Chicken Infectious Anemia in Taiwan : Virus Isolation and Antibody Survey

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SUMMARY An outbreak of anemia caused by chicken anemia virus (CAV) infection was diagnosed in a broiler flock in 1991 in Taiwan. The affected chicks had yellowish bone marrow and severe depletion of lymphocytes in the lymphoid organs were observed in most birds examined. Twelve CAV isolates were obtained in MDCC-MSB1 cells from livers of the affected chicks. Anemia was induced in specific-pathogen-free chicks inoculated with an isolate at one day of age. In total 209 (71.58 %) serum samples from nine breeder flocks of 292 serum samples from 11 breeder flocks had the antibody against CAV by an indirect immunofluorescent antibody test. This report is the first on the serological survey and isolation of CAV in Taiwan. [* Lu YS, Tsai HJ, Kwang MJ, Tseng CS Chicken infectious anemia in Taiwan : virus isolation and antibody survey. J. Chin Soc Vet Sci 19(3) : 137-146, 1993. * Corresponding author TEL 02-6212111, FAX 02-6225345]

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INTRODUCTION

Chicken anemia virus (CAV), previously called chicken anemia agent (CAA), was first isolated by Yuasa et al. in 1979 [24]. CAV is a non-enveloped, icosahedral virus with a diameter about 23 nm and contains a circular, single-stranded DNA genome[22]. A relationship to porcine circovirus[21]. was suggested because of their similarity in composition.

CAV produces marked anemia with aplasia of the bone marrow and atrophy of the lymphoid organs with a concomitant immunosuppression in susceptible chickens[19,20,24]. Thus, CAV-induced disease constitutes a serious economic threat. Serological evidence indicates that infection of chickens with CAV is globally widespread[13,27,29]. Many specific-pathogen-free (SPF) flocks were also contaminated with CAV [3,13,14,17,29]. Because the virus can be transmitted vertically [28]. Avian vaccines can be contaminated with CAV if the eggs for vaccine production originate from CAV -infected flocks.

We describe here the clinical, histological, and virologic features of a spontaneous outbreak of CAV-associated disease. The experimental production of anemia in SPF chicks inoculated with the isolated virus is also given. The result of a serological survey is reported.

MATERIALS AND METHODS

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Clinical examinations

Blood samples were taken from the wing vein into a heparinized capillary tube from the moribund chicks. The hematocrit (Ht) value (%) was read after the samples were centrifugated at 12,500 xg for 5 min. Following post-mortem examination, tissue samples were collected, fixed in buffered formalin solution (10 percent) and embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Parts of livers from the moribund chicks were collected for examination of pathogens and stored in a freezer at −70 ℃.

Cell culture and assay of CAV MSB1

MSB1 cells were obtained at passage 30 from Dr. N. Yuasa (National Institute of Animal Health, Japan) and were used between passages 31 and 34. Cells were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. The material was placed in an incubator at 37 ℃ in an atmosphere with 5 % CO_2. One fifth of the cells inoculated were transferred each day. Successful transfer was judged from the color of the culture medium. The culture showing a red color and failing to transfer was regarded as CAV -positive. The microtest method for the titration of CAV using 96-well microplates was the method of Imai and Yuasa [11]. The infective titer was expressed as a median tissue culture infective dose ($TCID_{50}$).

Preparation of inoculum and CAV isolation

The livers from the affected chickens were homogenized and prepared as 20 % suspensions in RPMI-1640 medium (Hyclone Lab, Inc., Utah, USA) with kanamycin sulfate (100 μg/mL) and fungizone (1 μg/mL). After centrifugation at 2, 000 x g for 10 min, the supernatants were used as an inoculum. The CAV wsa isolated from the inoculum using MDCC-MSB1 cells by the method reported by Yuasa [25].

Virus strains

The Gifu-1 strain of CAV [24] was supplied by Dr. N. Yuasa. The virus had been passaged in MDCC-MSB1 (MSB1) cells [1] 28 times before arrival in this laboratry. Twelve isolates of CAV, designated Ilan-1 to Ilan-12, were made at this laboratory from the naturally occurring case described in this paper.

Electron microscopy

CAV was grown in MSB1 cells, the cell suspension was collected and centrifuged at a speed of 10,000 x g for 15 min. The supernatant was harvested and centrifuged at 80,000 x g for 10 min. The sediments were mounted on carbon -coated 400-mesh grids, stained with 2 % potassium tungstic phosphate, and examined with a transmission electron microscope (Hitach H600). Chicks from a SPF flock maintained at The Branch Institute of Animal Drugs Inspection, Taiwan Provincial Research Institute were used.

