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（鶏伝染性気管支炎ウイルス共通抗原の検出
およびＤＮＡプローブを用いたウイルス株の型別）

Hideki NAGANO

(1)

Demonstration of a common antigen in chickens infected with infectious bronchitis virus and differentiation of the virus strains with a DNA probe

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Contents

Abbreviations

Avian infectious bronchitis virus	IBV
Chicken embryo kidney	CEK
Eagle's minimum essential medium	EMEM
Enzyme-linked immunosorbent assay	ELISA
Ethylenediaminetetraacetic acid	EDTA
50% egg infectious dose	EID_{50}
50% tissue culture infectious dose	$TCID_{50}$
Membrane	M
Monoclonal antibody	Mab
Nonidet P-40	NP-40
Nucleocapsid	N
Phosphate-buffered saline	PBS
Sodium dodecyl sulfate	SDS
Specific pathogen free	SPF
Spike	S
1,000 daltons	kDa
Tris-buffered saline	TBS
Tryptose phosphate broth	TPB

Preface

Avian infectious bronchitis virus (IBV), a member of the family Coronaviridae, is the etiological agent of a highly contagious respiratory disease of young chickens and some strains of IBV also induce nephritis and nephrosis in chickens. The most characteristic signs in affected chickens are respiratory distress, tracheal rales, coughing, sneezing and nasal discharge with generalized weakness and depression as the disease progresses. The disease resulting from IBV infection is of considerable economic importance to the poultry industry not only as a result of high morbidity and, in some instances, mortality but also because of the debilitating nature of the disease with resulting poor utilization of feed by young chickens. The major economic loss is from ovarian damage and a precipitous and prolonged decrease in egg production in laying flocks (22).

The virions of IBV, with a diameter of 80 to 120 nm, are enveloped, pleomorphic particles with a distinctive 'corona' of club-shaped surface projections, 9 to 11 nm in length, on the distal end, and a large single-stranded RNA genome of positive polarity (56, 58, 59). In IBV-infected cells, in addition to genome-sized RNA, five subgenomic mRNA species, that have a common 3' terminus, but extend for different lengths in the 5' direction, forming a nested set, can be detected. These mRNA species are designated as mRNA 1 to 6 (previously referred to as mRNA F

to A, respectively), mRNA 6 being the smallest and mRNA 1 being of full genome length (7, 62, 63).

There are three structural proteins in IBV virions: the spike (S) and membrane (M) glycoproteins, and nucleocapsid (N) protein (10, 40, 65, 67, 70). In vitro translation studies have demonstrated that mRNA species 6, 4 and 2 code for N, M and the precursor of S protein, respectively (66). The N protein is within the lumen of the virus formed by the virus membrane while the other two proteins, S and M glycoproteins, are partially exposed at the virus outer surface. Only a small, glycosylated portion of M protein appears to be exposed at the virion surface (6, 17). In contrast, most of the S glycoprotein is exposed (11, 17).

The S protein comprises two copies of each of two glycopolypeptides, S1 of 90,000 daltons (90 kDa) and S2 of 84 kDa, containing N-linked oligosaccharide of the high-mannose type (11-13, 65) and derived by cleavage of a precursor glycoprotein (18, 64). S protein is anchored in the membrane by S2 while S1 forms the major part of the distal, bulbous end of S. This view is supported by a study using monoclonal antibody (46) and nucleotide sequence analyses of the S gene (3). Purified S protein, but not M or N protein, has the ability to induce both virus-neutralizing and hemagglutination-inhibiting antibodies in chickens (15). Furthermore, IBV lacking S1 was unable to induce virus-neutralizing or hemagglutination-inhibiting antibody (16) and was

no longer infectious or able to cause hemagglutination (14). Therefore, S, especially S1, carries a neutralization epitope, and is responsible for serotype specificity.

Demonstration of an acquired humoral antibody response to a pathogenic microorganism or of an etiological agent in the affected animal has traditionally been a diagnostic tool for many infectious diseases. In the case of IBV infection, the specific antibody has been demonstrated by several serological tests, such as the neutralization test conducted in embryonated chicken eggs (21, 28, 33, 54) or in tissue culture with a marker of plaque-reduction or inhibition of cytopathic effects (26, 35, 36, 74), the immunofluorescence test (42, 43, 74), the hemagglutination-inhibition test (1, 2) and the agar gel precipitin test (73). However, the former three methods require more than one strain for the precise demonstration of antibodies against IBV, and particularly the neutralization test, which is most usually used for the detection of antibody and identification of IBV isolates, requires considerable time to perform even a single assay. The latter method has a problem with its sensitivity to detect antibody. Moreover, numerous serotypes in IBV have been recognized, and intraserotypically antigenic variation has also been described mainly by the neutralization test (19, 20, 23-25, 31-33, 71, 72). As a result, it is likely that serological tests such as the neutralization test using a single antiserum or a single strain could provide equivocal or negative results when utilized

to confirm an outbreak by IBV infection in serum samples from convalescent chickens or to identify a virus isolate as IBV. Thus, a method which is rapid and simple to perform and can detect a common antigen of IBV is necessary for proper confirmation of the outbreak and identification of an isolate as IBV.

A rapid, sensitive, reproducible, and simple enzyme-linked immunosorbent assay (ELISA) technique was initially developed by Engvall and Perlmann (27) and Weerman and Schuurs (69) in 1971 and has been widely used to study antigen-antibody reactions. In addition, for the detection of antibody to IBV, a number of workers have devoted their efforts to the study of ELISA because of its simplicity (9, 29, 44, 45, 47, 51, 61).

In this study the detection of an antibody against a common antigen of IBV and detection of a common antigen of IBV by ELISA are discussed. Chapter I describes a simple ELISA technique for the detection of antibodies to IBV strains with different serotype specificities (47). On the other hand, in rapid diagnosis of IB in the acute phase, direct demonstration of the specific antigen in the tracheas of affected chickens appears to be more effective. Chapter II describes sensitive sandwich ELISA using a polyclonal antibody and a monoclonal antibody (Mab) directed to the nucleocapsid of strain B42 (48, 49). Thus, IBV-specific antibodies or antigens could be detected broadly by ELISA using a single virus strain or a single antiserum and Mab. After detection a virus isolate must be differentiated into a given sero-

type. Serotypes of IBV strains have been basically defined by cross-neutralization tests. In some cases unequivocal assignment of an isolate to a serotype is complicated by one-way neutralization and an ambiguous neutralization titer (24, 25). In an attempt to remedy this, cDNA complementary to the S gene was cloned from IBV strain M41 (Massachusetts serotype), and a probe was prepared from the recombinant cDNA clone complementary to the S1 region of the gene. Chapter III describes application of the probe to dot-blot hybridization of genomic RNA samples prepared from IBV strains of several serotypes and discusses the probability of the hybridization differentiating an isolate of IBV (50).

Chapter I

Cross-reactivity among the strains of infectious bronchitis virus in enzyme-linked immunosorbent assay

Introduction

Disease caused by IBV infection is usually diagnosed by clinical examination, virus isolation, and demonstration of an IBV-specific antibody in the sera of birds recovering from the disease. Confirmation of an outbreak by virus isolation or the serum neutralization test is occasionally made difficult by the existence of numerous serotypes of IBV (20, 23-25, 31-33, 71, 72). Use of a single IBV strain for testing serum samples from convalescent chickens as confirmation of a suspected IBV outbreak could lead to ambiguous results.

ELISA has widely been used for the detection of antibodies against IBV (9, 29, 44, 45, 51, 60, 61). Marquardt et al. (44) reported that cross-reactivity was observed by ELISA among three strains, M41, JMK and A5968, which were distinguished from each other by the neutralization test. Antibody titers obtained by ELISA are considerably higher because of its higher sensitivity

(44, 45, 61) and the antibody response after an infection or vaccination with IBV can be detected earlier by ELISA than by the neutralization test (9, 29, 44, 45, 51). In this study, ELISA for the detection of antibodies against IBV isolates in Japan is described. In addition the ELISA described herein is highly sensitive, requires only small amounts of reagents and serum, and permits rapid testing of a large number of samples by using a single hundredfold serum dilution.

Materials and Methods

Virus strains:

The IBV strains used in this study were B42, M41 (Massachusetts serotype), Gray (Delaware serotype), A5968 (Connecticut serotype), Iowa 97 (Iowa 97 serotype), Iowa 609 (Iowa 609 serotype), and six isolates from 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 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 until use.

Cell culture:

Primary monolayer cultures of chicken embryo kidney (CEK) cells were prepared from the kidneys of 18-day-old chicken embryos by dispersion with 0.1% trypsin. CEK cells were grown in Eagle's minimum essential medium (EMEM) containing 10% tryptose phosphate broth (TPB), 5% calf serum and 0.2% $NaHCO_3$. They were maintained in EMEM supplemented with 10% TPB, prepared in flat-bottomed microplates by seeding each with 0.1 ml of growth medium containing 0.2% cells, and incubated in an atmosphere of 5% CO_2 in air at 37°C for 48 hr.

Preparation of antisera:

Specific antisera against each IBV strain were prepared in 50-day-old SPF chickens by giving intranasal and intraocular 50% tissue culture infectious doses ($TCID_{50}$) of 10^7 to 10^8 of IBV at three-week intervals. Serum samples were obtained seven days after the last administration, inactivated at 56°C for 30 min and stored at -20°C until use.

Experimental infection of chickens with IBV strain B42:

Ten 50-day-old SPF chickens were intranasally inoculated with 10^7 $TCID_{50}$ virus of strain B42 and boosted at 28 days after the first inoculation. Serum samples were collected from each chicken at 0, 5, 10, 15, 20, 30, 40, 50 and 60 days postinfection. These sera were heat-inactivated and stored at -20°C until

use.

Serum samples:

One hundred forty-eight chicken serum samples were supplied to The Kitasato Institute from 14 farms geographically far away from each other for measuring antibody titers against IBV from May to November in 1985. These serum samples were used for examining the correlation of serum neutralization titers and ELISA values. The sera were heat-inactivated and stored at -20°C until use.

Serum neutralization test:

The test was carried out by the serum dilution method using CEK cell cultures prepared in flat-bottomed microplates. In the well of a plate, 25 μl of each of serial twofold dilutions of the serum inactivated at 56°C for 30 min was mixed with 25 μl of virus solution containing 200 $TCID_{50}$ 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 a CEK cell monolayer. The plates were incubated in an atmosphere of 5% CO_2 in air at 37°C for seven 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 (55).

