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# ISOLATION OF CHICKEN ANAEMIA VIRUS IN CELL CULTURES AND DETECTION OF VIRAL DNA BY POLYMERASE CHAIN REACTION IN NATURALLY INFECTED CHICKEN IN MALAYSIA

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## SUMMARY

Chicken anaemia virus is known to cause severe anaemia in newly hatched chicks. Although serological reactors to chicken anaemia infection have been detected in the country, the virus has never been isolated from chickens. This paper reports the first isolation of the virus in Malaysia and highlights the application of polymerase chain reaction (PCR) technique for rapid detection of the virus from field specimens.

Keywords: Chicken anaemia virus, virus isolation, PCR

## INTRODUCTION

Chicken anaemia virus (CAV) is a small virus with a diameter of approximately 23 nm and contains a circular single-stranded DNA of size 2.3 kb (Tod *et al.*, 1990). The virus is known to cause clinical and subclinical disease of economic importance in broiler chickens (McNulty, 1991). Immunosuppression arising from subclinical infection is due to the depletion of cortical thymocytes in young chickens (Yuasa *et al.*, 1979; Jewrissen *et al.*, 1989).

The virus has been reported in many countries and is considered world-wide in distribution (McNulty, 1991). Although a serological survey carried out previously had indicated the presence of CAV infection in Malaysia (Chai and Yuasa, 1989), the virus has never been isolated. The isolation of CAV in chicken T cell line MDCC-MSB1 transformed by Marek's disease virus is tedious and time consuming because it requires many passages (Yuasa *et al.*, 1983) and takes 2 to 3 weeks to complete. However, the development of polymerase chain reaction (PCR) technique has made it possible to detect the virus within one to two days (Soine *et al.*, 1993).

This report documents the first isolation of the CAV in Malaysia and highlights the PCR technique for virus detection from field specimens.

## MATERIALS AND METHODS

*Clinical history*

Six chicken broiler farms (A to F) with birds ranging from 14 to 33 days old were monitored for CAV infection (Table 1). Chicks in Farm A had general weaknesses while those in farms B to E showed spiking mortality syndrome (Davis *et al.*, 1995). At necropsy, no significant gross lesions were seen in the chicks from all farms, except for those from farm F in which marked swelling of the kidneys was evident.

**Table 1. Age of broiler chicks investigated**

| Farm | Age of chick (days) |
|---|---|
| A | 15 & 22 |
| B | 28 |
| C | 33 |
| D | 21 |
| E | 14 |
| F | 22 |

*Virus isolation*

Liver specimens of individual chicks from farms A to F were used for isolation of CAV. The liver samples were separately homogenised, diluted 10 to 20% in Ham's F10 growth medium with antibiotics and centrifuged at 14,000 rpm for 20 min at 4°C. The supernatants were used as inocula.

Suspension cultures of MDCC-MSB1 cells in aliquots of 0. 1 mL were seeded into a 24-well plastic tissue culture plates at $10^5$ cells per well. Viral samples were inoculated into the wells at 10 µL per well and were allowed to adsorb at 40°C for 1 h. Only one well of the MDCC-MSB1 cell suspension was inoculated for each viral sample. Positive samples consisting of CAV and negative samples consisting of culture fluid were also inoculated into the respective wells. Maintenance medium consisting of F10 medium in 10% foetal calf serum and 10% tryptose phosphate broth was dispensed into the wells in volumes of 0.9 mL per well. The cultures were incubated at 40°C in 5% carbon dioxide. Virus passages were carried out every 2 to 3 days by transferring 50 to 100 µL of the cell suspension into 0.9 mL of fresh culture medium. Inoculated and

control cultures were examined daily for cytopathic effect (CPE) as evidenced by alkaline medium and failure of the cells to multiply. A total of 6 to 8 passages were conducted to rule out the presence of CAV. All inoculated cultures were screened for the presence of CAV by the indirect immunofluorescence.

*Immunofluorescence test for detection of CAV antigen in MDCC-MSB1 cells*

Cell smears of inoculated and control MDCC-MSBI cells were prepared on 4 mm diameter wells of the 24-well teflon coated glass slides, allowed to air dry and fixed in cold acetone for 10 min. The indirect fluorescent antibody (IFA) test was carried out by reacting 10μL of 1:100 diluted positive and negative chicken CAV antiserum with the cell smears. Controls consisted of CAV infected and uninfected cell smears reacted with the positive and negative sera. Phosphate buffered saline (PBS) was used as conjugate control. The smears were incubated at 37°C for 30 min in a moist chamber and washed in 3 changes of PBS solution. Ten μL of fluorescein conjugated anti-chicken immunoglobulin G (IgG) diluted to 4 fluorescent units was added into each well and incubated at 37°C in a humid chamber for another 30 min. After washing in 3 changes of PBS solution, the cell smears were examined under an ultra-violet microscope for cell associated immunofluorescence.