Chicken susceptibility test

Four chicks aged one day were inoculated intramuscularly with $10^{7.75}$ $TCID_{50}$ / 0.1 mL of CAV per bird (high dose group). Another five chicks at the same age were inoculated with $10^{5.75}$ $TCID_{50}$ / 0.1 mL per bird (low dose group). Five more chicks were kept as the uninoculated control group. These three groups were reared separately and fed a commercial feed and tap water ad libitum. All chicks were observed daily until 42 days of age. when they were bled, sacrificed and necropsied; tissue samples were obtained. Chickens were considered susceptible to CAV when their Ht value was less than 29 % and lesions of CAV were found in the thymus, bursa of Fabricius (BF), and bone marrow [12].

Serum samples

Serum samples were collected from 11 breeder flocks in Taiwan during 1992- 1993. Antiserum against CAV, and SPF chicken serum free of antibodies against CAV were provided by Dr. N. Yuasa and were included in each indirect immunofluorescent antibody (IIF) test.

IIF test

The IIF test was used to detect CAV in MSB1 cells and to survey the CAV antibodies in breeder flocks in Taiwan. The test was performed as described by Yuasa et al.[29] For the antibody survey, the Gifu-1 strain of CAV grown in MSB1 cells were used. In brief, MSB1 cells infected with Gifu-1 CAV or local isolates were harvested at 48 h post inoculation and washed three times with phosphate buffer solution (PBS, pH 7.2). Ten μ L containing 100, 000 cells was placed in each well of a Teflon-covered glass microscope slide (Cel-Line Associated, Inc., N.J., USA). Uninfected control smears were prepared with 50,000 uninfected MSB1 cells per well on the same slide. The slides were dried in air at room temperature, fixed in cold acetone for 10 min. Ten ul of chicken serum, diluted 1:100 and 1:500 in PBS, were placed on CAV-infected cells and uninfected cells After 30 min of incubation at 37 ℃ in a humid chamber, the slides were rinsed in PBS three times each for 5 min at room temperature. Ten μ L of a 1:30 dilution of rabbit antichicken IgG conjugated with fluorescein inothiocyanate (Jackson Immuno Research Lab, Inc, USA) was then applied to the cells. The slides were washed three times as before, and mounted with tris-glycerol (pH 8.5). Samples were read as positive only when bright staining of small irregularly shaped granules was observed in the nuclei of CAV-infected MSB1 cells and no such staining was observed in the uninfected cells. When fluorescence was observed in the uninfected cells, the result was considered un-interpretable.

RESULTS

Case study

The disease occurred in Ilan county in August 1991 in a broiler flock composed of 30, 000 chickens at the age of 39 days. The mortality and culled rate was approximately 2-2.5 %. Dead or sick birds were submitted to our laboratory for diagnosis at the age of 48 days. The sick chickens had a low Ht value (15-20%), pale bone marrow, atrophied thymus and BF. The main histological lesions included aplasia and hyperplasia of bone marrow, lymphocytic depletion in thymus, bursa and spleen.

Virus isolation

Virus infection was suspected on the third or fourth passage of liver- homogenate-inoculated MSB1 cultures. Specific fluorescence was observed in Ilan isolates-infected MSB1 cells by the IIF test using specific antiserum prepared against Gifu-1 strain CAV (Fig 1). The fluorescence were scattered all over the cells as irregular, large or small granules. Specific fluorescence was not seen in control cell cultures. Electron microscopic examination of infected and processed MSB1 cells revealed many virus particles of diameter 23-26 nm that had isometric symmetry (Fig 2). In total 12 isolates were made and designated Ilan-1 to Ilan-12, respectively.

Pathogenicity of CAV for one-day-old chicks

Fourteen days after inoculation, two of four chicks of $10^{7.75}$ $TCID_{50}$/ 0.1 mL inoculated group became anemic with Ht values 21% and 11%. All birds in the $10^{5.75}$ $TCID_{50}$/0.1 mL inoculated group and control group were normal at this age, All birds in the $10^{7.75}$ $TCID_{50}$/0.1 mL inoculated group died between 26 and 30 days of age. All birds in $10^{5.75}$ $TCID_{50}$/ 0.1 mL inoculated group became anemic and three died. The control group remained normal until the end of this study (Table 1).

