Enzyme-Linked Immunosorbent Assay for Detection of Infectious Bronchitis Antibody in Chickens Using Local Isolate of PTS III
(Enzyme-linked Immunosorbent Assay Untuk Mendeteksi Antibodi Infectious Bronchitis pada Ayam dengan Isolat Local)

Risa Indriani & NLP. Indi Dharmayanti

Institute Centre Research of Veterinary, Jl. RE Martadinata no 30, Bogor 6114. **E-mail**: risain52@yahoo.com

Memasukan: September 2012, **Diterima:** April 2013

ABSTRACT

An indirect enzyme-linked immunosorbent assay (ELISA) was developed for screening of antibody to avian infectious bronchitis (IBV). Antigen was prepared from whole virus of infectious bronchitis local isolate PTS-III serotype. Optimum dilution with minimum background for antigen concentration, rabbit anti-chicken conjugate and sera in developed ELISA were determined 0.4µg/well, 1:2000 and 1:100, respectively. Correlation optical densities (OD) were compared with a standard commercial ELISA (R2=0.933). The developed ELISA has a better sensitivity to hemagglutination inhibition (HI) test. The developed local isolate ELISA can be used to detect antibody against infectious bronchitis virus and it is suitable for sample screening at the diagnostic laboratories.

Keywords: ELISA, antibody, chicken, IB PTS-III local isolate

ABSTRAK

Pengembangan Enzyme-linked immunosorbent assay (ELISA) untuk mendeteksi antibodi virus infectious bronchitis (IB) pada ayam. Antigen ELISA IB disiapkan dari virus IB serotipe isolat lokal PTS-III. Pengenceran optimal pada konsentrasi antigen, konjugat *rabbit anti-chicken*, dan serum dengan *background* minimum dalam uji ELISA adalah 0.4µg, 1:2000 and 1:100 secara berurutan. *Optical density* (OD) dari serum IB positif pada uji ELISA yang dikembangkan dan ELISA standard mempunyai tingkat korelasi yang tinggi (R2=0.933). ELISA yang dikembangkan mempunyai tingkat sensitifitas lebih baik terhadap uji hemagglutination inhibition (HI). Uji ELISA IB serotipe isolat lokal PTS-III dapat digunakan untuk screening sampel dalam laboratorium diagnostik.

Kata Kunci: ELISA, antibodi, ayam, IB isolat lokal PTS-III

INRODUCTION

Infectious Bronchitis virus (IBV) infects the respiratory tract, kidneys or oviduct of chicken of all ages causing retarded growth, mortality, reduced egg production and inferior egg shell quality (King & Cavanagh 1991). The virus belong to family of Coronaviridae. Corona viruses are enveloped, pleiomorphic, with a mean diameter of approximately 120 nm, club-shaped surface projections -the heavily glycosylate spike (S) glycoprotein. IBVs contain four structural proteins. The S1 and S2 glycoproteins, from the spike or peplomer (S) located at surface of virion. In the peplomer S, the S2 glycoprotein forms the stalk and is anchored in the membrane, whereas the S1 forms the outer bulbous part of the peplomer. The membrane (M) glycoprotein is embedded in the viral lipid bilayer and is only partially (10%) exposed on surface of virion. The nucleocapsid (N) protein is located inside the virion, associated with viral RNA (Cavanagh 1983a,b,c). The role of the S1 glycoprotein in induction of humoral antibody responses, and it induces virus neutralizing (VN) and haemagglutination inhibiting (HI) antibodies (Cavanagh *et al.* 1984, Koch *et al.* 1990; Kant *et al.* 1992). The S1 glycoprotein has been considered to be the most likely inducer of pro-

