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DETERMINATION OF SENSITIVITY AND SPECIFICITY OF IN-HOUSE SANDWICH ELISA FOR THE DETECTION OF INFECTIOUS BURSAL DISEASE VIRUSES

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ABSTRACT

The study was designed for the development of an In-House sandwich ELISA as a suitable serological method for the rapid detection of infectious bursal disease virus (IBDV). The test was also designed to compare and evaluate its sensitivity and specificity with other traditional methods used for the detection of IBDV from field outbreak cases prevalent among the poultry population of Bangladesh. To develop the In-House sandwich ELISA, hyper-immune serum was raised against live IBDV vaccine in rabbit which was used to coat each of the 96-well flat bottomed polystyrene microtitre plate whereas, hyper-immune sera raised in chickens against IBDV used as secondary antibody. The newly developed In-House sandwich ELISA was standardized by dispensing different dilutions (10^{-1} up to 10^{-4}) of rabbit serum. Among them, the 10^{-2} dilution of serum showed most suitable reading for the detection of IBD virus and used to coat the plate to evaluate its sensitivity and specificity. Sensitivity test was done by different dilutions (10^{-0} to 10^{-4}) of reference IBD virus. The virus dilution, 10^{-3} was the highest dilution having lowest capacity to bind with coated antibody of the ELISA plate which indicated that IBD viruses was absent in the dilutions of above 10^{-3}. The cut-off value of negative control samples was determined as 0.937 which indicated titer of tested samples >0.937 was positive and <0.937 was negative. Specificity test was performed using different known viruses (IBDV and NDV) using different dilutions (10^{-1} up to 10^{-4}). Only the IBDV showed positive result which indicated high specificity of newly developed ELISA plate. A total of 26 samples (feces, cloacal swab, spleen and bursa) from control group, experimental and natural IBDV outbreaks were used as field viral antigen for the evaluation of sensitivity and specificity of the newly developed In-House sandwich ELISA. In case of experimental infection, 5 (62.5%) of 8 feces sample but none of cloacal swab were positive for IBDV whereas, all bursa and spleen samples were positive by both In–House sandwich ELISA and AGIDT. In case of natural outbreak cases, 6 of 6 bursal samples and 4 of 6 spleen samples were positive by In-House sandwich ELISA whereas, AGIDT detected all bursal and 3 spleen samples. No virus was detected from the samples of control group. The result showed 92.85% specificity of the developed sandwich ELISA for detection of IBDV with AGIDT which indicated that the developed ELISA is a sensitive, specific, cost effective and reliable tool for the detection of IBDV antigen from a large number of field samples.

Key words: IBDV, sandwich ELISA, AGIDT

INTRODUCTION

Infectious bursal disease (IBD) also known as Gumboro disease, is one of the highly contagious viral diseases of young chickens caused by infectious bursal disease virus (IBDV) belongs to the genus *Avibirnavirus* of the family *Birnaviridae* having bi-segmented dsRNA genome (Jackwood *et al.,* 1984). The disease is characterized by immunosuppression, depression, debilitation, dehydration and high mortality generally at 3 to 6 weeks of age. The disease is economically important to the poultry industry worldwide due to increased susceptibility to other diseases and negative interference with effective vaccination. In recent years, very virulent strains of IBDV (vvIBDV) causing high mortality in chicken have emerged in South-East Asia, Europe, Latin America, Africa, and the Middle East. There are two distinct sero-types of the virus (McFerren *et al.*, 1980), but only sero-type 1 virus causes disease in poultry and includes the classical virulent (cv), very virulent (vv) and variant strains (Van den Berg *et al.*, 1991). At least six antigenic sub-types of IBDV serotype 1 have been identified by in-vitro cross-neutralization assay. Viruses belonging to one of these antigenic sub-types are commonly known as variants, which were reported to break through high levels of maternal derived antibodies in commercial flocks, causing up to 60 to 100 percent mortality in chickens.

