

Theileria sergenti抗体検出用ELISAの抗原性状に関する検討

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Antigenic Properties of *Theileria sergenti* in ELISA Serodiagnosis

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Japanese bovine theileriosis is caused by intraerythrocytic parasitism of *Theileria sergenti* (*T. sergenti*), and the most difficult problem in bovine grazing land in Japan, so there is a great require for the easily, rapidly serodiagnostic method in the field survey.

Although gel precipitation test [9, 11], indirect fluorescent antibody (IFA) method [4-6, 8] complement fixation test [7] are devised for serodiagnosis of various piroplasmosis, these are not commonly used in the feald survey.

In this report we describe the properties and reactivity of antigens, prepared from purified *T. sergenti* for enzyme-linked immunosorbent assay (ELISA) [1, 10] serodiagnosis.

A sprenectomized calf, infected with the parasites was exsanguinated for approximately 30%

of it's erythrocytes under anesthesia. The blood was collected and defibrinated. Leucocytes were removed by CF-11 cellulose colum.

The erythrocytes were washed five times by centrifugation at 2,000 G, after successive suspensions in phosphate-buffered saline pH 7.2 (PBS).

Subsequently, the erythrocytes were suspended in PBS at 20% (v/v). And equal volume of 0.5% saponin in PBS was poured into erythrocyte suspension, mixing to hemolysis.

The erythrocyte ghosts were broken to separate the parasites from them by passing through into Millipore support screen for micro sylinge filter holder. The released protozoa were collected by centrifugation for 30 min at 4,000 G, and were washed three times with PBS. The purified protozoa had strong infectivity to a sprenectomized calf. These protozoa were freeze-and-thawed, and centrifuged for 60 min at 11,000 G. The supernatant fluids were collected for

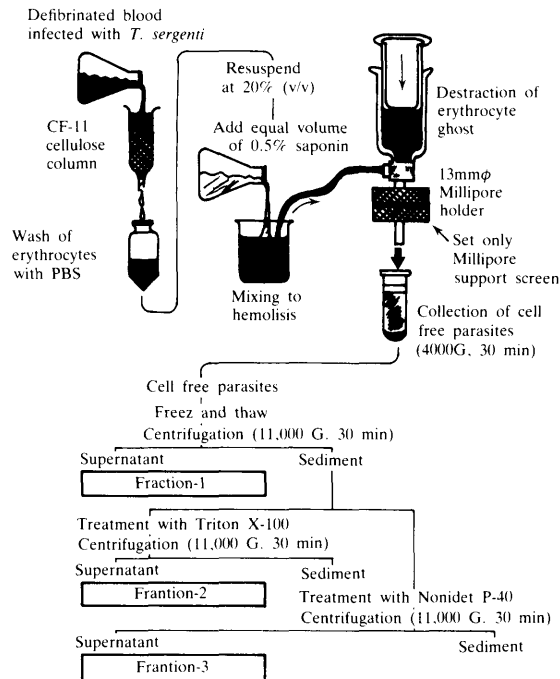


Fig. 1. Preparation method of cell free parasites, and three antigen fractions.

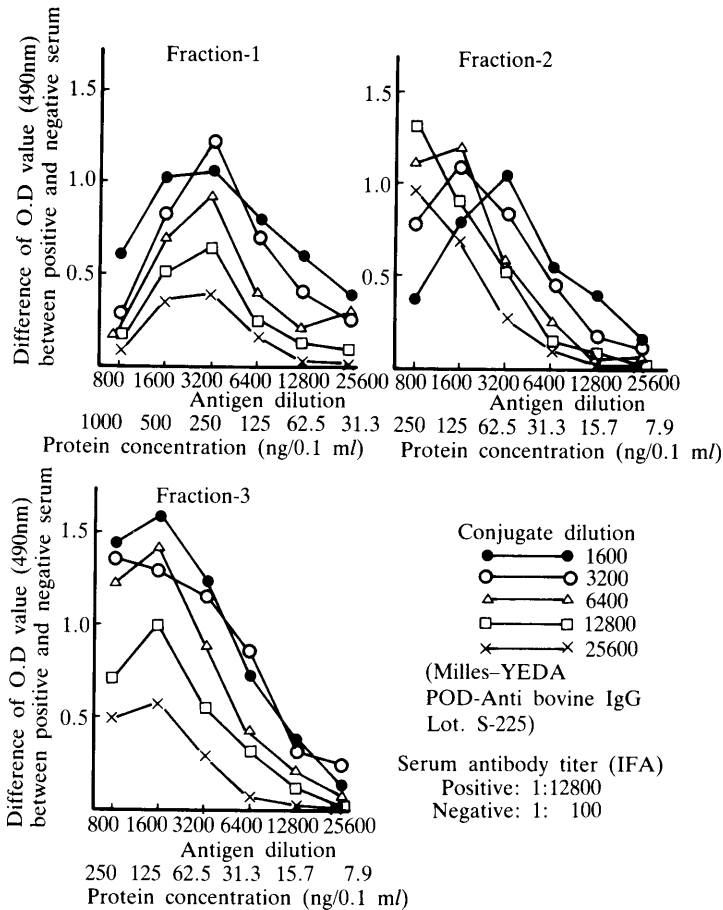


Fig. 2. Reactivity of each antigens in ELISA. These figures indicate the difference of optical density at wave length 490nm between positive and negative serum.