*Polymerase chain reaction (PCR)*

Liver homogenate samples obtained from chicks from farms A to F, diluted 10 to 20% and 100 μL aliquot were treated with 10% sodium dodecyl sulfate. The DNA was then extracted by the phenol-chloroform-isoamyl alcohol treatment and precipitated in ethanol containing 5M sodium chloride. For the PCR reaction, the Takara PCR kit (Takara Shuzo Company, Shiga, Japan) and primers 5-AATGAACGCTCTCCAAGAAG-3' and 5'-AGCGGATAGTCATAGTAGAT-3' were used. The PCR consisted of 30 repetitive cycles of amplification as follows

i. denaturation at 94°C for 45 seconds
ii. annealing at 55°C for 45 seconds
iii. elongation at 72°C for 90 seconds

The reaction was terminated by a final elongation step at 72°C for 5 min. The amplified PCR products (10 μL) were loaded into 2% agarose gel in Tris-acetic acid-EDTA buffer.

*Restriction enzyme digestion*

The method of enzyme digestion by Hind III has been described previously (Kono *et al.*, 1996). It involved purification of the PCR product by precipitation with sodium acetate in ethanol, extraction in phenol-chloroform mixture and a final precipitation step with sodium acetate in ethanol. Following confirmation of the purified PCR product in an adequate amount by agarose gel electrophoresis (AGE), Hind III enzyme digestion was carried out at 37°C for 3 h, followed by incubation at 4°C overnight. The reaction mixture consisted of

| | |
|---|---|
| purified PCR product | 4.0 λ |
| Hind III | 1.5 λ |
| 10x buffer | 1.0 λ |
| distilled water | 3.5 λ |

The digested fragments were resolved through AGE and examined under ultra-violet light.

## RESULTS

*Virus isolation*

One and five isolates of CAV were obtained from farms A and B respectively. Infected MDCC-MSB1 cells were degenerated and showed decrease in cell growth after 3 to 4 passages and by the 6th passage, the cell multiplication ceased completely. Control cells and those inoculated with liver specimens from the farms C, D, E and F continued to show exponential cell growth for up to the final 8th passage. The presence of CAV was detected by both IFA test (Table 2) and PCR (Fig. 1) in cultures showing CPE.

*Detection of CAV from liver samples by PCR*

Out of the 26 liver samples from farms A and B that were screened for CAV by the PCR technique, viral DNA was detected from only 6 samples (Fig. 2). The 6 samples were the same samples from which CAV were isolated earlier in MDCC-MSB1 cultures. Similarly, the samples that were screened negative by the PCR technique were the samples from which the virus could not be isolated in cell cultures (Table 3).

*Restriction enzyme analysis*

The PCR products of the 6 CAV isolates were cleaved by Hind III into 2 fragments of sizes 275 and 305 kb as expected in relation to the specific restriction sites present in the CAV viral genome (Fig. 3).

## DISCUSSION

The isolation of CAV viruses in MDCC-MSB1 cells resulted in cell degeneration and failure of the cells to multiply after 3 to 6 passages. The presence of CAV in the cells was confirmed by immunofluorescence staining. The PCR technique also detected the viral DNA in the infected cells and from the liver samples of chicks from farms A and B. Results of virus isolation in MDCC-MSB1 cells and detection by immunofluorescence concurred with those of PCR detection of viral DNA in infected MDCC-MSB1 cells and liver homogenates of chicks. Samples that were

**Table 2. Results of CAV isolation in MDCC-MSB1 cultures following detection by IFA test and PCR**

| | | Detection of CAV by IFA test | | | | | PCR detection |
|---|---|---|---|---|---|---|---|
| | | sample*+ PS+C | sample*+ INS+C | CAV+ PS+ C | CAV + NS + C | CAV + PBS + C | |
| Farm | A: 1/21 with CPE | 1/21+ve | 21/21 -ve | 1/1 +ve | 1/1 -ve | 1/1 -ve | 1/21 sample +ve |
| Farm | B: 5/5 with CPE | 5/5 +ve | 5/5 -ve | 1/1 +ve | 1/1 -ve | 1/1 -ve | 5/5 samples +ve |
| Farm | C: 11/11 no CPE | 11/11 -ve | 11/11 -ve | 1/1 +ve | 1/1 -ve | 1/1-ve | not tested |
| Farm | D: 7/7 no CPE | 7/7 -ve | 7/7 -ve | 1/1 +ve | 1/1 -ve | 1/1 -ve | not tested |
| Farm | E: 18/18 no CPE | 18/18 -ve | 18/18 -ve | 1/1 +ve | 1/1 -ve | 1/1 -ve | not tested |
| Farm | F: 5/5 no CPE | 5/5 -ve | 5/5 -ve | 1/1 +ve | 1/1 -ve | 1/1 -ve | not tested |

PS =positive serum NS = negative serum C = anti-chicken IgG fluorescent conjugate
CPE = cytopathic effect sample* = MDCC-MSB 1 cells inoculated with liver specimens