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When any outbreaks occur in a farm, initiatives are taken to confirm the diagnosis and measures are taken accordingly to reduce morbidity and mortality to prevent further spread of causal agent. So, it is necessary to diagnose the disease quickly and perfectly. In our country, clinical signs and post mortem lesions are commonly used as the main basis for the diagnosis of IBD. Though gross lesions found in IBD affected poultry is considered sufficient for diagnosis of IBD (Cosgrove, 1962), but the clinical signs, post mortem lesions and histopathological findings of IBD are very much similar with other bacterial and viral diseases of poultry (Banda, 2002). Various serological tests like agar gel immunodiffusion test (AGIDT) (Patnayak *et al.,* 1997), serum neutralization test (SNT), indirect hemagglutination (IHA) test, enzyme-linked immunosorbent assay and molecular test like RT-PCR are employed for confirmatory diagnosis of the disease (Liu *et al.,* 1998). The enzyme-linked immunosorbent assay (ELISA) is commonly used as a rapid and sensitive test for detection of IBDV antigen or antibody against IBDV (Marquardt *et al.*, 1980; Howie and Thorson, 1981 and Solano *et al.*, 1985). ELISA is now being used for sero-profiling of chicken flocks and examination of the efficiency of vaccines (Solano *et al.*, 1986). Studies of the molecular epidemiology of IBDV are important, and the DAS-ELISA could be an alternative technique for screening a large number of samples before testing (Tham *et al.,* 1995).

This paper describes the development of an In-House sandwich ELISA for rapid detection of IBDV antigens from large number of field samples and comparison of sensitivitiy with the agar gel immunodiffusion test (AGIDT) in detecting IBDV antigen using field and laboratory samples.

MATERIALS AND METHODS

Rabbit anti-IBDV reference serum: The New Zealand white rabbits (n=4) were vaccinated with live IBD vaccine (BAL-IBD EM from BESTAR) on day 7, 14 and 21 through S/C route @ 0.5 ml/rabbit and blood was collected prior to first and during each vaccination. The separated serum was checked with reference IBD virus by AGIDT for IBDV antibodies and positive sera were used as coating antibody and positive control. The serum collected from one non-vaccinated control rabbit was screened for the antibodies against IBDV and used as negative control.

Chicken anti-IBDV serum: ISA Brown chickens (n=4) of two months old were vaccinated followed by blood collection thrice at day 7, 14 and 21 with live IBDV vaccine through ocular route. The separated sera samples were checked for anti-IBDV antibodies by AGIDT against reference IBDV antigen and positive sera were pooled to use as secondary antibody and positive control. For negative control serum blood was collected at day 28 from two non-vaccinated birds and checked by AGIDT to confirm the absence of antibody. All the sera samples were preserved at -20^{0}C in the screw-capped vial until used.

Reference IBD virus: Virulent strain of IBDV was used as reference virus obtained from the Department of Microbiology and Hygiene, Bangladesh Agricultural University, Mymensingh.

Detection of anti-IBDV antibody and IBDV antigen by agar gel immunodiffusion test (AGIDT)

All the sera samples raised in rabbit and chicken using live IBDV vaccine and prepared antigen from the samples of experimental and natural IBD outbreak cases were tested using known IBDV antigen and raised hyper-immune serum in chicken against IBDV vaccine respectively. The test was performed following the procedures described by Wyeth (2000).

Coating of the microtitre plate with hyper-immune serum raised in rabbit against IBDV

For the development of an In-House sandwich ELISA, flat bottomed 96-well microtitre plates were used (Tanimura *et al.,* 1995). A 100 µl of hyper-immune serum of each dilution (10^{-1} to 10^{-4}) with coating buffer (pH 9.6) were dispensed on each well of microtitre plate (each dilution for two rows) and incubated at 37^{0}C for 1 h, followed by overnight at 4^{0}C. A 100 µl of block ace solution (4% BSA) was added to each well and incubated at 37^{0}C for 1 h to block unbinding sites. Then the plate was emptied and washed 3 times with 200 µl of washing buffer (0.5% $Tween_{20}$ in PBS) and the antibody coated plate was stored at -20^{0}C until use. A 20% bursal suspension was prepared from the sample of naturally and experimentally infected birds and used as a source of viral antigen for In-House sandwich ELISA.

