

# Newly Developed Seroimmunological Diagnosis (Enzyme-Linked Immunosorbent Assay) for Chicken Leucocytozoonosis

By TAKASHI ISOBE

Poultry Disease Laboratory, National Institute of Animal Health  
(Kurachi, Seki, Gifu, 501-32 Japan)

## Introduction

*Leucocytozoon caulleryi*, a causative agent of chicken leucocytozoonosis, was first discovered by Mathis and Leger from a chicken in Tonking in Southeast Asia<sup>12</sup>. Since then, outbreaks of this disease have been reported from various Asian countries such as Thailand<sup>3</sup>, Burma<sup>6</sup>, Malaysia<sup>16</sup>, Singapore<sup>4</sup>, Philippines<sup>11</sup>, India<sup>18</sup>, Sri Lanka<sup>17</sup>, Taiwan<sup>10</sup>, Korea<sup>2</sup>, Japan<sup>1</sup> and so on. Leucocytozoonosis has affected the productivity of chickens through a reduction in egg production, weight loss and sometimes death.

To date, direct microscopic examination of blood smears continues to be the method of choice for diagnosing chicken leucocytozoonosis. It is, however, time consuming to examine each slide, and a trained technician is required for accurate identification when low numbers of parasites are present. Moreover, the period of parasitemia is very short (for about 10 days), so that it is limited to detect merozoites or gametocytes of *L. caulleryi* in blood smears.

On the other hand, immunodiagnostic procedures such as the agar gel precipitation (AGP) test<sup>14</sup>, the counterimmuno-electrophoresis<sup>5</sup>, and the indirect immunofluorescent antibody (IFA) test<sup>8</sup> for detecting antibodies to *L. caulleryi* have been developed. These methods provide important information with regard to exposure to *L. caulleryi* owing to the persistence of antibodies after disappearance of merozoites and gametocytes from the blood. However, none of them is entirely satisfactory for the rapid and sensitive detection of antibodies in large number of samples. Recently, the

enzyme-linked immunosorbent assay (ELISA) has been developed to detect antibodies to *L. caulleryi* as a highly sensitive and specific seroimmunological diagnosis for chicken leucocytozoonosis<sup>9</sup>. In this paper, the procedure of the ELISA and advantages of the ELISA are described.

## Antigen preparation

Specific-pathogen-free (SPF) chickens were inoculated with *L. caulleryi* sporozoites intravenously. Thirteen to 15 days after inoculation, they were killed or died, and the spleens, kidneys and bursa of Fabricius were removed. These organs were homogenized with phosphate buffered saline (PBS, pH 7.2) at 3,000 rpm for 4 min in the homogenizer. The resulting homogenate was washed with PBS through two sieves of 30 and 60 mesh, respectively. The filtrate was again washed thoroughly with PBS through a 100-mesh sieve. Many schizonts were retained within the 100-mesh sieve. They were collected into PBS and centrifuged at 185 g for 5 min. The sedimented schizonts were then washed three times with PBS by centrifugation as above, and suspended in PBS. They were sonicated using an ultrasonic disruptor for 5 min and centrifuged at 8,000 g for 30 min at 4°C. The supernatant fluid was used as antigen. They were divided into aliquots or absorbed into microplate after dilution, and stored at -80°C until use. Antigen concentration should be determined by checker board titration, because it depends on serum and conjugate dilution.

