

# Enzyme-linked immunosorbent assay(ELISA)法による鶏 伝染性気管支炎ウイルス各株間の交差反応性

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## Cross-Reactivity among Infectious Bronchitis Viruses in Enzyme-Linked Immunosorbent Assay

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**ABSTRACT.** Enzyme-linked immunosorbent assay (ELISA) using B42 strain of infectious bronchitis virus (IBV) as an antigen was developed for detection of antibodies against IBV. Purified IBV solubilized with Triton X-100 served as the antigen. Although there was little cross-reactivity in the serum neutralization (SN) test, cross-reactivity was observed among IBV strains B42, Gray, and A5968 (Massachusetts, Delaware, and Connecticut serotypes, respectively). In addition, B42 strain reacted also with antisera against three other serotype strains (Iowa97, Iowa609, and M41) and those against six strains isolated in Japan (distinguishable from each other by the SN test) in ELISA. Thus a wide range of cross-reaction was observed with B42 strain of IBV. Corrected ELISA (cELISA) values of serum samples of chickens naturally infected with IBV in the field did not correlate with their SN titers. Those of chickens experimentally infected with a single strain of IBV (B42), however, increased with the rise of the SN titer. Therefore, our ELISA system seems to show an IBV-specific reaction. These results suggest that antibodies to several subtypes of IBV can be detected with only one type of virus as an ELISA antigen. The ELISA technique appears to be suitable for large-scale serological surveys and rapid and simple to perform.—**KEY WORDS:** crossreactivity, ELISA, IBV.

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Avian infectious bronchitis virus (IBV) is responsible for a highly contagious aerosol-transmitted acute respiratory disease affecting chickens of all ages [7, 14]. Morbidity is high among chickens of all ages, but high mortality is seen only among chicks less than 6 weeks of age [7]. The decreased feed consumption resulting in reduction in weight gain among broilers and decreased production among laying hens during infection is a major concern of the poultry industry.

Avian infectious bronchitis is diagnosed usually by clinical examinations, virus isolation, and demonstration of the antibody in the serum of birds recovered from disease. Confirmation of an outbreak by virus isolation and the serum neutralization (SN) test is occasionally perplexed by the existence of several subtypes of IBV [6–9, 11, 12, 20]. Use of a single antiserum leads to equivocal

or negative results if the virus isolate is not of a related type. Likewise, use of a single IBV strain for testing serum samples from convalescent birds as confirmation of a suspected IBV outbreak can lead to ambiguous results.

Enzyme-linked immunosorbent assay (ELISA) has widely been used to study antigen-antibody reactions in avian virology [18]. In this paper, we report ELISA for detecting circulating antibodies against several subtypes of IBV. ELISA we developed is highly sensitive, requires only small amounts of reagents and serum, and permits rapid testing of a large number of samples.

### MATERIALS AND METHODS

*Viruses:* The IBV strains used were B42, M41 (Massachusetts serotype), Gray (Delaware serotype), and Iowa97 (Iowa serotype).

ware serotype), A5968 (Connecticut serotype), Iowa97 (Iowa97 serotype), Iowa609 (Iowa609 serotype), and six strains isolated in Japan (designated ON, KH, S4W, 0353, Takeshima, and Shizuoka). All the strains were kindly supplied by Dr. Hitoshi Kawamura, National Institute of Animal Health. The viruses were grown in the allantoic cavity of 12-day-old embryonated specific-pathogen-free (SPF) chicken eggs. Allantoic fluid (AF) was harvested after incubation at 37°C for 18 hr, clarified by centrifugation at 9,300 xg for 30 min and stored at -20°C.

**Cell culture:** Primary monolayer cultures of chicken embryo kidney (CEK) cells were prepared from the kidneys of 18-day-old chicken embryos by dispersing with 0.1% trypsin. CEK cells were grown in Eagle's minimum essential medium (MEM) containing 10% tryptose phosphate broth (TPB), 5% calf serum and 0.2% NaHCO<sub>3</sub>. They were maintained in MEM containing 10% TPB. They were prepared in flat-bottomed microplates by seeding each with 0.1 ml of growth medium containing 0.2% cells and incubating in an atmosphere of 5% CO<sub>2</sub> in air at 37°C for 48 hr.