Fraction-1. In the next place, cell membrane sediment was washed with PBS, and suspended in 0.4% of Triton X-100 [Rohm & Haas] and Nonidet P-40 [Sigma].

After stirring for 16 hour at 4°C these suspensions were centrifuged for 60 min at 11,000 G. The supernatant fluid was collected for Fraction-2 and Fraction-3 respectively. These three fractions were used for ELISA antigens (Fig. 1). The modified method of Purnell [10] was used for this ELISA procedure.

Round-bottom 96-well micro ELISA plates (Immulon; Gleiner Inc.) were coated with 0.1 ml of antigen fractions in carbonate buffer (pH9.6).

After incubation, plates were washed 4 times with 0.02% of Tween-20 in PBS (pH7.4) (Tween-PBS).

Plates were then blocked with 0.25ml of Tween-PBS containing 0.3% of horse serum albumin (HSA) [Sigma, Fraction-V] for 16 hour at 4°C.

Plates were washed and 0.1 ml of bovine antiserum (IFA titer 1:12,800) or normal control serum (IFA titer < 1:100), diluted to 1/100 in Tween-PBS containing 0.02% of HSA were added. The plates were then incubated at 30°C for 1 hour. Washing was repeated 4 times and 0.1 ml of horseradish peroxidase conjugated anti bovine IgG [Miles-YEDA], diluted in Tween-PBS was added.

After 1 hour incubation at 30°C, washing was again repeated and 0.1 ml of substrate solution (o-phenyldiamine; 0.4 mg/ml in citrate buffer pH 5.2) was added and incubated at 30°C for 30

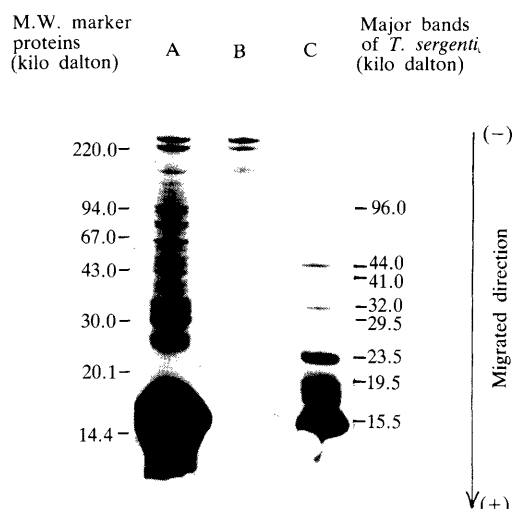


Fig. 3. Examination of *T. sergenti* whole cell purification. Lanes; (A), Normal bovine erythrocytes. (B), Erythrocyte ghosts, haemolized with saponin. (C), Purified *T. sergenti*.

min. Finally the enzymatic reaction was stopped by adding 0.1 ml of 2 M sulfuric acid, and measured for the absorbance at 490nm wavelength.

Fig.2 shows the difference of optical density (O. D.) between positive and negative serum by ELISA. Maximum difference of O. D. was observed using Fraction-3 at the condition of 125 ng protein/0.1 ml, showing 1.6.

On the other hand, Fraction-1 showed 1.2 at 250 ng/0.1ml, and then Fraction-2 showed 1.3 at the same condition as Fraction-1.

These antigens were analyzed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) in 4-30% of gradient gel slabs. Test samples were treated with 2.5% of SDS in sample-buffer (10 mM Tris-HCl; pH 8.0), for 16 hour at 25°C.

Ten μ l of each sample was loaded, and electrophoresis was continued for 4 hour at 150 volt. After the migration, the gel slab was stained with Coomassie brilliant blue R-250 (CBB) in 50% methanol and 7% acetic acid, and then destained electrophoretically in 10% acetic acid for 90 min.

The staining patterns in SDS-PAGE are shown in Fig. 3 and Fig. 4.

It was confirmed that erythrocyte elements contamination was negligible because normal bovine erythrocytes (Lane: A) and erythrocyte

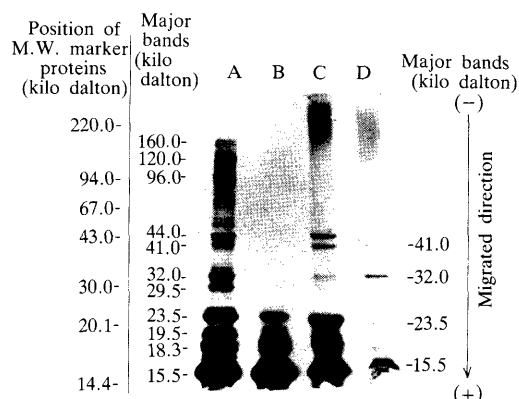


Fig. 4. Coomassie blue-stained SDS-PAGE of whole cells and each antigen fractions. Lanes: (A), purified *T. sergenti*. (B), Fraction-1. (C), Fraction-2. (D), Fraction-3.

ghosts (Lane: B) formed characteristic two bands around 220 kilo dalton (kd) in SDS-PAGE gel slab, whereas whole cells of *T. sergenti* (Lane: C) formed no band at the same position (Fig. 3).

