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Development of an in-house indirect ELISA kit for the serological detection of antibodies against infectious bursal disease in chickens

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Abstract

Infectious bursal disease is a highly contagious disease of young chickens caused by the infectious bursal disease virus. This disease poses an important threat to the commercial poultry industry globally. This study was designed to develop an In-House Indirect Enzyme-Linked Immune Sorbent Assay Kit for the serological detection of antibodies against infectious bursal disease viruses. An infectious bursal disease virus antigen dilution (1:2), sample serum (1:500), and mouse anti-chicken immunoglobulin G (IgG) labeled with horseradish peroxidase (HRP) (1:2,000) were used in this assay. The calculated cutoff value was 0.24. This in-house indirect ELISA method was compared with a commercial ELISA kit for the detection of antibodies against infectious bursal disease virus in chickens. The performance of the newly developed and commercial ELISA kit was evaluated as described by Samad et al. (1994). The sensitivity and specificity of the current ELISA method were 98% (95% CI: 92.96–99.76) and 97% (95% CI: 91.48–99.38), respectively. The average intra-assay % CV of the triplet of 2 samples was 7.6, and interassay comparisons indicated a CV of 5.45%. As indicated by the results, we described a valuable and cost-effective, sensitive and specific in-house indirect ELISA kit for the serological diagnosis of infectious bursal disease in Ethiopia.

Keywords *Antibody*, *Chicken*, *Infectious bursal disease virus*, *In-house indirect enzyme-linked immunosorbent assay*

Introduction

Infectious bursal disease (IBD), also known as Gumboro disease, is a highly contagious, immunosuppressive disease of young chickens [1] and accounts for global economic damage in the poultry industry. Infectious bursal disease virus (IBDV), which causes IBD, is a double-stranded RNA virus belonging to the genus *Avibirnavirus* of the family *Birnaviridae* [2]. Two serotypes of IBDV, namely, serotypes 1 and 2, have been described, although IBD is caused mainly by serotype 1, which has variable pathogenicity. Thus far, virulent, classical, variant, and very virulent (vv) strains of serotype 1 IBDV have been characterized [3, 4]. On the basis of the report by Jackwood and Saif (1987), various variant and vvIBDV strains have been isolated from disease outbreaks despite the presence of high levels of maternal antibodies to classic strains of IBDV [5]. This calls for the identification of local strains of IBDV involved in outbreaks and the design of suitable vaccination programs for

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well-organized prevention and control. Antigenic variation is linked with sequence variation in virus protein 2 (VP2) genes [6–8], and this variation has been used for strain characterization of IBDV [9].

Clinical cases of IBD can be diagnosed by a combination of characteristic signs and postmortem lesions. Subclinical cases can be confirmed only in the laboratory through the demonstration of anti-IBDV antibodies in unvaccinated chickens or the detection of viral antigens or viral genomes in tissues. The virus neutralization test (VNT) and agar gel immunodiffusion (AGID) are some of the commonly used tests for the detection of viral antigens in tissue samples. VNT requires the isolation of the virus in cell cultures. They are mostly useful for the evaluation of vaccine responses or variations between IBDV serotypes 1 and 2 [10]. The AGID test is not as sensitive as the VNT test. ELISA-based tests are preferable to VNT or AGID tests for rapid diagnosis; they are less costly in terms of labor, although the reagents are more expensive [10]. The existing commercial kits are expensive and need to be replaced by easily available kits locally after their performance is approved. Poultry producers and researchers often demand timely feedback on the results of antibody assays. To solve this problem, a homemade indirect ELISA kit was developed and evaluated as an alternative to the commercial ELISA kit.

Materials and methods

Preparation of the coating antigen

The LC-75 vaccine strain of the IBD virus was obtained from Pan African Vaccine Quality Control (PANVAC), with a titer of $TCID_{50} \times 10^6$*. The virus was grown on chicken fibroblasts prepared from 12-day-old embryonated eggs at the National Veterinary Institute (NVI). After the virus was harvested and pooled, the antigen was purified from the cell debris by centrifugation (Beckman Coulter Avanti J-E Centrifuge) at 14000 rpm, and the resulting supernatant was used for coating.*

Study animals

A total of 80 chickens were purchased from farms suspected of being infected with infectious bursal disease virus around Hawassa town, Ethiopia. Blood samples were collected aseptically from the wing vein. The blood was kept at room temperature for three hours, and clear serum was isolated from the clot and screened with a commercial ELISA kit.

