酵素抗体法による鶏伝染性気管支炎ウイルス抗原の検出

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Enzyme-Linked Immunosorbent Assay for the Detection of Infectious Bronchitis Virus Antigens

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ABSTRACT. Enzyme-linked immunosorbent assay (ELISA) to detection of infectious bronchitis virus (IBV) antigen was applied. IBV M41, A-5968 and L_2 strains showing strain specificity in virus neutralizing test showed marked cross-reactivity. IBV M41 antigen was detectable from both SPF eggs and chickens inoculated with the virus. Between the positive (purified IBV and infectious allantoic fluid) and negative controls (Newcastle disease virus B1 strain and allantoic fluid from SPF eggs), there were marked differences in ELISA titers. From these results, ELISA was considered to be a speedy and good technic for detecting IBV antigens from test samples infected with the virus experimentally.—KEY WORDS: ELISA, IBV.

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The virus isolation is a reliable diagnostic method for infectious bronchitis virus (IBV) infection. Isolation of IBV is most frequently accomplished by inoculating embryonated chicken eggs. The presence of the virus in the embryonating egg following inoculation is difficult to discern in the initial passage since there is little change in the majority of the inoculated embryos. The sample is necessary to passage several times in eggs. Therefore, easy and speedy methods for detection of IBV antigen from field samples is required.

Enzyme-linked immunosorbent assay (ELISA) is a speedy method for detection of antigen or antibody in general [16]. In additon to the virus neutralizing test [10, 11], the haemagglultination inhibition test [1, 2] and the double immunodiffusion test [12, 17], the ELISA has been employed for detection of IBV antibody [7, 8, 14, 15, 16]. However, the ELISA has not been employed for detection of the IBV antigen. therefore, the basically studies are required to carry out by employing several egg-passaged strains of IBV for the establishment of the IBV antigen detection by the ELISA.

MATERIALS AND METHODS

Virus: IBV M41 strain (Massachusetts type), A-5968 strain and L_2 strain (Connecticut type) [6, 13] and NDV B1 strain were used. These viruses were propagated in the allantoic cavity of 10-day-old eggs. The eggs incubated for 48 hr at 37°C and then chilled. The allantoic fluid was harvested from the eggs and clarified by centrifugation at 5,000 rpm for 30 min at 4°C.

Purification of virus: This was carried out according to Snyder *et al.* [15] with a slight modification. Infectious allantoic fluid was centrifuged at 4°C for 1 hr at 64,300×g in a Hitachi RP70T rotor. The virus was then resuspended in PSB (pH7.4). Next, virus suspension was layered over a discontinous gradient composed of 20%, 30% and 55% (wt/wt) sucrose in saline. The gradient was centrifuged at 4°C for 4 hr at 134, 500×g in a Hitachi RPS27–3 rotor. Virus band was resuspended in PBS. The virus was then sedimented at 73,000×g for 1 hr in a Hitachi RPS27–3 rotor and resuspended to about

0.5 m*l* of PBS.

Anti-IBV chicken serum preparation: Antiserum to IBV M41 strain was prepared in 30-day-old SPF chickens (Line M, Nisseiken Co. Ltd., Japan). Each chicken received intratracheally 0.1 m*l* of infectious allantoic fluid containing about $10^{6.0}EID_{50}$ of IBV M41 strain twice at intervals of two weeks. All chickens were bled out at two weeks post-inoculation, and antisera were pooled.

Anti-IBV rabbit serum preparation: A rabbit received of inactivated purified IBV M41 strain (50 μg protein) with Freund's complete adjuvant (Miles Lab. Inc., USA) intramuscularly twice at intervals of three weeks. Two weeks post-inoculation, the inactivated purified virus was injected intravenously twice at intervals of four weeks. The rabbit was bled ten days post-inoculation, and natiserum was made.

Preparation of IgG: Rabbit serum was inactivated at 56°C for 30 min and absorbed with packed chicken red blood cells for three times. The rabbit and chicken sera were fractioned with DEAE-cellulose (Seikagakukogyou Co. Ltd., Japan) and Sephadex G-200 (Pharmacia, Sweden) [4, 5] columns.

The conjugation method for peroxidase: This was done according to one-step glutaraldehyde method [16]. For peroxidase conjugations, anti-M41 rabbit IgG, anti-M41 chicken IgG and rabbit anti-chicken IgG (Miles Lab. Inc., USA) were employed.