Ten major bands (15.5, 18.3, 19.5, 23.5, 29.5, 32, 41, 44, 96 and 120 kd) were observed in whole cells of *T. sergenti*. On the other hand, 4 major bands (15.5, 18.3, 19.5 and 23.5 kd) were observed in Fraction-1, and 4 major bands (15.5, 23.5, 32 and 41 kd) in Fraction-3 (Fig. 4).

These antigens were analyzed using western blotting by the method of Bittner and others [2, 3, 12], using nitrocellulose membrane (0.2 μ m). The transfer took place at 12V (4 V/cm) overnight at 4°C. After the transfer to nitrocellulose, the sheet was blocked with Tris-buffered saline pH 7.2 (TBS) containing 0.3% of HSA. And then, sheet strips were reacted with γ -globulin fraction obtained from the antiserum of infected cattle (IFA titer 1:25,600) in TBS-HSA for a hour, washed two times in 0.02% Tween-TBS, and reacted with horseradish peroxidase conjugated anti-bovine IgG. Reacted membranes were washed three times as described above and treated with the substrate solution: 3,3'-diaminobenzidine in 0.05 M Tris buffer pH 7.6 and 0.04% of 30% hydrogen peroxide solution.

As a result of this western blotting analysis, seven major bands were observed at 23.5, 29.5, 32, 55, 64, 70 and 96 kd in whole cells of *T. sergenti*, and 23.5 kd in Fraction-1, 32 and 96 kd in Fraction-2, and 32, 64, and 96 kd in Fraction-3 (Fig. 5).

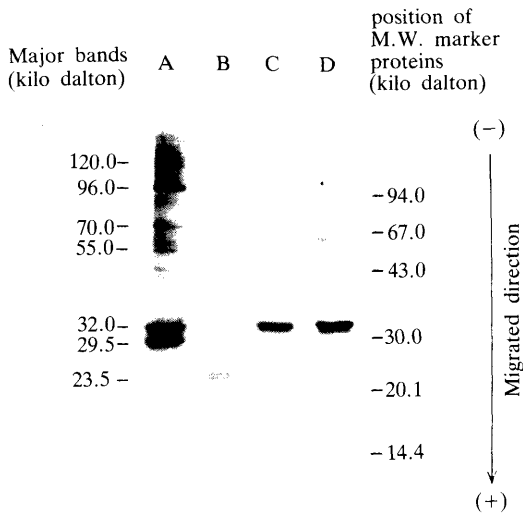


Fig. 5. Western blotting of whole cells and each antigen fractions. Lanes: (A), purified *T. sergenti*. (B), Fraction-1. (C), Fraction-2. (D), Fraction-3.

As described above, Fraction-3 extracted with Nonidet P-40 showed the most distinctive reactivity in ELISA among these three antigen fractions.

It is considered that Fraction-3 produces no antigenic competitive inhibition at the absorption to the wells of the ELISA plates, because the small amount of non-reactive low molecular weight components; less than 20 kd, are probably derived from the protoplasm in the protozoa. Especially the 15.5kd protein is considered to be hemoglobin, or hemozoin which is similar to malaria parasites components.

On the other hand, 15.5, 32, 64, and 96 kd proteins reacted in western blotting are considered to be from the cell membrane of protozoa.

要 約

Theileria sergenti 抗体検出用 ELISA の抗原性状に関する検討 (短報) : 扇谷年昭, 岡部達二, 佐々木文存, (株)微生物化学研究所) — 牛のタイレリア病の血清学的診断用に, 精製した *Theileria sergenti* から 3 種の ELISA 抗原を抽出し, 抗原性状を検討したところ ELISA に於て最も良好な反応性を示したのは Nonidet P-40 による可溶性抗原であった。抗体により認識される抗原を高度免疫牛血清との western blotting 法で, 解析したところ, 主たる抗原は分子量 32,000 の蛋白質であった。

As the reaction to 23.5, 64, and 96 kd proteins are minor, major antigen recognized by ELISA and western blotting is considered to be 32 kd protein.

CF, GP, and IFA method have been used previously for serodiagnosis [4-8]. These methods have some weakness in sensitivity and objectivity. So it seems that the ELISA using this antigen fraction will develop into one of the most useful method in serodiagnosis of the protozoan disease because of its high sensitivity, rapidity, and especially its excellent objectivity.

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