tection (Cavanagh *et al*. 1986, Ignjatovic & Galli 1995) and cross-ractive epitopes are the most likely to be involved in protection. The S2 glycoprotein carries epitop which induce cross-reactive antibodies (Kusters e*t al.* 1989; Lenstra *et al*. 1989; Koch *et al.* 1990). Immunity to IBV has most often been assessed using traditional serological assay; however, the enzyme-linked immunosorbent assay (ELISA) is used on a more frequent basis to measure IBV antibodies (Case *et al.*1982). The technique initially developed by Engvall & Perlmann (1971) and has been widely used. The use of ELISA offers a number of advantages compared with traditional serological assays, including increase sensitivity and simplicity of automation (Garcia & Bankowski 1981). The cross reactivity of the IB ELISA with several strains of the virus and detection antibodies against other serotypes (Zellen &Thorsen 1986 ; De Wit 2000) support the idea that ELISA is a promising tool for serological studies, especially for use in evaluating the efficacy of vaccination regimens and monitoring the immune status of birds in a flock (Cavanagh & Naqi 2003; Wing *et al.* 2002). Indirect ELISAs were adapted and standardized for detecting antibodies against whole virus, and S1, S2 and N protein of IBV (Ignjatovic & Galli 1995; Wing *et al*. 2002) and compared with other serological test (De Wit *et al.* 1997; Perrotta *et al.* 1988; Thater *et al.* 1987). This paper describes study of indirect ELISA for detection of antibody against IBV by using local isolate virus (PTS-III), which its according to the virus circulating in the field.

MATERIAL AND METHODS

The local isolate IBV of PTS III as described by Darminto (1992) was propagated in specific pathogenic free emberyonated chicken eggs at day 10 (Biofarma, Indonesia) by infecting the virus to allantoic fluid then incubated at 37^{0}C for 72 hours. A thousand ml allantoic fluid was harvested and clarified at 8000g for 30 minutes then EID_{50} was calculated. It was inactivated 0.05% β-PL and centrifuged at 90.000g for 90 minute in a sorvall AT- 629 (32ml) at 4^{0}C. The pooled result was pelleted in TEN buffer (150 mM NaCl, 10mM Tris-hydrochloride, and 1mM EDTA, pH 7.4) and analyzed for protein content by using Spectrophotometer/Nano Drop Tecnolo -gies ND-1000 base on Bradford's method. The solution was finally divided into several aliquots and frozen at 70^{0}C.

Serum IBV was prepared from 20 of specific pathogenic free chickens that reared under controlled conditions in the isolator (BSL-3) and vaccinated day 10 with live H120 (vaccine commercial), day 44 with inactivated oil-emulsion vaccine PTS-III (BBALITVET) and day 57 challenge with 10^7EID_{50} of the same virus. The chickens were bled at day 10, 24, 34, 44, 57, 70, 78 and 89 of age. A group of specific pathogenic free chicken were reared as negative control and bled at the same time.

Ortho-phenylene diamine (OPD) (Sigma P.23938) was used. Horeseradish peroxidase conjugated rabbit anti-chicken IgG (Sigma A.9046) was used.

Antigen was diluted at concentrations 4 mg/ml, 2 mg/ml and 1 mg/ml in carbonate buffer. Known positive IBV and negative sera was diluted at 1:100, 1:200 and 1:500 in dilution buffer. Conjugate was diluted at 1: 1000, 1:2000, 1:3000 and 1:4000. Six replicated reaction were done. The color of reaction was obtained from Ortho-phenylene diamine. The Optical density were measured at 450 nm using an automatic ELISA reader (Multiskan EX, Thermo Lybsystems) and the signal-to noise (S/N) ratio at the same dilution were evaluated.

