Bangladesh Society for Veterinary Microbiology and Public Health Since 2011

Microbes and Health

ISSN: 2226-0153 (Print) 2305-3542 (Online)

http://journal.bsvmph.org/

Microbes and Health, December 2012, 1(2): 65-71
DOI: 10.3329/mh.v1i2.14093

Sensitivity and Specificity of an In-house Sandwich ELISA Kit for Newcastle Disease Virus Antigen Detection

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[**Received**: 03 December 2012, **Revised**: 10 December 2012, **Accepted**: 12 December 2012]

ABSTRACT

Of all serological tests enzyme-linked immunosorbent assay (ELISA) is still considered the gold standard for the detection of antigens and antibodies of either macro or micro-organisms worldwide. The ELISA kits for serum antibody detection against many viruses and other micro-organisms of both man and animals are available in the market. Whereas, antigen detection ELISA kits for Newcastle disease virus (NDV) is not yet available in Bangladesh. The Present study was designed for the development of an economically feasible In-house Sandwich ELISA and to test its sensitivity and specificity for the detection of NDV antigens from clinically suspected field samples. 96-well flat bottom polystyrene plates coated with hyperimmune polyclonal serum against NDV raised in rabbits was used to capture NDV antigens. The anti-rabbit IgG and DAB with 30% H_2O_2 were used as conjugate and substrate respectively for standardization of the test method. The plate coated with serum diluted 10^{-3} was found suitable for capturing maximum antigen of NDV by the In-house Sandwich ELISA. The cut-off value of the present ELISA test was calculated as 0.855 and was able to capture the viral antigen present in the 10^{-4} fold dilution of allantoic fluid (AF) which is equivalent to 1HA unit, indicating the highest degree of sensitivity of the newly developed ELISA. In case of field samples, the newly developed ELISA kit was able to detect 100% viral antigens of NDV present in the feces, 95.50% of the brain tissue and oro-nasal swab and 94.12% of colon swab samples of either naturally and experimentally infected birds in this study. The ND virus specific polyclonal antibody used in the kit bind only with ND virus without any cross reactive antigens of other viruses of chicken like Avian influenza virus (AIV) and Infectious bursal disease viruses (IBDV). Therefore, findings of the present study clearly indicates that the newly developed In-house Sandwich ELISA kit can be used for rapid confirmatory diagnosis of Newcastle disease (ND) with minimum cost, using any kind of field samples from either sick or dead birds.

Keywords: In-house, Sandwich ELISA, Newcastle disease virus, Antigen detection, Polyclonal serum

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Introduction

Newcastle disease (ND) is a deadly viral disease of poultry all over the world since the time of first isolation in England in 1926 to today. This disease is caused by a single-stranded, enveloped, non-segmented, negative-sense RNA virus belonging to the genus Avulavirus of the sub-family Paramyxovirinae and family Paramyxoviridae under the order Mononegavirales (Barbezange and Jestin, 2005; Mayo, 2002). Depending on pathogenicity, NDV has been distinguished into five pathotypes; viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic, and asymptomatic viruses (Alexander, 1997; Westbury, 1984). In Bangladesh, frequent outbreaks of ND is mostly due to velogenic strain of NDV (Chowdhury *et al*., 1982). The members of lentogenic strains of NDV are B1, LaSota, and F (Hitchner and Johnson, 1948; Winterfield *et al*., 1957).

ND is considered as one of the major threats to the poultry industry in Bangladesh because of its high morbidity, mortality and reduced productivity of eggs this results in remarkable economic losses every year since first isolation and identification of the virus in 1978 in Bangladesh by Chowdhury *et al*., (1982). According to Chowdhury *et al*., (1982), ND alone is responsible for at least 40-60% mortality of the total population of poultry in Bangladesh.