Determination of sensitivity and specificity of in-house sandwich ELISA

Standardization of optimal concentration of hyper-immune serum to coat the newly developed In-House sandwich ELISA plate

A 100 µl of each diluted (10^0 to 10^{-3}) known IBDV antigen were dispensed on A & B, C & D, E & F as well as G & H rows respectively up-to 10 number well of microtitre plate (11 was negative control and 12 positive control) of newly developed In-House sandwich ELISA plate and incubated at 37^0C for 1 hour. Excess antigen was removed and washed with $PBST_{20}$. A 100 µl of sera raised in chickens (10^0, 10^{-1} to 10^{-3}) were dispensed on all wells A & B, C & D, E & F as well as G & H respectively and incubated at 37^0C for 1 h. Excess antibody was removed and washed with $PBST_{20}$. A 100 µl of conjugate solution was added in each well and incubated for 1 h at 37^0C followed by emptying and washing of the plate. Then, 100 µl of substrate solution (OPD) was added and incubated for 30 minute at room temperature. Orange color was developed in case of positive reaction and the reaction was stopped by adding 100 µl of stop solution (1N H_2SO_4). The OD (optical density) value was determined using an ELISA reader at 405 nm filter.

Coating of microtitre plate with hyper-immune sera at 10^{-2} dilution to develop an In-House sandwich ELISA

A 100 µl of diluted (10^{-2}) anti-IBDV rabbit hyper-immune sera were dispensed on each of the 96-wells flat bottomed microtitre plate and incubated at 37^0C for 1 hour, followed by overnight incubation at 4^0C. A 100 µl of block ace solution was added to each well and incubated at 37^0C for 1 hour. Excess blocking solution was discarded and the plate was washed 5 times with $PBST_{20}$. Finally the antibody coated plate was stored at 4^0C until use.

Standardization of sensitivity of the In-House sandwich ELISA

A 100 µl of reference IBDV antigen (10^{-1} to 10^{-4}) were added into the wells of A & B, C & D, E & F, as well as G & H rows of previously coated plate with 10^{-2} diluted rabbit hyper-immune serum and incubated at 37^0C for 1 h. Excess antigen was removed and the plate was washed. A 100 µl of 10^{-2} diluted sera raised in chickens were dispensed on each well except column 11 (negative control) and 12 (positive control) and incubated at 37^0C for 1 h. Excess antibody was removed and washed with $PBST_{20}$. A 100 µl of conjugate solution was added and incubated at 37^0C for 1 h followed by emptying and washing of the plate. Then, 100 µl of substrate was added and incubated for 30 minutes at room temperature. The reaction was stopped by adding 100 µl of stop solution (1N H_2SO_4) and OD value was determined using an ELISA reader at 405 nm filter.

Standardization of specificity of the newly developed In-House sandwich ELISA

The specificity test of the plate coated with anti-IBDV serum (10^{-2}) was performed using known IBDV and NDV antigens. A 100 µl of each antigen (10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}) were dispensed on all wells of the plate except 11th (Negative control) and 12th (positive control) column and incubated at 37^0C for 1 h. Excess antigen was removed and the plate was washed 5 times with $PBST_{20}$. A 100 µl of undiluted (10^0) and diluted (10^{-1}, 10^{-2}, 10^{-3}) secondary antibody were dispensed on all wells except column 11 & 12 and incubated at 37^0C for 1 h. Excess antibody was removed and washed with $PBST_{20}$. A 100 µl of conjugate was added and incubated at 37^0C for 1 h followed by emptying and washing of the plate. Then, 100 µl of substrate was added and incubated for 30 minutes at room temperature. The reaction was stopped by adding 100 µl of stop solution and OD value was determined using an ELISA reader at 405 nm filter.

Evaluation of sensitivity and specificity of In-House sandwich ELISA

Three, 25-day-old local chickens were bought from KR market, BAU Mymensingh for experimental infection. Various samples (8 feces, 8 cloacal swab, 2 spleen and 2bursa) from experimentally infected and natural outbreaks cases (6 bursa and 6 spleens) from three layer farms (Mymensingh, Muktagacha and Sirajgonj) were subjected to prepare 20% inoculums with PBS to evaluate the newly developed ELISA. A 100 µl of undiluted (10^0) inocula were dispensed into all wells of the rows A to H respectively coated with anti-IBDV antibody (10^{-2}) and incubated at 37^0C for 1 h. Excess antigen was removed and the plate was washed with $PBST_{20}$. Addition of 100 µl sera raised in chickens (10^0, 10^{-1} to 10^{-3}) on A to H rows respectively and incubated at 37^0C for 1 h. Excess antibody was removed and washed with $PBST_{20}$. A 100 µl of conjugate was added to all wells and incubated at 37^0C for 1 h followed by emptying and washing. Then, 100 µl of substrate was added and incubated for 30 minutes at room temperature. Orange color was developed in case of positive reaction and the reaction stopped by adding 100 µl of stop solution (1N H_2SO_4). The OD value was determined using an ELISA reader at 405 nm filter.