Preparation of ELISA antigen:

Zinc acetate was added to allantoic fluid at a final concentration of 25 mM. After neutralization by adding 1 N NaOH, the allantoic fluid was stirred at 4°C for 30 min. The precipitate was dissolved in saturated ethylenediaminetetraacetic acid (EDTA) buffer. This solution containing virus served for virus purification using 30% and 60% sucrose cushion centrifugation. The purified virus sample in Tris-buffered saline (TBS, pH 7.4) was solubilized with 0.1% Triton X-100 (Eastman Kodak Co., New York, U.S.A.) and dialyzed against the TBS buffer. The protein concentration of the dialysate was determined by the method of Lowry et al. (41).

Normal allantoic fluid 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 TBS, solubilized in the same way as above, and coated on an ELISA plate as a negative control antigen.

ELISA procedure:

ELISA was performed in polystyrene microplates (Toyoshima Co. Ltd., Tokyo) with 96 flat-bottomed wells. Purified IBV and negative antigens were each diluted with carbonate buffer (0.05 M, pH 9.6). The diluted antigen (0.1 ml) was added to each well. The plate was allowed to stand overnight at 4°C and then washed with a washing buffer (0.85% NaCl and 0.02% Tween 20) six times.

Serum samples were diluted 100-fold in dilution buffer consisting of 0.1% Nonidet P-40 (NP-40) and 10% inactivated calf serum phosphate-buffered saline (PBS, pH 7.4) and 0.1 ml aliquots of each sample were added to positive and negative antigen-coated wells. The composition of the dilution buffer was defined so as to eliminate non-specific binding of the serum protein to the solid phase. The plates were incubated for 1 hr at room temperature. After incubation, the wells were washed and 0.1 ml of horseradish peroxidase-conjugated rabbit anti-chicken IgG (heavy and light chains, Zymed Labs. Inc., California, U.S.A.) diluted in the dilution buffer was added to each well. The plates were incubated for 30 min at room temperature and washed as described above.

One-tenth ml of the substrate solution (40 μg of o-phenylenediamine dihydrochloride in 100 mM Na_2HPO_4 and 50 mM citric acid, pH 4.8) containing 0.02 μl of 30% H_2O_2 was added to each well and the plates were incubated at room temperature for 20 min in the dark. The coloring reaction was stopped by adding 0.1 ml of 3 N H_2SO_4 to each well. Color degree was read in a Microplate photometer Model MTP-22 (Corona Electric Co., Ibaraki) with dual-wavelength reading at 492 nm and a reference at 610 nm, blanked against a reagent control. For positive control serum (the neutralization antibody titer against IBV strain B42 is 90) the conjugate was diluted so as to give an ELISA value (absorbance of the positive antigen-coated well minus that of the nega-

tive antigen-coated well) of 1.00. ELISA values of serum samples were corrected by the following formula:
ELISA value = ELISA value of test serum X (1.00/ELISA value of positive control serum).

Results

Determination of optimal concentration of ELISA antigen:

A viral antigen (strain B42), prepared as described in Materials and Methods, was coated in quantities of 5, 2.5, 0.5, 0.25, 0.125, and 0.06 μg per well (Fig. 1). The absorbance (A_{492}/A_{610}) of positive serum was almost constant between 5 and 1 μg of antigen/well and diminished at concentrations lower than 1 μg/well. No reactivity of strain B42 with negative serum collected from SPF chickens was observed ($A_{492/610}$ less than 0.08). Therefore, 1 μg of the protein was employed to coat each well.

Comparison of cross-reactivity of the neutralization test and ELISA among IBV strains:

Cross-reactivity among three IBV strains B42, Gray, and A5968 observed in the neutralization test was compared with that in ELISA. In the neutralization test, strain B42 reacted with each antiserum against strains Gray and A5968 at titers of 16 and 8, respectively. Strain Gray reacted with the antiserum against

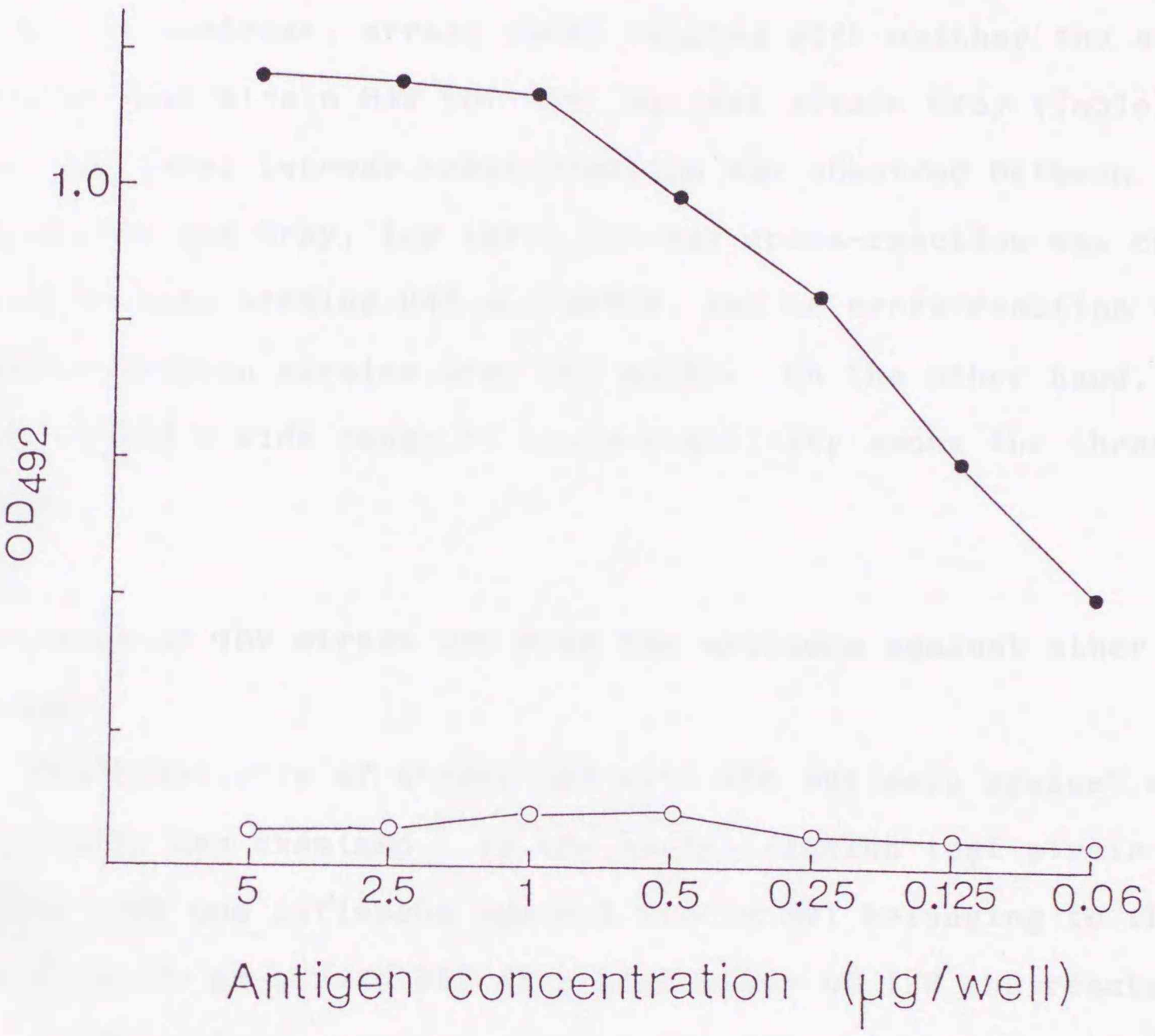

Fig. 1 ELISA value of sera reacted with different concentrations of antigen (strain B42). Dilution of positive serum (●) and negative serum (○) was 1:100.

strain B42 at a titer of 13 but not with that against strain A5968. In contrast, strain A5968 reacted with neither the antiserum against strain B42 nor that against strain Gray (Table 1). Thus, low level two-way cross-reaction was observed between strains B42 and Gray, low level one-way cross-reaction was observed between strains B42 and A5968, and no cross-reaction was observed between strains Gray and A5968. On the other hand, ELISA showed a wide range of cross-reactivity among the three strains.

Reactivity of IBV strain B42 with the antisera against other IBV strains:

The reactivity of strain B42 with the antisera against other IBV strains was examined. In the neutralization test strain B42 reacted with one antiserum against strain M41 belonging to the same serotype as strain B42 at a high titer of 128 and reacted with three antisera against strains ON, S4W and Shizuoka at low titers of 4, 4 and 8, respectively, but did not react with five other antisera. In contrast, in ELISA, strain B42 showed high reactivity with all antisera tested against not only two other serotype strains (Iowa 97 and Iowa 609) but also six isolates designated as KH, ON, Takeshima, S4W, 0353 and Shizuoka in Japan (Table 2).

Table 1 Cross-reactions between different types of antigens and their antisera by the neutralization test and ELISA

Method	Antigen	Antiserum against: B42	Gray	A5968
Neutralization test	B42	90[1)]	16	8
	Gray	13	128	<2
	A5968	<2	<2	38
ELISA	B42	1.00[2)]	0.81	0.41
	Gray	0.73	0.79	0.41
	A5968	0.66	0.70	0.44

1) Reciprocal of the highest serum dilution showing 50% neutralization.
2) ELISA value.

Table 2 Reactivity of strain B42 to antisera of chickens to infectious bronchitis virus in ELISA and the neutralization test

Antiserum against:	Methods		
	ELISA values against strain B42	Neutralization antibody titers against strain B42	Neutralization antibody titers against a homologous strain
M41	1.13[1)]	128[2)]	512[2)]
Iowa 97	0.80	<2	90
Iowa 609	0.81	<2	56
KH	0.86	<2	90
ON	1.25	4	128
Takeshima	1.36	<2	128
S4W	1.26	4	256
Shizuoka	1.09	8	128
0353	1.31	<2	256

1) ELISA value.
2) Reciprocal of the highest serum dilution showing 50% neutralization.