Reference serum

The positive control serum was collected from chickens naturally infected with the infectious bursal disease virus. The collected sera were screened with a commercial ELISA kit (ID-vet IBDV indirect ELISA kit), and those with strongly positive sera were selected and used as positive controls. Sera collected from noninfected chickens whose results were negative upon screening with a commercial ELISA kit were used as a negative control. The same batch of kits was used for selecting negative and positive controls and comparing the in-house ELISA kits.

Indirect-Enzyme-Linked immunosorbent assay steps

The indirect ELISA method was used for the detection of antibodies against a specific antigen in a two-step incubation process. The vaccine strain of the IBD virus with a titer of $TCID_{50} \times 10^6$ was diluted 1:2, 1:4 and 1:8 with bicarbonate buffer, which was prepared from sodium carbonate (Na_2CO_3) and sodium bicarbonate ($NaHCO_3$) with a pH of 9.4 (Medicago AB Uppsala, SEWEDEN), and 200 µL of the diluted antigen was added to micro plate wells (Thermo Scientific™ Nunc™ 96-Well Polystyrene Round Bottom Micro well Plates, Denmark) and incubated overnight at + 4 °C. Following overnight incubation, the content was discarded, and the samples were washed three times with ELISA washing buffer, which was made from 0.05 M Tris, 0.138 M NaCl, and 0.0027 M KCl, pH 8.0, at 25 °C, with 0.05% Tween 20, and the contents were dissolved in one liter of deionized water.

The nonbinding sites of the wells were blocked with 5% skim milk (SIGMA-ALDRICH, SWITHERLAND) and incubated for 2 h. The serum samples that were diluted with 5% skim milk (1:500) were added to the wells and incubated for 30 min at room temperature. During the first incubation step, specific antibodies to the coated antigen, if present in the serum, are bound to the solid-phase precoated antigens [18], washed to remove the unbound serum proteins, and anti-chicken antibodies conjugated to the enzyme horseradish peroxidase (HRP), Sigma–Aldrich) were diluted to a concentration of 1:2000, after which 100 µl was added to each well. During the second incubation procedure, these HRP-conjugated antibodies are bound to any antigen–antibody complexes previously formed, and the unbound HRP conjugate is then removed by washing [19]. A solution of tetramethylbenzidin (TMB) substrate/chromophore (IDEXX, Switzerland) was then added and incubated for 15 min in the dark, resulting in the development of a blue color. The reaction was stopped with 0.5 M sulfuric acid (IDEXX, Switzerland), and the absorbance was measured with an ELISA plate reader (Thermo Scientific, Multiskan EX) at 450 nm. Wells containing negative samples were colorless [20]. The ELISA procedure was conducted in duplicate, with one replicate utilizing a commercially available ELISA kit and the other replicate employing an in-house indirect ELISA kit.

Table 1 Determination of the sensitivity and diagnostic specificity of the homemade ELISA kit

		The gold standard (ID-vet Indirect ELISA)		
		Positive	**Negative**	**Total**
In-House Indirect ELISA	Positive	a	b	a + b
	Negative	c	d	c + d
Total		a + c	b + d	a + b + c + d = N

Adopted from Samad et al. (1994)

Explanation of the above method:

a = number of samples positive for both in-house and gold standard tests:

b = number of samples positive to in-house but negative to the gold standard test

c = number of samples negative to in-house but positive to the gold standard test

d = number of samples negative for both in-house and gold standard tests

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

Sensitivity: Compared with the gold standard, the test can detect positive samples (a/a + c × 100)

Specificity: Compared with the gold standard test, the test can detect negative samples (d/b + d × 100)

Determination of the cutoff value

The cutoff value was determined on the basis of the method published by Kumar and Rao (1991) via the mean absorbance of negative controls plus three times the standard deviation [12]. Twenty known negative serum samples were used to determine the cutoff value, which was calculated as follows:

Cutoff value = (mean ± 3 × standard deviation) of the negative control.