**Antisera:** Specific antisera against each IBV strain were prepared in 50-day-old SPF chickens by giving intranasally and intraocularly doses of 10<sup>7</sup> to 10<sup>8</sup> TCID<sub>50</sub> of IBV at 3-week intervals. Serum was obtained 7 days after the last administration.

**Serum samples from field chickens:** Serum samples in the field were collected from chickens suspected of IBV infection. The sera were inactivated at 56°C for 30 min and stored at -20°C until use.

**Serum neutralization (SN) test:** This was carried out by the serum dilution method using CEK cell cultures prepared in flat-bottomed microplates. In a well of a plate, 0.025 ml of each of serial twofold dilutions in a diluent (MEM containing 10% TPB) of the serum inactivated at 56°C for 30 min was mixed with 0.025 ml of the diluent contain-

ing 200 TCID<sub>50</sub> of the virus. Two or three wells were used per serum dilution. The virus-serum mixtures were incubated at 37°C for 60 min and transferred to wells of microplates containing CEK cell monolayers. The plates were incubated in an atmosphere of 5% CO<sub>2</sub> in air at 37°C for 7 days. The antibody titer was expressed as the reciprocal of the highest serum dilution showing 50% neutralization of the virus calculated by the method of Reed and Muench [17].

**Preparation of ELISA antigen:** After thawing and centrifuging AF, zinc acetate was added to a final concentration of 25 mM. After neutralization by adding 1 N NaOH, AF was stirred at 4°C for 30 min and then centrifuged at 9,300 xg for 30 min. The precipitate was dissolved in saturated ethylenediaminetetraacetic acid (EDTA) buffer. This solution was centrifuged at 9,300 xg for 20 min and the supernatant layered onto a 30% and 60% sucrose cushion, which was centrifuged at 70,000 xg for 2 hr in a Beckman SW 25.1 rotor (Beckman Instruments, Fullerton, California) at 4°C. The band between the 30% and 60% sucrose layers was collected and diluted with NTE buffer (100 mM NaCl, 10 mM Tris-HCl, and 1 mM EDTA, pH 6.8). The diluted virus was centrifuged at 100,000 xg for 60 min in a Beckman SW 28 rotor at 4°C to remove sucrose. The virus pellet was suspended in a small amount of NTE buffer, solubilized with 0.1% Triton X-100 (Eastman Kodak Co., Rochester, New York) and dialyzed against phosphate buffered saline (PBS; 100 mM NaCl, 10 mM phosphate buffer, pH 7.2). Protein concentration of the dialysate was determined by the method of Lowry *et al.* [15].

Normal AF from non-infected SPF eggs was directly concentrated by centrifugation at 60,000 xg for 3 hr in a Beckman Type 21 rotor at 4°C. The pellet was suspended in NTE buffer, solubilized in the same way as

above, and coated on an ELISA plate as a negative antigen.

**Conjugate and substrate for ELISA:** The conjugate used was horseradish peroxidase (HP)-conjugated immunoglobulin G (IgG) fraction, rabbit anti-chicken IgG, heavy and light chains (Lot 50318, Zymed Labs, San Francisco, California). The optimal dilution of the conjugate was determined so as to give an ELISA value of 1.00 (see below) with positive control serum (PCS, SN titer 1:90). In our ELISA system, the conjugate dilution was  $1.5 \times 10^{-4}$  in a dilution buffer (0.13 M NaCl, 9 mM  $\text{Na}_2\text{HPO}_4$ , 0.8 mM  $\text{NaH}_2\text{PO}_4$ , 1.5 mM  $\text{KH}_2\text{PO}_4$ , 10% inactivated calf serum, 0.1% NP-40, pH 7.4). The coloring reagent was o-phenylenediamine dihydrochloride (OPD) obtained from Wako Pure Chemical Industries, (Osaka).