Statistical Analysis

The statistical analysis to compare the specificity between the newly developed In-House sandwich ELISA and agar gel immunodiffusion tests was done according to the statistical formula given by Samad *et al.* (1994). The statistical formula was used as described below.

		Gold standard test (agar gel immunodiffusion test)		Total
		Positive	Negative	
In-House sandwich ELISA	Positive	a	b	a+b
	Negative	c	d	c+d
Total		a+c	b+d	a+b+c+d=N

The notations used above are defined as under.

a =Number of samples positive to both conventional and the gold standard tests

b = Number of samples positive to conventional but negative to the gold standard test

c = Number of samples negative to conventional but positive to the gold standard test

d = Number of samples negative to both conventional and the gold standard tests

a+b+c+d=Total number of samples (N)

Specificity: It is the capacity of the test to detect non-diseased cases, when compared with the gold standard test (d/b+d x 100).

RESULTS AND DISCUSSION

Confirmation of anti-IBDV antibody raised in rabbit and chicken by AGIDT

Prominent white line of precipitation was formed between bursal homogenates of the central well and known positive anti-IBDV hyper-immune serum of each of the five of the six peripheral wells due to antigen and antibody reaction. The band was more distinct incase of the serum collected after 28th days of vaccination than the others. Similar findings were reported by Joshi and Shakya (1996), Thevathasan and Jayawardana (1997), Umapathi *et al.* (2002) and Makadiya (2004).

Plate 4: AGIDT slide showing positive result for the prepared hyper immune serum of rabbit against IBDV. Well 1= Control serum, 2&6= Serum before 1st vaccination, 3=Serum after day 7 of 1st vaccination,4= Serum after day 7 of 2nd vaccination, 5= Serum after day 7 of 3rd vaccination.

Plate 5: AGIDT slide showing positive result for the prepared hyper immune serum of chicken against IBDV. 1= Control serum, 2&6= Serum before 1st vaccination, 3=Serum after day 7 of 1st vaccination,4= Serum after 7 day of 2nd vaccination, 5= Serum after 7 day of 3rd vaccination.

Determination of sensitivity and specificity of in-house sandwich ELISA

Standardization of optimal concentration of hyper-immune serum to coat the newly developed In-House sandwich ELISA plate

Among various dilutions (10^{-1} up to 10^{-4}) of hyper-immune serum raised in rabbit, the serum dilution 10^{-2} showed suitable reading for the detection of IBD virus. For this reason, the developed sandwich ELISA was further coated with 10^{-2} dilution of hyper-immune serum to evaluate the sensitivity and specificity of the newly developed In-House sandwich ELISA (Table 1).

Table 1. OD values for the standardization of newly developed in-House sandwich ELISA

	Hyper-immune serum dilution to coat the plate	Dilution of known IBD virus	OD value of sandwich ELISA											
			1	2	3	4	5	6	7	8	9	10	11*	12**
A	10^{-1}	10^{0}	2.814	2.879	2.868	2.807	2.763	2.707	2.789	2.677	2.683	2.715	0.639	1.976
B	10^{-1}	10^{0}	2.868	2.838	2.829	2.820	2.778	2.869	2.650	2.757	2.657	2.747	0.568	2.189
C	10^{-2}	10^{-1}	2.667	2.646	2.637	2.677	2.631	2.569	2.595	2.568	2.693	2.664	0.624	2.213
D	10^{-2}	10^{-1}	2.650	2.621	2.676	2.668	2.706	2.658	2.694	2.689	2.635	2.598	0.695	2.234
E	10^{-3}	10^{-2}	1.854	1.971	2.014	1.962	1.864	1.924	2.015	1.675	2.139	1.629	0.761	2.378
F	10^{-3}	10^{-2}	1.964	2.202	1.958	1.729	1.857	2.034	1.824	1.546	1.964	1.846	0.772	2.563
G	10^{-4}	10^{-3}	1.686	1.490	1.486	1.481	1.476	1.675	1.428	1.414	1.356	1.426	0.786	2.265
H	10^{-4}	10^{-3}	1.468	1.465	1.446	1.437	1.430	1.309	1.192	1.464	1.452	1.449	0.798	2.365

