Available online at www.scholarsresearchlibrary.com

Scholars Research Library

Der Pharmacia Lettre, 2016, 8 (9):295-298
(http://scholarsresearchlibrary.com/archive.html)

Scholars Research Library
ISSN 0975-5071
USA CODEN: DPLEB4

Detection of infectious bronchitis virus by Enzyme Linked Immunosorbent Assay (ELISA) in chickens

Bellam Praveen and G. Narasimha

Department of Virology, Sri Venkateswara University, Tirupati – 517 502, A.P, India

---

ABSTRACT

The purpose of present study is to evaluate the symptoms exhibiting avian infectious bronchitis virus (IBV) in infected chickens in poultry farms by standard enzyme linked immunosorbent assay (ELISA) by produced polyclonal antibodies. The optimized polyclonal antibodies serve as potential agents for detection of IBV infection in field level.

Keywords: Infectious bronchitis virus, ELISA, Dot Blot, antibody, Massachusetts vaccine strain.

INTRODUCTION

Infectious bronchitis is an acute, highly contagious disease of major economic importance in commercial chicken flocks throughout the world. Infectious bronchitis virus (IBV) is an enveloped *coronavirus* that contains an unsegmented, single-stranded, positive-sense RNA genome. Clinical symptoms include respiratory signs of gasping, coughing, sneezing, tracheal rales, nasal discharge, growth retardation in broilers, drop in egg, production losses are due to poor weight gains and heavy mortality in poultry [1,2,3]. Over hundreds of known serotypes, new viral variants have emerged due to rapid viral evolution and antigenic variation in avian coronaviruses [4,1]. Despite regular vaccine use, IBV outbreaks occur frequently and owners of infected poultry farms suffer from tremendous economic losses. A number of ELISA tests for IBV antibody detection have been described [5,6,7,8,9,10,11], Because it is a simple, rapid, sensitive, and large-scale evaluation tool. ELISA has been used widely in IBV serological profiling. Immunity to IBV has most often been assessed using traditional serological assays; however, the enzyme-linked immunosorbent assay (ELISA) is used on a more frequent basis to measure IBV antibodies [12]. The technique was developed by Engvall and Perlmann [13] and has been widely used with some modifications according to the work study. The major developments that have been taken place over the past 25 years, it is obvious that the enzyme-linked immunosorbent assay (ELISA) is the most significant advance, especially in virus detection. The ELISA offers a number of advantages compared with traditional serological assays, including sensitivity and specificity [14, 15]. Without these techniques, many immunoassays and protocols for molecular testing would simply never have been developed. In the present study the purpose of understanding field IBV infection, we standardized DAC-ELISA by producing polyclonal antibodies and evaluated with field samples collected from poultry farms.

MATERIALS AND METHODS

Test antigen/ antibody samples

Infectious bronchitis virus, commercial live Massachusetts strain (Mass type strain $\geq 10^{3.5}$ EID_{50}) vaccine, purchased from Vertri biological, vaccine division, pure, India were used for producing polyclonal antibodies. For

Screening, the IBV samples were collected from poultry farms of Tirupathi, Andhra Pradesh, India.

Production of polyclonal antibodies

The New Zealand white rabbit was immunized with 4 regular and 1 booster dosages, each dose containing purified commercial Massachusetts strain (Mass type strain ≥ $10^{3.5}$ EID_{50}) administered into the rabbit intramuscularly. Initial dose was made by Freund's complete adjuvant (1:1 v/v) and the subsequent doses with incomplete adjuvant. Three intramuscular injections were given after the initial dose at weekly intervals. Three weeks after the last dose, booster dose was given and the blood was drawn at weekly interval from the ear veins. The collected blood was allowed to clot by incubating at 4^0C and antiserum was collected by centrifugation at 5000 rpm for 10 min. Aliquots of antiserum were stored at -20°C after adding sodium azide at 0.002% conc.