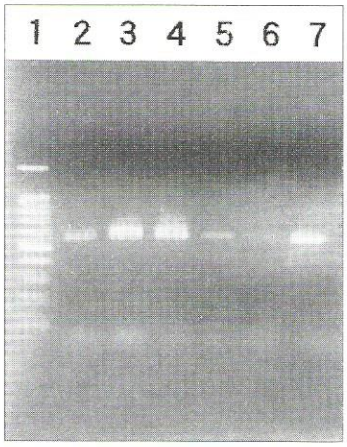


**Fig. 1. PCR detection of CAV in MDCC-MSB1 cells inoculated with liver samples of chicks from farms A and B. Lane 1=1 00 bp marker; Lanes 2 to 5=CAV infected cells (farm B); Lane 6=CAV infected cells (farm A); Lane 7=CAV positive control cell**

positive by virus isolation were also positive for detection of CAV DNA in infected cell cultures and liver samples. Vice versa, those that were negative for virus isolation were also negative by the PCR technique.

Further to the PCR method of detection based on the size of the product amplified (fragment of CAV genome), results of identification by restriction enzyme analysis showed the presence of 2 digested fragments in AGE that corresponded to the expected sizes of CAV genome cleaved by Hind III digestion. These findings confirm the first isolation of CAV in Malaysia and highlight the possible application of the PCR for diagnostic use. Although the infected chicks of ages 22 and 28 days old in farms A and B respectively had complaints of general weaknesses and spiking mortality syndrome, no characteristic pathological changes due to CAV infection were seen in the chicks. Age of the infected chicks could be an important factor relating to pathological changes (Rosenberger and Cloud, 1989).

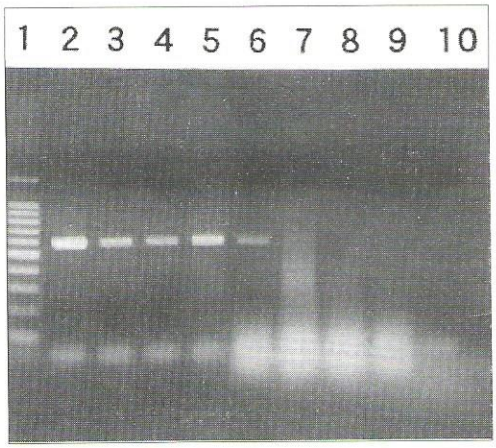


**Fig. 2. PCR detection of CAV from liver homogenates of chicks from farms A and B. Lane 1=100 bp marker; Lane 2=Reference CAV; Lanes 3-5=positive chick liver (farm B); Lane 6=positive chick liver (farm A); Lanes 7-9=negative chick liver (farm A); Lane 10=Cell culture fluid (negative control)**

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**Table 3. Isolation of CAV and detection of viral DNA by PCR**

| Farm | Age of chicks | CAV isolation in MDCC-MSB1 cells | PCR detection of CAV in MDCC-MSB1 cells | PCR Detection of CAV in liver samples from chicks |
|---|---|---|---|---|
| A | 15 days | 0/10* | 0/10** | 0/10** |
| A | 22 days | 1/11 | 1/11 | 1/11 |
| B | 28 days | 5/5 | 5/5 | 5/5 |
| C | 33 days | 0/11 | not tested | not tested |
| D | 21 days | 0/7 | not tested | not tested |
| E | 14 days | 0/18 | not tested | not tested |
| F | 22 days | 0/5 | not tested | not tested |

* = No. isolated/Total No. of samples inoculated; ** = No. detected/Total no. tested by PCR

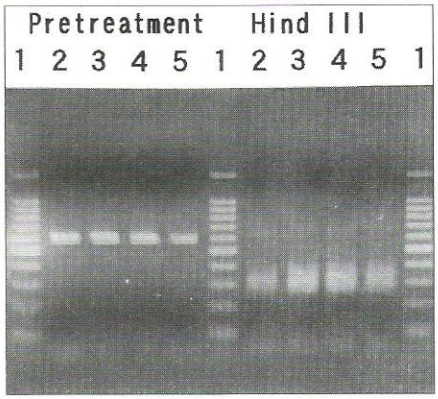


**Fig. 3. Digestion of CAV PCR product with restriction enzyme Hind III. Two fragments corresponding to the expected sizes of 275 and 304 bp were obtained. Lane 1=100 bp marker; Lane 2=Reference CAV; Lane 3=CAV isolate from farm A; Lanes 4 & 5=CAV isolates from farm B**

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**RINGKASAN**

*PENGASINGAN VIRUS ANEMIA AYAM DALAM KULTUR SEL DAN PENGESANAN DNA VIRUS MELALUI TINDAKBALAS RANTAIAN POLIMERASE PADA AYAM YANG TERJANGKIT SECARA SEMULAJADI DI MALAYSIA*

*Virus anemia ayam diketahui menyebabkan anemia teruk pada anak ayam baru menetas. Walaupun tindakbalas serologi terhadap jangkitan anemia ayam dikesan di negara ini, virus tersebut tidak pernah diasingkan daripada ayam. Kertas ini melAporkan pengasingan julungkali virus ini di Malaysia dan menengahkan kegunaan teknik tindakbalas rantaian polimerase bagi mengesan virus dari spesimen lapangan secara pantas.*