

Enzyme-linked immunosorbent assay(ELISA)法による equid herpesvirus type 1(EHV-1)に対する抗体の検出

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—NOTE—

Enzyme-Linked Immunosorbent Assay for the Detection of Antibodies to Equid Herpesvirus Type 1 (EHV-1)

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ABSTRACT. The enzyme-linked immunosorbent assay (ELISA) method was used to detect antibodies to equid herpesvirus type 1 (EHV-1) in horses. The antigen was a preparation obtained by solubilizing virus-infected cells with Nonidet P-40 (NP-40). The ELISA values were almost constant in the antigen protein concentration range of 5–1 $\mu\text{g}/\text{well}$ in the reaction with EHV-1 positive serum. The average ELISA value of the negative serum in 11 cases was 0.236. Changes in ELISA values and neutralizing antibody titers were compared using sera collected at intervals of about 1 week after inoculation of EHV-1 inactivated vaccine. The ELISA values and neutralizing antibody titers showed similar changes, but the detection sensitivity of ELISA was excellent in sera collected 1–2 weeks after inoculation. From these results, it might be possible to apply ELISA for the detection of EHV-1 antibodies.—**KEY WORDS:** ELISA, equid herpesvirus type 1, soluble antigen.

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For serological diagnosis and seroepidemiology of EHV-1 infections in horses, the complement fixation (CF) test, serum neutralization (SN) test, immunodiffusion (ID) test and immunofluorescence (IF) test have been used [3, 4, 13].

The ELISA shows a sensitivity similar to that of the radioimmunoassay (RIA), can be assayed of large numbers of serum samples in rapid and permits objective evaluations. Therefore, it is widely used in the field of veterinary medicine [1, 2, 7, 9, 10, 12]. There have been few reports on the application of ELISA to EHV-1, the only one being that of Dutta *et al.* [5]. They used virus particles prepared by centrifugation of frozen and thawed material of EHV-1 infected cell cultures for 3 hours at $60,000\times g$ as the ELISA antigen. However, a solubilized antigen should be adsorbed more efficiently on the plate and also be more stable than when the virus particles are adsorbed directly on the plate. Among the animal herpesviruses, satisfactory results were

obtained using soluble antigen prepared by treatment of virus infected cells by NP-40 in the ELISA test by Nanba *et al.* (Nanba, K., Kobayashi, K., and S Shimizu, Y. 1982. pp. 156, *In: Proc. 93rd Meeting of Japanese Society of Veterinary Science*, abstract.) in detection of antibody to suid herpesvirus type 1 and by Edwards *et al.* [6] to bovid herpesvirus type 4. The authors attempted to detect the EHV-1 antibody using soluble antigen prepared from EHV-1 infected cells as antigen for ELISA.

Soluble antigen for ELISA was obtained from infected cell cultures prepared by inoculating at $\text{moi} \approx 1$ of EHV (HH-1 strain) into BHK-21 cells, and incubated at 37°C . The culture fluid was discarded after about 17 hours of incubation, the cell surface was washed three times with phosphate buffered saline (PBS), a small amount of PBS was added and the cells were collected using a rubber policeman. After centrifugation at $1,000\times g$ for 10 minutes, RSB buffer (0.01 M NaCl, 0.0015 M MgCl_2 , 0.01 M

Tris-HCl buffer, pH 7.4) containing 0.2% NP-40 was added to the precipitate so that the cell concentration was adjusted about 2×10^7 cells/ml, and the mixture was gently shaken for 30 minutes at 4°C. The cytoplasmic fraction of the supernatant obtained by centrifuging the mixture at $1,000 \times g$ for 10 minutes was collected. It was then centrifuged at $77,000 \times g$ for 1 hour, and the supernatant obtained was dialyzed against PBS. Control antigen was prepared by treating BHK-21 cells in the same way but not inoculating the virus. The protein contents of these antigens were measured by Lowry's method [8] and they were stored at -80°C until use.