ELISA method: This was done according to Voller *et al.* [16] with a slight modification. The anti-M41 IgG was diluted with coating buffer (pH9.6). The IgG was added to wells on a ELISA plate (Sankoujunyaku Co. Ltd., Japan) in 0.2 m*l* amount. The plate was incubated for overnight at 4°C and washed with PBS-Tween (0.05% tween 80, pH7.4). BAS-PBS (1% BSA) was added to wells on a plate in about 0.3m*l* amount. The plate was incubated for 4 hr at room temperature, and washed with PBS-Tween three times and then employed in the test.

Test samples were added NP-40 (Sigma, USA) for a final concentration of 1% and incubated for 2 hr at room temparature. These samples were diluted with BSA-PBS-Tween (1% BSA, 0.02% Tween 80, pH7.4) (BPT) ten times. Two tenth of each sample dilution was added to a well on a plate. The assays were then done at room temparature, after which the plates were washed with PBS-Tween. Following addition of 0.2 m*l* of anti-M41 conjugate diluted to 1:1000 in BPT to a well on a plate, the plates were incubated again at room temperature, and then washed as before, Two tenth of o-phenylene diamine solution (40 mg/100 m*l*) was added to a well of each plate. The plate contents were incubated for 30 min at room temperature. Finally, 0.05 m*l* of 2.5M H_2SO_4 was added to a well. The absorbance of each wells was measured at 490 nm and 620 nm in a E-J reader (Sankoujunyaku Co. Ltd., Japan). The reading absorbances were employed for the ELISA titer.

Determination of optimal test conditions for ELISA.: For coating solid phase and for peroxidase labeling, antibodies from two animal species were investigated. Four pairs of antibodies, 1) Chicken-Rabbit (antibody for coating solid phase-antibody for peroxidase labeling), 2) Rabbit-Chicken, 3) Chicken-Chicken and 4) Rabbit-Rabbit, were tested. For positive control and for negative control, infectious allantoic fluid from SPF eggs infected with M41 strain and normal allantoic fluid from SPF eggs were employed, respectively.

For determination of optimal incubation times, the changes of the ELISA titers were examined at the laps of both the sample incubation times and the conjugate incubation times (5, 10, 30, 60, 120 and 180 min).

Specificity test: Both virus and IBV strain specificitities were investigated. For positive control, purified IBV M41 and A-5968

Table 1. Combination of the antibodies for coating solid phase and for enzyme rabeling.

Test No.	Species of Animal		Positive control	Negative control	
	Antibody for coating solid phase	Antibody for enzyme rabeling	AF[a] (M41 strain infected)	AF No virus infected)	PBS
1	Chicken	Rabbit	+++[b]	+	−
2	Rabbit	Chicken	+++	++	−
3	Chicken	Chicken	+++	+++	+++
4	Rabbit	Rabbit	+++	++	−

a) Allantoic fluid.
b) Value of ELISA titer against control/ELISA titer against M41 strain. +++: ≧1, ++: 1~3/4, +: 3/4~1/2, −: 1/2~1/4, −: ≦1/4.

strains, and allantoic fluid from SPF eggs infected with IBV M41 or L_2 strain were used. For negative control, allantoic fluid from SPF eggs infected with NDV B1 strain, normal allantoic fluid from SPF eggs, and rabbit anti-M41 sera absorbed with chicken red blood cells were employed.

Virus recovery test: The $10^{1.8}EID_{50}$ of IBV M41 strain was inoculated into the allantoic cavity of SPF eggs and into SPF chickens intranasally. The samples were harvested at the laps of the times. From twenty five embryonating eggs, the allantoic fluids were harvested at 6 hr, 1day, 2days (d), 3d and 4d after virus inoculation. From fourteen chicken, the respiratory tracts were harvested at 6 hr, 1day, 2d, 3d, 4d, 5d and 6d after virus inoculation. These respiratory tracts were homogenized with 10 m*l* PBS (1% BSA, 100U penicillin, 1 mg streptomycin, 1 mg kanamycin, pH7.4) per 1g sample. These sample were assayed by both the ELISA and the virus titration in embryonating eggs.