Comparison of neutralization titers and ELISA values in sera from chickens reared on 14 different farms:

Neutralization antibody titers and ELISA values against IBV strain B42 were measured in 148 serum samples of chickens from different 14 farms. The results of neutralization tests were expressed as the geometric mean value ± standard deviation and those of ELISA were expressed as the mean value ± standard deviation as shown in Table 3. Serum samples of chickens from farm A having no detectable neutralizing antibody showed very low ELISA values (0.21 ± 0.16) whereas those from farm B showed moderate ELISA values (0.70 ± 0.18), though with no detectable neutralizing antibody as with farm A. Similar results were observed in chicken sera showing low neutralization antibody titers. Sera of chickens from farms C to F showed low titers of neutralizing antibody (from 12 ± 1.2 to 21 ± 1.7) but their ELISA values varied from 0.30 ± 0.19 to 1.10 ± 0.16. These results indicated that serum samples without or, if any, with low neutralizing antibodies had variable ELISA values, that is, a low neutralizing antibody titer was not always parallel with its ELISA value. On the other hand, serum samples of chickens from farms H to N with high neutralization antibody titers (from 64 ± 1.1 to 240 ± 1.6) showed moderate or high ELISA values (from 0.82 ± 0.08 to 1.25 ± 0.28). In addition, tentatively, a positive ELISA rate was calculated for each farm when the ELISA value was 0.5 or more, and was thus regarded as a positive reaction. The positive rates

Table 3 Comparison of neutralization titers and ELISA values in serum samples collected from chickens reared on 14 different farms

Farm (number)	Neutralization antibody titer	ELISA value	Positive rate (%) by ELISA
A(4)	<8	0.21 ± 0.16[2)]	0[3)]
B(4)	<8	0.70 ± 0.18	100
C(8)	12 ± 1.2[1)]	0.30 ± 0.19	25
D(10)	21 ± 1.5	0.46 ± 0.21	50
E(10)	21 ± 1.7	0.79 ± 0.12	90
F(12)	14 ± 1.5	1.10 ± 0.16	100
G(10)	37 ± 1.8	0.75 ± 0.24	90
H(11)	64 ± 1.1	0.86 ± 0.07	100
I(13)	119 ± 1.8	0.88 ± 0.13	100
J(13)	137 ± 1.5	0.86 ± 0.08	100
K(14)	240 ± 1.6	0.82 ± 0.08	100
L(13)	90 ± 2.0	1.20 ± 0.17	100
M(14)	128 ± 2.4	1.25 ± 0.28	100
N(12)	181 ± 1.7	1.07 ± 0.15	100

1) Reciprocal of the highest serum dilution showing 50% neutralization is shown as the geometric mean value ± standard deviation.
2) ELISA value is shown as mean value ± standard deviation.
3) Positive rate by ELISA is shown as percentage when the positive ELISA value was 0.5 or more.

of farms A, C, D, E and G were 0, 25, 50, 90 and 90%, respectively, and that of other farms was 100%.

Correlation between ELISA values and neutralization antibody titers in 148 chicken sera:

The correlation between the neutralization test and ELISA was examined in all 148 sera as shown in the scatter diagram in Fig. 2. Of eight serum samples having no detectable neutralization antibody, four sera had ELISA values of less than 0.5 and the others 0.5 or more and less than 1.0. Serum samples with detectable neutralizing antibody were divided into two groups; one group contained samples with low neutralization antibody titers of less than 64 and the other consisted of samples with high neutralization antibody titers of 64 or more. Of 49 serum samples showing low neutralization antibody titers, 13 samples (26.5%) had ELISA values of less than 0.5, 24 samples (49.0%) had ELISA values of 0.5 or more and less than 1.0, and 12 samples (24.5%) had ELISA values of 1.0 or more. In contrast to 91 serum samples showing high neutralization antibody titers, none of the serum samples showed ELISA values of less than 0.5, 60 samples (65.9%) showed ELISA values of less than 1.0 and 31 samples (34.1%) showed ELISA values of 1.0 or more. From these data, the positive threshold line in ELISA was put at 0.5. Based on the cut-off point of ELISA, four of 148 samples (2.7%) were positive by ELISA but negative by the neutralization test and 13 samples

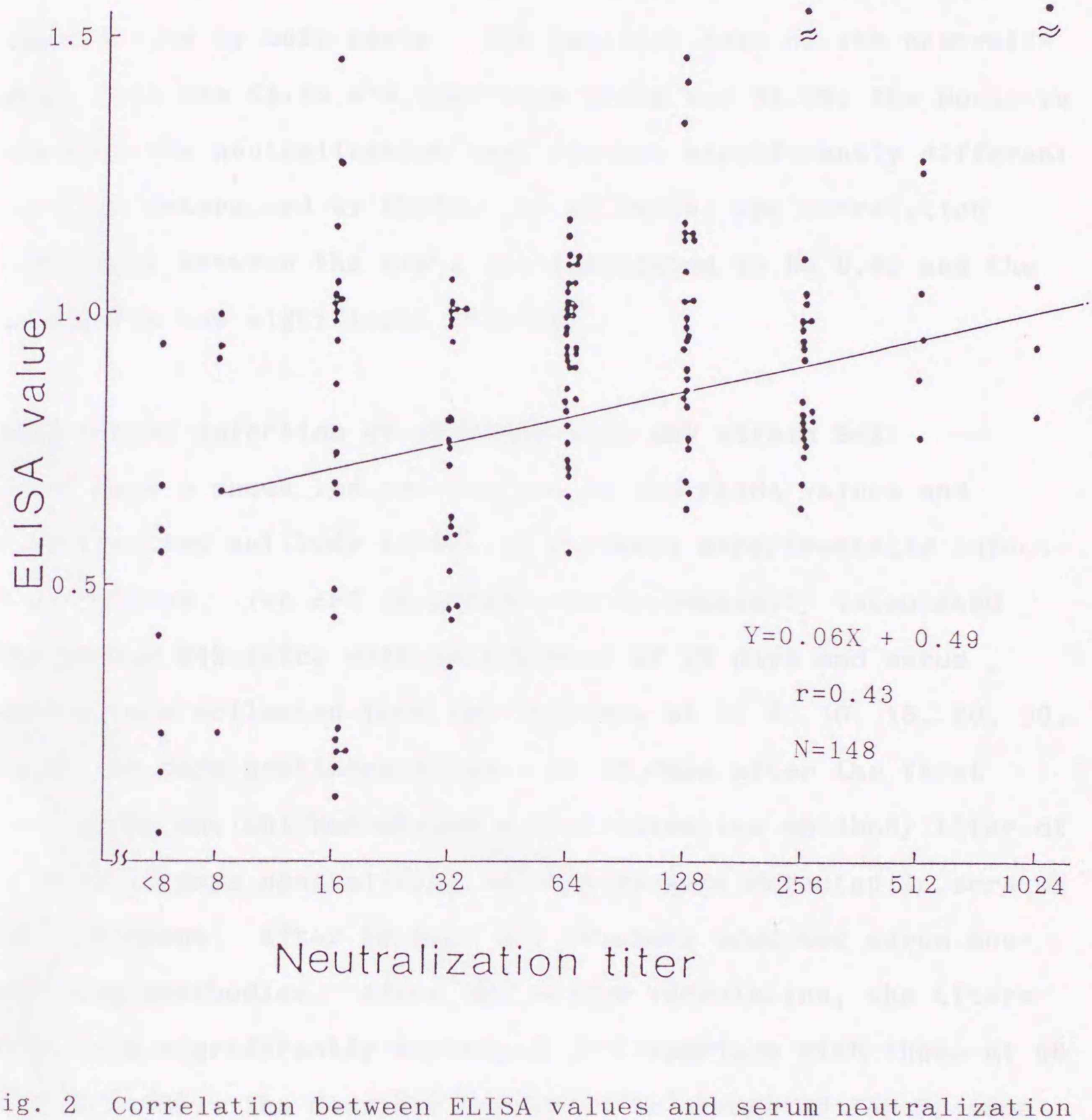

Fig. 2 Correlation between ELISA values and serum neutralization titers of individual chicken sera against strain B42.

(8.8%) were negative by ELISA but positive by the neutralization test. Four samples (2.7%) were negative and 127 samples (85.8%) were positive by both tests. The positive rate of the neutralization test was 94.6% and that with ELISA was 91.2%; the positive rate with the neutralization test was not significantly different from that determined by ELISA. In addition, the correlation coefficient between the tests was calculated to be 0.43 and the correlation was significant ($P<0.05$).

Experimental infection of chickens with IBV strain B42:

Figure 3 shows the fluctuation of the ELISA values and neutralization antibody titers in chickens experimentally infected as follows. Ten SPF chickens were intranasally inoculated with strain B42 twice with an interval of 28 days and serum samples were collected from the chickens at 0, 5, 10, 15, 20, 30, 40, 50, 60 days postinoculation. At 10 days after the first inoculation one chicken showed a neutralization antibody titer of 4 and at 15 days neutralizing antibodies were detected in sera of eight chickens. After 20 days all chickens produced serum neutralizing antibodies. After the second inoculation, the titers at 60 days significantly decreased in comparison with those at 50 days ($P<0.05$). The decrease seemed to be accounted for by some of the features of strain B42, which is completely attenuated by more than 200 passages through embryonated eggs and/or the fact that its S1 polypeptide responsible for the production of neutral-