The ELISA procedure was standardized on the method developed by Case *et al* (1982) with

some modification. IBV local isolate PTS-III of antigen was assay at concentrations 0.4 µg/50 µl/ well in carbonate buffer (0.1M $NaCO3_{,}$ 0.02% NaN3, pH 9.6). Flat bottomed micro plates (Nunc) were coated antigen at same time. After incubate at 4^0C for one night, the wells were washed using washing buffer (0.15M NaCl, 2.5 mM KCL, 1.5 mM NaH_2PO_4 H_2O , 9.0 mM Na_2HPO_4 and 0.05% Tween 20, pH 7.4) with soaking for 5 minutes at room temperature in each time and trapped out onto absorbent paper. Fifty µl blocking buffer (0.15M NaCl, 2.5 mM KCL, 1.5 mM NaH_2PO_4 H_2O, 9.0 mM $Na_2HPO_{4,}$ 1mM EDTA, 0.5% Casein, and 0.05 % Tween 20, pH7.4) was added to each well. The plate was washed after incubation at 37^0C for 120 minutes. Serum samples were prepared by diluting 1:100 in phosphate buffer saline (0.15M NaCl, 2.5 mM KCL, 1.5 mM NaH_2PO_4 H_2O , 9.0 mM $Na_2HPO_{4,}$ 1mM EDTA, 0.1% Casein, and 0.05 % Tween 20, pH7.4) then 50 µl was added to each well. The plate was washed after incubation at 37^0C for 60 minute. Horseradish peroxidase conjugated rabbit anti-chicken IgG was prepared by diluting 1:2000 µl of conjugate buffer (0.5M NaCl, 2.5 mM KCL, 1.5 mM NaH_2PO_4 H_2O, 9.0mM $Na_2HPO_{4,}$ 1mM EDTA, 0.1% Casein, and 0.05 % Tween 20, pH7.4) then 50 µl was added to each well. The plate was washed after incubation at 37^0C for 60 minute. A hundred µl of substrate (0.04% OPD, 0.04% H2O2, 0.2M NaHPO4, 0.1 M Citric acid, pH5) was added and incubated at room temperature for 45 minute then stopped by 1M H_2SO_4. Optical density of each serum was determined by using ELISA reader.

Cut-off value of the ELISA was determined by using optical density (OD) serum from unvaccinated group (as negative control). The average value of OD serum was added three of standard deviation (STDEV) as cut-off value of the ELISA.

The specificity of the developed ELISA was calculated as the percentage of negative in unvaccinated group and the sensitivity was calculated as percentage of positives in vaccinated group. The result of serum obtain by the Elisa (local isolate PTS-III) were compared with those obtained by a commercial kit (IDEXX).

Infectious Bronchitis antigen which tested by using ELISA was evaluated to determine antigen binding reaction and non specific antibodies against other respiratory viral diseases (Infectious Laryngotrachitis).

HI test which conducted by M41 antigen (kindly provided by BPMSOH), was used for the preparation of haemagglutinating antigen as described by King and Hopkins (1983) and Alexander *et al* (1983).

RESULTS

Antigen, serum and conjugate dilution

The protein content of prepared antigen of IBV local isolate PTS-III was 8.8 mg/ml base on Bradford's method (Spectrophotometer/Nano Drop Tecnologies ND-1000). We obtained minimal nonspecific binding at 8 mg protein /well. According to titration result by 0,4 µg/well antigen (dilution 1:1000/well), 1:100 chicken serum dilution and 1:2000 conjugate HRPO in S/N ratio which showed expensiveness of conjugate, was chosen. Optical density of ELISA value for positive sera was 6 times greater to negative sera (Figure 1.).

Cross-reaction with other respiratory viral infection

Non specific antibody reaction other respiratory disease (ILT) in local isolate ELISA IB test the average value OD was 0.144, with STDV

0.022, while the antigen control was 0.101 to 0.002 STDV (Tabel 1.)