* = Negative control, **= Positive control

Sensitivity of the newly developed In-House sandwich ELISA

The known IBD virus dilution, 10^{-3} appeared to be the highest dilution, which had the lowest capacity to bind with the coated antibody of the ELISA plate (Table 2). This result indicated that IBD viruses absent in the dilution which was above 10^{-3} resulting that they failed to bind with the coated antibody of the ELISA plate which agree with the earlier report of Barman *et al.* (2003). The column 11 containing negative control serum did not show any binding where as, the column 12 showed type specific binding with positive control serum.

Table 2: OD values of the results of sensitivity of newly developed in-House sandwich ELISA.

	Dilution of hyper-immune serum for coating of the plate	Dilution of known IBD virus	OD value of sandwich ELISA											
			1	2	3	4	5	6	7	8	9	10	11*	12**
A	10^{-2}	10^{-1}	2.598	2.586	2.497	2.486	2.473	2.435	2.558	2.531	2.564	2.537	0.598	2.169
B	10^{-2}	10^{-1}	2.561	2.581	2.565	2.561	2.536	2.496	2.483	2.538	2.567	2.546	0.637	2.198
C	10^{-2}	10^{-2}	2.475	2.469	2.547	2.434	2.416	2.402	2.461	2.438	2.443	2.436	0.689	2.276
D	10^{-2}	10^{-2}	2.432	2.416	2.401	2.494	2.479	2.445	2.423	2.505	2.473	2.501	0.765	2.265
E	10^{-2}	10^{-3}	1.548	1.596	1.653	1.587	1.584	1.579	1.616	1.608	1.616	1.568	0.628	2.258
F	10^{-2}	10^{-3}	1.596	1.612	1.598	1.584	1.593	1.595	1.539	1.601	1.565	1.623	0.773	2.349
G	10^{-2}	10^{-4}	0.781	0.651	0.534	0.673	0.679	0.614	0.661	0.589	0.568	0.713	0.783	2.456
H	10^{-2}	10^{-4}	0.578	0.627	0.583	0.617	0.698	0.609	0.671	0.576	0.768	0.715	0.791	2.537

* = Negative control, **= Positive control

Determination of cut-off value from negative control readings

The cut-off value of present ELISA system was determined by using the mean absorbance of negative control (Table 1 & 2) plus three times the standard deviation (Kumar & Rao, 1991). The cut-off value of present ELISA system is calculated as 0.940 (Table 3). If the titer of tested samples with the newly developed In-House sandwich ELISA is greater than 0.940, indicates IBDV positive whereas, less than 0.940 indicates negative.

Calculation Factor: Cut-off Value = (Mean ± 3 × standard deviation) of the negative control serum.

Table 3: Cut-off value for newly developed In-House sandwich ELISA

Serial no	OD value	Mean±SD	Mean+3SD	Cut-off value
1	0.639			
2	0.568			
3	0.697			
4	0.772			
5	0.695			
6	0.624			
7	0.786			
8	0.798			
9	0.598	0.703±0.079	0.703±0.237	0.940
10	0.637			
11	0.689			
12	0.765			
13	0.628			
14	0.773			
15	0.783			
16	0.791			

Specificity of the newly developed In-House sandwich ELISA by using known IBD and ND viruses

The IBD virus was found to bind with the coated antibody instead of ND virus in the plate, which revealed the high specificity of the newly developed In-House sandwich ELISA against IBD (Table 4).

Table 4. OD values of the result of specificity of newly developed in-House sandwich ELISA