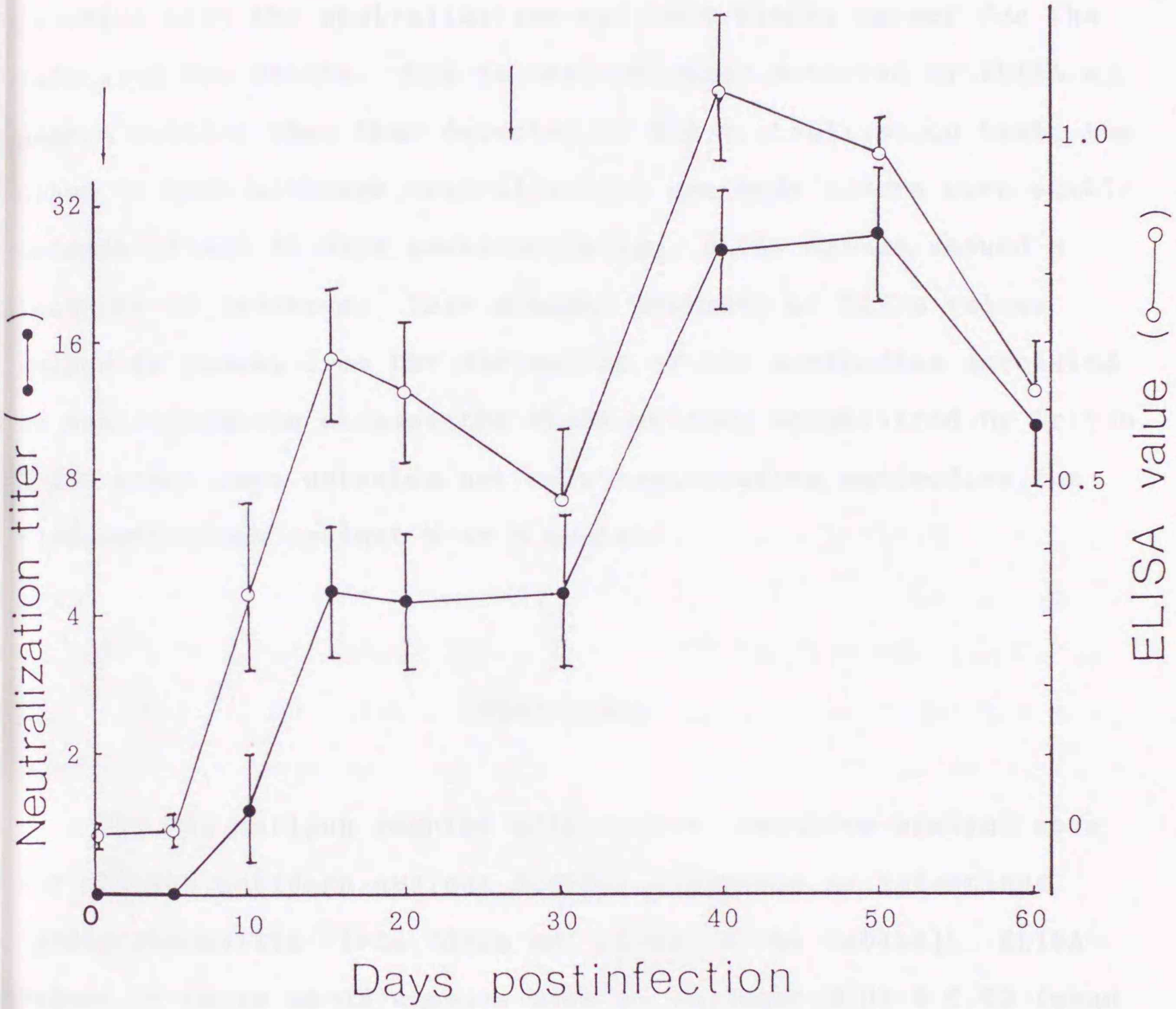

Fig. 3 Correlation between ELISA values and serum neutralization titers in serum samples collected from 10 chickens intranasally inoculated with strain B42 of IBV. Both tests were done with strain B42 as the antigen. Arrows show the day of inoculation and bars standard errors.

izing antibody is easily dissociated from the virion (64). On the other hand, ELISA values of the chicken sera fluctuated in parallel with the neutralization antibody titers except for the following two points. One is that antibody detected by ELISA appeared earlier than that detected by the neutralization test; the other is that although neutralization antibody titers were stable between 15 and 30 days postinoculation, ELISA values showed a tendency to decrease. This gradual decrease of ELISA values seemed to result from the diminution of the antibodies unrelated to neutralization because the ELISA antigen solubilized by Triton X-100 might have detected not only neutralizing antibodies but also antibodies against N or M protein.

Discussion

The B42 antigen reacted with neither negative control sera nor chicken antisera against Newcastle disease or infectious laryngotracheitis virus (data not shown in the Tables). ELISA values of these serum samples were as follows: 0.03 ± 0.03 (mean value ± standard deviation) in 100 serum samples from SPF chickens, 0.02 ± 0.01 in 10 antisera to Newcastle disease virus and 0.03 ± 0.01 in 10 antisera to infectious laryngotracheitis virus. Therefore, the ELISA reported in this study seemed to be an IBV-specific reaction. The reason why the B42 strain was employed as

an ELISA antigen is that it is the prototype of IBV and propagates in embryonated eggs to a higher titer than other IBV strains.

As shown in Fig. 2 all the chicken sera with neutralization antibody titers of 64 or more showed ELISA values of 0.5 or more. In addition, in experimental infection of SPF chickens with strain B42 (Fig. 3), most serum samples from chickens with detectable neutralizing antibody two weeks after inoculation also showed ELISA values of 0.5 or more. Based on these results, the positive threshold line in this ELISA seemed to be about 0.5. On the other hand, chicken sera with high levels of neutralizing antibodies had a tendency to converge near 1.0 in ELISA in field cases (Fig. 2) and in experimental infection the ELISA values reached a peak of around 1.0 after the second inoculation (Fig. 3). Judging from these results, serum showing an ELISA value of more than 1.0 might contain antibodies resulting from an infection with IBV.

Chickens from farm A seemed not to have received any IBV vaccines nor to be infected with any IBV strains because there was no antibody detectable by either the neutralization test or by ELISA. In contrast, chickens on farms B and F, with no detectable or low neutralizing antibody levels but with high ELISA values, were not vaccinated but may have been infected with some IBV strains not neutralized by the antiserum to strain B42. These observations coincided with the fact that chicken flocks

experience a series of exposures to several serotypes of IBV (31). These findings and the results of the cross-reactions among IBV strains observed in ELISA showed that antibodies to heterologous IBV strains with different serotype specificity in Japan could be detected by ELISA. However, it is also conceivable that the high ELISA values observed on farm B might reflect the existence of small amount of neutralizing antibody (less than 8) and that the high ELISA values observed in sera, for example, of chickens on farms E and F, with low levels of neutralizing antibody might result from the existence of antibodies not responsible for neutralization. The latter hypothesis is supported by the finding that internal components of IBV particles are likely to be one of major reacting antigens in ELISA (61).

As shown in Table 3, farms with high neutralization antibody titers, 64 or more in mean value, showed high positive rates (100%) in ELISA. Similarly, as shown in Fig. 2, there were no ELISA values of less than 0.5 in serum samples with neutralization antibody titers of 64 or more. Therefore, in chicken groups with high neutralization antibody titers, positive rates obtained by ELISA corresponded with the neutralization antibody titers. In contrast, on farms with low neutralization antibody titers, the positive rates corresponded with the mean ELISA values but not with the neutralization antibody titers. As shown in Fig. 2, 13 of 49 serum samples (26.5%) with neutralization antibody titers of 8 to 32 were negative in ELISA (ELISA values of less

than 0.5). Thus, a mismatch between neutralization antibody titers and ELISA values was observed in some sera. However, these 13 samples corresponded to 8.8% of the 148 serum samples. Therefore, when serum samples of chickens with low immune status are examined by ELISA, special attention must be paid to the interpretation of the ELISA values obtained. Similar observations were reported by Garcia and Bankowski (29) but they did not show exact data. However, no significant difference was observed between positive rates obtained by the neutralization test (94.6%) and by ELISA (91.2%). Furthermore, these findings also indicate that the ELISA reported herein is suitable for qualitative rather than for quantitative usage.

Cross-reactivity among certain IBV strains has been reported in the hemagglutination inhibition test (2). King and Hopkins (37), however, serotyped IBV isolates by the test. This method, therefore, requires several IBV strains for demonstration of antibodies to unknown IBV strains. In this ELISA, strain B42 of IBV had the ability to react with chicken antisera against not only four strains belonging to a different serotype (Gray, A5968, Iowa 97 and Iowa 609) but also against six isolates designated as KH, ON, Takeshima, S4W, 0353, and Shizuoka in Japan (Tables 1 and 2). Thus, cross-reactivity in ELISA was proven among more IBV strains than tested before (29, 44, 45).

On the other hand, the neutralization test, which has been widely used for detection of antibody to IBV, is very useful for

monitoring immune responses to vaccines in chicken flocks. The test, however, requires considerable time to perform even a single assay and various strains to confirm an outbreak of IB in sera from convalescent chickens. From this point of view, ELISA is rapid to perform and requires only one strain even to detect antibodies against serologically heterologous strains of IBV.

Most workers using ELISA for the detection of antibodies to IBV have employed the end-point method for the expression of ELISA titers. Case et al. (9) employed a high-ionic-strength serum diluent to eliminate nonspecific binding of the serum protein to the solid phase. For this purpose, a negative antigen and a diluent containing 0.1% NP-40 and 10% inactivated calf serum were employed in the ELISA system described in this paper. However, the possibility that the moderate ELISA values observed in some serum samples with low neutralization antibody titers resulted from non-specific reactions could not be denied because ELISA is a highly sensitive reaction. Therefore, when moderate ELISA values are obtained, for example, in evaluation of the responses of aged chickens to IBV vaccine, it may better to accompany it with the neutralization test.

In this study, thus, a large number of serum samples could be handled at one time by employing a single serum dilution. ELISA in the present study, therefore, seems to be easier to perform and to employ to screen a large number of chickens in a serological survey.

Brief Summary

ELISA using strain B42 of IBV as an antigen was developed for the detection of antibodies against IBV. Purified IBV solubilized with Triton X-100 served as the antigen. In this method conspicuous cross-reactions were observed among IBV strains B42, Gray, and A5968 (Massachusetts, Delaware, and Connecticut serotypes, respectively) in comparison with the neutralization test. In addition, in ELISA strain B42 also reacted with antisera against two other serotypes (Iowa 67 and Iowa 609) and with those against six isolates from Japan. Thus a wide range of cross-reaction was observed with strain B42. On the other hand, examination of the correlation between ELISA and the neutralization test in 148 serum samples from chickens in field cases and those experimentally infected with strain B42 indicated that the positive threshold line should be put at 0.5. The ELISA technique reported in the present study, thus, appears to be suitable for large-scale serological surveys because it is rapid and simple to perform. Moreover, it is useful for the confirmation of a suspected IB outbreak in chicken flocks.

Chapter II

Detection of the common antigen shared among strains of infectious bronchitis virus by a highly sensitive enzyme-linked immunosorbent assay using a monoclonal antibody

Introduction

An outbreak due to infection with IBV is usually confirmed by isolation of the causative agents and detection of antibodies to the agents. The isolated causative agents have been mainly identified as IBV by the neutralization-test method, which is the most reliable and easily available. However, a large number of IBV strains have been isolated from IB-suspected chickens, and attempts have been made to classify these strains by using a serological approach. On the basis of neutralization tests, numerous serotypes of IBV strains have been recognized (20, 23-25, 31-33, 71, 72). The existence of such numerous serotypes of IBV appears to require repeated neutralization tests for precise identification of the agent as IBV.