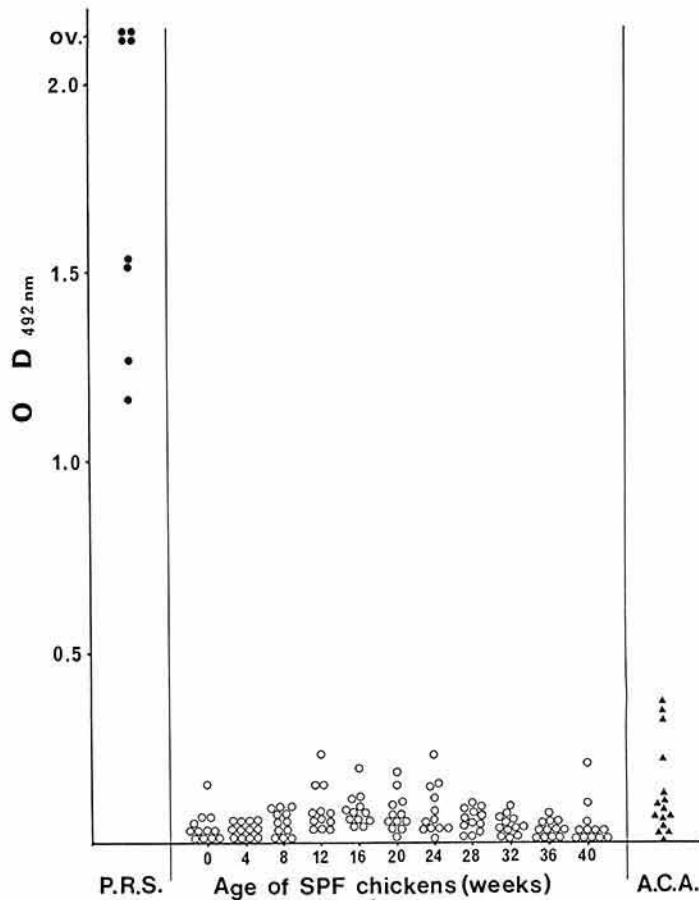


Fig. 1. Optical density (OD) values for SPF chicken sera, antisera against various kinds of chicken disease agents (A. C. A.) and positive reference serum (P. R. S.)

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## ELISA

### 1) Conjugate

Anti-chicken immunoglobulin G or M labelled with horseradish peroxidase were used. They were diluted 10 times with PBS containing 1% bovine serum albumin (BSA) and stored in aliquots at  $-80^{\circ}\text{C}$  until use. Conjugate concentration also should be determined by checker board titration.

### 2) Substrate

Orthophenyldiamine (OPD) was dissolved in ethanol at 1%. Immediately before use, 0.6 ml of

OPD solution was added to 10 ml of 0.1 M phosphate buffer (pH 7.2) with 0.01%  $\text{H}_2\text{O}_2$ .

### 3) ELISA procedure

The ELISA was performed in 96-well, flat-bottomed polystyrene micro-ELISA plates. One hundred  $\mu\text{l}$  of the antigen, diluted with 0.1 M carbonate-bicarbonate buffer containing 0.01 M ethylenediamine-tetraacetate (CBB-EDTA, pH 9.6), was added to each well and the plates incubated in a moist chamber for 1 hr at  $37^{\circ}\text{C}$ . After coating, the antigen was discarded and 200  $\mu\text{l}$  of CBB-EDTA containing 1% BSA was added to each well, and the plate incubated for 1 hr at  $37^{\circ}\text{C}$  for