**ELISA procedure.** The enzyme immunoassay was performed in polystyrene microplates (Toyoshima, Tokyo) with 96 flat-bottomed wells. Highly purified IBV and negative antigens were each diluted with carbonate buffer (0.05 M, pH 9.6). The antigen dilution (100  $\mu\text{l}$ ) was added to each well. The plate was covered and allowed to stand overnight at 4°C. The wells were washed with a washing buffer (0.85% NaCl, 0.02% Tween 20) six times with a Titertek Microplate Washer Model 120 (Flow Labs., Inc.) and excess solution was tapped out onto filter paper.

Serum samples were diluted 100-fold in dilution buffer and 100- $\mu\text{l}$  portions of each were added to positive and negative antigen-coated wells. The plates were incubated for 1 hr at room temperature. After incubation, the wells were washed and 100 $\mu\text{l}$  of HP-conjugated rabbit anti-chicken IgG diluted in the dilution buffer was added to each well. The plates were incubated for 30 min at room temperature and washed as above.

The substrate (100  $\mu\text{l}$ ) (0.4 mg of OPD per ml, 100 mM  $\text{Na}_2\text{HPO}_4$ , and 50 mM citric

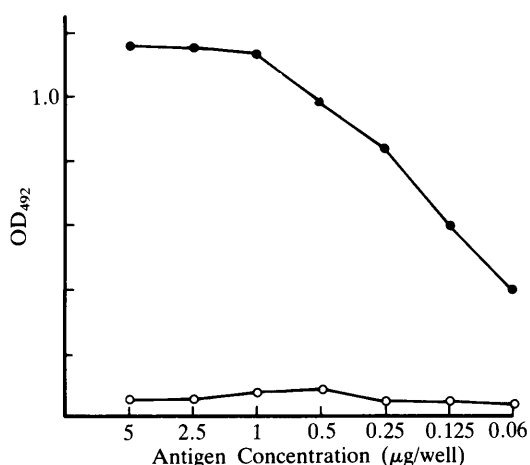


Fig. 1. Determination of optimal concentration of ELISA antigen (B42 strain). Dilution of positive serum (●—●) and negative serum (○—○) was 1:100.

acid, pH 4.8) containing 0.2  $\mu\text{l}$  of 30%  $\text{H}_2\text{O}_2$  per ml was added to each well and the plates were incubated at room temperature for 20 min in dark. The enzyme reaction was stopped by adding 100  $\mu\text{l}$  of 3N  $\text{H}_2\text{SO}_4$  to each well. Color reactions were read in a Microplate photometer Model MTP-22 (Corona electric Co., Ibaraki, Japan) with dual-wavelength at  $\lambda_{\text{test}}(492\text{nm})/\lambda_{\text{reference}}(610\text{nm})$ , blanked against a reagent control. For PCS (SN titer 1:90), the conjugate was so diluted as to give an ELISA value (absorbance of the positive-antigen-coated well minus that of the negative-antigen-coated well) of 1.00. ELISA values of serum samples were corrected by the following formula:

$$\text{Corrected ELISA (cELISA) value} = \frac{\text{ELISA value of test serum} \times 1.00}{\text{ELISA value of PCS}}$$

## RESULTS

**Determination of optimal concentration of ELISA antigen:** Antigen was coated in quantities of 5  $\mu\text{g}$ , 2.5  $\mu\text{g}$ , 1  $\mu\text{g}$ , 0.5  $\mu\text{g}$ , 0.25  $\mu\text{g}$ , 0.125  $\mu\text{g}$ , and 0.06  $\mu\text{g}$  per well (Fig. 1). The absorbance ( $A_{492}/A_{610}$ ) of positive

serum was almost constant between 5  $\mu$ g and 1  $\mu$ g of antigen/well and diminished at lower concentrations than 1  $\mu$ g/well. No reactivity of B42 strain with negative serum was observed ( $A_{492/610}$  less than 0.08). Therefore, 1  $\mu$ g of protein was employed to coat each well.

**Cross-reactivity among IBV strains in ELISA:** Cross-reactivity was compared in ELISA and SN among IBV strains B42, Gray, and A5968 (Table 1). In ELISA, apparent cross-reactivity was observed among these three strains but in the SN test little cross-reactivity. Since the SN titer of anti-A5968 serum to the homologous strain was very low (at a titer of 38), cELISA values of the antiserum to heterologous strains (B42 and Gray) were higher than that to the homologous strain. In addition, B42 strain of IBV used as an ELISA antigen reacted with

antisera against three other serotype strains (Iowa97, Iowa609, and M41) and six strains isolated in Japan (KH, ON, Takeshima, S4W, 0353, and Shizuoka) in ELISA (Table 2).