As described in Chapter I, ELISA is a rapid and simple method in comparison with the neutralization test and can detect

antibodies to heterologous strains of IBV with different serotype specificities (47). However, there are a few reports about the detection of IBV antigen; Yagyu and Ohta (75) recently reported the detection of IBV antigen with a polyclonal antibody in allantoic fluid of infected embryonated chicken eggs and tracheal emulsions of infected chickens. They, however, insisted that the sensitivity of the ELISA they developed was not satisfactory. Furthermore, Mabs to IBV have been prepared and characterized in some laboratories (38, 46, 53). In this study production of Mabs to IBV strain B42 and application of a Mab directed to N protein for the detection of IBV antigens by ELISA are described.

Materials and Methods

Viruses used in this study:

The IBV strains used were B42, Gray, A5968, Iowa 609, ON, KH, Nerima, SS, Takeshima, Shizuoka, S4W and 0353. Viruses other than strains SS and Nerima are the same as described in Chapter I. Strain Nerima is commercially available as a vaccine strain and strain SS isolated from sick chickens was kindly provided by Dr. H. Kawamura of the National Institute of Animal Health. Strains B42, SS and Nerima were classified into the Massachusetts serotype (see Chapter III). These viruses were propagated as described in Chapter I.

Primary cell culture:

Primary CEK cell culture was carried out as described in Chapter I.

Preparation of monoclonal antibodies against IBV strain B42:

Purified B42 virus was disrupted in 0.1% Triton X-100 and used for the inoculum to BALB/c mice. Hybridoma cells were prepared from the immunized mice as described by Hohdatsu et al. (34). Briefly, BALB/c mice, five weeks of age, were inoculated intraperitoneally with a mixture of 50 μg of the viral antigen prepared as described above and 10^9 cells of pertussis adjuvant, and boosted three times at two-week intervals in a similar manner. Eight weeks after the initial immunization they were boosted intravenously with the same amount of disrupted antigen without adjuvant. Spleen cells from the mice were fused with myeloma cell line P3/X63-Ag8.653 three days after the last immunization. Hybridoma cells were screened for antibody production by ELISA, and antibody-secreted cells were cloned on soft agar (34). Immune ascitic fluid was made in BALB/c mice primed with pristane (2,4,10,14-tetramethylpentadecane; Aldrich, Wisconsin, U.S.A.).

Preparation of chicken antiserum against IBV strain B42 and its IgG fraction:

Virus samples for the immunogen were purified as follows. Clarified allantoic fluid containing virus was concentrated by

centrifugation at 50,000 xg for 2 hr. The concentrated virus sample was applied to a Sephacryl S-1000 (Pharmacia, Uppsala, Sweden) column (1.0 by 90 cm) equilibrated with 0.02 M phosphate buffer (pH 7.2) containing 0.15 M NaCl. The column was eluted with the same buffer at a flow rate of 6 ml per hour and each 3 ml of the eluate was fractionated. Fractions containing virus particles were pooled and concentrated by centrifugation (48).

SPF chickens aged 50 days were intranasally and intraocularly infected with a purified virus sample whose virus dose was 10^7 to 10^8 $TCID_{50}$ at three-week intervals. They were intravenously infected with the virus seven days before being bled. Serum samples were collected from the chickens and inactivated at 56°C for 30 min. Chicken IgG was prepared from the serum samples by 50% ammonium sulfate precipitation and gel chromatography through a Sephacryl S-300 (Pharmacia, Uppsala, Sweden) column equilibrated with PBS. The prepared IgG was stored at -20°C until use.

ELISA procedure:

ELISA was performed according to the method described in Chapter I with slight modifications. One microgram of chicken IgG against IBV strain B42 was coated on ELISA plates (Sankojunyaku, Tokyo). After washing with washing buffer, 0.1 ml aliquots of the viral samples treated with 1% NP-40 and diluted 10-fold with dilution buffer were added to the wells of the ELISA plates and they were incubated at 37°C for 1 hr. After washing, Mab 1F

was added to the plate and incubated at 37°C for 1 hr. Following the addition of horseradish-conjugated rabbit anti-mouse IgG to the wells, the plates were incubated again at 37°C for 1 hr, and then washed. Peroxidase binding was visualized by 2-2'-azinobis-3-ethylbenzthiazoline sulfonic acid (ABTS; Sigma, U.S.A.) at 25°C for 10 min and the reaction was stopped by the addition of 0.1 ml of 0.1 N NaOH. Absorption was measured at 405 nm in a microplate photometer (Corona Electric, Ibaraki).

Recovery experiment from IBV-infected embryonated eggs:

One-tenth ml of the PBS containing a 50% egg infectious dose (EID_{50}) of 10^2 of IBV strain B42 or A5968 was inoculated in the allantoic cavities of SPF eggs. The allantoic fluid was harvested from five eggs each 5 hr and 1, 2, 3, and 4 days after inoculation. These samples served for both ELISA and virus titration in CEK cell cultures.

Recovery experiment from tracheas of IBV-infected chickens:

SPF chickens were inoculated intranasally with 10^3 EID_{50} of IBV strain M41 or A5968. The respiratory tracts were harvested from three chickens once a day for one week . They were homogenized to 10% (w/v) emulsions with PBS. The samples served for both ELISA and virus titration in embryonated chicken eggs.

Results

Monoclonal antibodies against IBV strain B42:

After being cloned three times on soft agar, five hybridoma cell lines secreting antibodies against a subunit (S2) of spike, N or M protein were established. Mabs secreted from these cell lines were tested for reactivity with other strains in ELISA. Mabs 1F and 3H reacted with all of the strains tested (Table 1). Mab 1F was employed for the following experiments because of its higher ELISA value (1.98) than Mab 3H (0.58). On the other hand, two strains, Nerima and SS, as well as homologous strain B42 belonging to the same serotype, reacted with all five Mabs with high titers.

Recovery experiment from IBV-infected embryonated eggs:

To determine the negative threshold line, ELISA values of allantoic fluid collected from 95 SPF embryonated chicken eggs 14 days old were measured. The mean value of the samples was 0.17 and the standard deviation was 0.03. Therefore, the negative threshold line for 1% probability of misclassification (the mean value plus three times the standard deviation) was set at 0.26. Infectious IBV was recovered from embryonated eggs inoculated with strain B42 or A5968 one day after inoculation. Virus titers of the embryonated eggs infected with strain B42 declined slightly at three days whereas those inoculated with strain A5968 did

Table 1 Cross-reactivity of monoclonal antibodies to IBV-B42 with other IBV isolates

Viral strains	Monoclonal antibodies (Protein specificity)[1]				
	3G (S2)	1F (N)	1E (M)	3H (M)	1C (M)
B42	+++[2]	+++	+++	+++	+++
Nerima	+++	+++	+++	+++	+++
SS	+++	+++	+++	+++	+++
Gray	−	++	+++	+	−
A5968	−	++	++	+++	−
Iowa 609	++	++	−	++	−
ON	+++	+++	+++	+++	+
KH	+++	+++	+	++	−
Takeshima	+++	+++	+	++	−
Shizuoka	++	+++	−	++	−
S4W	++	++	−	++	−
0353	+	++	−	+	−

1) Analysed by Western blotting.
2) The amount of monoclonal antibody used was twice the amount which gives maximal binding to IBV strain B42. Binding to the heterologous virus is expressed as the percentage of the absorbance obtained with IBV-B42. +++:75-100%, ++:50-75%, +:25-50%, and −:less than 25%.

not change throughout the observation period. The ELISA values of the samples also became positive one day after inoculation. There was no significant fluctuation in ELISA values of any viral strains tested. The limit of the virus titer detectable by ELISA was calculated as approximately 10^4 $TCID_{50}$ for strain B42 and 10^5 $TCID_{50}$ for strain A5968 (Fig. 1).

Recovery experiment from tracheas of IBV-infected chickens:

Similar to the case of the allantoic fluid used to determine the negative threshold line, the ELISA values of tracheal emulsions of 10 two-week-old SPF chickens were measured. Based on the results, the negative threshold line was set at 0.24. Strain M41 was employed as the inoculum since strain B42 was no longer infectious to chickens and because it belongs to the same serotype as strain B42. Infectious IBV was recovered from tracheal emulsions from the chickens inoculated with strain M41 or A5968 two days after inoculation. The titers of the emulsions reached their peaks at four to five days after inoculation in strain M41 and at three to four days in strain A5968. ELISA values of the emulsions from chickens inoculated with strain M41 became positive four to five days after inoculation and three to four days after inoculation with strain A5968. The time at which the ELISA value was converted to positive coincided with the peaks of the virus titers of each strain. The limit of the virus titer detectable by ELISA was calculated as 10^5 EID_{50} for both strains