	Hyper-immune serum dilution to coat the plate	Dilution of known ND & IBD viruses	OD value of different dilution of ND and IBD viruses											
			1	2	3	4	5	6	7	8	9	10	11*	12**
A	10^{-2}	10^{-1}(IBD)	2.659	2.647	2.629	2.698	2.684	2.753	2.661	2.685	2.653	2.647	0.647	2.173
B	10^{-2}	10^{-1}(ND)	0.556	0.528	0.564	0.543	0.527	0.545	0.561	0.532	0.554	0.516	0.505	2.189
C	10^{-2}	10^{-2}(IBD)	2.413	2.494	2.461	2.512	2.497	2.445	2.443	2.513	2.456	2.501	0.758	2.267
D	10^{-2}	10^{-2}(ND)	0.664	0.613	0.681	0.618	0.521	0.615	0.582	0.528	0.613	0.548	0.701	2..246
E	10^{-2}	10^{-3}(IBD)	1.693	1.638	1.614	1.598	1.657	1.601	1.643	1.576	1.563	1.511	0.607	2.348
F	10^{-2}	10^{-3}(ND)	0.769	0.758	0.689	0.696	0.615	0.569	0.583	0.561	0.572	0.531	0.554	2.469
G	10^{-2}	10^{-4}(IBD)	1.501	1.568	1.593	1.558	1.521	1.538	1.614	1.589	1.567	1.576	0.782	2.563
H	10^{-2}	10^{-4}(ND)	0.781	0.726	0.686	0.649	0.596	0.674	0.661	0.567	0.572	0.512	0.662	2.581

*=Negative control, **= Positive control

Determination of sensitivity and specificity of in-house sandwich ELISA

Evaluation of sensitivity and specificity of newly developed In-House sandwich ELISA for the diagnosis of field samples

In case of experimental infection, 5 (62.5%) of 8 fecal samples and none of the cloacal swab samples were positive for IBDV by newly developed In-House sandwich ELISA assay. The fecal samples collected after day 2 to 4 of post infection was positive for the detection of virus and was absent in the sample of day first of infection. Both the bursa and spleen of experimentally infected dead birds were positive for the diagnosis of IBD (Table 5 & 7). The result partially supports the findings of Kanani (2000).

In case of natural outbreak cases in the layer farms of Muktagach, Sirajgonj and Mymensingh areas, 6 (100%) of 6 bursal samples and 4 (66.66%) of 6 spleen samples were positive for the diagnosis of IBD. Spleen sample of each bird of Sirajgonj and Mymensingh district was negative for IBDV (Table 6 & 8).

Table 5. OD values of the test samples of experimentally infected group of bird

	Sample description	OD value of sandwich ELISA											
		1	2	3	4	5	6	7	8	9	10	11	12
		Feces sample						Cloacal sample					
A	Day 1	0.714	0.726	0.665	0.701	0.711	0.698	0.679	0.765	0.723	0.709	0.679	0.598
B	Day 2	2.797	2.815	2.896	2.869	2.856	2.745	0.621	0.693	0.764	0.768	0.676	0.667
C	Day 3	2.839	2.853	2.814	2.832	2.783	2.616	0.824	0.736	0.746	0.597	0.539	0.648
D	Day 4	2.867	2.851	2.845	2.783	2.869	2.589	0.865	0.798	0.711	0.756	0.765	0.636
E	Control	0.656	0.723	0.712	0.665	0.663	0.598	0.701	0.645	0.543	0.653	0.789	2.269
		Bursal samples						Spleen					
F	Dead birds	2.756	2.769	2.813	2.746	2.778	2.801	2.815	2.798	2.764	2.758	2.637	2.223
G	Control	0.742	0.563	0.495	0.521	0.563	0.498	0.475	0.421	0.391	0.403	0.567	0.456
H	Blank	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	0.783	2.465

Table 6. OD values of the samples from natural outbreak cases

	Sample description	OD value of different dilution of IBD virus											
		1	2	3	4	5	6	7	8	9	10	11	12
A	Bursa (Sirajgonj)	2.768	2.776	2.713	2.745	2.764	2.756	2.189	2.789	2.636	2.713	2.786	2.169
B	Spleen (Sirajgonj)	2.745	2.789	2.506	2.896	2.856	2.895	0.881	0.874	0.835	0.736	0.702	0.798
C	Bursa (Mymensingh)	2.738	2.856	2.858	2.865	2.835	2.619	2.876	2.859	2.765	2.739	2.689	2.213
D	Spleen (Mymensingh)	2.912	2.869	2.764	0.781	2.765	2.732	0.764	0.778	0.637	0.864	0.765	0.665
E	Bursa (Muktagacha)	2.902	2.754	2.812	2.896	2.873	2.737	2.756	2.868	2.832	2.731	2.732	2.198
F	Spleen (Muktagacha)	2.712	2.598	2.543	2.832	2.843	1.776	1.785	1.736	1.813	1.636	2.698	2.256
G	Blank	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL
H	Blank	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL	BL