Determination of the sensitivity and specificity of the homemade ELISA kit

The sensitivity and diagnostic specificity of the homemade ELISA were determined using the method described by Samad et al. (1994), in comparison with a commercial kit [11]. Accordingly, the sensitivity and diagnostic specificity of the homemade ELISA kit were calculated as follows (Table 1).

Comparison of the assay consistency within wells and between the plates

Interassay and interassay evaluations were carried out to validate the consistency of the assay. Similar samples were tested under the same conditions, and precision was analyzed through the factors of standard deviation and coefficient of variation (CV). The results were then compared across different runs.

Data analysis

The data analysis was performed via GraphPad Prism 5 and Microsoft Excel on Windows 10.

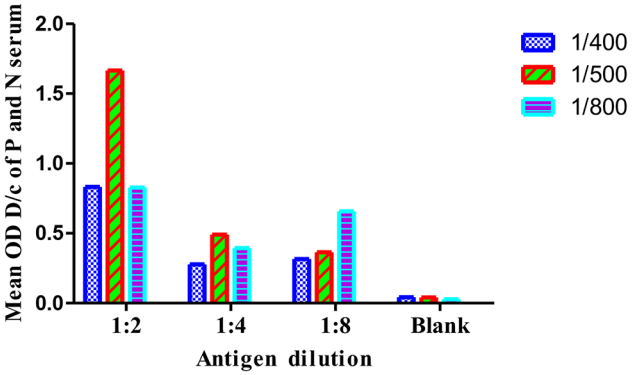

Fig. 1 This figure reveals the optimization of the antigen concentration and serum dilution. D/ce: difference; OD: optical density; P: positive; N: negative; Ag: antigen, nm: nanometer

Results

Optimization of the coating antigen

A known titer of the vaccine strain of IBDV, $TCID_{50} \times 10^{6,}$ was used for coating the ELISA plate. Serial twofold dilutions of the antigen were made at ratios of 1:2, 1:4, and 1:8 using coating buffer, and 200 µL of the diluted antigen was added to each well and incubated overnight at + 4 °C. After overnight incubation, the content was discarded, and the samples were washed three times with washing buffer containing Tween 20, blocked with 5% skim milk and incubated for 2 h. The contents were again discarded, the samples were washed three times, and indirect ELISA procedures were applied. Finally, a 1:2 dilution was considered the best antibody-capturing dilution of the antigen (Fig. 1).

Optimization of serum Dilution and conjugate

Different dilutions of sera (1:400, 1:500, 1:600, and 1:800) were prepared, and 100 µL of each dilution was added to the microwells and incubated for 30 min at 25 °C. The contents were discarded, and the wells were washed three times with washing buffer. The conjugate was diluted at 1:2000 and 1: 4000, and 100 µL of the diluted conjugate was added to each well and incubated for 30 min at 25 °C. The results revealed that 1:500 and 1:2000 dilutions of the serum and conjugate, respectively, were considered the optimum dilutions (Fig. 2).

Optimization of incubation time and temperature

The optimized antigen was prepared and coated onto microplates following the procedure outlined in Fig. 1. The serum samples were diluted at a concentration of 1:500, and 100 µl of the diluted serum was added to the wells of three separate microplates. The plates were then incubated at different times and temperatures: 30 min at 25 °C, 30 min at 37 °C, 1 h at 25 °C, and 1 h at 37 °C (Fig. 3). After the specified incubation period, the contents of the wells were discarded, and the wells were