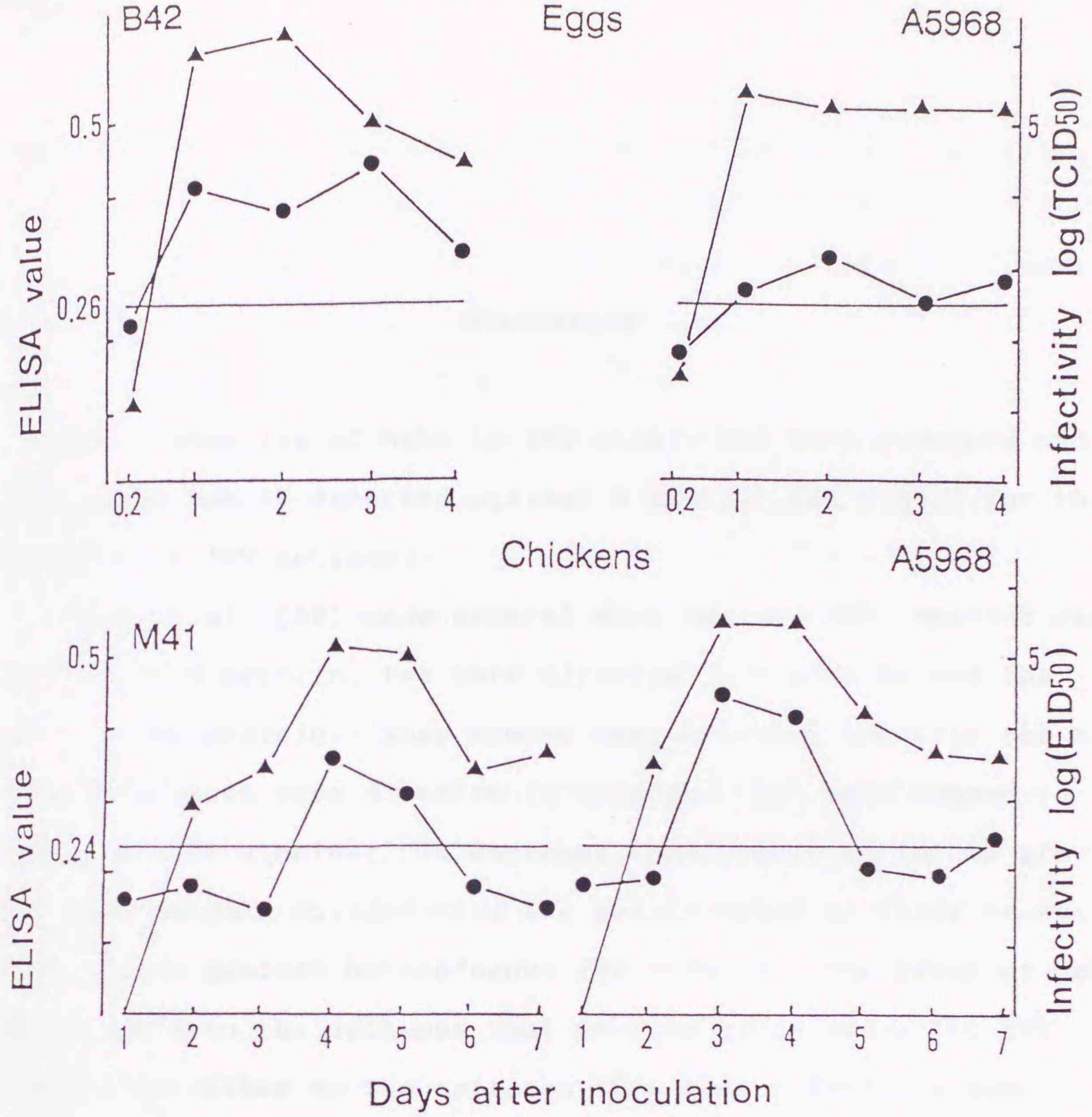

Fig. 1. Virus detection from SPF embryonated chicken eggs infected with IBV-B42 or A5968 and from respiratory tracts of chickens infected with IBV-M41 or A5968. Samples were tested with ELISA (●) and virus titration (▲). Straight lines (at point of 0.26 and 0.24) represent the negative-threshold lines estimated with ELISA values of allantoic fluids from 95 non-infected SPF eggs and of respiratory tracts of 10 non-infected chickens, respectively.

(Fig. 1).

Discussion

The properties of Mabs to IBV strain B42 were examined and ELISA using Mab 1F directed against N protein was tested for the detection of IBV antigen.

Koch et al. (38) made several Mabs against IBV; one Mab was directed to M protein, two were directed to N protein and the others to S2 protein. They showed that the Mabs specific for M or for N protein were directed to epitopes that were common to almost all IBV strains. In contrast, Mabs specific for S2 protein were roughly divided into two groups based on their cross-reactivities against heterologous IBV strains. One group of Mabs was directed to the epitopes that were variable among the IBV strains; the other to the epitopes that were relatively conserved. In this study Mab 3G specific for S2 protein reacted with nine of the 11 strains used. Therefore, Mab 3G, similar to the latter group, seemed to be directed to a relatively well-conserved region on S2 protein. In contrast, Mabs specific to M protein showed a heterogeneous pattern of cross-reaction; Mab 3H reacted with all IBV strains tested although Mabs 1E reacted with seven strains and 1C with only three strains. This suggests that there are variable epitope(s) as well as common epitope(s) on M

protein. Thus, the cross-reactive pattern of Mab 3H coincided with that reported by Koch et al. (38) but those of Mabs 1E and 1C were different. It is interesting that three strains reacting with Mab 1C at high titers were in the same serotype. This may suggest that there is serotype specific region on M protein as well as on S1 protein. However, Mab 1F specific for N protein, isolated in the present study, reacted with all IBV strains tested. In addition, Brown et al. (8) reported that the ^{32}P-labeled probe complementary to the nucleotide sequence of the IBV genome RNA encoding N protein hybridized with all RNA samples prepared from IBV strains. These observations seem to suggest that the N protein of IBV has a common epitope. Accordingly Mab 1F was employed to capture the IBV antigen in ELISA.

As shown in Fig. 1, a notable decrease of the virus titers was not observed only in strain A5968 propagated in embryonated chicken eggs. The precise reason why the virus titers of strain A5968 did not decrease in embryonated eggs is unknown. It is conceivable, however, that this is caused by the difference of the growth property of strain A5968 in embryonated eggs because of lesser adaptation of the strain to eggs than strain B42.

Yagyu and Ohta (75) reported that IBV antigen had been detected from strain M41-infected SPF embryonated eggs by ELISA and that the limit of the virus titer detectable by ELISA had been calculated to be about 10^6 EID_{50}. In this study it was shown that more sorts of IBV antigens in different serotypes could be

detected by ELISA because Mab 1F, reactive with every strain, was employed in this ELISA system. Furthermore, the ELISA reported in the present study was shown to be a more sensitive assay than that reported by them (75).

Until now, up-to-date confirmation of outbreaks of IBV infection has been made by virus isolation and succeeding identification of the isolates by the neutralization test. It is, however, time-consuming for virus isolation because at least three blind passages are necessary for the proper confirmation of the result concerning virus isolation. In addition, the isolates must be sequentially identified, usually by repeated neutralization tests, as IBV. The greatest advantage of ELISA is that the IBV antigen can be rapidly detected and simultaneously identified as IBV. If necessary, samples positive by ELISA can serve for virus isolation. In addition, as shown in Fig. 1, since the time of the antigen detectable in tracheal emulsions is limited, tracheal samples may be better passed through eggs to increase sensitivity for the detection of antigen. The limitation for the detection of IBV antigen in the tracheal emulsions might be due to inhibitors contained in the tracheal tissue. Thus, the results show that it may be possible to apply ELISA for the confirmation of an outbreak by IBV infection in chickens by antigen detection.

Brief Summary

ELISA was developed for the detection of IBV antigen with a Mab. Five Mabs reacting with IBV strain B42 by ELISA were obtained. There were differences in the cross-reactivities of the Mabs to other IBV strains with ELISA. Mab 1F directed against N protein with a molecular weight of 50 kDa was employed for ELISA because N protein was regarded as a common antigen via antigenic analysis using various Mabs and because of its nucleotide sequence. ELISA plates coated with anti-B42 chicken IgG were provided for the reaction. When strain B42 or A5968 was inoculated into the allantoic cavity of 11-day-old SPF embryonated chicken eggs, each viral antigen was detected by ELISA one day after inoculation and later. The limits of the virus titers of strains B42 and A5968 detectable by ELISA were calculated to be about 10^4 and 10^5 $TCID_{50}$, respectively. Viral antigens were also detected from two-week-old SPF chickens infected with strain M41 or A5968 at two days by the culture method, but at three to four days by ELISA. The limit of the virus titer was also calculated to be about 10^5 EID_{50}. These results show that this ELISA system has the ability to react with IBV strains of several serotypes with high sensitivity, is useful to identify isolates as IBV, and is rapid and simple to perform.

Chapter III

Dot-blot hybridization using a digoxigenin-labeled cDNA probe complementary to the S1 region of the S gene of infectious bronchitis virus permits differentiation of virus strains

Introduction

It is well known that there exists extensive antigenic diversity among IBV strains as detected mainly by the neutralization test (20, 23-25, 31-33, 71, 72), although this diversity is not observed in most other coronaviruses. The existence of various serotypes in IBV has made it difficult to precisely and rapidly confirm outbreaks by serological methods. Chapters I and II described a rapid and sensitive ELISA for the detection of antibody and IBV antigens regardless of serotype specificity that showed wide reactivity against IBV strains of several serotypes.

As the next step in the diagnosis of IBV, differentiation of an isolate is necessary for protection of a flock against infection by, for example, establishment of a vaccination program. ELISA is not suitable for this purpose. Classification of an

isolate into a given serotype has been attempted by the neutralization test. However, all isolates are not always serotyped clearly by this test; some European strains of IBV, for example, react with both antisera against strains belonging to different serotypes (24).

In contrast, S protein (15), especially its S1 polypeptide region (16), carries a neutralization epitope and is responsible for serotype specificity. In addition, the nucleotide sequence of the gene coding for S protein has been determined in some IBV strains (3, 4, 52, 68). By utilizing the cDNA coding for the entire S gene of IBV, I prepared a digoxigenin-labeled cDNA probe complementary to the S1 region. This chapter describes the application of the cDNA probe to dot-blot hybridization for differentiating IBV strains.

Materials and Methods

Viruses:

The following 19 IBV strains were employed in this study: i) strains M41, B42, Nerima, SS and 7073 (Massachusetts serotype), ii) strain A5968 (Connecticut serotype), iii) strain Holte (Holte serotype), iv) strain Gray (Delaware serotype), v) strain Iowa 609 (Iowa 609 serotype), vi) strain Iowa 97 (Iowa 97 serotype), vii) Japanese isolates Takeshima, KH, ON, Tochigi, 0353, Shizuoka

and S4W, and viii) isolates Fe and J19 recently isolated in our laboratory. Significant cross-reactivity among the above-mentioned five strains of the Massachusetts serotype has been confirmed by our recent cross-neutralization tests (data not shown).

Virus growth and isolation of viral RNA:

For cDNA cloning, strain M41 (Massachusetts serotype) was grown in the allantoic cavity in three hundred 12-day-old embryonated chicken eggs for 36 to 48 hr at 37°C. The virus was pelleted by centrifugation at 50,000 xg for 2 hr, and the virus pellet was suspended in TBS. The virus suspension, after clarification by low-speed centrifugation, was applied to a Sephacryl S-1000 (Pharmacia, Uppsala, Sweden) column equilibrated with TBS (48). The virus-containing eluate was concentrated by centrifugation and the pellet was resuspended in TBS. Other virus strains used for hybridization tests were grown and purified in a similar manner, except that 10 eggs were used for virus growth and that column chromatographic purification was omitted.