Most farmers of Bangladesh confuse ND with other infectious diseases of poultry during endemic and epidemic outbreaks like Avian influenza (AI), Infectious bursal disease (IBD), Infectious bronchitis (IB), and Infectious laryngotracheitis (ILT) due to lack of rapid and confirmatory diagnostic facilities. Traditionally, the methods of diagnosis of ND practiced at the field level of Bangladesh are very much limited. Recording of the clinical signs and post-mortem findings manifested by the affected birds being the only method. Recently serology based diagnostic methods like the haemagglutination inhibition test (HIT) (Miers *et al*., 1983), virus neutralization test (VNT) (Beard *et al*., 1980), fluorescent antibody technique (FAT), plaque reduction neutralization test (PRNT) (Offit *et al.* 1983), agar gel immunodiffusion test (AGIDT) (Tsai and Lee, 2006, Gelf and Cianci, 1987) and virus isolation have been introduced in the field of diagnosis all over the world. Although very few of the above mentioned methods are being practiced in the field for diagnosis of ND in this country, they are not so popular because

To cite this article: Kamal MM, MM Ali, ME Haque, MMI Chowdhury, MA Haque, A Aktar, MJ Ara, ME Haque and MM Islam, 2012. Sensitivity and specificity of an in-house sandwich ELISA kit for Newcastle disease virus antigen detection. Microbes Health, 1(2): 65-71.

of low reliability, authenticity and the difficulty to perform due to high consumption of media, reagents, chemicals and time. Several molecular techniques like polymerase chain reaction (PCR), reverse transcriptase polymerase chain reaction (RT-PCR), multiplex reverse transcriptase polymerase chain reaction (MRT-PCR) are available for rapid diagnosis of ND using samples collected directly from field cases. But these are expensive and require valuable equipment and skilled personnel to perform to develop a kit like sandwich ELISA for the detection of viral antigen, it is also a prerequisite to have purified virus particles. Because, purified virus has the ability to produce type specific antibody against specific viral antigen such as the HN, NA, F and M protein of NDV, AIV, IBDV respectively. The sandwich ELISA is relatively easy to perform following simple dilution, incubation and washing protocols. There is no need for secondary reactions like complement fixation and the result can be read visually on the basis of color change due to interaction between the enzyme and substrate with the ELISA reader. This assay is rapid, easy and its reagents are safe and relatively inexpensive with long shelf-life and able to detect a very low concentration of antigen (NDV). It can also be performed within 4-5 hours. It had been shown to be more sensitive than haemagglutination, haemagglutination inhibition, complement fixation, plaque reduction, virus neutralization and other serological tests (Nazerian *et al*., 1990). Commercial sandwich ELISA kits are available in many laboratories to determine the level of serum antibody not the viral antigens of NDV.
Therefore, keeping the above points in view, the present study was undertaken to develop a reliable, rapid and cost effective serological tool for the detection of ND viral antigen directly from field samples and to evaluate its sensitivity and specificity to avoid confusion with other deadly diseases (ND/AI/IBD) of poultry in the commercial and breeder flock of either layer or broiler farms in Bangladesh.

Materials and Methods

Reference NDV
Previously characterized velogenic strain of NDV was obtained from the repository of the Dept. of Microbiology and Hygiene, BAU, Mymensingh and used for large scale production of purified antigens for preparation of hyper immune serum in rabbits.

Embryonated eggs
Sero-negative eggs from 65 week old parent stock of broiler (Cob 500) chickens were used for the propagation the velogenic NDV.

Carrier adjuvant
Freund’s complete adjuvant (Sigma, USA) was used to bind the inactivated purified antigen of NDV for the production of hyper immune serum (polyclonal antibody) in rabbits.

96-well Microplate
Flat bottomed polystyrene 96-well plate was used to coat each well of the plate with primary antibody (anti-NDV) raised in the rabbit.

Rabbits
American white male, young (4 months old) and healthy rabbits were used for the production of hyperimmune serum against NDV.

Anti-Rabbit IgG
Horse radish conjugated commercial anti-rabbit IgG was used as a source of conjugate (Sigma, USA).

Substrates
3,3'-Diaminobenzidine (DAB) with 30% H_2O_2 was used as a source of substrate

ELISA Reader
ELISA reader (Thermo ELECTRON CORPORATION, ORIGINAL *MULTISKAN EX, Japan)* at 405 nm filter

Important reagents and chemicals
6% PEG-6000, 2.2% Sodiun chloride, Sucrose density gradient solution used for the purification of NDV and Dulbacco’s Phosphate buffer solution (dPBS), 1N (H_2SO_4), 0.5% $PBST_{20}$ (PBS with Tween20) and coating buffer (Na_2CO_3 2.795 gms, $NaHCO_3$ 3.465 gms , NaN_3 1.1 gms and 500 ml DW) used for ELISA

