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Takele Tesgera Hurisa

takele.tesgera@gmail.com

National Veterinary Institute

Amde Zeleke

National Veterinary Institute

Takele Abayneh

National Veterinary Institute

Hika Waktole

Addis Ababa University College of Veterinary Medicine and Agriculture

Bersissa Kumsa

Addis Ababa University College of Veterinary Medicine and Agriculture

Berecha Bayisa

National Veterinary Institute

Hunduma Dinka

Adama Science and Technology University

Mirtneh Akalu

National Veterinary Institute

Getu Ayele

National Veterinary Institute

Kedir Sherefa

National Veterinary Institute

Dawit Abdisa

National Veterinary Institute

Birhanu Abebe

National Veterinary Institute

Abinet Legesse

National Veterinary Institute

Adunya Geresu

Adama Science and Technology University

Mulatu Mokonnin

National Veterinary Institute

Warkisa Chala Bult

National Veterinary Institute

Article

Keywords: Antibody, Antigen, Chicken, ELISA kit, Fowl cholera, Pasteurella multocida

Posted Date: June 1st, 2026

DOI: https://doi.org/10.21203/rs.3.rs-9715455/v1

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Additional Declarations: No competing interests reported.

Development of an in-house indirect ELISA kit for the serological detection of antibodies against Fowl cholera in chickens

Takele Tesgera Hurisa[1]*, Amde Zeleke[1], Takele Abayneh[1], Hika Waktole[2], Bersissa Kumsa[2], Berecha Bayisa[1], Hunduma Dinka[3], Mirtneh Akalu[1], Getu Ayele[1], Kedir Sherefa[1], Dawit Abdisa[1] Birhanu Abebe[1], Abinet Legesse[1], Adunya Geresu[1], Mulatu Mokonnin[1], Warkisa Chala Bult[1].

[1]National Veterinary Institute

[2]Addis Ababa University College of Veterinary Medicine and Agriculture

[3]Adama Science and Technology University

Corresponding author: Takele Tesgera Hurisa

Phone number: +251931524792; Email: takele.tesgera@gmail.com

Abstract

Fowl cholera (FC) is an infectious disease of birds caused by *Pasteurella multocida. This* disease is prevalent across the world and a major threat to all kinds of birds. FC is endemic to Ethiopia and has caused major economic losses due to mortality and loss of production. Accurate diagnostic techniques are considered useful tools in the management and control of FC in endemic areas. Owing to the lack of appropriate diagnostic methods, the exact circulation of FC in Ethiopia has not been well studied and documented. The present study aimed to develop an in-house indirect ELISA kit that can detect antibodies produced against FC in chickens. For this purpose, a freeze-dried FC seed obtained from NVI stock was allowed to grow on tryptose media. The purity and identity of the *P. multocida* strain was confirmed after being subjected to Gram stain and PCR, respectively. The bacterial cells were lysed via high-speed centrifugation by washing with phosphate-buffered saline (PBS), the antigen was purified, and the protein content was estimated via the bicinchoninic acid (BCA) assay method. In this study, all the data were analyzed via the Microsoft Excel spreadsheet of Windows 10. The working antigen concentration, serum dilution and secondary antibody dilutions were optimized at 1.5 mg/ml, 1:600 and 1:2000, respectively. The incubation time and temperature were optimized at 25°C (room temperature) for 30 minutes. The cutoff value was determined to be 0.28. The test sensitivity and diagnostic specificity of this assay were 97.4 % and 97.5%, respectively. The intra- and interassay comparisons within the wells and between the plates fell within the acceptable range. The newly developed ELISA kit is reliable, sensitive, and specific and can be used for the detection of antibodies against FC.

Keywords: *Antibody, Antigen, Chicken, ELISA kit, Fowl cholera, Pasteurella multocida.*

INTRODUCTION

Fowl cholera (FC) is a commonly occurring infectious disease caused by the bacterium *Pasteurella multocida,* which belongs to the *Pasteurellaceae* family and affects all types of birds globally [1]. The bacterial species that belong to this family are classified into five serogroups (A, B, D, E, and F) and 16 somatic serovars. *P. multocida*, which typically consists of strains of capsular serogroup A and serovars 1, 3, and 4, causes FC disease in chickens [2]. *P. multocida* subspecies *septica* and *gallicida* may also cause FC-like disease to some extent [3]. In 1881, *P. multocida* was first identified as the causative agent of fowl cholera disease by Louis Pasteur, after which the bacterium was identified as the causative agent of many other economically important diseases in a wide range of hosts [4]. The outbreak of FC often occurs as acute fatal septicaemia, commonly in adult birds [5]. Mainly it colonizes the respiratory tract of birds and causes stress under respiratory conditions. Fever, anorexia, mucus discharge, feather ruffling and watery diarrhea are common clinical signs. Chronically, it causes lameness, swollen wattles and torticollis, but it can also be asymptomatic [6].