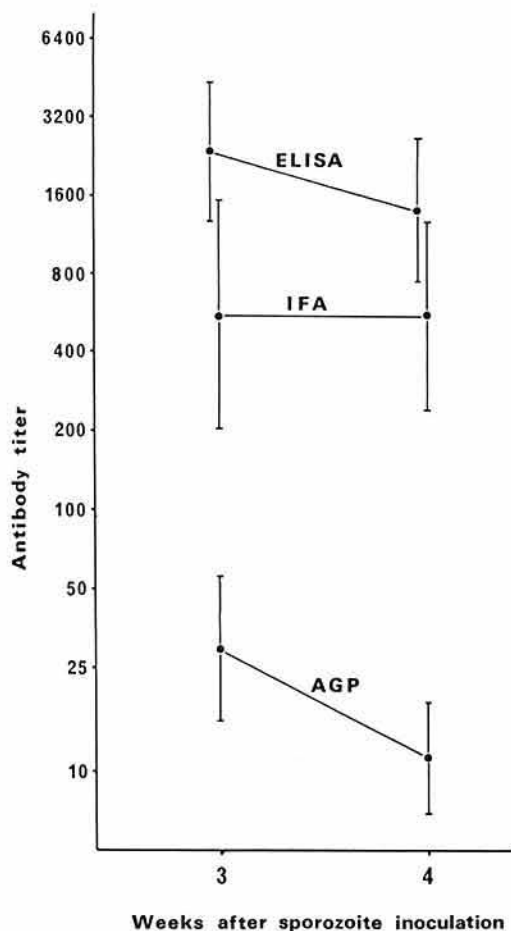


Fig. 2. Antibody titers measured by ELISA, AGP test and IFA test using sera collected from experimentally infected chickens with sporozoites of *L. caulleryi*

blocking the remaining adsorptive sites. After incubation, the plate was washed three times with PBS containing 0.05% Tween 20 (PBS-Tween). Then, 100  $\mu$ l of a serum sample diluted with PBS-Tween containing 0.25% BSA was added to each of antigen coated wells and incubated for 1 hr at 37°C in a moist chamber. The plate was then washed three times as before. One hundred  $\mu$ l of diluted conjugate was added to each well and incubated for 1 hr at 37°C in a moist chamber. After incubation, the plate was washed five times as before, and 100  $\mu$ l of the substrate was added to each well, and reacted for 30 min at 37°C in the

dark. The reaction was stopped by adding 100  $\mu$ l of 2.0 M  $H_2SO_4$  to each well. Absorbance reading was made at 492 nm with a microplate photometer.

### Specificity of the ELISA

Twelve SPF chickens were bled every 4 weeks from 0 to 40 weeks. Antisera against *Eimeria tenella*, *Mycoplasma gallisepticum*, *M. synoviae*, *Hemophilus paragallinarum*, Marek's disease virus, herpesvirus of turkey, infectious laryngotracheitis virus, infectious bronchitis virus, infectious bursal disease virus, avian adeno virus, avian reovirus, chicken anemia agent<sup>19)</sup>, avian nephritis virus<sup>7)</sup>, avian leukosis virus and egg drop syndrome 1976 virus were used for the specificity test. SPF chicken sera and antisera against various kinds of chicken disease agents showed no nonspecific reaction and no cross-reaction (Fig. 1).

### Sensitivity of the ELISA

The ELISA was compared with IFA and AGP tests using sera obtained from chickens experimentally infected with *L. caulleryi*. SPF chickens were inoculated with sporozoites intravenously and serum samples were collected from 3 and 4 weeks after inoculation. Fig. 2 shows that the ELISA is more sensitive than the AGP and IFA tests. The ELISA titers were approximately 100 to 400 times higher than those of AGP and about 3 to 10 times higher than those of IFA.

### ELISA for sera collected from experimentally infected chickens

ELISA IgM antibodies were detected in some sera on the day 10th after sporozoite inoculation (Fig. 3) and reached highest optical density (OD) values 3 weeks after inoculation (Fig. 4). After that, they declined suddenly and kept the low level until 10 weeks after inoculation (Fig. 4). Contrary to this, ELISA IgG antibodies to *L. caulleryi* were detected on the day 14th in some infected chicken sera (Fig. 3) and later, the OD value of them gradually went up. They reached high level 8 to 10