**Antibody responses to IBV infection:** Fig. 2 shows correlation between cELISA values and SN titers of sera collected from chickens naturally infected with IBV in the field. The cELISA values did not correlate with the SN titers as shown in Fig. 2 ( $r=0.43$ ). In sera collected from birds experimentally infected with a single strain (B42), the cELISA value increased with the rise of the SN titer (Fig. 3).

Table 1. Comparison in cross-reactivity of serum neutralization (SN) test with ELISA

Method	Antigen	Antiserum against:		
		B42	Gray	A5968
SN test	B42	90 <sup>a)</sup>	16	8
	Gray	13	128	<2
	A5968	<2	<2	38
ELISA	B42	1.00 <sup>b)</sup>	0.81	0.41
	Gray	0.73	0.79	0.41
	A5968	0.66	0.70	0.44

a) Reciprocal of the highest serum dilution showing 50% neutralization.

b) ELISA values.

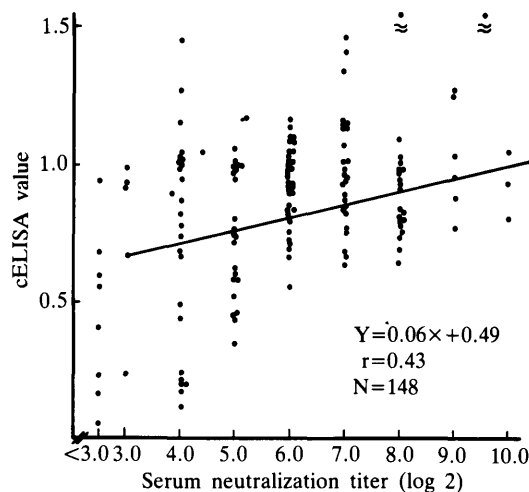


Fig. 2. Correlation between cELISA value and serum neutralization titer of individual chicken sera against B42 strain.

Table 2. Reactivity of strain B42 to anti-infectious bronchitis virus chicken sera in ELISA and the SN test

Method	Antiserum against:								
	M41	Iowa97	KH	Iowa609	ON	Takeshima	S4W	Shizuoka	0353
ELISA with B42 strain as an antigen	1.13 <sup>a)</sup>	0.80	0.86	0.81	1.25	1.36	1.26	1.09	1.31
SN test against B42 strain	128 <sup>b)</sup>	<2	<2	<2	4	<2	4	8	<2
SN test against homologous strain	512 <sup>b)</sup>	90	90	56	128	128	256	128	256

a) cELISA values.

b) Reciprocal of the highest serum dilution showing 50% neutralization.

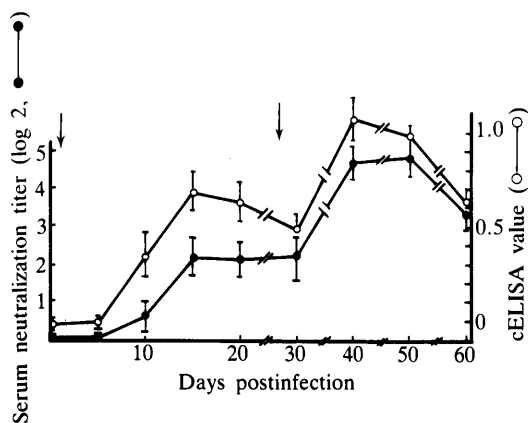


Fig. 3. Correlation between cELISA value and serum neutralization titer in serum samples collected from 10 chickens intranasally inoculated with just B42 strain of IBV. Both tests were done with B42 strain as an antigen. Arrows show the day of inoculation and bars standard errors.