Sensitivity and specificity of AGIDT to diagnose clinical and post-mortem samples of experimentally and naturally infected chickens

AGID has been reported by a number of workers to be useful in easy screening of the field samples, prior to either isolation or detection and characterization of the virus (Parthiban *et al.,* 2000 and Kadam, 2001). In case of experimental infection, 5 (62.5%) of 8 fecal samples but no cloacal swab samples and all the postmortem samples (2 bursa and 2 spleen) were positive by AGIDT (Table 7). The positive fecal samples were collected within day 2 to 4 of post infection and sample of first day was negative. The result correspond with the findings of Dash *et al.* (1991) and Kanani, (2000) who detected IBDV antigen in BF from one to sixth day of PI using AGIDT. Among the 12 post mortem samples (6 bursa + 6 spleens) of natural outbreaks, all the bursal samples

(100%) but 3 spleen samples (50%) revealed distinct white line of precipitation with anti-chicken/rabbit serum against IBDV (Table 8). The result is in good agreement with the findings of Prajapati and Jalnapurkar (1982), Panisup *et al.* (1989), Snyder *et al.* (1992), Vijaya Praveen *et al.* (1995) and Parthiban *et al.* (2001).

Table 7. IBDV positive cases by developed In-House sandwich ELISA and AGIDT from the samples of experimentally infected cases

Experimental infection	No. of birds	Type of samples	No. of samples tested	No. of IBDV Positive samples	
				In-House ELISA	AGIDT
Day-1	2	Feces	2	0	0
		Cloacal swab	2	0	0
Day-2 (Sick)	2	Feces	2	1	1
		Cloacal swab	2	0	0
Day-3 (Sick)	2	Feces	2	2	2
		Cloacal swab	2	0	0
Day-4 (Dead)	2	Feces	2	2	2
		Cloacal swab	2	0	0
		Bursa	2	2	2
		Spleen	2	2	2
Control (Dead)	1	Faeces	1	0	0
		Cloacal swab	1	0	0
		Bursa	1	0	0
		Spleen	1	0	0

Table 8. IBDV positive cases by developed In-House sandwich ELISA and AGIDT from the samples of naturally infected cases

Places of sampling	No. of dead birds	Type of samples	No. of samples tested	No. of IBDV Positive samples	
				In-House ELISA	AGIDT
Sirajgonj	2	Bursa	2	2	2
		Spleen	2	1	1
Mymensingh	2	Bursa	2	2	2
		Spleen	2	1	1
Muktagacha	2	Bursa	2	2	2
		Spleen	2	2	1

Comparison between newly developed In-House sandwich ELISA and Agar gel immunodiffusion test (AGIDT)

The result of AGIDT from different experimental IBDV infection using known hyper-immune serum against IBDV showed 100% similarity with the results of newly developed In-House sandwich ELISA (Table 7). In case of natural outbreak, the result was almost similar except one splenic sample of Muktagacha which was negative by AGIDT but was positive by In-House sandwich ELISA (Table 8). The result was correspondent with the findings of Ajinkya *et al.* (1980) who reported that bursal suspensions were more reliable source for IBDV antigen. Out of total 32 tested samples except control, 19 (9 experimental infection and 10 natural outbreak) were

positive for IBDV by newly developed In-House sandwich ELISA whereas, 18 (9 experimental infection and 9 natural outbreak) by AGIDT which revealed 92.85% specificity of newly developed In-House sandwich ELISA method with AGIDT (Table 9).

Table 9. Results of specificity test

		Gold standard test (agar gel immunodiffusion test)		Total
		Positive	Negative	
In-House sandwich ELISA	Positive	18	1	19
	Negative	0	13	13
Total		18	14	32
Specificity (d/b+d x 100)				92.85%

From the above findings the present study may be concluded that studies of the molecular epidemiology of IBDV are important and the In-House sandwich ELISA could be used as an alternative technique for screening a large number of samples before testing (Tham *et al.,* 1995) and also for the confirmation of the IBDV quickly from a large number of IBD suspected field samples. If it is produced commercially in a country it can be a valuable tool for the detection of IBDV virus with minimum cost and it is highly reliable like other procedures of IBDV isolation and detection such as AGIDT, molecular detection.

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