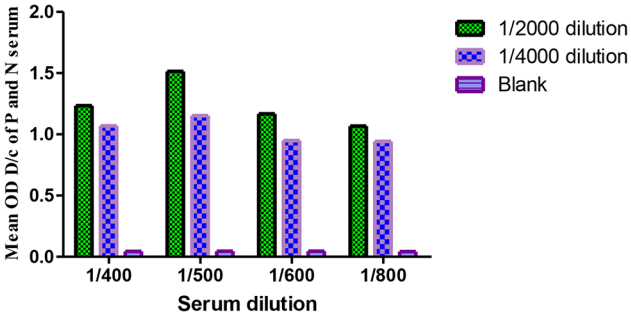

Fig. 2 Optimization of serum dilution and secondary antibodies (conjugate). D/ce: difference; OD: optical density; P: positive; N: negative; nm: nanometer

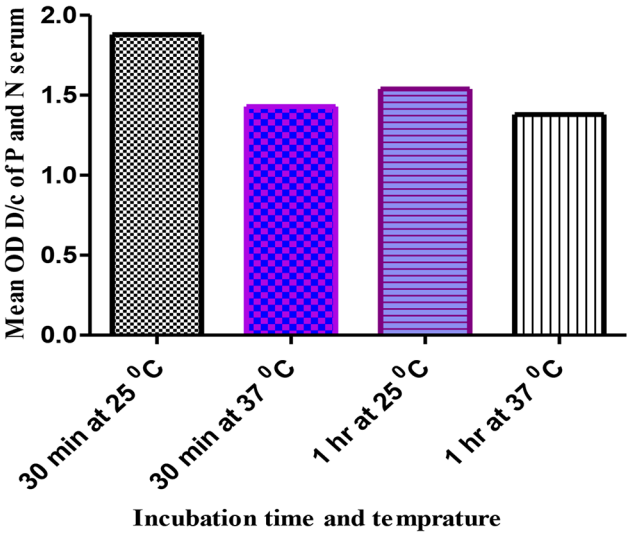

Fig. 3 Schematic representation of the optimization of the incubation time and temperature. D/ce: Difference; P: Positive; N: Negative; nm: nanometer; min: minute

washed three times to remove any unbound material. Next, the conjugate was diluted at a ratio of 1:2000, and 100 µl of the diluted conjugate was added to each well. The plates were incubated under the same conditions as mentioned earlier. Following the completion of the indirect ELISA method, a 30-minute incubation at 25 °C provided the best results.

Determination of the cutoff value

To determine the cutoff value, the optical density (OD) values of 20 negative controls were analyzed. The cutoff value was determined via the method described by Kumar and Rao (1991), which involves calculating the mean absorbance of the negative control and adding three times the standard deviation [12]. In this study, the cutoff value was calculated as 0.24 on the basis of the formula cutoff value = (mean ± 3 × standard deviation) of the negative control. This value, 0.24, was established as the cutoff value for the assay and was used to distinguish between positive and negative results (Table 2).

Determination of test sensitivity and diagnostic specificity

The sensitivity and diagnostic specificity of the currently developed ELISA kit were evaluated via a set of two hundred (200) pretested samples, which were also tested with a commercial kit for comparison. The method described by Samad et al. (1994) was employed to determine the test sensitivity and diagnostic specificity by comparing the results with those of the gold standard [11]. The sensitivity of the currently developed ELISA kit was 98% (95% CI: 92.96–99.76), indicating its ability to correctly identify positive samples (Figs. 4 and 5). The diagnostic specificity, on the other hand, was 97% (95% CI: 91.48–99.38), indicating its ability to identify negative samples correctly. Importantly, these values were derived from comparisons with commercial kits. These results demonstrate the effectiveness of the currently developed ELISA kit in accurately detecting antibodies against the infectious bursal disease virus (IBDV).

Cross-reactivity

The specificity of the indirect ELISA kit was checked after a variety of positive sera against Newcastle disease virus, *Mycoplasma gallisepticum*, Fowl typhoid, and Fowl cholera were tested. No cross-reactivity was detected against any of the sera, as all of them tested values below the defined cutoff values (data not shown). This evidence revealed that the coated antigen has good diagnostic specificity.