Purification of reference ND viruses
The velogenic starin of NDV was obtained from the repository of the Dept. of Microbiology and Hygiene, BAU, Mymensingh and inoculated into 10 day-old embryonated eggs through the allantoic cavity route of inoculation and incubated at 37^oC until death of the embryos. The allantoic fluid (AF) from dead embryos was harvested and the presence of NDV was confirmed by slide HA test. The virus particles were separated from non viral elements present in the AF by high speed centrifugation at 5,000 rpm for 30 minutes at 4^oC. The pellet was discarded and the clear supernatant was collected and treated with PEG-6000 (6.6%) and NaCl (2.2%) and the AF was allowed to stir overnight at 4^oC in the refrigerator. The PEG treated virus was further concentrated by high speed centrifugation at 12,000 rpm for 1 hour maintaining an internal temperature 4^oC. The supernatant was discarded and the pellet was resuspended with 1 X dPBS. A column of gradient solution was prepared using different concentrations of sucrose solution in dPBS (50%-15%). The PEG treated crude concentrated virus was overlayed on the top layer (15%) of the column containing different concentration of sucrose and was centrifuged at 16,000 rpm for 2 hours. Each band of virus particles which appeared between the columns was collected in glass test tubes using a manual fraction collector. More than one glass tube containing viruses of similar size and concentration were pooled, and the sucrose solution was diluted with dPBS. To settle the virus particles from the sucrose solution by centrifugation at 16,000 rpm for 2 hours at 4^oC. The purified and concentrated viral pellet was resuspended with dPBS and the presence of NDV was reconfirmed by slide HA test. The purified and concentrated virus was collected in cryo-vials and stored at -86^oC until used for vaccination of rabbits and chickens to prepare hyperimmune sera as a source of coating antibody and secondary binding antibody respectively against NDV to develop an In-house sandwich ELISA kit.

Adjuvanted vaccine preparation with NDV
The purified and concentrated NDV was inactivated with 0.1% formaldehyde and the inactivated virus particles were mixed at a ratio of 1:1 with the carrier adjuvant (Freund’s complete adjuvant) for the preparation of vaccine.

Production of hyperimmune serum
The vaccine prepared with the purified inactivated NDV was inoculated into rabbits at day 0, 14 and 28 for the production of hyperimmune serum. At 7 days of post vaccination blood samples from rabbits were collected for the preparation of hyperimmune serum. Antibody levels of each serum were screened by HI and AGID tests.

Coating of 96-well flat-bottomed microtitre plate with hyperimmune serum
The prepared hyperimmune serum (raised in rabbits) was diluted at 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} with coating buffer (P^H=9.6). 100 µl of each diluted serum was dispensed on A & B, C & D, E & F and G & H rows of the 96-well flat bottomed microtiter plate respectively. The coated plate was incubated for 1 hour at 37^oC, and then moved to 4^0 C overnight. Uncoated wells were blocked with 100 µl of 4% Block Ace solution. After blocking the plate was incubated for 1 hour at 37^oC. Blocking solution was discarded and the plate was washed 5 times with 200 µl of washing buffer solution ($PBST_{20}$) for each well. Then the antibody coated microtiter plate was stored at -20^oC until use.

Standardization of sensitivity of the newly developed In-house sandwich ELISA kit
The known reference NDV was diluted to 10^0 (undiluted), 10^{-1}, 10^{-2} and 10^{-3} with sample diluent to standardize the different

concentration of viral antigen to be detected by the newly developed In-house sandwich ELISA kit. For standardization of the newly developed In-house sandwich ELISA kit, 100 µl of each known undiluted (10^0) and diluted (10^{-1}, 10^{-2} and 10^{-3}) test antigens (NDV) were dispensed on A & B, C & D, E & F as well as G & H rows upto No. 10 well of the plate respectively. The negative and positive control samples were dispensed onto for numbers 11 and 12 rows well. After adding viral antigen the plate was incubated for 1 hour at 37ºC. Excess antigen was removed and the plate was washed 5 times with 200 µl of washing buffer ($PBST_{20}$). After that 100 µl of diluted (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) hyperimmune sera raised in rabbit was dispensed on A & B, C & D, E & F and G & H rows of the microtiter plate respectively. The plate was incubated at 37ºC for 1 hour. Hyperimmune serum was removed and the plate was washed 5 times with 200 µl of washing buffer ($PBST_{20}$). A 100 µl conjugate solution was added to each well of the ELISA plate. The plate was incubated again for 1 hour at 37ºC. The conjugate solution was removed and the plate was washed 5 times with 200 µl of washing buffer ($PBST_{20}$). Finally 100 µl of substrate solution (DAB) was added to each well of the ELISA plate. The plate was incubated for 30 minutes at room temperature. After the establishment of positive reaction between substrate and conjugate, an orange colour developed and the reaction was stopped by adding 100 µl of stop solution with 1N H_2SO_4 to each well of the ELISA plate. The intensity of color development was measured by determination of the OD (optical density) value using an ELISA reader (Thermo ELECTRON CORPORATION, ORIGINAL *MULTISKAN EX, and Japan)* at 405 nm filter.