The ELISA was performed as follows. Virus antigen and control antigen diluted with carbonate buffer (0.05 M, pH 9.6) were placed in 96-well flat bottom Microelisa plates, 100 μ l in each well, and the plates were let stand at 4°C overnight. Plates were washed four times with washing solution (0.85% NaCl containing 0.02% Tween 20). Then 100 μ l of the test serum diluted 100-fold with diluent (10% calf serum, 0.05% Tween 20-PBS) was added, and then it was reacted for 1 hour at 37°C. The plates were then washed again, 100 μ l of horseradish peroxidase conjugated rabbit anti-horse IgG serum diluted to the optimal concentration with diluent was added to each well, and it was reacted for 30 minutes at 37°C. After the reaction, the plates were washed, 100 μ l of the substrate solution was added to each well and it was reacted at 25°C for 20 minutes in a dark room. The substrate solution was prepared by dissolving o-phenylenediamine dihydrochloride at a concentration of 0.4 mg/ml in 0.1 M citric acid - 0.2 M Na_2HPO_4 buffer (pH 4.8) and adding 0.2 μ l/ml of 30% H_2O_2 . After completion of the reaction, the reaction was stopped with 3N H_2SO_4 solution, and the absorbance was measured at a wave length of 492 nm. The ELISA value was obtained

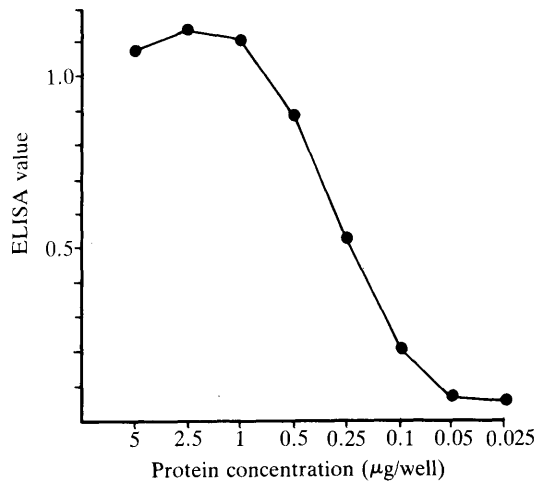


Fig. 1. Determination of optimal concentration of the ELISA antigen. ELISA value: The absorbance obtained with the control antigen was subtracted from the absorbance of the viral antigen.

by subtracting the absorbance of the control antigen from that of the virus antigen.

The SN test was performed by the microplate method. The test serum was diluted in two-fold steps and an equivalent amount of 200 TCID₅₀ of the virus (EHV, HH-1 strain) was added. After reacting at 37°C for 1 hour, the virus-serum mixture was inoculated into MDCK cells and incubated in an atmosphere of 5% CO₂ in air at 37°C for 5 days. The neutralizing antibody titer was expressed as the reciprocal of the maximum dilution of the serum which completely inhibited the appearance of cytopathic effect (CPE).

To decide the optimal concentration of ELISA antigen, the virus and control antigens were subjected to serial dilutions so that the protein concentrations ranged from 5 μ g to 0.025 μ g/well. The positive horse serum (SN titer; 1:32) immunized with EHV-1 inactivated vaccine, was diluted 100-fold and reacted. The results indicated that there was an almost constant reactivity in the protein concentration range of 5 μ g-1 μ g/well, and there was a sharp decrease in the ELISA values at concentra-