The disease is prevalent in most regions of Ethiopia and results in major economic losses due to decreased production and high mortality [7]. In natural outbreaks, FC is mostly diagnosed by conventional methodologies, including isolation and identification by serotyping and biochemical characterization, which demonstrate the presence of various serogroups/types in different geographical regions. However, conventional diagnostic methods are not sensitive enough to identify and differentiate agents involved in natural infection [4]. Accurate and timely diagnoses are regarded as effective tools for controlling infectious diseases such as FC. Different serological tests, such as agglutination, agar gel immuno-diffusion assay (AGID) and passive hemagglutination, have been employed to detect antibodies against FC in serum from chicken hosts. However, none of these methods are highly sensitive [5]. ELISA is valuable because of its rapidity, sensitivity and specificity. Nevertheless, commercially available ELISA kits are very expensive, making delivery difficult for professionals. Thus, identifying an alternative diagnostic

method that can detect antibodies against FC is a paramount task for researchers in developing countries. Therefore, this study aimed to develop an in-house indirect ELISA kit that can detect antibodies against Fowl Cholera in chickens.

3. MATERIALS AND METHODS

3.1. Description of the study area

All the experiments were performed at the National Veterinary Institute (NVI), which is located at Bishoftu, Ethiopia. Bishoftu is located 47 km southeast of Addis Ababa at 9°N and 40° E. It has a bimodal rainfall season: the long rainfall season from late June to late September and the short season from February to April, with a mean annual rainfall range of 450 mm to 1,000 mm and a temperature range of 17°C to 30°C (http://www.mwud.gov.et/web/bishoftu/home).

3.2. Culturing of the bacteria and antigen preparation

One vial freezes dried local isolate seeds of *P. multocida* type obtained from NVI, was reconstituted in sterile tryptose broth (TB) media (*HIMEDIA, India*) and streaked on tryptose agar (TA) (*SIGMA-ALDRICH, German*). The streaked plate was incubated overnight at 37°C to achieve bacterial growth from freeze-dried culture (NVI FC vaccine production protocol). Once growth was confirmed through visualization of the colonies, purity was also checked by Gram staining for the presence of pure gram-negative coccobacilli. After the purity of *Pasteurella multocida* was confirmed, the second passage was carried out by inoculating fresh colonies from TA media into sterile TB media and incubating at 37°C for 7 hrs. The growth and purity of the bacteria were subsequently checked via turbidity and the Gram-stain technique, respectively. Consequently, the seeds were inoculated into 2 bottles of 1-liter sterile inoculums containing 5 % horse serum and glucose and incubated at 37 °C for 18 hrs. In addition, following confirmation of growth, the purity was checked via Gram staining and pH (NVI FC vaccine production protocol).

3.2.1. DNA extraction

DNA extraction was performed via a Qiagen extraction kit according to the manufacturer's instructions (https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6425773/). Briefly, 200 µl of

bacterial suspension, 20 µl of proteinase K and 200 µl of AL buffer were added, homogenized by vortexing for 15 seconds and then incubated at 60 °C for 10 minutes. A total of 200 µl (96–100%) of ethanol was added, and the mixture was homogenized. Then, the suspension was transferred to a mini spin column and centrifuged at 8,000 rpm for 1 minute. The flow through was removed, and 500 µl of washing buffer (AW1) was added and centrifuged at 8,000 rpm for 1 minute. The flow through was discarded, and 500 µl of AW2 washing buffer was added, after which the mixture was centrifuged at 14,000 rpm for 3 minutes. The mini spin column was placed in a 2 ml collection tube and centrifuged again for 1 minute at 14,000 rpm to dry the column matrix. The mini spin column was placed in labeled Eppendorf tubes, and the DNA was eluted by adding 50 µl of elution buffer and centrifuging at 8000 rpm for 1 minute. The eluted DNA was stored at -20°C until processing.