Specificity test of the newly developed In-house sandwich ELISA kit

The specificity test of the newly developed In-house sandwich ELISA kit was performed using two known viruses NDV and IBDV. Samples were diluted 10^{-1}-10^{-4} to show the specificity of the newly developed In-house sandwich ELISA kit designed for specific binding of viral antigen of NDV only. For the specificity test, 100 µl of 10^{-1},10^{-2}, 10^{-3} and 10^{-4} concentration of prepared inoculum of known ND & IBD suspected field samples were dispensed on A & B, C & D, E & F and G & H columns up to 10 rows of antibody coated microtiter plate respectively. The negative and positive control samples were dispensed on rows 11 and 12. After adding the viral antigen the plate was incubated for 1 hour at 37ºC. Excess antigen was removed and the plate was washed 5 times with 200 µl of washing buffer ($PBST_{20}$). After that 100 µl of 10^{-3} diluted hyperimmune serum raised in rabbit was dispensed on the A & B, C & D, E & F and G & H rows of the microtiter plate. The plate was incubated at 37ºC for 1 hour. Hyperimmune serum was removed and the plate was washed 5 times with washing buffer ($PBST_{20}$). A 100 µl conjugate solution was added to each well of the ELISA plate. The plate was incubated again for 1 hour at 37ºC. The conjugate solution was removed and the plate was washed 5 times with washing buffer ($PBST_{20}$). Finally, 100 µl of substrate solution was added in each well of the ELISA plate. The plate was then incubated for 30 minutes at room temperature until the color reaction appeared. After establishment of positive reaction between the substrate and conjugate an orange colour developed and the reaction was stopped by adding 100 µl of stop solution with 1N H_2SO_4 to each well of the ELISA plate. The intensity of color development was measured by determination of OD (optical density) value using an ELISA reader (Thermo ELECTRON CORPORATION, ORIGINAL *MULTISKAN EX, Japan)* at 405 nm filter.

Results

Purification of reference NDV by sucrose density gradient centrifugation

During purification of reference NDV virus by high speed centrifugation the sub-cellular elements were found in suspension between 45% and 50% fractions of the sucrose solution. The large virus particles were found in suspension between 40% and 45%, medium virus particles were found in suspension between 30% and 35% and the lipid and other non-viral particles were found in suspension between 15% and 25% of the sucrose solution.

HI titer of hyperimmune serum of rabbits vaccinated with purified inactivated NDV

The micro-plate HI test was conducted to determine the HI titer of hyperimmune serum samples collected from each of the two rabbits before and after vaccination with the purified NDV in this study. Sera samples of vaccinated and non-vaccinated control rabbits were tested with the serum samples collected at day 14 and day 28 after the 1st and 2nd vaccinations, and also with the hyperimmune serum collected at day 7th post vaccination by microtiter HI test. The HI titer of hyperimmune serum of rabbits vaccinated with the third dose of purified inactivated NDV revealed the highest HI titer compared to the serum of first and second vaccinations, 1536±724.1, 192±90.5 and 384±181.0 respectively in this study.

Table 1. Standardization of In-house sandwich ELISA kit

	Dilution of hyperimmune serum for coating of the plate	Dilution of known ND 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	0.647	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	0.505	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	0.758	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	0.701	2.234
E	10^{-3}	10^{-2}	2.454	2.424	2.414	2.487	2.469	2.499	2.465	2.513	2.496	2.489	0.607	2.378
F	10^{-3}	10^{-2}	2.504	2.425	2.458	2.484	2.420	2.439	2.448	2.465	2.425	2.489	0.554	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	0.782	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	0.662	2.365