Comparison of the assay consistency within wells and between the plates

To assess the consistency of the assay, both intraassay and interassay comparisons were conducted. The intraassay comparison involved running the same samples within wells, whereas the interassay comparison involved running the same samples between plates (Tables 3 and 4). The coefficient of variation (CV) was used to evaluate the consistency of the assay. The CV was calculated from the mean and standard deviation of the optical density (OD) values. Ideally, the interassay CV should be less than 15%, indicating good consistency between different runs. Notably, the intraassay CV is typically lower than the interassay CV because the variation between runs is greater than that within the same run(17).

The formula for calculating the coefficient of variation (CV) is as follows:

$$CV(\%) = \left(\frac{S\tan dard\,deviation}{Mean}\right) \times 100$$

Table 2 Determination of the cutoff value

0.11	0.10	0.08	0.09	0.08	0.08	0.07	0.11	0.15	0.23	Mean	STDEV	Cutoff value
0.11	0.07	0.11	0.09	0.09	0.18	0.07	0.10	0.16	0.10	0.11	0.04	0.24

Two different samples were tested on the same plate in different wells. The OD values of each sample were compared and the % CVs for each sample were 8.56% and 6.76%, respectively. STDEV: standard deviation; CV: coefficient of variation.

Seventeen (17) different samples were tested on two plates of different wells, the OD value of each sample was compared, and the average %CV was 5.45%. STDEV: standard deviation; CV: coefficient of variation.

Discussion

This study aimed to develop and optimize an in-house indirect ELISA kit for the detection of antibodies against the infectious bursal disease virus (IBDV). The analysis of 200 serum samples via the newly developed in-house indirect ELISA kit and a commercial kit revealed good overall agreement between the two methods. These findings indicate that the results obtained from the newly developed in-house ELISA closely align with those of the commercial kit. The agreement can be attributed to the antibody-capturing ability of the IBDV, as well as the meticulous optimization of various factors influencing the test reactions, such as the antigen concentration, serum dilution, HRP-conjugated antibody, incubation time, and temperature. The use of skim milk for blocking and antibody dilution was found to reduce nonspecific binding. This optimization method for ELISA has been demonstrated in previous studies [13, 14].

The ELISA method has been widely employed as a diagnostic tool to evaluate the immune response against IBDV in various studies [13, 15, 16]. The results of the present study further support previous findings, as it exhibited excellent performance in detecting antibodies against IBDV in chickens.

The cutoff value for the present ELISA method was determined by calculating the mean absorbance of the negative controls (Table 2) plus three times the standard deviation [12], resulting in a value of 0.24. This value closely matches the cutoff value of the ID-vet indirect commercial ELISA kit used for comparison. A titer greater than 0.24 in the tested samples via the newly developed in-house indirect ELISA indicates IBDV positivity, whereas a titer less than 0.24 indicates negativity (Fig. 6).

The selection of a specific and immunogenic antigen is crucial in ELISA development because of its ability to produce and detect antibodies. Studies by Gómez et al. (2020) and Wang et al. (2008) utilized IBDV subviral particles (SVPs) and the VP3 protein, respectively, as coating antigens in their ELISA kits for detecting antibodies against IBDV [13, 14]. Another study by Saha et al. (2010) developed a sandwich ELISA with 100% sensitivity and 92.85% specificity for detecting IBDV antibodies [15]. To improve the sensitivity of this ELISA method, we purified