Standardization of DAC-ELISA

A checkerboard titration was performed to determine the optimum antigen concentration and primary antibodies (polyclonal Abs) dilution. Serial dilutions (1:10 to 1:1000 (v/v)) of the virus antigen (Massachusetts strain) were made in bicarbonate-carbonate antigen coating buffer, pH 9.6 (0.43 g $NaHCO_3$, 0.53 g Na_2CO_3, and 100 ml distilled water, pH 9.6). 100 µl of each respective sample was loaded into wells of ELISA plates (Nunc Maxi Sorb; Denmark) and incubated at 37^0C for 60 min. The antigen coated plates were washed five times with PBS-T (PBS, pH 7.4, containing 0.05% Tween20). The plates were then blocked with blocking buffer (PBS-TPO with 5% skimmed milk powder) and incubated at 37^0C for another 60 min. The plates were then washed with PBS-T as above. The produced polyclonal antisera of Massachusetts vaccine strain of five bleeds were used as primary antibodies at 1:100 to 1: 40,000 (v/v) dilutions in antibody buffer (v/v), were coated and incubated for 60 min at 37^0C and washed five times with PBS-T as above. Goat anti rabbit-ALP conjugate at 1:2000 (v/v) dilution in antibody buffer (Genei, Bangalore) was added and incubated at 37^0C for 60 min. Paranitrophenyl phosphate (pNPP; Sigma, Germany) was used as a substrate at 5 mg / 10 ml of substrate buffer (diethanolamine buffer, pH 9.8). Absorbance values were recorded in ELISA plate reader (Bio-Rad, USA) at 405 nm after 15-30 min of substrate addition. The reactions were terminated with 3 N NaOH (50 µl/well).

Dot-blot hybridization assay

As described in above experiment the fifty microliters serial dilutions of the coating antigen was pipetted on Pure nitrocellulose blotting membrane. The membrane was dried by incubating at 37^0C for 30 min. The nitrocellulose membrane was then washed with washing buffer (PBS containing 0.05% Tween 80) three times for 10 min each time. The membrane was then transfer to a petriplate and add blocking solution till the membrane is fully immersed; Nonspecific binding sites were minimized by the addition of blocking buffer (5% dry skimmed milk in PBS-T pH7.4) for 1 hr at room temperature. The membrane was washed again in the manner described above. Transfer the membrane from blocking solution to 1: 20,000 (v/v) diluted antiserum (3 bleeds) in a blocking buffer and kept at 37^0C for 1 hr. The membrane was washed again as described earlier. Fifty microliter of a 1:50,000 dilution of HRP ladled Anti-Chicken IgY (IgG) (whole molecule)−Alkaline Phosphatase antibody conjugate produced in rabbit (Sigma Immunochemicals, St. Louis, MO) was makeup in dilution buffer, then added and incubated for 1hr at 37^0C. Washing was repeated. Next, 5-Bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium (BCIP/NBT) substrate solution specific to the enzyme was added to membrane, and color development was observed for 20 min at room temperature. Excess substrate was removed by washing three times with distilled water. The membrane was then air dried in the dark, and the results interpreted visually.

Evaluation of IBV infected farm by DAC- ELISA

Screening of IBV samples by DAC-ELISA

For the screening of infectious bronchitis virus the optimized polyclonal antiserum and antigen source were used for detection of IBV. For that, total of 66 serum samples was collected from the broiler breeder flocks located around Tirupati of Andhra Pradesh, India. The positive serum samples collected from which chicks exhibiting IBV symptoms like gasping, coughing, sneezing, tracheal rales, and nasal discharge were taken in consideration. The optimum dilution of stock IBV antigen was used, which was established in a preliminary experiment using checkerboard titration, as described above. The appropriate dilution 1:10 of IBV-positive field sera, IBV reference vaccine strain positive control and negative control SPF sera were made in dilution buffer. Basically, the ELISA tests were run as described, in checkerboard titration (above), except that a single antigen 1:100 dilution (v/v) was used in coating buffer all of the respective wells. Third bleed antiserum at 1:20000 (v/v) was routinely used for detection of virus. Results were expressed as either positive or negative based on color development