Optical density of the local isolate ELISA

Optical density obtained by local isolate PTS-III and commercial ELISAs were measured from 180 specific pathogenic free chickens sera and 140 specific pathogenic free chickens sera infected IBV (Table 2)

The HI titer (log2) of the positive sera were 0.19, 3.45, 4.05, 3.95, 6.35, 8.3, 8.45, and 8.6 respectively. In non-vaccinated group, HI titer at first and the end of test period were 2.05 and 2.1 respectively. The peak titer in vaccinated group was recorded at day 44 or after 34 days post vaccinated with H120 live vaccine and the titer increased at day 57 (after 2 weeks booster vaccinat-

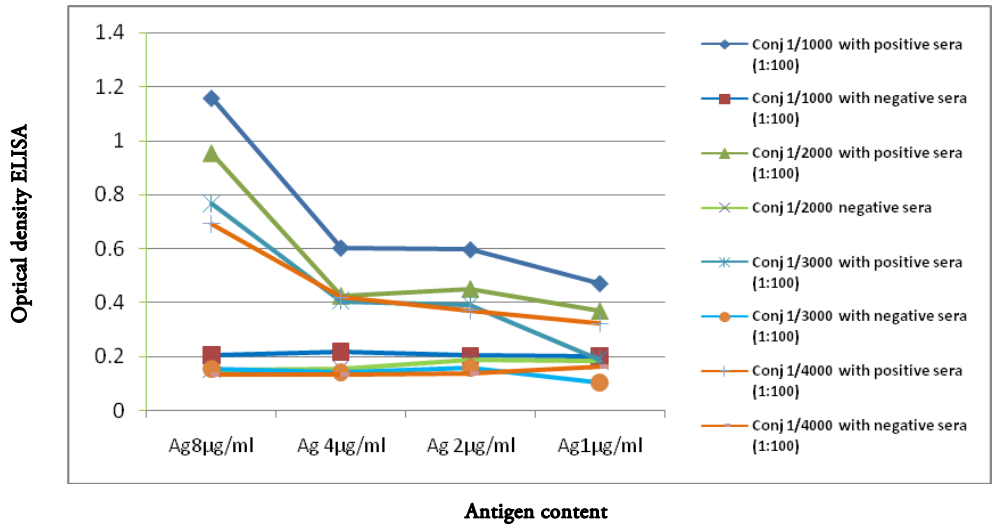

Figure 1. Optimum dilution of antigen, sera and conjugate

Tabel 1. Idetification reaction of non specific other respiratory virus (ILT)

			IBV Antigen ELISA		Control Antigen	
? negative sera	? IB sera	? ILT sera	Mean OD	STDV	Mean OD	STDV
16			0.162	0.001	0.155	0.101
	16		0.993	0.005	0.144	0.002
		10	0.144	0.022	0.101	0.002

Table 2. The result of 140 positive and 180 negative sera tested for IB antibody using local isolate PTS-III and commercial ELISAs

	Specific pathogenic free chicken sera infected IBV								Spesific pathogenic free chicken sera							
Optical density	10 $	24 #	34 #	44 #	57 ##	70 *	78 *	89 *	10	24	34	44	57	70	78	89
local isolate Elisa	0.144	0.313	0.504	0.521	0.880	0.904	0.933	0.930	0.142	0.146	0.141	0.162	0.135	0.135	0.131	0.135
Standard commercial Elisa	0.052	0.090	0.154	0.239	0.766	0.877	0.868	0.917	0.052	0.054	0.055	0.052	0.052	0.053	0.052	0.054

= days chicken age after live vaccine, ## = days chicken after booster kill vaccine,
*= days chicken age after challenge , $= days before vaccinated

ed). Also the titer was increased rapidly after challenge in ELISAs. Correlation between the local isolate ELISA test and standard commercial ELISA was very high (R2=0.933) in vaccinated and challenged chicken sera, while at the unvaccinated chicken sera has no correlation (R2=0.010)

Cut off optical density of the ELISA

The cut off for the standardized ELISA test was determined as mean of negative sera plus thrice of the standard deviation 0.201 [0.141+ (3x0.020)] (Figure 2.)

Specificity and Sensitivity of the local isolate ELISA

Sensitivity and specificity were evaluated using sample positive (S/P) ratio (Table 2). Percentage of sensitivity local isolate PTS-III ELISA was slightly higher than hemaglutination inhibition test, i.e. 96.42% and 90% respectively. While percentage of specificity between local isolate PTS-III ELISA and hemaglutination inhibition test were 100%.