* = Negative control, **= Positive control

Table 2. Determination of cut-off value for negative control samples

No. of samples	Name of samples	OD value	Mean±SD	Mean+3SD	Cut-off value
Sample-1	Faeces*	0.782	0.705±.05	0.705+0.15	0. 855
Sample-2	Oro-nasal swab*	0.751			
Sample-3	Brain*	0.703			
Sample 4	Lung*	0.697			
Sample-5	Spleen*	0.667			
Sample-6	Colon*	0.665			
Sample-7	Thymus*	0.662			
Sample-8	Kidney*	0.666			
Sample-9	Bursa*	0.654			
Sample-10	Faeces**	0.769			
Sample-11	Oro-nasal swab**	0.756			
Sample-12	Brain**	0.764			
Sample-13	Lung**	0.746			
Sample-14	Spleen**	0.701			
Sample-15	Colon**	0.734			
Sample-16	Thymus**	0.668			
Sample-17	Kidney**	0.667			
Sample-18	Bursa**	0.638			

*= control bird 1, **= control bird 2

Table 3. Sensitivity and detection limit of the In-house sandwich ELISA kit

	Dilution of hyperimmune serum for coating of the plate	Dilution of known ND virus	OD value of sandwich ELISA											
			1	2	3	4	5	6	7	8	9	10	11*	12**
A	10^{-3}	10^{-2}	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^{-3}	10^{-2}	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^{-3}	10^{-3}	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^{-3}	10^{-3}	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^{-3}	10^{-4}	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^{-3}	10^{-4}	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^{-3}	10^{-5}	0.781	0.551	0.534	0.673	0.579	0.514	0.661	0.589	0.568	0.713	0.783	2.456
H	10^{-3}	10^{-5}	0.578	0.627	0.583	0.517	0.698	0.509	0.671	0.576	0.768	0.715	0.791	2.537

* = Negative control, **= Positive control

Table 4. Specificity of the In-house sandwich ELISA kit

	Dilution of hyperimmune serum for coating of 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^{-3}	10^{-1}(ND)	2.659	2.647	2.629	2.698	2.684	2.753	2.661	2.685	2.653	2.647	0.639	2.173
B	10^{-3}	10^{-1}(IBD)	0.556	0.528	0.564	0.543	0.527	0.545	0.561	0.532	0.554	0.516	0.568	2.189
C	10^{-3}	10^{-2}(ND)	2.413	2.494	2.461	2.512	2.497	2.445	2.443	2.513	2.456	2.501	0.624	2.267
D	10^{-3}	10^{-2}(IBD)	0.664	0.613	0.681	0.618	0.521	0.615	0.582	0.528	0.613	0.548	0.695	2..246
E	10^{-3}	10^{-3}(ND)	1.693	1.638	1.614	1.598	1.657	1.601	1.643	1.576	1.563	1.511	0.761	2.348
F	10^{-3}	10^{-3}(IBD)	0.769	0.758	0.689	0.696	0.615	0.569	0.583	0.561	0.572	0.531	0.772	2.469
G	10^{-3}	10^{-4}(ND)	1.501	1.568	1.593	1.558	1.521	1.538	1.614	1.589	1.567	1.576	0.786	2.563
H	10^{-3}	10^{-4}(IBD)	0.781	0.726	0.686	0.649	0.596	0.674	0.661	0.567	0.572	0.512	0.798	2.581

* = Negative control, **= Positive control

Table 5. Sensitivity of In-house Sandwich ELISA kit for antigen detection of clinical and post-mortem samples of experimentally infected layer chickens

Experimental infection	No. of birds	Type of samples	No. of samples tested	Positivity of NDV	
				No. of positive samples	%
Day-1	5	Faeces	5	0	0
		Oro-nasal swab	5	0	0
Day-2 (Sick)	5	Faeces	5	5	100
		Oro-nasal swab	5	4	80
Day-3 (Dead)	5	Faeces	5	5	100
		Oro-nasal swab	5	5	100
		Brain	5	5	100
		Spleen	5	3	60
		Lungs	5	5	100
		Colon	5	5	100
		Bursa	5	0	0
Control bird	1	Faeces	1	0	0
		Oro-nasal swab	1	0	0
		Brain	1	0	0
		Spleen	1	0	0
		Lungs	1	0	0
		Colon	1	0	0
		Bursa	1	0	0

Table 6. Specificity of In-house Sandwich ELISA kit for antigen detection of clinical and post-mortem samples of suspected ND and IBD from natural outbreak