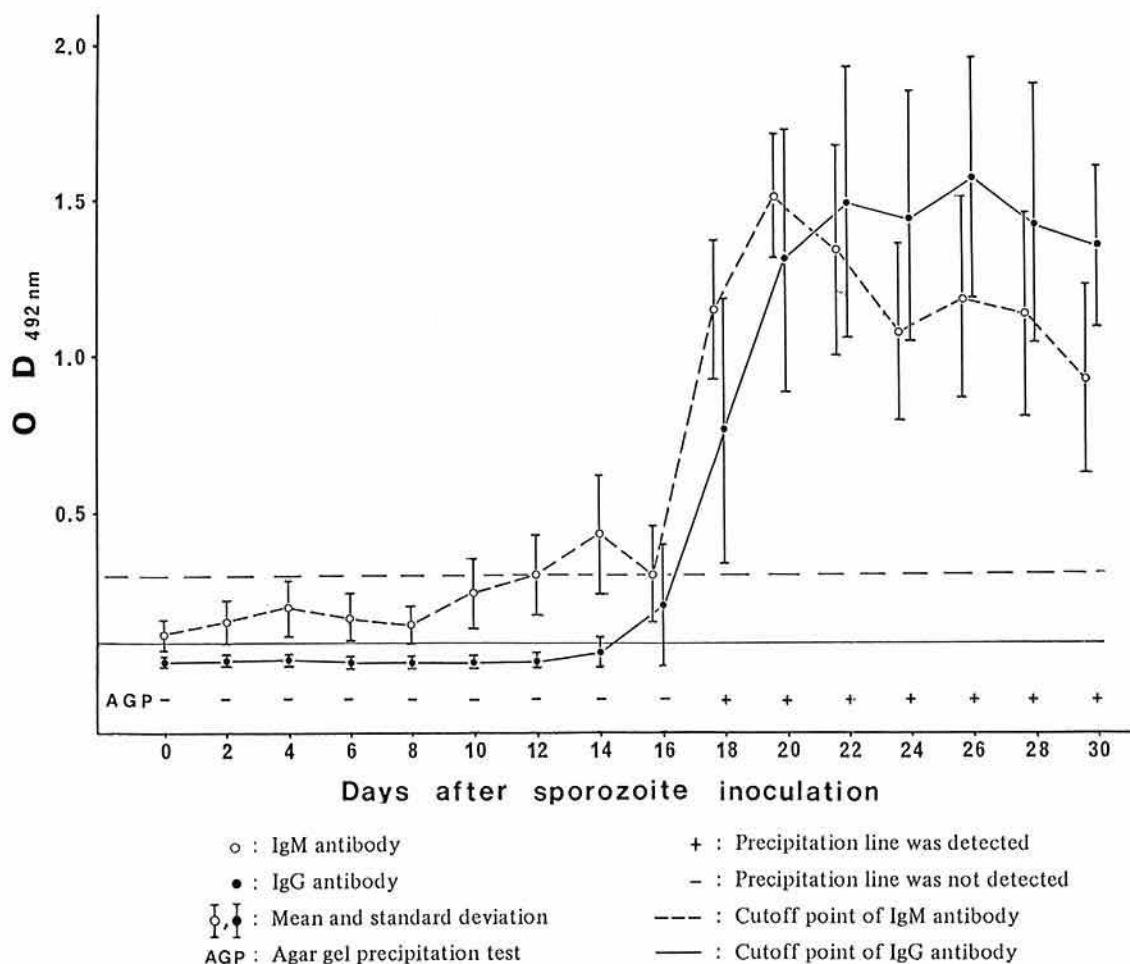


Fig. 3. Antibody response to *L. caulleryi* measured by ELISA in sera from experimentally infected chickens

weeks after sporozoite inoculation (Fig. 4). In AGP test, the antibody to *L. caulleryi* was detected from 17 days after sporozoite inoculation<sup>14)</sup> and the precipitin antibodies in the sera were of 7S class of immunoglobulin<sup>15)</sup>. From these results, the ELISA detects the antibody response to *L. caulleryi* earlier than AGP test and could diagnose the disease earlier.

### ELISA for sera collected from naturally infected chickens

ELISA antibodies were detected with/after detection of merozoites and/or gametocytes in

blood smears. After that, they persisted for nearly one year showing various levels (Fig. 5). Then ELISA antibodies were detected every time after their arising. On the other hand, AGP antibodies could not be detected sometimes. Chickens recovered from sporozoite infection with *L. caulleryi* show a strong resistance to reinfection with sporozoite<sup>13)</sup>. From these results, the infected chicken with *L. caulleryi* could be differentiated from the non-infected chicken by detection of antibodies using ELISA. Therefore, it would be easy to plan the prevention for chicken leucocytozoonosis in the flock against next epizootics.