RESULTS

Initial evaluation of produced polyclonal antibodies

The A_{405} values greater the twice the value of the negative serum (SPF serum) sample were generally considered as positive. Positive reactions were observed in all the antibody dilutions of 3rd, 4th and 5th bleeds. The strong values were recorded up to 1:1000 antibody dilutions in the 1st and up to 1:5000 dilutions in the 2nd bleeds. The mean absorbance (A_{405}) values of various antibody dilutions in DAC-ELISA are given in the table .1. A_{405} values were observed in the 1:15000 and 1:20000 antibody dilutions of 3rd and 4th bleeds are stronger than the other dilution and respective bleeds shown in the **table. 1.** Hence the antiserum of these bleeds was further used for routine diagnosis of IBV samples by DAC-ELISA. Whereas at A_{405} the optimal concentration of antigen of different dilutions the 1:10 shown best among the other dilutions as shown in the **table. 2.**

Table: 1 Determination of optimal concentration of IBV antibody for ELISA

Antibody dilutions (v/v)	Bleeds									
	I		II		III		IV		V	
	pAbs	control	pAbs	Control	pAbs	Control	pAbs	Control	pAbs	Control
1:100	0.52	0.09								
1:500	0.62	0.10								
1:1000	0.45	0.10	2.42	0.43						
1:2000			2.69	0.36						
1:3000			2.38	0.22	2.26	0.20	2.35	0.20		
1:5000			1.53	0.23	2.31	0.19	2.30	0.20		
1:7000					2.66	0.13	2.10	0.15	2.00	0.24
1:10000					2.79	0.22	2.10	0.12	2.10	0.24
1:15000					3.42	0.13	3.40	0.16	2.00	0.18
1:20000					**3.46**	0.14	**3.33**	0.16	**2.01**	0.16
1:30,000					2.72	0.16	3.10	0.13	1.56	0.16
1:40,000					2.39	0.16	2.90	0.15	1.33	0.21

Table: 2 Determination of optimal concentration of IBV antigen for ELISA of IIIrd bleed (1:20000) antiserum

Antigen dilutions										
1:10		1:50		1:100		1:500		1:1000	
Positive Antigen	SPF serum control	Positive Antigen	SPF serum control	Positive Antigen	SPF serum control	Positive Antigen	SPF serum control	Positive Antigen	SPF serum control
2.16	0.19	2.10	0.21	2.06	0.20	1.89	0.16	0.95	0.12

Dot blot assay procedure for optimal antigen concentration

A result was considered positive when well defined purple to brown spots were observed shown in the fig. 1.The highest dilution of antigen that gave the best visible color difference compared to the negative serum. The 1:10 dilution was considered to be optimum antigen concentration for subsequent use in the experiment.

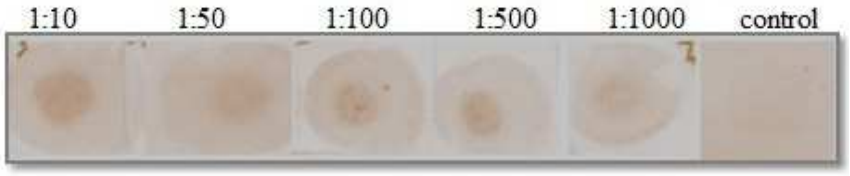

Fig. 1 Detection of IBV antigens titer (1:10, 1:50, 1:100, 1:500 and 1:1000 dilutions (v/v) against 1:20000 dilution (v/v) of IIIrd bleed antiserum by DOT-BLOT

Evaluation of IBV infected farms by DAC- ELISA

In DAC-ELISA tests the 66 suspected IBV field serum samples collected from different locations in Tirupati region of Andhra Pradesh, where the strong positive reactions were observed in seven samples with the antisera of IBV Massachusetts strain. Only those samples whose ELISA A_{405} values were twice or greater than twice the value of negative SPF serum and almost equal to the positive IBV reference strain sample were considered as virus infected **(table. 3).**