#### DISCUSSION

In our ELISA system, 1  $\mu$ g of highly purified antigen of B42 per well was adequate (Fig. 1). This amount coincided with that reported by Soula and Moreau [18] but did not with that reported by Case *et al.* [4]. This diversity may have been due to the different methods of purification of the antigen and assay systems. This antigen reacted with neither negative control serum nor chicken antisera against Newcastle disease or, infectious laryngotracheitis virus. All antisera showed cELISA values lower than 0.041 (data not shown). Therefore, the ELISA system using this antigen seems to be IBV-specific reaction. B42 strain was employed as an ELISA antigen since it is the prototype of IBV and propagates in embryonated eggs to a higher titer than other IBV strains.

Case *et al.* [4] employed a high-ionic-strength serum diluent to eliminate nonspecific binding of the serum protein to the solid phase. In our ELISA system, a negative antigen and a diluent containing 0.1% NP-40 and 10% inactivated calf serum were

employed to eliminate nonspecific reaction (data not shown). Furthermore, the use of positive control serum in each plate may have eliminated variance in absorbance.

Cross-reactivity among a certain IBV strains has been reported in the hemagglutination inhibition (HI) test [1] and immunofluorescence [16]. In the former method, cross-reactivity was observed among a few strains and King and Hopkins [13] serotyped IBV isolates by the HI test. In the latter method, a wide range of cross-reactivity was observed but Lucio and Hitchner [16] insisted that conjugates prepared against two strains (Iowa97 and Massachusetts) were able to detect antigens of all strains of IBV tested. It seems that both methods using one type of IBV strain have limitation for diagnosis of IBV infections. In our ELISA system, cross-reactivity was apparently observed among three IBV strains, B42, Gray, and A5968 (Table 1). In addition, B42 strain of IBV used as an ELISA antigen had an ability to react with chicken antisera against not only three other serotype strains (Iowa97, Iowa609, and M41) but also six strains isolated in Japan (designated as KH, ON, Takeshima, S4W, 0353, and Shizuoka) which were distinguishable from each other by the plaque-reduction test [8]. With only one strain of IBV (B42 in our system) as an antigen, the circulating antibody against any strain of IBV included in this study can be detected by highly sensitive ELISA (Tables 1 and 2). It seems, therefore, that ELISA using solubilized antigen may solve the diagnostic problems arising from the existence of subtypes of IBV.

Although the ELISA value did not correlate with the SN titer in some serum samples in the field, the ELISA value increased with the rise of the SN titer in serum samples collected from chickens infected with a single IBV strain (B42) (Figs. 2 and 3). This indicates that chickens in the field were

exposed to several subtypes of IBV and ELISA detects circulating antibody against any subtype strain of IBV. Therefore, ELISA should be used for qualitative tests in the field since our ELISA system does not quantitate of IBV-specific antibody. The SN titer of a chicken from field can not be estimate from the cELISA value of the serum.

It was suggested that common antigenic determinant(s) exist among IBV strains by ELISA and some other serological tests [1, 14]. Recently, genetical analysis of IBV genomic RNA has progressed [2, 3, 5]. Bournsnel *et al.* [3] reported similar nucleotide sequences of nucleocapsid genes of two IBV strains (Beaudette and M41), each containing a single long open reading frame of 1227 bases. The 3' non-coding region of the Beaudette strain contains a 184-base segment, which is not found in M41 strain [3]. Thus, nucleocapsid protein may be the common antigenic determinant of IBV. More detailed antigenic analysis will be needed on a protein or genetical level.