Place of sampling of natural outbreak	No of birds	Type of birds	Type of samples	No. of samples tested for virus isolation	Positivity of NDV	
					No. of positive samples	%
Gazipur (commercial layer farm)	2	Sick birds	Faeces	2	2	100
			oro-nasal swab	2	2	100
			Brain	2	2	100
			Spleen	2	1	50
			Lungs	2	1	50
			Colon	2	1	50
			Bursa	1	0	0
	2	Dead birds	Faeces	2	2	100
			Oro-nasal swab	2	2	100
			Brain	2	2	100
			Spleen	2	2	100
			Lungs	2	2	100
			Colon	2	2	100
			Bursa	1	0	0
Muktagacha (commercial layer farm)	2	Sick birds	Faeces	2	2	100
			Oro-nasal swab	2	2	100
			Brain	2	2	100
			Spleen	2	1	50
			Lungs	2	1	50
			Colon	2	2	100
			Bursa	1	0	0
	2	Dead birds	Faeces	2	2	100
			Oro-nasal swab	2	2	100
			Brain	2	2	100
			Spleen	2	2	100
			Lungs	2	2	100
			Colon	2	2	100
			Bursa	2	0	0
Kishurganj (Backyard layer farm)	2	Sick birds	Faeces	2	2	100
			Oro-nasal swab	2	2	100
			Brain	2	2	100
			Spleen	2	1	50
			Lungs	2	2	100
			Colon	2	2	100
			Bursa	1	0	0
	2	Dead birds	Faeces	2	2	100
			Oro-nasal swab	2	2	100
			Brain	2	2	100
			Spleen	2	2	100
			Lungs	2	2	100
			Colon	2	2	100
			Bursa	2	0	0

Discussion

The research work was undertaken with a view to develop and evaluate the sensitivity and specificity of a newly developed In-house sandwich ELISA kit for the detection of Newcastle disease viral antigen from field outbreak cases. For the development of the In-house sandwich ELISA kit, purified and concentrated NDV antigen was used. The NDV was purified using a high speed centrifugation method (Folitse *et al*., 1998) followed by sucrose density gradient purification (Williams *et al*., 1997). As an alternate method, robotic RBC capturing was used. In sucrose density gradient centrifugation method, the purified NDV was found in suspension between the 40% and 45% fraction of the gradient solution. The result of concentration of purified virus particles of this study closely agrees with the findings of Saha *et al. (*1998) and JianMing *et al.* (2008). For further confirmation of the presence of NDV, the purified and concentrated NDV was tested by AGIDT. Hyperimmune serum preparation was done by inoculating inactivated purified NDV into rabbits with three consecutive injections at day 0, 14 and 28 respectively. Antibody level of hyperimmune serum raised in rabbits was determined by HIT. The last vaccination of rabbit with inactivated adjuvanted ND vaccines induced better immune response (higher HI titer) than the immune response of primary and secondary vaccinations. The serum antibody titre of the present findings supports the findings of Gosh *et al.* (2003).

Precipitating antibody levels of hyperimmune serum raised in rabbits vaccinated thrice with inactivated adjuvanted ND vaccine were determined by AGIDT. The result of the AGIDT in the present study revealed that a clear distinct white band of precipitation due to type-specific antigen and antibody reaction of NDV appeared on the 1% agarose gel. The serum of rabbits collected before vaccination as control serum failed to show any band. The hyperimmune serum raised in rabbits after each vaccination revealed very clear and distinct bands.

The present study clearly indicated that band of precipitations reaction appeared in those cases where the antibody was specific to the antigen whereas absence of the band indicated that the antibody was not present against type specific antigen to the supplied serum samples of non-vaccinated rabbits. Similar types of findings were also reported by Saha *et al.* (1998) and Gosh *et al.* (2003).

Standardization of the newly developed In-house sandwich ELISA kit was performed by dispensing different dilutions (10^0, 10^{-1}, 10^{-2}, 10^{-3}) of the known reference ND viruses on the newly

developed In-house sandwich ELISA kit coated with different dilutions (10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}) of hyperimmune serum raised in rabbits. The serum dilution 10^{-3} showed suitability for the development of the sandwich ELISA kit to bind the concentration of viral antigens present in the tested samples.

The cut-off value of the ELISA system is determined by using 18 negative control samples from two control birds. The cut-off value of the present ELISA system is calculated as 0.855. If the titer of tested samples with the newly developed In-house sandwich ELISA kit is greater than 0.855, it indicates a positive result of tested samples. Less than 0.855 indicates a negative result of tested samples.