The chicks inoculated with the Ilan-1 virus became depressed. reluctant to move, and anemic (Fig 3). Grossly, anemia throughout the body, yellowish bone marrow (Fig 4), atrophic thymus (Fig 5) and BF, and enlarged yellowish liver were observed in most chicks that died. In some chicks the associated changes were hemorrhagic lesions in skeletal muscles and / or proventricular mucosa (Fig 6). Histologically, almost all hematopoietic tissue was replaced by adipose tissue in anemic chicks (Fig 7). Thymic lymphocytes were destroyed and diminished in all severely infected chicks, and the lobules were replaced by reticular cells. The hepatic sinusoid were dilated and contained serous exudates with swollen endothelial cells. Fatty degeneration and hyaline necrosis were observ-

ed in the hepatocytes around the central vein in some dead chicks. No inclusion bodies were found in hepatic cells.

CAV was recovered from the liver of incoulated chicks by method described above.

IIF antibody survey

In total 292 serum samples from 11 breeder flocks were collected and tested. Antibodies aganist CAV were found in 209 serum samples (71.58%) from 9 flocks (Table 2).

Table 1 The pathogenicity of the Ilan-1 isolate of CAV to 1-day-old SPF chicks

Groups[a]	14 days PI			30 days PI
	Ht value (%) Mean (SD)	B.W.(g) Mean (SD)	Anemia[b] induced	Mortality[c]
High dose	25.3 (11.4)	61.9 (11.7)	2 / 4	4 / 4
Low dose	36.0 (1.4)	58.9 (5.0)	0 / 5	3 / 5
Control	37.8 (2.0)	56.6 (6.3)	0 / 5	0 / 5

[a] Each bird in the high-dose group was inoculated with $10^{7.75}$TCID$_{50}$ Illan-1 isolate of CAV in 0.1 mL intramuscularly, whereas each bird in the low-dose group received $10^{5.75}$TCID$_{50}$ in 0.1 mL.
[b] No. of birds become anemic/ No. birds inoculated. Birds were considered anemic when their Ht value was less than 29 %.
[c] No. of birds dead/ No. of birds incoulated

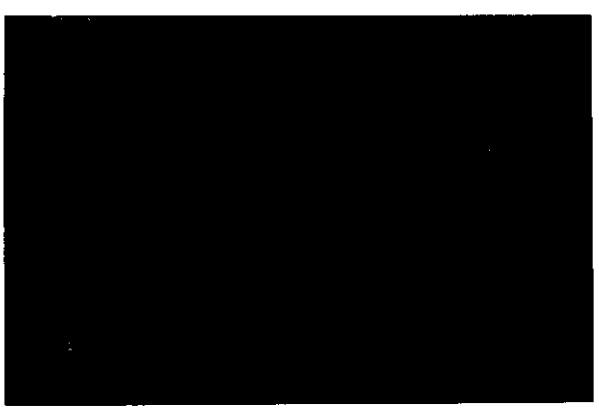


Fig 1 Specific fluorescence shown in Ilan-1 isolate-infected MSB1 cells by FITC-conjugated antibodies against Gifu-1 strain chicken anemia virus, x 400

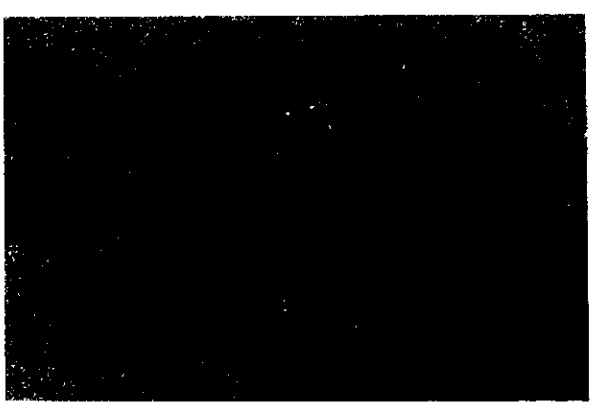


Fig 2 Ilan-1 strain CAV particles negatively stained with potassium tungstic phosphate. (bar = 100 nm) x 200,000.

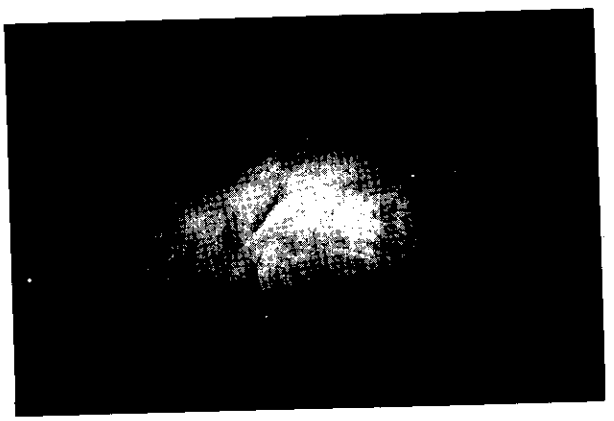


Fig 3 Fourteen days after inoculation with Ilan-1 isolate, the SPF chick became depressed, reluctant to move, and anemic.