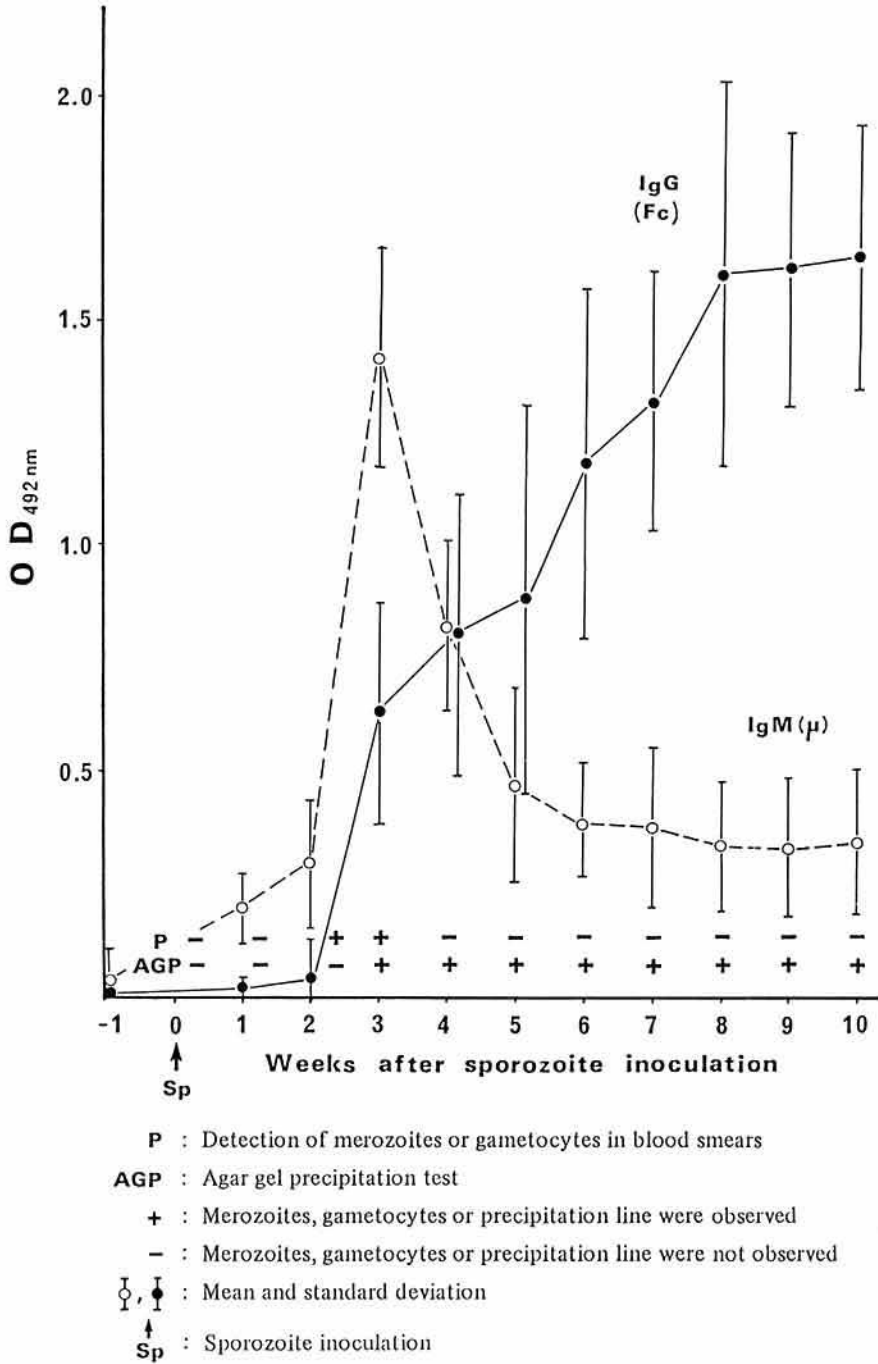


Fig. 4. Antibody response to *L. caulleryi* measured by ELISA in sera from experimentally infected chickens