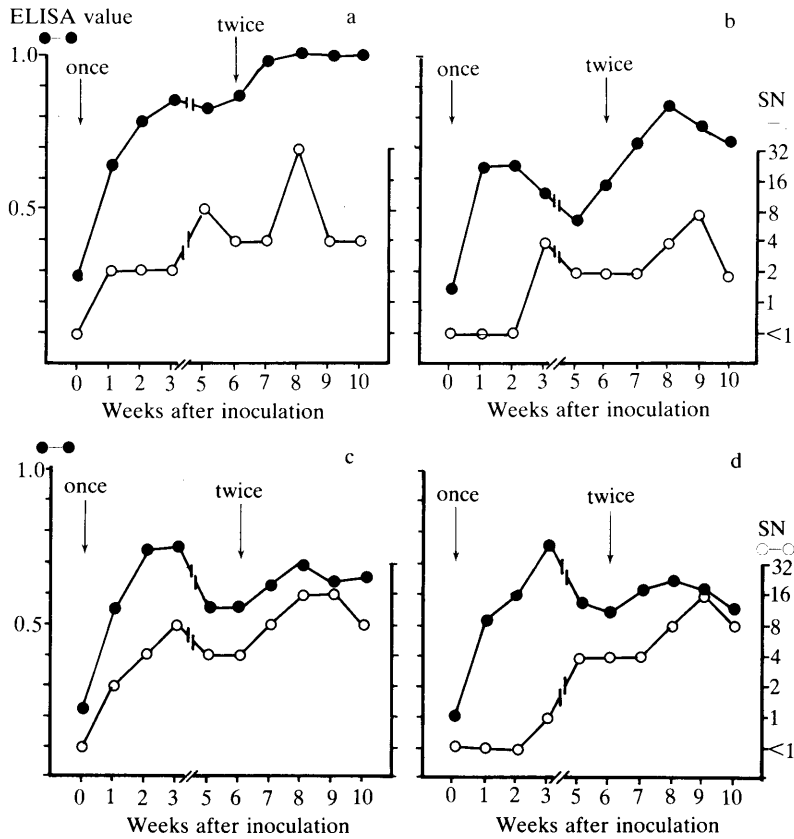


Fig. 2a, b, c and d. Comparative development of antibodies revealed by the serum neutralization test or by ELISA in the serum of a horse injected twice with the EHV-1 inactivated vaccine.

tions below $1 \mu\text{g}/\text{well}$ (Fig. 1). Therefore, the antigen protein was diluted to be $2.5 \mu\text{g}/\text{well}$ in the experiments.

The ELISA values of total of 11 sera collected from 11 horses with negative neutralizing antibodies against EHV-1 virus were determined in order to decide the normal range of ELISA values. The results showed that the average ELISA value was 0.236. In the future, it will be necessary to investigate the reactivity to more horse sera at different ages and which were inoculated tissue culture vaccine.

To investigate changes in antibody titers in ELISA and the SN test, four antibody negative horses were inoculated intramuscularly twice with EHV-1 inactivated vaccine

(HH-1 strain) and the sera collected at about 1 week intervals were used (these sera were kindly provided by Dr. T. Tokui of National Institute of Animal Health of Japan). As can be seen in Figs. 2a, b, c and d, the neutralizing antibody titers and ELISA values showed similar changes. However, there was always an increase in the ELISA value in the 1st week after the 1st inoculation, while there were no increases in the antibody titers of two horses in the SN test (Figs. 2b and d).

The ELISA test using the soluble antigen prepared from virus infected cells is considered to be useful in investigation for antibodies in large numbers of sera in the field. Furthermore, virus inactivation is not

necessary since the soluble antigen does not contain virus particles with infectivity because NP-40 was used in the process of antigen preparation. The detection sensitivity is the same as that of the SN test or even better for detection of the antibodies at an early stage. However, in the future, it will be necessary to study the relation between the SN test and ELISA values using naturally infected sera or sera collected periodically after experimental infections. It is considered especially necessary to investigate the relation with the SN test in the presence of complement [11] using sera collected in the early stage of infection.

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要 約

Enzyme-linked immunosorbent assay (ELISA) 法による equid herpesvirus type 1 (EHV-1) に対する抗体の検出 (短報): 宝達 勉・栄木隆郎・井出誠弥・山岸郭郎 (北里研究所家畜衛生研究所)——ウイルス感染細胞可溶性抗原を用いた ELISA 法により EHV-1 に対するウマの抗体検出を試みた。陽性血清との反応で、抗原タンパク濃度 5 μg ~1 μg /well で ELISA 値はほぼ一定値を示した。EHV-1 不活化ワクチン接種後、経過的に採取した血清で ELISA 値と中和抗体価の推移を比較した。ELISA 値と中和抗体価は、ほぼ類似した推移を示したが、接種後、1~2 週後の血清では ELISA 法の検出感度が優れていた。