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#### REFERENCES

- Alexander, D. J., Bracewell, C. D., and Gough, R. E. 1976. Preliminary evaluation of the haemagglutination and haemagglutination inhibition tests for avian infectious bronchitis virus. *Avian Pathol.* 5: 125-134.
- Binns, M. M., Bournsnel, M. E. G., Cavanagh, D., Pappin, D. J. C., and Brown, T. D. K. 1985. Cloning and sequencing of the gene encoding the spike protein of the coronavirus IBV. *J. Gen. Virol.* 66: 719-726.
- Bournsnel, M. E. G., Binns, M. M., Foulds, I. J., and Brown, T. D. K. 1985. Sequences of the nucleocapsid genes from two strains of avian infectious bronchitis virus. *J. Gen. Virol.* 66: 573-580.
- Case, J. T., Ardans, A. A., Bolton, D. C., and Reynold, B. J. 1983. Optimization of parameters for detecting antibodies against infectious bronchitis virus using an enzyme-linked immunosorbent assay: Temporal response to vaccination and challenge with live virus. *Avian Dis.* 27: 196-210.
- Cavanagh, D., Binns, M. M., Bournsnel, M. E. G., and Brown, T. D. K. 1984. Genetically engineered vaccine against avian infectious bronchitis virus with the advantages of current live and killed vaccines. pp. 215-218. *In: Modern approaches to vaccines; molecular and chemical basis of virus virulence and immunogenicity* (Chanoock, R. M. and Lerner, R. A. eds.) Cold Spring Harbor Lab., New York.
- Chomiak, T. W., Luginbuhl, R. E., and Steele, F. M., 1963. Serological differences between the Beaudette and Massachusetts strains of infectious bronchitis virus. *Avian Dis.* 7: 325-330.
- Cunningham, C. H. 1970. Avian infectious bronchitis. *Adv. Vet. Sci. Comp. Med.* 14: 105-148.
- Doi, M., Yamagami, T., Koimaru, H., Yoshimura, M., Masu, S., Shirai, J., and Kawamura, H. 1982. Serotypes of avian infectious bronchitis virus isolates from field cases in Japan. *Avian Dis.* 26: 946-956.
- Hitchner, S. B., Appleton, G. S., and Winterfield, R. W. 1964. Evaluation of the immunity response to infectious bronchitis virus. *Avian Dis.* 8: 153-162.
- Hitchner, S. B., Winterfield, R. W., and Appleton, G. S. 1966. Infectious bronchitis types in the United States. *Avian Dis.* 10: 98-102.
- Hofstad, M. S. 1958. Antigenic differences among isolates of avian infectious bronchitis virus. *Am. J. Vet. Res.* 19: 740-743.
- Hofstad, M. S. 1961. Antigenic and immunological studies on several isolates of avian infectious bronchitis virus. *Avian Dis.* 5: 102-106.
- King, D. J., and Hopkins, S. R. 1984. Rapid serotyping of infectious bronchitis virus isolates with the hemagglutination-inhibition test. *Avian Dis.* 28: 727-733.
- Leine, P. P., and Hofstad, M. S. 1947. Attempts to control airborne infectious bronchitis and Newcastle disease of fowls with sterilamps. *Corneil Vet.* 37: 204-211.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L., and Randall, R. J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193: 265-275.
- Lucio, B., and Hitchner, S. B. 1970. Differentiation and detection of infectious bronchitis virus subtypes by immunofluorescence. *Avian Dis.* 14: 9-24.
- Reed, L. J., and Muench, A. 1938. A simple method of estimating 50 percent end points. *Am. J. Hyg.* 27: 493-497.
- Snyder, D. B. 1986. Latest development in the enzyme-linked immunosorbent assay (ELISA). *Avian Dis.* 30: 19-23.

19. Soula, A., and Moreau, Y. 1981. Antigenic requirements and specificity of a microplate enzyme-linked immunosorbent assay (ELISA) for detection of infectious bronchitis viral antibodies in chicken serum. *Arch. Virol.* 67: 283-295.
20. Winterfield, R. W., Hitchner, S. B., and Appleton, G. S. 1964. Immunological characteristics of variant of infectious bronchitis isolated from chickens. *Avian Dis.* 8: 40-47.

#### 要 約

Enzyme-linked immunosorbent assay (ELISA) 法による鶏伝染性気管支炎ウイルス各株間の交差反応性：長野秀樹・宝達 勉・土本まゆみ・井出誠弥・山上 正・山岸郭郎・藤崎優次郎（北里研究所附属家畜衛生研究所）——精製ウイルスをトライトンX-100で可溶化したものを抗原とする ELISA 法により中和試験でほとんど交差性がみられなかったB42株（マサチューセッツ型）、グレイ株（デラウェア型）およびA5968株（コネチカット型）の3株間に強い交差反応がみられた。ELISA 法ではB42株は血清学的に相互に異なると思われる上記3株以外の8株と強く反応し、鶏血清のB42株に対する ELISA 抗体価は中和抗体価と平行して推移した。