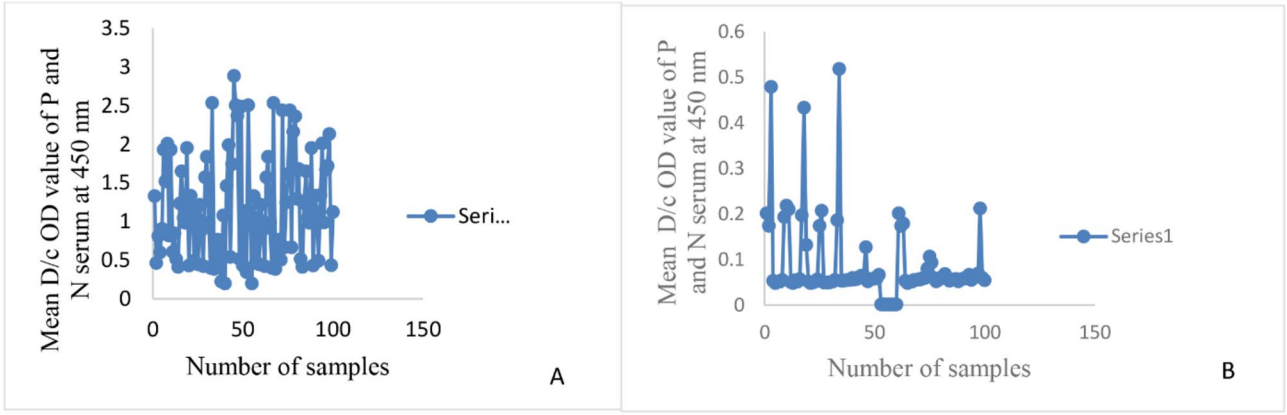

Fig. 4 Sensitivity and specificity test. Known positive ($n=100$) and negative ($n=100$) sera were tested with a newly developed ELISA kit. Except for 2 samples, all positive sera tested positive (Fig. 6A). Among the negative control samples, 3 tested positive (Fig. 6B). n: number of samples, P: positive control serum, N: negative control samples, nm: nanometer

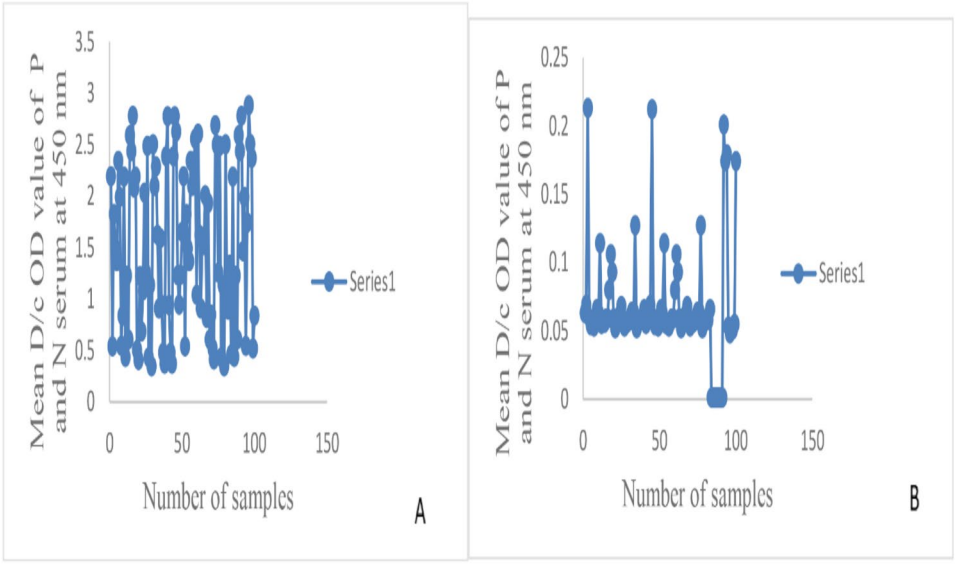

Fig. 5 Known positive ($n=100$) and negative ($n=100$) sera were tested with a commercial ELISA kit. All positive sera tested positive (Fig. 4A); similarly, all negative samples were also tested correctly (Fig. 4B). n: number of samples, P: positive control serum, N: negative control samples, nm: nanometer

Table 3 Intraassay comparison after testing with the newly developed ELISA kit

Triplated samples					
1.526	1.458	1.289	Mean	STDEV	CV %
			1.424333	0.122034	8.56
Triplated samples					
0.885	0.84	0.773	Mean	STDEV	CV %
			0.832667	0.056359	6.76