DISCUSSION

Protective immunity to avian infectious bronchitis is not reflected by humoral antibodies, as shown by Davelaar & Kouwenhoven (1980). Nevertheless, monitoring antibodies after vaccination is a valuable procedure for indicating that responses have occurred. The local isolate ELISA test for measuring antibody level against IBV was developed in this study. The test was standardized in term of reagent to obtain the significant S/N ratio. ELISA test has been developed to monitor

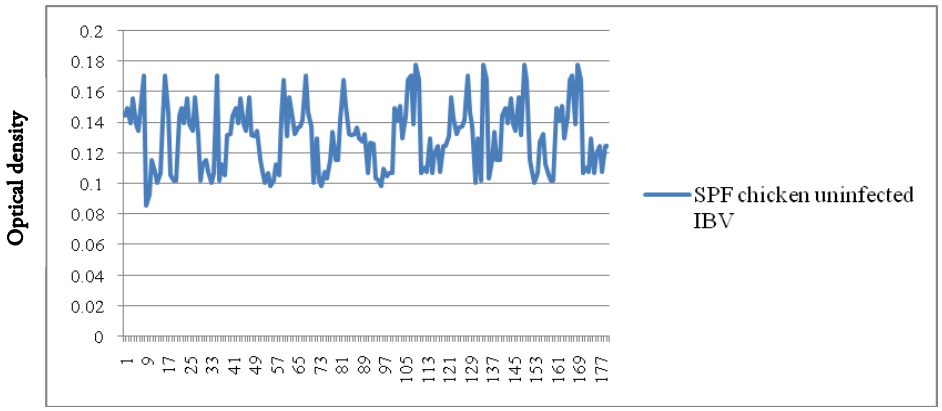

Figure 2. The cut off for standardized ELISA of local isolate PTS-III

Table 4. Sensitivity and specificity the ELISA local isolate PTS-III dan HI

ELISA reaction	Chicken Sera	SPF chicken infected IBV	SPF chicken uninfected	Precentation
Local isolate IB Elisa	Positive	135 (a)	0 (b)	Sensitivity 96,42%
	Negative	5 (c)	180 (d)	Specificity 100%
Hemaglutination Inhibition (HI) test	Positive	126 (a)	0 (b)	Sensitivity 90%
	Negative	14 (c)	180 (d)	Specificity 100%

Sensitivity = $\frac{(a)}{(a)+(c)}$; Specificity = $\frac{(d)}{(d)+(b)}$ (BALDOCK 1988)

antibody respond to vaccination against IB (Monreal *et al.* 1985, Mockett and Darbyshire 1981, Soula &Moreau 1981). Case *et al.* (1982) optimized the parameter of ELISA for detecting antibody against IBV. They obtained minimal nonspecific binding and very high sensitivity using purified IB as antigen 50 ng protein /well and final NaCl concentration 1.0M in buffer. In this study it was obtained minimal nonspecific binding at 0.4 µg protein/well. According to titration result base on 0.4 µg protein/well at 1:2000 conjugate showed similar significant difference in S/N ratio, and cheaper conjugate was chosen. To eliminate non specific binding in the local isolate -III ELISA, we used blocking buffer (0,15M NaCl, 2,5 mM KCL, 1,5 mM KH_2PO_4, 9,0 mM $NaHPO_4$, 0.5% Casein, and 0.05 % Tween 20, pH7.4) after coated antigen. The local isolate ELISA indicated low to moderate level after live vaccination and moderate to relatively high level of antibody IB after injection of inactivated vaccine as well as commercial one. This data was agreed with HI results. In non-vaccinated group OD was lower then that the cut off (0.201) in the local isolate ELISA. Also, the titer of HI test which negative in this group parallel with the ELISAs results. The OD of the ELISAs and HI titer increased slowly following live H120 vaccination and more following oil vaccine injection.