Table: 3 DAC-ELISA screening of field collected IBV samples

Nature of antigen	Range of A405 values*	Number of samples confirmed
Field collected IBV positive samples	1.36-1.53	7/66*
IBV SPF serum control	0.21-0.26	
IBV positive antigen control	1.43-1.62	
Buffer control	0.08-0.16	

** A405 values given are the average values of triplicate wells*
** Number of samples confirmed/total number of samples tested*

DISCUSSION

IBV is a highly infectious pathogen and the infected birds usually develop clinical signs very rapidly, within 36-48 hours. The ELISA is considered a useful tool for routine laboratory diagnosis of IBV antigen. The IBV antigen is currently detected by the use of a commercial ELISA kit, which is very expensive. Therefore study was taken up to standardize ELISA test for diagnosis of IBV and serum samples were collect from clinically affected farms with symptoms of coughing, sneezing, tracheal coarse crackles, nasal discharge, decrease of feed intake and conversion, loss of body weight and poor growth in broilers. In the current study, we produced specific IBV polyclonal antibodies, which can be used for detect IBV in chickens. And standardized the DAC-ELISA with simple, reliable, sensitive assay for the detection of field suspected IBV samples. As indicated in the results section, the produced antibodies were found to be highly specific in the detection of infectious bronchitis virus isolates in field serum samples (1:100 dilution w/v) at 1:20000 dilution of 3th bleed antiserum. This shows that the produced antiserum can economically be used for detection of infectious bronchitis virus filed level.

CONCLUSION

In the present study, the produced antiserum against IBV can be successfully used for the early and rapid screening of IBV infection, particularly where large numbers of sera samples to be tested. It is a cost saving diagnostic procedure with minimum laboratory facilities for the assay.

REFERENCES

[1] D. Cavanagh, S.A. Naqi,. In: Saif, A.M., Fadly, Y.M., McDougald, L.R., Swayne, D.E. (Eds.), Diseases of Poultry, (Iowa State University Press, Ames, IA, **2003**) 101–119.
[2] D. Cavanagh, *Vet. Res.*, **2007,** 38, 281–297.
[3] J. Cook, In: Jordan, F., Pattison, M., Alexander, D., Faragher, T. (Eds.), Poultry diseases, (WB Saunders, New York, **2002**) 298–306.
[4] D. Cavanagh, *Avian Pathol.*, **2005**, 34, 439-448.
[5] R.V. Bronzoni, M. Fatima, S. Montassier, G. T. Pereira, N. M. Gama, V. Sakai, H. J. Montassier, *Viral Immunol.*, **2005**, 18, 569–578.
[6] J. J. De Wit, *Avian Pathol.*, **2000**, 29, 71-93.
[7] J. J. De Wit, D. R. Mekkes, B. Kouwenhoven, J. H. Verheijden, *Avian Pathol*., **1997**, 26, 105-118.
[8] W. W. Marquardt, D.B. Snyder, B. A. Schlotthober, *Avian Dis.*, **1981**, 25, 713–722.
[9] C. Perrotta, C. Furtek, R.A. Wilson, B.S. Cowen, R. J. Eckroade, *Avian Dis.*, **1988**, 32, 451–460.
[10] G. K. Zellen, Thorsen, *J., Avian Dis.*, **1986**, 30, 695–698.
[11] G. K. Zellen, Thorsen, *J., Avian Dis.* **1987**, 31, 455–458.
[12] J. T. Case, A. A. Ardans, D. C. Bolton, and B. J. Reynolds, *Avian Diseases*, **1982**, 27, 196-210.
[13] E. Engvall, P. Perlmann, *Immunochemistry***, 1971**, 8, 871-874.
[14] Z. Garcia, R. A. Bankowski, *Avian Diseases*, **1981**, 25, 121-130.
[15] D. B. Synder, W. W. Marquardt, E. T. Mallinson, P. K. Savage, and D. C. Allen, *Avian Diseases*, 1**984**, 28, 12-24.