Polyacrylamide gel electrophoresis (PAGE): This was done according to Cavanagh *et al.* [3]. Purified IBV M41 strain was disrupted with 1% sodium dodecylsulphate (SDS) and 1% 2-mercaptoethanol for 6 hr at room temperature. For protein molecular weight (M. W.) maker, protein test mixture 4 (SERVA, USA) (M. W. 92,500, 68,000, 45,000 and 29,000 dalton) was used.

The disrupted virus samples and M. W. marker were layered on a polyacrylamide slab gel containing 10% acrylamide and electrophoresed with 9mA for 16 hr.

Electrophoretic transfer: This was done according to Ogata *et al.* [9]. The transfer buffer consisted of 25mM Tris, 95nM glycine and 15% (V/V) methanol at pH8.3. A vortage gradient of 10 v/cm was applied for 6 hr. For substrate, 3–3 diaminobenchidin HC1 salt was employed.

RESULTS

Determination of optimal antibodies for coating solid phase and for enzyme labeling: The differences of ELISA titers between positive control and negative controls were marked in 1) and 2) tests (Table 1), In 3) test, PBS showed high ELISA titers which were similar to those of positive control. In 4) test, allantoic fluids from SPF eggs showed higher ELISA titers than those in 1) or 2) test. Therefore, chicken IgG was employed for coating solid phase, and rabbit IgG was employed for enzyme labeling.

Determination of optimal incubation times for ELISA: Over 60 min incubation times at room temperature, ELISA titers were

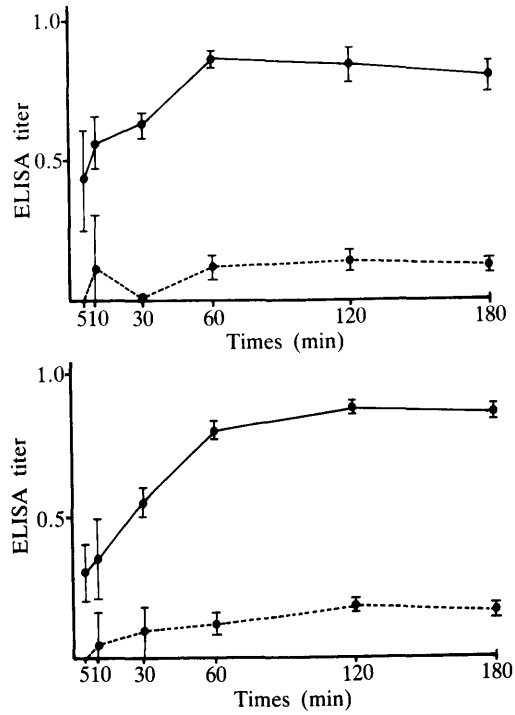

Fig. 1. Changes of ELISA titers at the laps of the reaction times. Upper graph shows the changes of titers at the laps of the sample incubation times. Lower graph shows the changes of titers at the laps of the conjugate incubation times. The solid line shows the ELISA titers of the normal allantoic fluid from SPF eggs.

stable in both the sample incubation times and the conjugate incubation times (Fig. 1). Therefore, sample incubation times of 2 hr and conjugate incubation times of 2 hr at room temparature were employed.

Virus specificity and strain specificity test: The ELISA showed the marked differences in the titers between the IBV positive and negative samples (NDV B1 strain) (Fig. 2). Both purified IBV M41 strain and A-5968 strain showed same ELISA titers at the same protein content (8 μg). Allantoic fluid from SPF eggs infected with IBV M41 strain or L_2 strain, showed the same virus titer ($10^{7.0}EID_{50}$) as well as the same ELISA titers. These positive reactions were abolished by the absorption with rabbit anti-M41 hyperimmune sera in the ELISA (Fig. 2).

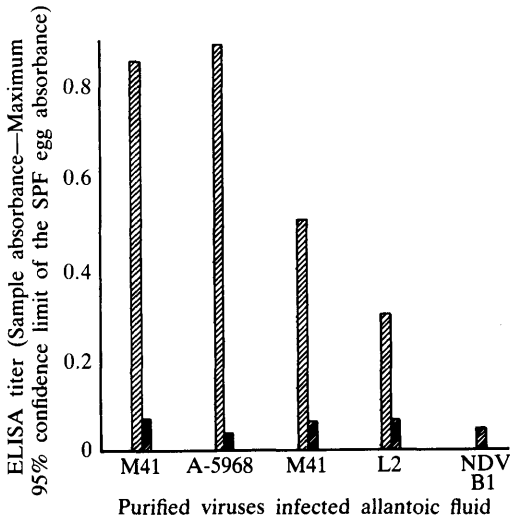

Fig.2. ELISA titers of purified virus, virus-infected allantoic fluids and antibody-absorbed virus samples. ELISA titer=sample absrobance −0.52 (Maximum 95% confidence limit of the SPF egg absorbance which was culculated from 200 SPF eggs absorbance). ▨:Virus sample titer ▩:Titers of virus samples absorbed with rabbit anti M41 serum.