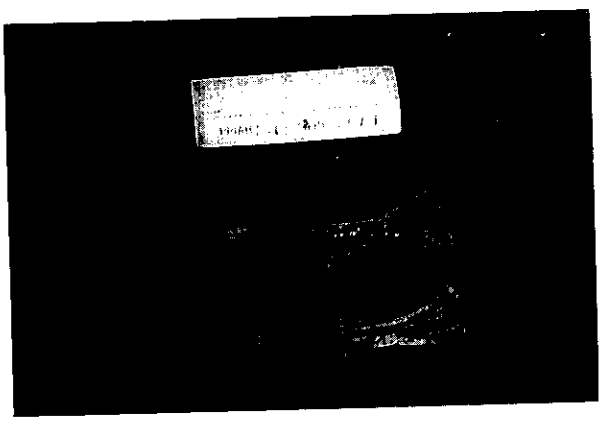


Fig 4 The yellowish femoral bone marrows (bottomm four) were observed in SPF chicks 26 days after inoculation with Ilan-1 isolate. The bone marrows of the control birds (top four) were red.

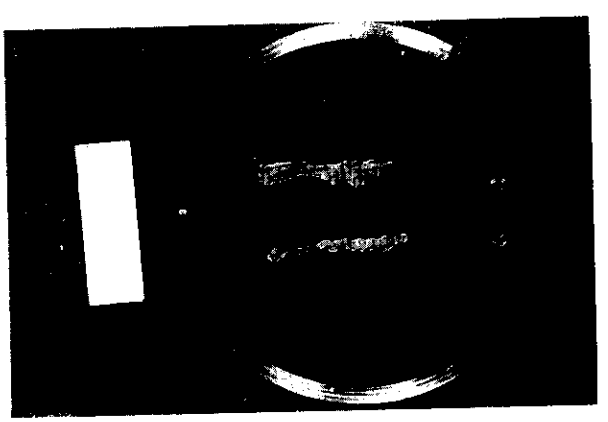


Fig 5 Twenty-six days arter inoculation with Ilan-1 isolate, the thymus of the SPF chicks became extremely atrophic (top). The thymus at the bottom is normal.

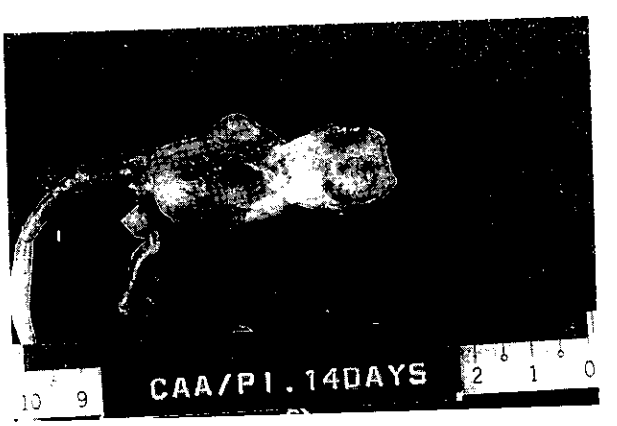
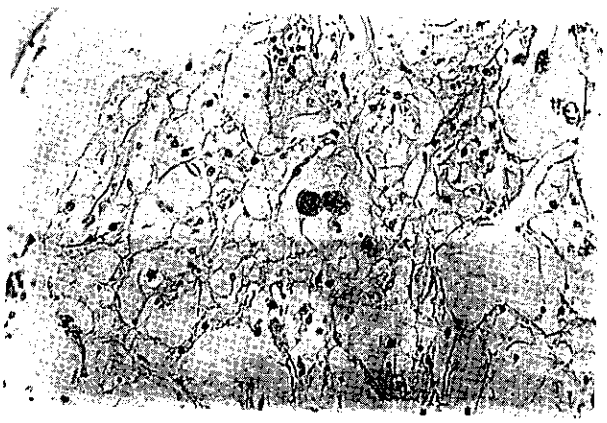


Fig 6 The proventriculus was swollen and the mucosal hemorrhage was observed in SPF chicks 14 days after being inoculated with Ilan-1 isolate.