3.2.2. PCR assay and gel electrophoresis

Following DNA extraction, PCR amplification of *P. multocida* type A causing Fowl cholera was carried out by targeting a specific fragment of the hyaD-hyaC gene of *P. multocida* via the primer pairs CAPA-FWD (5′- TGC-CAA-AAT-CGC-AGT-CAG -3′) and CAPA-REV (5′-TTG-CCA-TCA-TTG-TCA-GTG -3′), as described by WOAH (2021), with a PCR product of approximately 1044 bp. A total reaction volume of 22 µl was used, which contained 3 µl of nuclease-free water, 10 µl of the IQ super mix, 2 µl of forward primer, 2 µl of reverse primer and 5 µl of template DNA. The thermal profiles were as follows: initial denaturation at 95 °C for 5 minutes; 30 cycles of 95 °C for 30 seconds, 55 °C for 30 seconds, and 72 °C for 30 seconds; and a final extension at 72 °C for 5 minutes. After completing the PCR, 5 µL of the PCR product along with the red gel and 6x loading dye were loaded onto a 2 % agarose gel (w/v) for electrophoresis in 1× Tris acetate/EDTA buffer at 1200 °C for 1 hr (EC 2060, USA). A 100 bp molecular ladder Biotechrabbit GmbH Berlin, Germany) was also loaded and finally read via a UVitec Trans illuminator.

3.2.3. Antigen purification and protein estimation

Five hundred milliliters (500 ml) of the live bacterial culture was taken and centrifuged at 10,000 rpm for 20 minutes. The sediments were washed three times with phosphate-buffered saline (PBS)

through centrifugation and reconstituted with 5 ml of PBS. The protein content was estimated via the bicinchoninic acid (BCA) assay method with a standard (bovine albumin), copper sulfate ($Cuso_4$), distilled water, and BCA [9].

3.3. Reference serum preparation

3.3.1. Positive serum

Known positive controls were obtained from naturally infected chickens that were stored in the serum bank of the NVI serology laboratory.

3.3.2. Negative serum

Negative control serum was collected from noninfected chickens, and the absence of antibodies against *P. multocida* was tested via a commercial ELISA kit; negative control samples were used.

3.4. Preparation of working solutions

3.4.1. Washing buffer

The washing buffer used in this study was prepared by mixing 500 µl of tween 20 in one liter of PBS solution to obtain a final concentration of 0.05%, which was subsequently homogenized via a vortex mixer (https://sharebiology.com/pbst-buffer-pbs-with-tween-20/).

3.4.2. Blocking buffer

Five percent (5 %) blocking buffer was prepared by dissolving 5 g of skim milk (*SIGMA-ALDRICH, German)* in 100 ml of distilled water, and the mixture was homogenized by using a vortex mixer (NVI protocol).

3.4.3. Coating buffer

The coating buffer was prepared by dissolving 3.7 gm of sodium bicarbonate ($NaHco_3$) and 0.64 gm of sodium carbonate (Na_2co_3) in 1L of distilled water. Its pH was adjusted to 9.4-9.6 (https://www.fortislife.com/products/antibody-resources/sandwich-elisa-protocol/protocol020).

3.5. Optimization of antigen coating and serum dilution

Following protein estimation, the antigen was diluted with coating buffer (bicarbonate buffer) at concentrations of 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml and 2 mg/ml, coated into flat-bottomed 96-well

microplates and incubated at +4°C overnight. Following overnight incubation, the microplate wells were washed four times with 300 µl of wash buffer containing 0.05% phosphate-buffered saline with Tween 20 (PBST). Two hundred microliters (200 µl) of blocking solution (5 % skim milk) was added to each well and incubated at 37 °C for 2 hrs to block nonspecific binding. The plate was subsequently emptied and washed four times as described above, and the antigen-coated plate was stored at +4 °C until use (https://www.bosterbio.com/blog/post/how-to-use-checkerboard-titration-to-optmize-your-elisa-immunoassays).