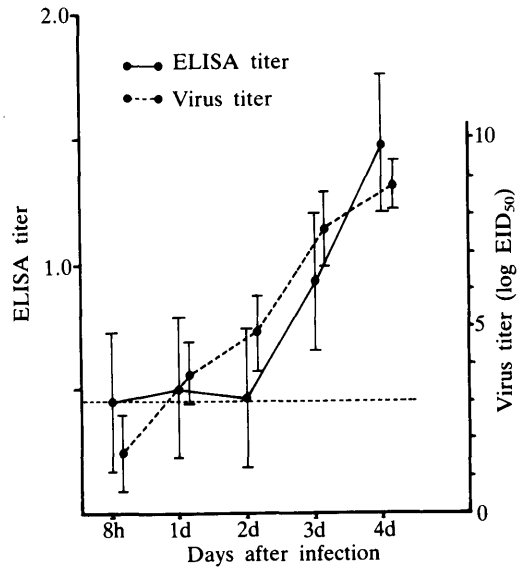

Fig. 3. Virus recovery from SPF eggs infected with IBV M41 strain. Longitudinal solid lines show the 95% confidence limits of titers. A straight dotted line shows the maximum 95% confidence limit of ELISA titer of allantoic fluids from twenty-five SPF eggs.

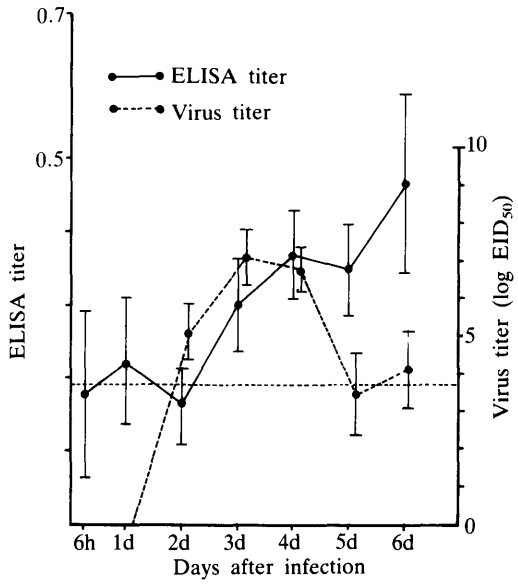

Fig. 4. Virus recovery from chickens infected with IBV M41 strain. Longitudinal solid lines show the 95% confidence limits of titers. A straight dotted line shows the maximum 95% confidence limit of ELISA titer of the respiratory tract emulsion from fourteen SPF chickens.

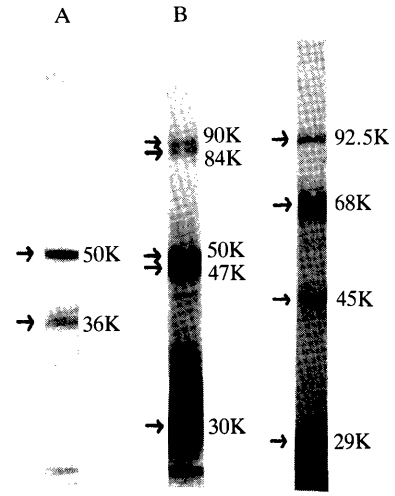

Fig. 5. Detection of the component protein of IBV M41 strain by rabbit (A) and chicken (B) IgG. Virus was distrupted with 1%SDS-1%2ME at room temparature for 6 hr. The rabbit IgG were labeled with peroxidase. For detection of the chicken IgG, peroxidase labeled rabbit anti-chicken IgG was employed.

Normal allantoic fluid from 200 SPF eggs from 4 company was tested. Average ELISA titer from three of four company was about 0.4. However, a few allantoic fluids from one company had high ELISA titers (over 1.5).