Ig 7 The depletion of erythrocytic and granulocytic series of myelocytes and infiltration of adipose cells were observed in bone marrow of SPF chicks 14 days after being inoculated with Ilan-1 isolate, x 400

Table 2 Serological survey of antibody against chicken anemia virus in the broiler breeder flocks in Taiwan

Flocks	No. of birds investigated	No. of birds positive	positive rate (%)
C	42	0	0
E	18	0	0
F	20	18	90.0
H	40	38	95.0
L	40	37	92.5
M	20	16	80.0
O	12	9	75.0
P	40	36	90.0
R	20	17	85.0
T	20	18	90.0
Y	20	20	100.0
Total	292	209	71.6

DISCUSSION

CAV is suspected to be ubiquitous in all major chicken-producing countries[4]. Only a few countries such as Japan[24,26]. Germany[2]. Sweden[6], USA[8], UK[5] and Australia [7] have successfully isolated the CAV. One reason is that CAV caused no particular lesions that can be considered pathognomonic, and hence the disease is generally masked by other complicating disease due to immunosuppression effect of CAV[4]. Another reason is that CAV produces no cytopathic effect (CPF) and fails to grow in any cultured monolayer cells that were prepared from chicken tissue and chicken embryos[24,26]. It growa in only some lymphoblastoid cell line such as MSB1 and 1104b1[26,29]. In our work, after three or four blind passages the specific immunofluorescent (IF) positive antigens were proved in MSB1 cells infected with CAV, The SPF chicks inoculated with the virus isolates became anemic and died. It was surprising to see the birds inoculated with higher dose of Ilan isolate had highest weight. But no significant difference was found among these groups by statistical analysis ($P > 0.01$). Thus it might due to the quantity of birds used in each group was too small. This report is the first on the isolation and identification of CAV from clinically ill chickens in Taiwan.

The immunofluorescence test revealed that antigenic similarity exists among Gifu-1 Japanese isolates and twelve Taiwan isolates. Two American isolates [8] and eleven Japanese isolates [30] serologically resemble each other. Also one American isolate resembles one UK isolate [30]. It is generally considered that CAV isolates appear to have only one serotype [15,16]. although minor variations among CAV isolates were shown by DNA hybridization and polymerase chain reaction [18]. Additional comparison of the similarities and differences among Taiwan isolates and between Taiwan isolates and foreign isolates may be needed.

The neutralization (NT) and IIF tests have been used in the survey of an antibody against CAV but the NT teat requires much more time to obtain the results. In contrast the IIF test is conducted more quickly and easily than the NT, and with the same degree of sensitivity [30]. Thus IIF test has been widely used in antibody surveys[3,13,14,29]. Todd et al,[23] developed an enzyme-linked immunosorbent

assay (ELISA) to detect a serum antibody against CAV. The test used a CAV-specific monoclonal antibody (NAb) to capture virus antigen, and 98.5% agreement was achieved between the results of the ELISA and the IIF test As no MAb against CAV is available in Taiwan, the IIF test was employed in this study.

The result of the antibody survey showed a high positive rate among the breeder flocks in Taiwan. The result agrees with surveys conducted in many countries such as USA[11]. UK[13] and Japan[29]. The antibody to CAV has been detected in many SPF chicken flocks in various countries [3,13, 14,17,29]. CAV infections are likely to have occurred in chickens in Taiwan for a long time. A retrospective project may be needed to trace the epizootiological history of CAV-infected chickens in Taiwan.

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雞傳染性貧血病毒分離與抗體調查

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摘要 1991 年 8 月宜蘭縣某肉雞場發生雞傳染性貧血病例，病雞呈貧血，胸腺及華氏囊萎縮，骨髓變黃，骨髓細胞被脂肪細胞所取代等症狀及病變。經以 MDCC-MSB1 細胞由病雞肝臟共分離到 12 株雞貧血病毒，分離病毒接種 1 日齡 SPF 小雞可引起雞傳染性貧血之病變。以間接螢光抗體法調查 11 個種雞場 292 例血清，結果發現 9 場 209 例血清具有雞貧血病毒抗體，總陽性率爲 71.58 %，本文爲台灣首次分離雞貧血病毒成功及抗體調查之報告。〔*呂榮修、蔡向榮、鄺懋勁、曾俊憲。雞傳染性貧血病毒分離與抗體調查。中華獸醫誌 19 (3) : 137-146, 1993. *聯絡人 TEL 02 -6212111, FAX 02-6115345〕

關鍵詞：雞傳染性貧血，雞貧血病毒

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