The positive sera obtained from naturally infected chickens that were stored at the serology laboratory of NVI and negative sera were diluted with blocking buffer at concentrations of 1:400, 1:500, 1:600, and 1:1000, and 100 µl of the diluted sera was dispensed into each well vertically and incubated at room temperature for 30 minutes. The excess antibodies were removed, and the samples were washed with PBST. One hundred microliters (100 µl) of horseradish peroxidase (HRP)-conjugated solution prepared at a 1:2000 dilution was added to each well, and the mixture was incubated for 30 minutes at room temperature, followed by emptying and washing of the plate. Then, 100 µl of substrate mixture (*TMB; Sigma–Aldrich*) was added, and the mixture was incubated for 10 minutes at room temperature (in the dark). The reaction was stopped by adding 100 µL of sulfuric acid (1 N H_2SO_4). The optical density (OD) values were determined via an ELISA reader with a 450 nm filter. The greatest difference between positive and negative serum OD values was observed for the antigen concentration and serum dilution. (https://www.bosterbio.com/blog/post/how-to-use-checkerboard-titration-to-optimize-your-elisa-immunoassays).

3.6. Optimization of the secondary antibody (conjugate)

In this study, the secondary antibody was optimized after being diluted 1:2000 and 1:4000 with blocking buffer. The indirect ELISA method was applied as described above (3.5).

3.7. Optimization of incubation time and temperature

For optimization of the incubation time and temperature, positive and negative controls were used, and the indirect ELISA technique was applied. The incubation times were compared after being

incubated for 30 minutes and 1hr both at room temperature and at 37°C (https://pubmed.ncbi.nlm.nih.gov/296 73915/).

3.8. Determination of the cutoff value

The cutoff value of the present ELISA technique was determined on the basis of the method reported by Kumar and Rao (1991), using the mean absorbance of the negative control plus three times the standard deviation. Thus, to determine the cutoff value, the formula was as follows:

X+ (3*SD), where:

X= the mean of the tested samples

SD= Standard deviation

3.9. Evaluation of test sensitivity and diagnostic specificity

The method described by Samad et al. (1994) was used for the determination of test sensitivity and diagnostic specificity by comparison with the gold standard. The sensitivity and specificity of the newly developed in-house indirect ELISA against the commercially available indirect ELISA kit were determined, as shown in Table 1.

Table 1: **Determination of the sensitivity and specificity of the in-house developed ELISA kit**

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

Explanation of the above method:

a = number of samples positive according to both conventional and gold standard tests

b = number of samples positive to the conventional test 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 for both conventional and gold standard tests

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

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

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

3.10. Validation of the kit

3.10.1. Evaluation of the consistency of the assay within and between the plates (intra- and interassay)

The consistency of the assay was checked by comparing intra- and intraassay results after the same samples were run within the wells and between the plates. The acceptable interassay coefficient of variation (CV) should ideally be less than 15 %. Usually, the intra-assay CV value is lower than the interassay CV because the variation between runs is greater than that in the same run. The coefficient of variation was formulated using the mean and standard deviation of the OD values (https://www.abbexa.com/analysing-elisa-data) as follows.