The sensitivity of local isolate PTS-III ELISA was slightly higher than hemaglutination inhibition test, i.e. 96.42% and 91,42% respectively. The results showed specificity was equal 100% between local isolate PTS-III ELISA and hemaglutination inhibition test. Some researcher demonstrated the high sensitivity of the ELISA and correlation of results obtained from the HI test in chicken sera infected intratarcheally with IB strain M41 (Mockett & Darbyshire 1981; Soula & Moreau 1981).

The result of antibodies titration showed that the developed local isolate PTS-III ELISA could be reliable, repeatable and more sensitive for monitoring of vaccination schedules and for detection of early rising of antibodies against IB rapidly.

CONCLUSION

This study concluded that local isolate IB PTS III ELISA could be reliable, repeatable and sensitive for monitoring of vaccination schedules and detection of early rising of antibodies against IB rapidly.

AKNOWLEDGMENTS

This research was supported by funds from a researcher budget of BBALITVET DIPA in 2012. We thank Heri Hoerudin and Apipudin, and all collaborators helped for implementation of this study

REFERENCES

Alexander, DJ., WH. Allan, PM. Biggs, CD. Bracewell. JH. Darbyshire, PS. Dawson, AH. Harris. FTW. Jordan, I. Macpherson, JB. McFerran, CJ. Randall, O. Swarbrick, & GP. Wilding. 1983. A satandard technique for haemagglutination inhibition test for antibodies to avian infectious bronchitis virus. *Vet. Rec.* 113:64

Baldock, FC. 1988 . Epidemiological evaluation of immunological test. *Elisa technology in diagnosis and research*. JCU, Townsville, Australia. 90-95.

Cavanagh, D. 1983a. Coronavirus IBV glycopolypeptides: size of their polypeptide moieties and nature of their oligosaccharides. *J. Gen. Vir.* 64: 1187-1191.

Cavanagh, D. (1983b) Coronavirus IBV: further

evidence that the surface projections are associated with two glycopolypeptides. *J. Gen. Vir.* 64: 1787-1791.

Cavanagh, D . (1983c) Coronavirus IBV: structural characterization of the spike protein. *J. Gen. Vir.* 64: 2577-2583.

Cavanagh, D., JH. Darbyshire, P. DAVIS, & RW. Peter. 1984. Induction of humoral neutralizing and haemagglutination-inhibiting antibody by the spike protein of avian infectious bronchitis virus. *Avian Path.* 13: 573-583.

Cavanagh, D., PJ. Davis, JH. Darbyshire, & RW. Peters. 1986. coronavirus IBV: virus retaining spike glycopolypeptide S2 but not S1 is unable to induce virus-neutralizing or haemagglutination-inhibiting antibody, or induce chicken tracheal protection. *J. Gen. Vir.* 67: 1435-1442

Case, JT., AA. Ardans, DC. Bolton, & BJ. Reynolds. 1982. Optimization of parameter for detecting antibodies against infectious bronchitis virus using an enzimed-linkes Immunosorbent Assay: Temporal respon to vaccination and challenge with live virus. *Avian Diseases* 27:196-210

Davelaar, PG. & Kouwenhoven, B. 1980. Vaccination of DOC broiler against infectious bronchitis by eye drop application or coarse droplet spray and the effect of revaccination by spray. *Avian Pathology* 9:499-510

Darminto. 1992. Characterization of subtype Infectious Bronchitis virus local isolate. *J. Penyakit Hewan* 24: 78-81

De Wit, JJ., DR. Mekkes, B. Kouwenhoven, & JHM. Verheijden.1997. Sensitivity and specificity of serological tests for infectious bronchitis virus antibodies in broilers. *Avian Path.* 26:105-118.

De Wit, JJ.(2000). Detection of infectious bronchitis virus. *Avian Pathology* 29:71-93.

Engvall, E., & P. Parlmann. (1971). Enzyme linked immunosorbent assay (ELISA) quantitative assay of immunoglobulin G. *Immun.* 8:871-874.