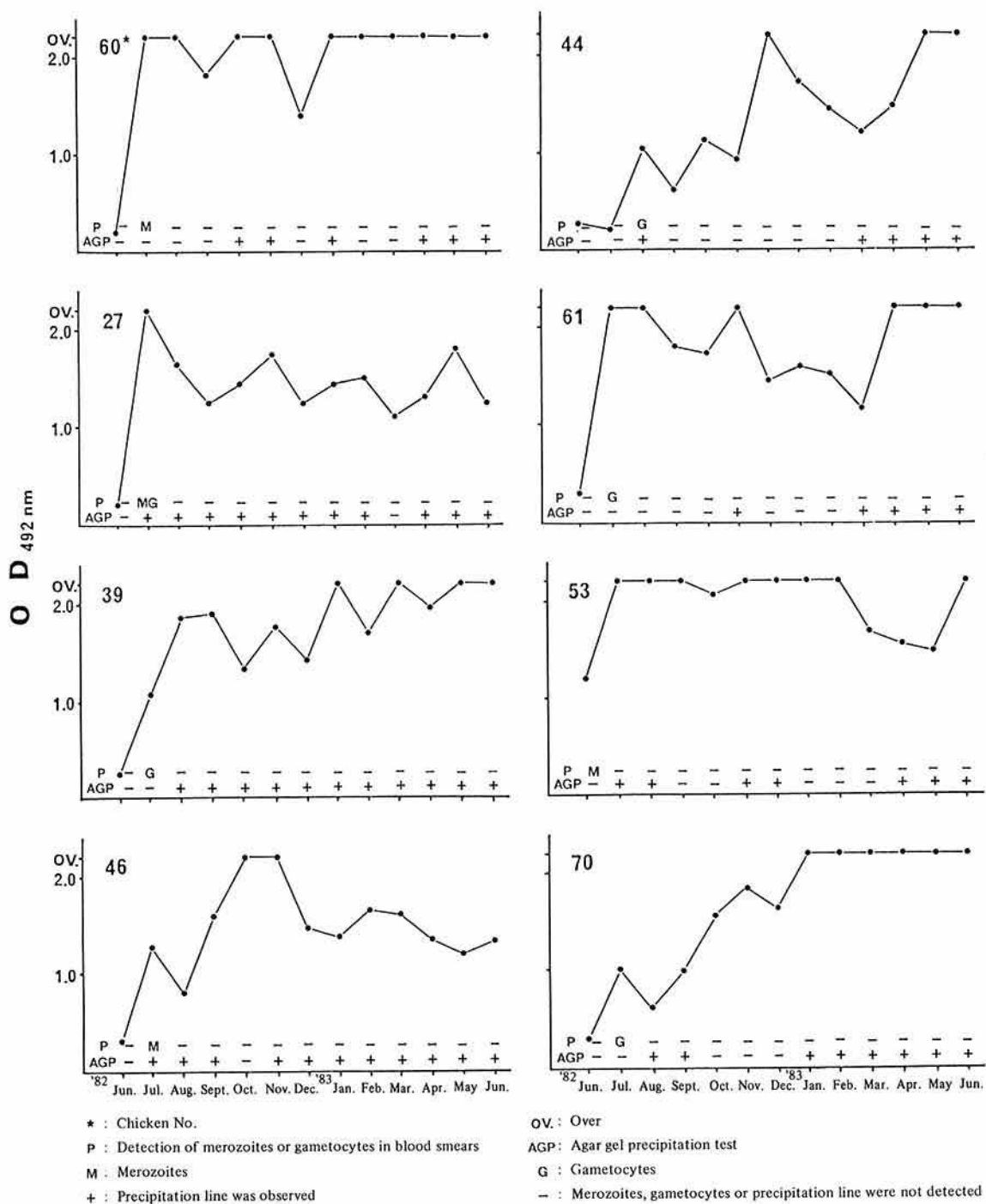


Fig. 5. ELISA antibody response to *L. caulleryi* in naturally infected chickens

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