Virus recovery test: Although the IB virus was recovered from embryonating eggs as early as one day after virus inoculation, the ELISA titers of the IBV infected allantoic fluid were found at three days after virus inoculation.

The IB virus was also recovered from chickens from two days after virus inoculation (Fig. 4). The titer reached to a peak at three days after virus inoculation and reduced there after. The ELISA titers of the emulsions of the resperatory tracts were raised from three days after virus inoculation and increased there after.

Detection of virus component proteins by antibodies for coating solid phase and for emzyme labeling: Chicken anti-M41 IgG used for coating solid phase detected M. W. 90,000 (90K), 84K, 50K, 47K and 30K dalton protein of IBV M41 strain (Fig. 5. B). Rabbit anti-M41 IgG used for enzyme labeling detected M. W. 50K and 36K dalton protein of IBV M41 strain (Fig. 5. A). Only 50K dalton protein was detected by both IgG for coating and for labeling.

DISCUSSION

The result showed that the ELISA was a good method for detection of IBV from test samples infected with the virus experimentally.

In the ELISA for detecting the IBV antigen, the combination of IgG may be important (Table 1). By employing IgG from different animals for coating and for labeling, the ELISA showed a good result in 1) test. In 4) test, a nonspecific reaction was found in normal allantoic fluid. This nonspecific reaction may be due to the reaction of the rabbit anti-M41 IgG with some

proteins in normal allantoic fluid. In 3) test, nonspecific reaction was found in both normal allantoic fluid and PBS. When the plate without coating chicken IgG was employed, no nonspecific reaction was seen in PBS. From the result, the nonspecific reaction in 3) test may be due to the results of chicken IgG for coating and for labeling being fixed together.

In this study, only 50K virus protein was detected by both IgG used for coating and for labeling (Fig. 5). The 50K virus protein is nucreoprotein (NP) [13], and the ELISA detect only NP antigen.

M41 strain and A-5968 strain or L2 strain showed very low cross-reaction in virus neutrarizing test [10, 11]. But, these strains were showed same ELISA titers in purified virus and in infected allantoic fluid at the same virus content (Fig. 2). From these result, IBV NP may be a common antigen of IBV.

The ELISA was able to detect the IBV antigen from respiratory tracts of chickens inoculated with IBV and to detect the antigen for a long terms compared with the virus recovery test using embryonating eggs. In virus inoculation test, virus titer of chicken respiratory tract showed the peak at three days after inoculation and decreased there after. However, the ELISA titers increased continuously from three days after inoculation. By electron microscopes, more virus particles were seen in the respiratory tract examined in seven day than those in three days after inoculation (Ohta. unpublished data), therefore, the ELISA may be detecting these viruses.

The sensitivity of the ELISA was inferior to that of virus recovery test using embryonating eggs. The virus was recovered as early as eight hour after virus inoculation from virus-inoculated embryos and two days from virus-inoculated chickens. But, the virus was detected from three days after virus inoculation by the ELISA. The limit of virus titer detectable by ELISA was culculated to be about $10^{6.0}EID_{50}$.

In this study, the sensitivity of ELISA is not still satisfaction. We now established several monoclonal antibodies against IBV M41 strain. For increasing the sensitivity of ELISA, a further study is in progress by using these antibodies instead of IgG from-chicken or rabbit.

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要　　約

酵素抗体法による鶏伝染性気管支炎ウイルス抗原の検出：野牛一弘・太田修一（農林水産省動物医薬品検査所）——鶏伝染性気管支炎ウイルス抗原のELISAによる検出の確立を図るために，発育鶏卵継代株を用いて基礎検討を行い，以下の成績を得た。ウイルス中和法で株特異性が強く認められたM41株とA—5968株及びL_2株の間で高い交差反応性が認められた。$10^{1.8}$ EID^{50}のM41株を接種された発育鶏卵および鶏から採取した尿膜腔液および気管乳剤から，ウイルス感染価が$10^{6.0}$ EID_{50}以上になった接種3日目から，ELISAにより抗原が検出された。陰性の対照のNDVB1株感染尿腔液及びSPF卵尿腔液の反応は微弱で，陽性対照に比して明らかな差が認められた。以上の成績から，本法はIBV実験感染材料からIBVを分離確認する際の正確かつ迅速な手段であるものと考えられた。