the virus from cell debris by centrifugation and optimized the time, temperature, and reagents while shaking the plate during each incubation time. In our study, we developed an ELISA kit that can substitute for a commercial ELISA kit for the detection of antibodies against IBDV in chickens by using the whole virus as a coating antigen. The virus was first grown on a primary cell line, then its titer was determined, and finally, the antigen was coated onto ELISA plates before the assay was performed. The sensitivity and specificity of our ELISA kit were evaluated via a formula adopted from Samad et al. (1994) with the gold standard method, which resulted in a sensitivity of 98% (95% CI: 92.96–99.76) and 97% (95% CI: 91.48–99.38), respectively. The specificity of this ELISA technique was confirmed by positive sera collected from experimentally and naturally infected chickens against viral and bacterial pathogens, including Newcastle disease virus, *Mycoplasma gallisepticum*, fowl pox and fowl cholera. Eighty (80) serum samples were tested for each pathogen, and no cross-reactivity was observed against any of the pathogens, which supports the accuracy of this ELISA kit to specifically detect antibodies produced against IBDV. However, further improvement can be

Table 4 Interassay comparison and results of the average coefficient of variation after testing with the newly developed ELISA kit

List of samples	Plate 1	Plate 2	Mean	STDEV	CV %
Result 1	0.046	0.042	0.044	0.002828	6.427273
Result 2	0.05	0.048	0.049	0.001414	2.885714
Result 3	0.045	0.045	0.045	0	0
Result 4	1.075	1.353	1.214	0.196576	16.19242
Result 5	2.925	2.997	2.961	0.050912	1.719419
Result 6	2.84	2.73	2.785	0.077782	2.79289
Result 7	2.909	2.892	2.9005	0.012021	0.414446
Result 8	2.915	2.81	2.8625	0.074246	2.593747
Result 9	1.613	1.565	1.589	0.033941	2.135997
Result 10	0.061	0.075	0.068	0.009899	14.55735
Result 11	1.926	2.072	1.999	0.103238	5.164482
Result 12	2.893	2.808	2.8505	0.060104	2.108542
Result 13	2.717	2.647	2.682	0.049497	1.845526
Result 14	3.019	2.65	2.8345	0.260922	9.205221
Result 15	2.697	2.824	2.7605	0.089803	3.253143
Result 16	0.532	0.559	0.5455	0.019092	3.499908
Result 17	0.949	1.235	1.092	0.202233	18.51951
				Average	5.45%

achieved by the use of engineered immunogenic IBDV proteins.

To assess the precision of the current ELISA, intra- and interassay comparisons were conducted. An intraassay comparison of the two samples revealed % CVs of 8.56 and 6.76 for samples 1 and 2, respectively (Table 3). Interassay comparisons of 17 different samples revealed a CV of 5.45% (Table 4), which falls within the acceptable range for new ELISA methods [18].

On the basis of a report by the WOAH Terrestrial Manual 2024, the ELISA technique has been suggested as a preferable tool to detect the immune response against IBDV [21]. In this study, the calculated cutoff value presented extraordinary test sensitivity and diagnostic specificity. Additionally, this assay revealed worthy repeatability and is guaranteed to be beneficial for the study of the prevalence of IBDV. ELISA kits detect and measure antibodies produced against IBDV unambiguously in the blood of chickens and can be used at universities and research institutes to study the prevalence of IBDV, which will help in monitoring the immune response precisely and designing control strategies for the prevention of IBDV.

Conclusion

ELISA is the most commonly used serological tool for the detection and screening of subclinical samples. The ELISA method developed in the present study is sensitive, specific, and reliable. This technique can be used for the detection of antibodies against infectious bursal disease in chickens. Moreover, using this method avoids the extra costs that are frequently incurred for the purchase of a commercial ELISA kit in Ethiopia.

Abbreviations

%	Percent
µl	microliter
^{0}C	Degree Celsius
Ag	Antigen
AGID	Agar gel immuno diffusion
CV	Coefficient of variations
D/ce	Difference
ELISA	Enzyme-Linked Immunosorbent Assay
HRP	Horse radish peroxidase
HVR	Highly variable region
IBD	Infectious bursal disease
IBDV	Infectious bursal disease virus
Min	Minute
N	Negative
nm	Nanometer
NVI	National Veterinary Institute