Sensitivity of the newly developed In-house sandwich ELISA kit was done by adding different dilutions (10^{-2}, 10^{-3}, 10^{-4} and 10^{-5}) of the known reference ND viruses to the plate which was coated with a 10^{-3} dilution of hyperimmune serum of rabbits. The virus dilution 10^{-4} appeared to be the highest dilution with capacity to bind the coated antibody of the ELISA plate. This indicated that ND viruses absent in the dilution above 10^{-4} failed to bind with the specific antibody coated ELISA plate. The sensitivity of the newly developed In-house sandwich ELISA revealed that the ND suspect samples were negative in direct slide haemagglutination (HA) test; not gave positive results in the newly developed In-house sandwich ELISA kit. The newly developed In-house sandwich ELISA kit can measure a minimum of 1 HA unit of ND virus present in the tested samples. This finding supports the results of Hu *et al*. (2002).

Specificity of the newly developed In-house sandwich ELISA kit was performed by adding various dilutions of two different known viruses like NDV and IBDV. The viruses were diluted from 10^{-1} to10^{-4} for the newly developed In-house sandwich ELISA kit which was coated with 10^{-3} dilution of hyperimmune serum raised in rabbits. The ND virus was found to bind with the coated antibody instead of IBDV in the plate which revealed the high specificity of the newly developed In-house sandwich ELISA kit for NDV. Specificity of viral antigen binding properties of the polyclonal serum of rabbit raised against NDV in this study support the findings reported by Fengying *et al*. (2008).

The development of the In-house sandwich ELISA kit for the detection of Newcastle disease virus is new in Bangladesh. This study has created the opportunity to study the serological investigations of ND virus which is one of the most important viral diseases of poultry now prevalent in Bangladesh. This will ultimately helps to take special measure to control and prevent the frequent outbreak of ND among the poultry population of Bangladesh.

All the samples of sick or dead birds either from natural or experimental infection were tested to detect the NDV antigen using the newly developed sandwich ELISA kit. Results of the present study clearly indicated that the newly developed sandwich ELISA kit which was used for the detection of NDV was a highly sensitive, specific, rapid, and economic tool compared to the technique of virus isolation and other conventional serological tests for the detection of NDV both from the clinical and post-mortem samples, although the virus is present in very minimum concentrations in the field samples. Among the clinical samples, higher rate of NDV detection by the newly developed In-house sandwich ELISA kit was noticed from faeces (100%) followed by oro-nasal swab (95.50%). In the case of post-mortem samples higher rates of NDV detection by the newly developed In-house sandwich ELISA kit was noticed from brain (100%) followed by colon (94.12%). Higher rates of NDV detection from faeces in this study might be the maximum release of the free virus in faeces during the course of infection as well as in case of brain, a higher rate of NDV is found which might be due to the site of its predilection. On the other hand, clinical samples revealed relatively lower rates of virus detection in oro-nasal swabs which might be due to the lower number of virus particles in discharge at the time of collection or failure to maintain cold chain parameters during transportation of the samples to the laboratory.

The goal of the development of an In-house sandwich ELISA kit is to achieve a simpler, more reliable, sensitive, specific, economic, rapid serological test, that is combined with maximum operator safety.

Conclusion

Commercially available sandwich ELISA kits which are just for the detection of serum antibody are highly expensive. To import these types of kits for the detection of anti NDV antibody in the serum of vaccinated birds in Bangladesh will require 45,000 BDT for 450 samples only. The newly developed sandwich ELISA kit is economically feasible and requires less than 500 BDT for 450 samples. The In-house Sandwich ELISA kit is designed for the detection of viral antigens from field samples and is cheaper than other commercially available sandwich ELISA kits. It is hoped the farmers will used the kit for rapid diagnoses of a large number of ND suspected samples at the farm level. The Government of the People's Republic of Bangladesh and a number of semi-government and non-government organizations can easily earn foreign currency by marketing and exporting this newly developed In-house sandwich ELISA kit designed for NDV after fulfilling the demand for this country.

Acknowledgement

The authors would like to express their sincere appreciation, gratitude and special thanks to Md. Mukhlesul Islam, Managing Director, FnF Pharmaceuticals Ltd, Bangladesh for providing eggs, rabbit and financial support for the completion of the research work in time.

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