The virus suspension was solubilized by addition of sodium dodecyl sulfate (SDS) at a final concentration of 1%. After repetitive phenol/chloroform extractions, the viral RNA in the aqueous phase was precipitated by ethanol.

cDNA cloning:

The three synthetic primers used were: primer I 5'GATTAAA-TGACCACCATGACCTACAC3' (26 mer), primer II 5'GGTATAGCACCAGCTGCA-GTAATAC3' (25 mer) and primer III 5'TTGCTTGCAACCACCTTGAAGAGG3' (24 mer). The positions of the synthetic primers are shown in Fig. 1. First-strand cDNA synthesis, following the annealing of a synthetic oligonucleotide on heat-denatured genomic RNA, was carried out with reverse transcriptase (Seikagaku Kogyo, Tokyo). Second-strand synthesis was according to the method of Gubler and Hoffman (30). The double-stranded cDNA thus obtained was cleaved with appropriate restriction endonucleases and ligated into the corresponding restriction sites of pUC18 or pUC19. The resulting recombinant plasmids were subsequently used for transformation of *E. coli* (strain TB-1). Colonies containing recombinant plasmids were screened by measurement of the size of the plasmid DNA by agarose gel electrophoresis. The cDNA for the IBV S gene was identified by digestion with appropriate restriction endonucleases, and by sequencing some of the DNA fragments by the dideoxy chain termination method (57) after subcloning the fragments into phage M13.

cDNA probe:

DNA labeling with digoxigenin-dUTP, hybridization and immunological detection of hybridized blots were carried out basically according to the manufacturer's protocol (DNA labeling and

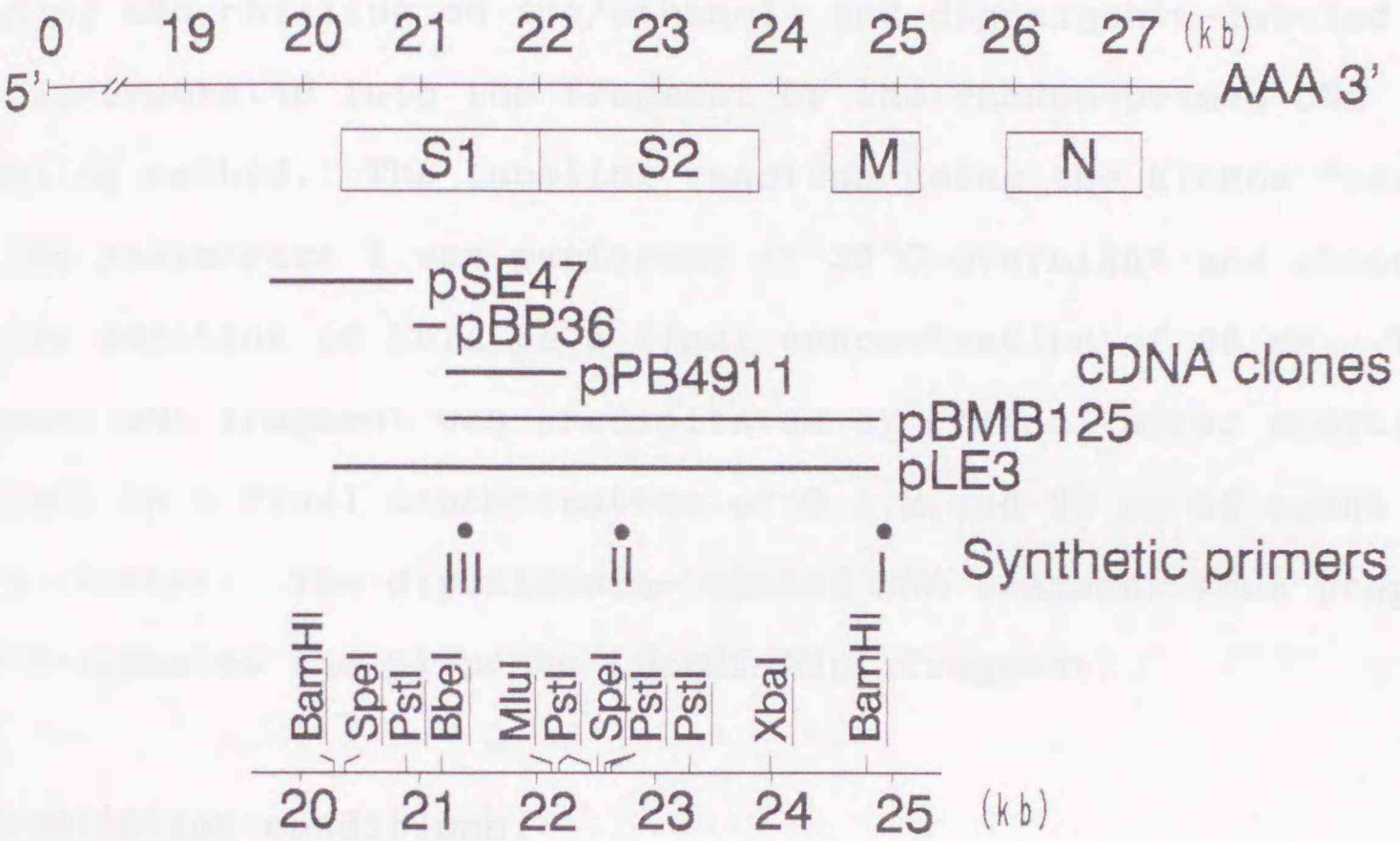

Fig. 1 Positions of the synthetic oligonucleotides used to prime cDNA synthesis and the derived cDNA clones. Cleavage sites with some restriction endonucleases are shown at the bottom. A *Bam*HI region was inserted nine nucleotides upstream from the initiation codon ATG by using a synthetic *Bam*HI linker.

detection kit-nonradioactive; Boehringer Mannheim GmbH, Penzberg, Germany) unless otherwise specified.

A *Bam*HI-*Mlu*I (1809 base pairs) fragment of pLE3 containing the whole S gene of IBV (Fig. 1) was purified from low-melting-point agarose gels. The fragment (500 ng) was denatured by heating and chilling on ice/ethanol, and digoxigenin-labeled dUTP was incorporated into the fragment by the random-primed DNA labeling method. The labeling reaction using the Klenow fragment of DNA polymerase I was performed at 37°C overnight and stopped by the addition of EDTA to a final concentration of 20 mM. The labeled DNA fragment was precipitated by ethanol after addition of NaCl to a final concentration of 0.1 M and 20 μg of yeast tRNA as a carrier. The digoxigenin-labeled DNA fragment thus prepared was designated the S1 probe (*Bam*HI-*Mlu*I fragment).

Hybridization conditions:

Genomic RNA (50 to 100 ng) purified from each IBV strain was dot blotted onto nylon membranes (Magnagraph nylon 0.45μ, Micron Separation Inc., Massachusetts, U.S.A.). The membranes were then incubated at 68°C for 1 hr in a prehybridization solution containing 50% formamide, 5X SSC (20X SSC equals 3 M NaCl and 0.3 M sodium citrate), 5% blocking reagent (contents in the kit), 0.1% N-lauroylsarcosine sodium salt (Wako Pure Chemicals, Osaka) and 0.02% SDS. The solution was replaced with a new one containing the freshly denatured labeled cDNA probe, and the membranes were

incubated for 18 hr at 56°C or 68°C. The membranes were washed twice for 5 min at room temperature in 2X SSC, 0.1% SDS and then twice for 15 min at 68°C with 0.1X SSC, 0.1% SDS. The hybridization signals on the membranes were immunologically detected. The following incubations were all performed at room temperature. In order to reduce background reactions, the membranes were incubated for 30 min in 0.5% blocking reagent, 0.1 M Tris-HCl and 0.15 M NaCl, pH 7.5 followed by brief washing with washing buffer (0.1 M Tris-HCl, and 0.1 M NaCl, pH 7.5). The membranes were immersed for 30 min in a polyclonal sheep anti-digoxigenin Fab-fragment conjugated to alkaline phosphatase (150 mU/ml) diluted with the washing buffer. Unbound conjugate was removed by washing twice for 15 min with the washing buffer. The color was developed by overnight incubation with nitroblue tetrazolium salt (338 ng/ml) and 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (175 ng/ml in 0.1 M Tris-HCl, 0.1 M NaCl and 50 mM $MgCl_2$, pH 9.5). The membranes were washed for 5 min with 10 mM Tris-HCl, 1 mM EDTA, pH 8.0, and were photographed.

Results

DNA cloning:

The strategy for cDNA cloning was devised on the basis of the sequence data of the S gene (strain M41) reported by Niesters et al. (52) and those of the M protein gene and mRNA 3 reported by Boursnell et al. (5, 6). By using three oligonucleotide primers to prime cDNA synthesis from viral RNA, four recombinant DNA clones were obtained (Fig. 1) and further characterized by restriction site mapping and by sequence determination after subcloning into M13 phage vectors. Recombinant plasmid DNA pLE3 containing the complete S gene insert of IBV was constructed by using the four cDNA recombinant clones. In pLE3, a *Bam*HI site was inserted nine nucleotides upstream from the initiation codon ATG by using a synthetic *Bam*HI linker.

The *Bam*HI-*Mlu*I (S1) fragment derived from pLE3 was labeled with digoxigenin as described in the section of Materials and Methods. Figure 2 shows the relative location of the S1 probe. The probe (1809 bases) includes the S1 gene accompanied by eight nucleotides (5'GATCCACC3') upstream from the initiation codon ATG and the 5' region (190 bases) of the S2 gene. The digoxigenin-labeled probe was evaluated for dot-blot hybridization assays using IBV strains representing six different serotypes and Japanese IBV strains. The specificity of the hybridization assay was confirmed by the failure of the probe to hybridize with yeast

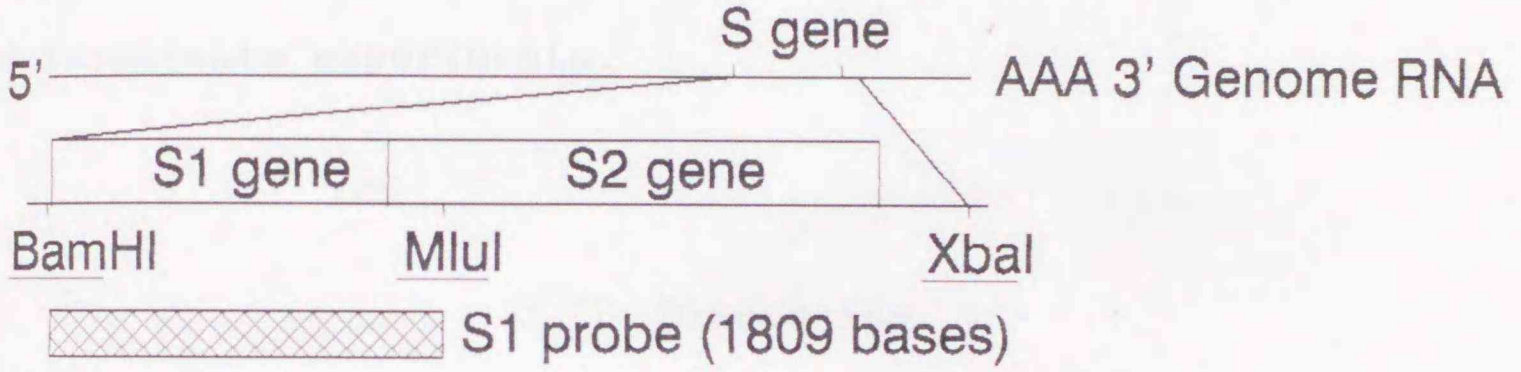

Fig. 2 Location and size of the S1 probe used for dot-blot hybridiation assays.

tRNA (see below).