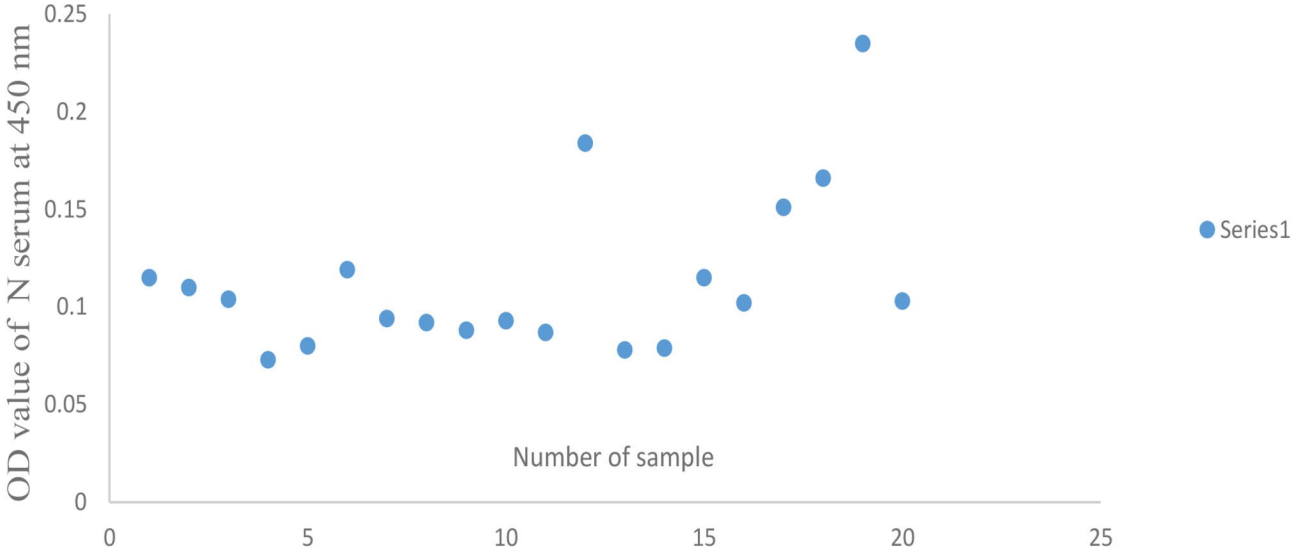

Fig. 6 Schematic representation of the cutoff value of negative serum tested by the newly developed in-house indirect ELISA kit

OD	Optical density
PANVAC	Pan African Veterinary Vaccine Center P: Positive
RNA	Ribonucleic acid
STDEV	Standard deviations
$TCID_{50}$	Tissue Culture Infection dose 50
TMB	Tetramethylbenzidin
VP2	Viral protein 2
vv	Very virulent
vvIBDV	Very Virulent Infectious Bursal Disease Virus: WOAH: World Organization for Animal Health

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Author contributions
Study concept and design: Takele Tesgera Hurisa; Analysis and interpretation of data: Takele Tesgera Hurisa; Critical revision: Takele Abayneh, Teshale Sori, Guohua Chengh, Yongxiang Fang, Berecha Bayisa, and Hunduma Dinka; Statistical analysis: Takele Tesgera Hurisa and Teshale Sori Tolera; Technical and material support: Mirtneh Akalu, Kedir Sherefa, Getu Ayele, Adunya Geresu, Mulatu Mokonnin, Warkisa Chala Bulto, Teferi Degefa Negi, Berhan Demeke, Megersa Mindaye Yami, Mohammed Jemal, Abinet Legesse, Yeneneh Tesfaye, Amde Zeleke.

Funding
Not applicable.

Data availability
Yes, available from the corresponding author upon request.

Declarations

Ethical approval
The experiment was performed in accordance with the international guidelines for animal experiments. All the chickens included all the necessary veterinary care and acceptable animal welfare regulations. Ethical approval for the chickens used in the study was obtained from the Animal Research Ethics Review Committee of Addis Ababa University (Reference: VM/ERC/36–37/07/13/2023). Since the chickens used were institutional, informed consent was not needed.

Consent to publish
Not applicable.

Competing interests
The authors declare no competing interests.

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