Examination of CAEV infection by TEM. Figures 5A and 5B indicate the cluster of extracellular virions at the syncytium of a primary goat synovial cell (arrow; bar = 200 nm, 12,000× magnification). High magnification of virions (C) showed the ultrastructure of the smooth antigenic external surface of virions, and budding-like virions with stalk-like structure from the surface of the syncytium were examined (D) (arrow; bar = 100 nm., 40,000× magnification).

indicated that the primary goat synovial cells were not infected with CAEV due to the fact that there were no CAEV-infected macrophages from the seronegative goat. This result also suggested a high specificity of the cultured cells as a diagnostic tool. Furthermore, typical expressions of fluorescence in synovial cells were faint, and finely bright granular appearance had been observed, as also demonstrated by a previous study (1). This indicated that CAEV could infect both the nucleus and cytoplasm of synovial cells, as previously described (22–24).

The TEM results revealed the character of viral infection in primary goat synovial cells as a cluster of 5–10 spike-covered viral particles, consistently 80–110 nm in length. Most of these virions were located in the extracellular space of the cytoplasm, while some mature stalk-like budding virions were observed, especially located in epithelial cells or surfaces of syncytial cells. Though the number of budding viruses was small, this specific finding indicated that viral multiplication was in an active stage and was appropriate to produce large number of virions

(6,15,23,24). Therefore, it was again confirmed that our primary goat synovial cells and cultivating system were appropriate.

In conclusion, the present study successfully applied primary goat synovial cells and the cultivating system as an appropriate method for CAEV cultivation and isolation. The characteristics of CPE, expression of viral antigen in synovial cells by positive fluorescence, and the ultrastructure of virions by TEM were predominantly observed. These results explained the acceptance of primary goat synovial cells for CAEV infection. This report was also the first described for cultivation and isolation of CAEV in Thailand. Though a previous report (25) described a suspected retrovirus infection in goats by the seropositive result of a CAE-like virus and retrovirus particles found in the spinal cord by electron microscopy from the first outbreak in Ratchaburi Province in 1985,

an exact CAEV infection was not clarified. Furthermore, this established in vitro study model for CAEV, to our knowledge, will enable a fundamental technique for subsequent experimental study of CAEV, especially without using animal models.

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