Garcia, Z., &. RA. Bankowski. 1981. Comparation of a tissue-culture virus-netraliz ation test and the enzyme-linked immunosorbent assay for measurement of antibodies to infectious bronchitia. *Avian Diseases* 25:121-130.

Ignjatovic, J., & L. Galli. 1995. Immune respon to structural protein of avian infectious bronchitis virus. *Avian Path.* 24:313-332

Kusters, JG., EJ. Jager, JA. Lenstra, G. Koch, WPA. Posthumus, RH. Meloen, & BAM. Van der Zeist. 1989. Analysis of an immunodominant region of infectious bronchitis virus. *J. Immun.* 143**:** 2692-2698.

Kant, A., G. Koch, DJ. van Roozelaar, JG. Kusters, FAJ. Poelwijk, & BMA. van der Zeijst, G. Koch, L. Hartog, A. Kant, & DJ. van Roozelaar. 1990. Antigenic domains on the peplomer protein of avian infectious bronchitis virus: correlation with biological functions. *J. Gen. Vir.*71: 1929-1935.

Kant, A., G. Koch, DJ. van Roozelaar, JG. Kusters, FAJ. Poelwijk, & BMA. van Der Zeijst 1992 Location of antigenic sites defined by neutralizing monoclonal antibodies on the S1 avian infectious bronchitis virus glycopolypeptide. *J. Gen. Vir.,* 73, 591-596.

King, DJ., & SR. Hopkins 1983. Evaluation of the hemaggutination-inhibition test for measuring the response of chickens to avian infectious bronchitis virus vaccination, *Avian Diseases* 27:100-112

King, DJ. & D. Cavanagh. 1991. Infectious bronchitis, in: BW. Calnek, HJ. Barnes, CW. Beard,WM. Reid & HW. Yoder (Eds) *Diseases of Poultry,* 9th edn, pp. 471-484

Koch,G., L. Hartog, A. Kant & DJ. van Roozelaar. 1990. Antigenic domains on the

peplomer protein of avain influenza bronchitis virus: correlation with biological fungtion. *J. Gen. Vir.* 71: 1929-1935.

Lenstra, JA., JG. Kusters, G. Koch. & BAM. van Der Zeijst. 1989 Antigenicity of the peplomer protein of infectious bronchitis virus. *Mol. Immun.* 26: 7-15.

Monreal, G., HJ. Baure & Wiegman. 1985. Comparasion of the enzyme-Linked Immunosorbent Assay (ELISA). Hemagglutination inhibition test and agar gel precipitation test for detection of antibodies to avian infectious bronchitis virus. *Avian Path.* 14:421 -434.

Mockett,APA., & JH. Darbyshire. 1981. Comapration studies with an enzyme-linked immunosorbent assay (ELISA) for antibodies to avian infectious bronchitis virus. *Avian Pathology* 10:1-10.

Perrotta, C. C. Furtek, RA. Wilson, BS. Cowen & RJ. Eckroade. 1988. A standardization enzyme-linked immunosorbent assay for infectious bronchitis virus: Comparison with hemagglutination-inhibition and virus-neutralization assays for measuring protective antibody levels in chickens. *Avian Diseases* 32:451-460.

Soula, A. & Y. Moreau. (1981). Antigen requirements and specificity of a microplate enzyme -linked immunosorbent assay (ELISA) for detecting infectious bronchitis viral antibodies in chicken serum. *Archives Vir.* 67:283-295

Thater, SG., P. Villegas. & OJ. Fletcher. (1987). Comparison of two commercial enzyme-Linked Immunosorbent Assay and conventional methods for avian serology. *Avian Diseases* 31:120-124.

Zellen, GK., &. J. Thorsen. (1986). Standardization and application of the Enzyme-Linked Immunosorbent Assay for infectious bronchitis. *Avian Diseases* 30:695-698.

Wing, CH., CC. HONG, & JCH. SEAK. (2002). An ELISA for antibodies against infectious bronchitis virus using an S1 spike polypeptide. *Vet. Micro.* 85:333-342.