Hybridization of the S1 probe:

Figure 3 shows the result of the hybridization assay using the S1 probe. At 56°C the probe reacted with RNA samples from strains B42, Nerima, SS and 7073 (spots B, J, M and P in Fig. 3, respectively) of the Massachusetts serotype as well as with that from the homologous strain. In contrast, the probe reacted only with the homologous strain at 68°C. The same result was obtained in triplicate experiments.

Discussion

S protein forms the petal-shaped peplomer or spikes of IBV. It consists of amino-terminal glycopeptide S1 and carboxy-terminal glycopolypeptide S2. In the present study, the cDNA (pLE3) coding for the entire S gene of IBV (strain M41) was generated, and DNA fragments representing S1 regions were prepared from the pLE3 clone. The cDNA probe labeled with digoxigenin was employed for the dot-blot hybridization.

The S1 probe at 68°C hybridized exclusively to RNA from the homologous strain, M41: the probe lacked the ability to hybridize even with strains B42, Nerima, SS and 7073 of the Massachusetts serotype. This suggests that there is genetic variation in the

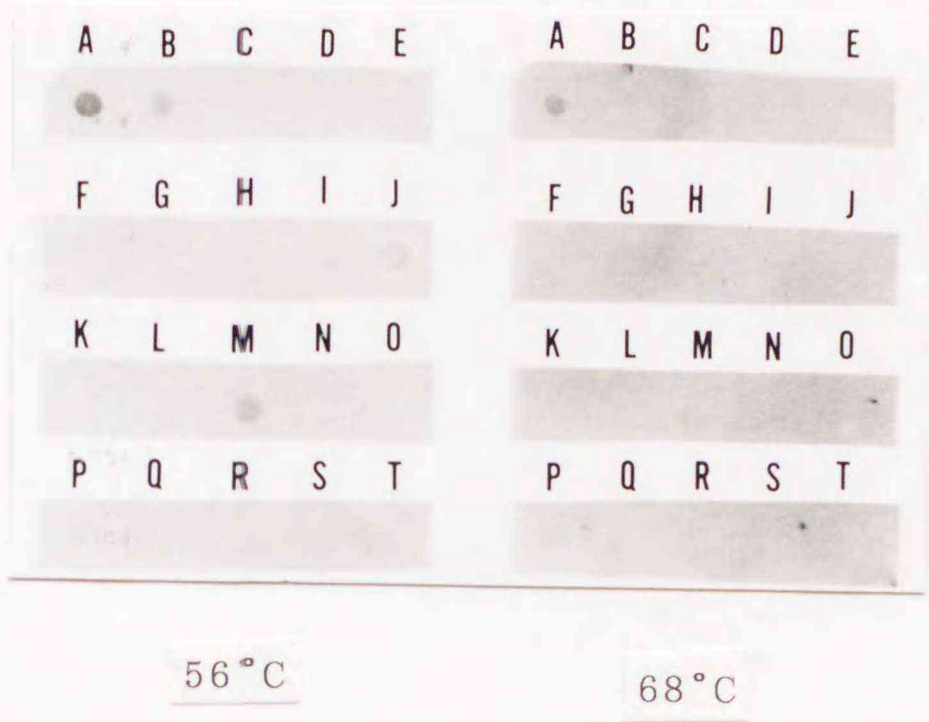

Fig. 3 Dot-blot hybridization of the digoxigenin-labeled S1 probe with RNA samples from various IBV strains. RNA samples for testing were extracted from IBV strains M41 (A), B42 (B), Gray (C), Iowa 609 (D), Iowa 97 (E), Holte (F), A5968 (G), Takeshima (H), KH (I), Nerima (J), ON (K), Tochigi (L), SS (M), 0353 (N), Shizuoka (O), 7073 (P), S4W (Q), Fe (R), J19 (S). As a negative control yeast tRNA (T) was employed.

S1 gene even among strains belonging to the same serotype. This finding also seems to reflect the various degrees of antigenic diversity observed among IBV strains in the same serotype (23).

On the other hand, classification of IBV strains has been based on serology, particularly on the neutralization test. In some cases unequivocal assignment to a serotype of the IBV strains is complicated by the phenomenon of one-way neutralization or the ambiguous results of cross-neutralization (24). An attempt was made to classify IBV strains into clusters by T1 fingerprinting using whole-genome RNA (39). In that study IBV isolates not clearly belonging to serotypes were apparently classified into three genotypes regardless of their serological relatedness (39). In contrast, the S1 probe used in this study, when reacted at 56°C, showed a serotype-specific reactivity pattern; the probe exhibited a positive reaction with strains B42, Nerima, SS and 7073 as well as homologous M41, all of which were found to belong to the Massachusetts serotype. Moreover, the probe did not react with the RNA sample from strain Tochigi, which showed unclear cross-reactivity with strains SS and 7073 (25). This implies the utility of the S1 probe for clearly differentiating IBV strains into a given serotype.

This technique may be applicable for diagnosis of IB. For diagnosis resulting from virus isolation it seems to be necessary to identify isolates as IBV and then differentiate them. Two methods recently developed are applicable to confirming the

identity of isolates with different serotype specificities as IBV even when the neutralization test is ineffective: one is a hybridization test using a ^{32}P-labeled cDNA probe complementary to the genomic RNA encoding the N protein of IBV strain B42 (8), and the other is an ELISA using a monoclonal antibody directed to the N protein of strain B42 (49). These two methods, however, are not suitable for the differentiation of isolates. The results of the present study show the differentiation of IBV strains to be possible. Furthermore, the use of nonradioactive digoxigenin-dUTP instead of ^{32}P-dATP for DNA labeling has several advantages in respect to cost, safety, and facilities.

Brief Summary

The cDNA clone complementary to the S gene of IBV strain M41 was obtained. A DNA probe labeled with digoxigenin-dUTP by the random-primed method was prepared from the cDNA clone; the S1 probe carries the coding regions of the S1 gene. The probe was applied to a dot-blot hybridization assay with RNA samples prepared from 19 IBV strains to differentiate the IBV strains. At 56°C the S1 probe reacted exclusively with four strains that were grouped in the same serotype by cross-neutralization tests and at 68°C reacted only with the homologous strain. These results suggested that hybridization using the S1 probe was serotype-spe-

cific at 56°C and strain-specific at 68°C. Therefore, it was shown that the S1 probe was applicable to the differentiation of IBV strains. Thus, this technique should be applied to determining serotypes of virus isolates.

Conclusion

For diagnosis of IB several serological techniques such as neutralization, hemagglutination-inhibition, agar gel precipitin and immunofluorescence tests have been employed. These methods, however, require several type strains or antisera of different serotypes for accurate diagnosis because of the existence of numerous serotypes in IBV. Recently ELISA was developed for the detection of antibody to IBV. In the present study, a simple and dependable diagnostic method for outbreaks of IBV using ELISA was investigated.

In comparison with the results obtained by the neutralization test, in ELISA strain B42 reacted at high titers with various antisera against heterologous IBV strains, not only four type strains with different serotype specificities but also six Japanese isolates distinguished from each other by the neutralization test. The results indicated that antibodies against IBV strains belonging to several serotypes can be detected by ELISA using a single strain, B42, as an antigen. The positive threshold line for ELISA was defined as 0.5. Furthermore, by using one-point dilution of serum samples the present ELISA was rapid and simple to perform and, therefore, appears to be suitable for surveying a large number of chickens.

In addition, ELISA using a Mab directed to the nucleocapsid of strain B42 was obtained. The Mab showed a wide-range of reac-

tivity with several IBV strains. Accordingly, ELISA using the Mab was developed for the detection of IBV antigens. By this ELISA both homologous and heterologous IBV strains were detected in the allantoic fluid of virus-inoculated embryonated chicken eggs and the tracheal emulsion of infected chickens with a high sensitivity. Thus the present ELISA, a rapid and simple method, developed in this study was proven to be able to detect antibodies to heterologous IBV strains in different serotypes or IBV strains belonging to different serotypes by using a single antigen or a single Mab, respectively.

Virus isolates could be identified as IBV by ELISA as mentioned above. At the next step, the virus isolates have to be differentiated. Many IBV strains have been classified into a serotype by neutralization tests but some strains are difficult to assign to a clean-cut classification. Accordingly, differentiation of IBV strains was attempted by the genetic approach. Initially, a cDNA clone was obtained from the S gene of IBV strain M41 and a digoxigenin-dUTP-labeled S1 probe containing the S1 region of the S gene was prepared. The probe was used for dot-blot hybridization to genomic RNA of IBV strains belonging to different serotypes. At 56°C the S1 probe exclusively hybridized RNA samples from four strains, B42, Nerima, SS and 7073, of the Massachusetts serotype, as well as that from homologous strain M41. In addition, it is noteworthy that the probe did not react with RNA of strain Tochigi, which ambiguously cross-reacts with

strains Nerima and SS. The results suggest that IBV strains can be differentiated by dot-blot hybridization using the S1 probe. By preparation of an S1 probe from the type strain the hybridization assay would be simple to perform and rapidly provide results compared with the neutralization test.

This study reported the application of ELISA to diagnosis of IB by detection of antibodies or antigens with a single antigen or a single Mab, respectively. Furthermore, the possibility of differentiation of IBV isolates on the basis of the dot-blot hybridization assay was suggested. Thus, this study introduces a rapid and simple procedure for diagnosis, from identification to differentiation, of an isolate.

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