*% Coefficient of variation = (standard deviation/mean) *100*

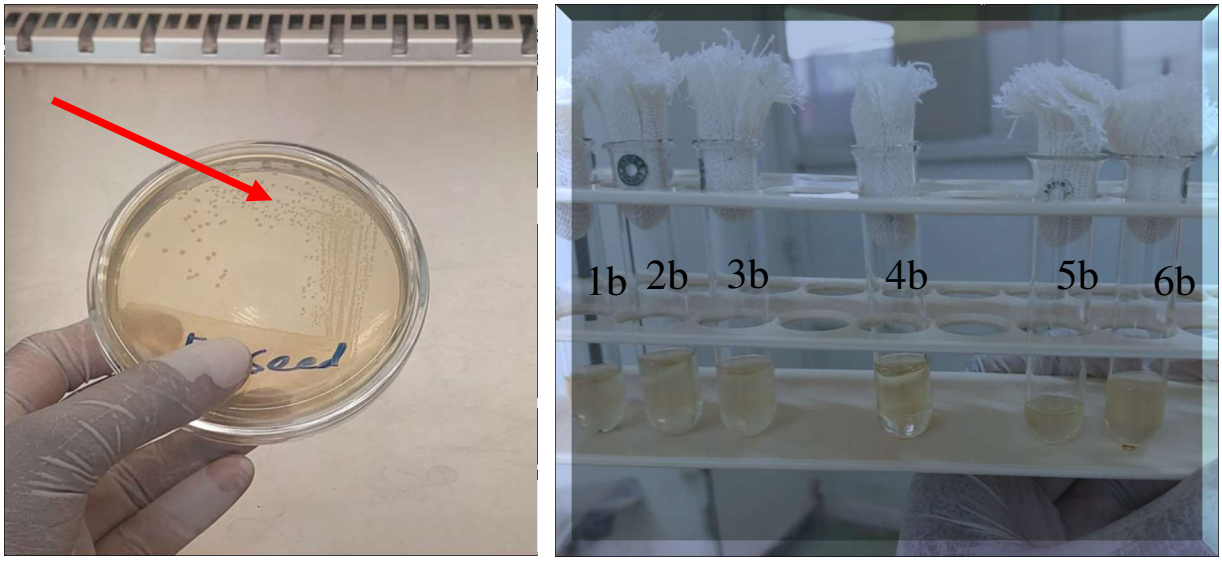
3.11. Data Management and Analysis

All the data obtained from the ELISA reader were entered into the MS Excel spreadsheet program to create a database. Microsoft Excel window 10 was used for data analysis, and the results are presented in the form of tables and graphs.

4. RESULTS

4.1 Culturing of bacteria and antigen preparation

Following the culture of the freeze-dried isolate of *P. multocida* type A seeds, the growth of TA was demonstrated as a spherical and smooth (mucoid) colony, which was observed by turbidity in broth medium (Figure 1a and 1b).



a b

Figure 1: Fresh colonies of *Pasteurella multocida* on tryptose agar media (a) and turbidity of the broth due to bacterial growth (1b, 2b, 3b and 4b), negative control (4b) and positive control (6b).

The purity was determined via Gram's stain method, which revealed that the morphology of the specific pathogen was identical (Figure 2).

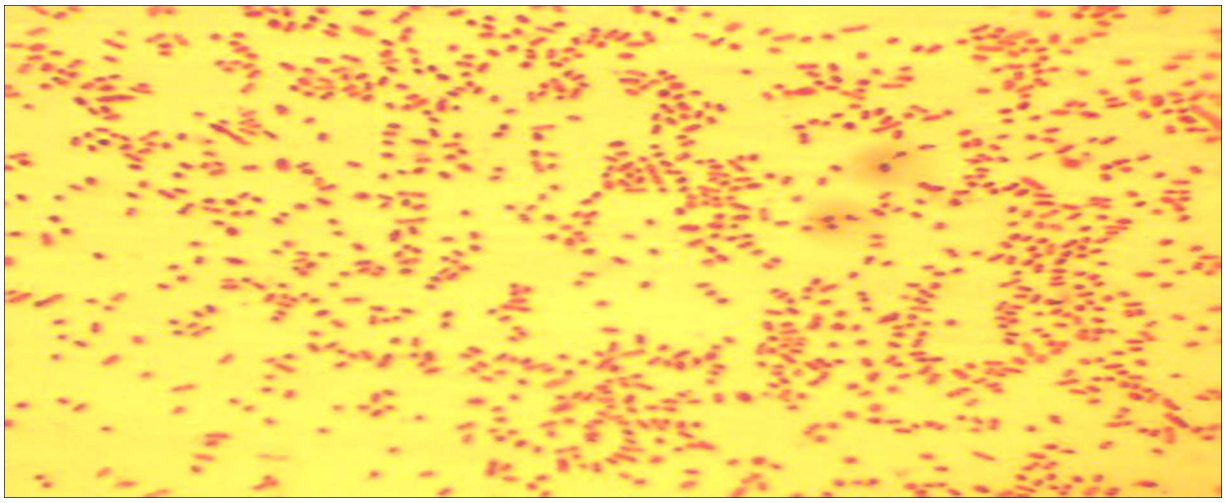


Figure 2: Gram stain showing the typical morphology of *Pasteurella multocida* coccobacillary samples from tryptose agar.

4.2. Molecular identification of *Pasteurella multocida*

The bacterial sample from a purely grown colony was characterized via polymerase chain reaction (PCR). Accordingly, the agarose gel electrophoresis results revealed a 1044 bp band, which was considered the size of the gene of antigen of interest (*Pasteurella multocida*) (Figure 3).

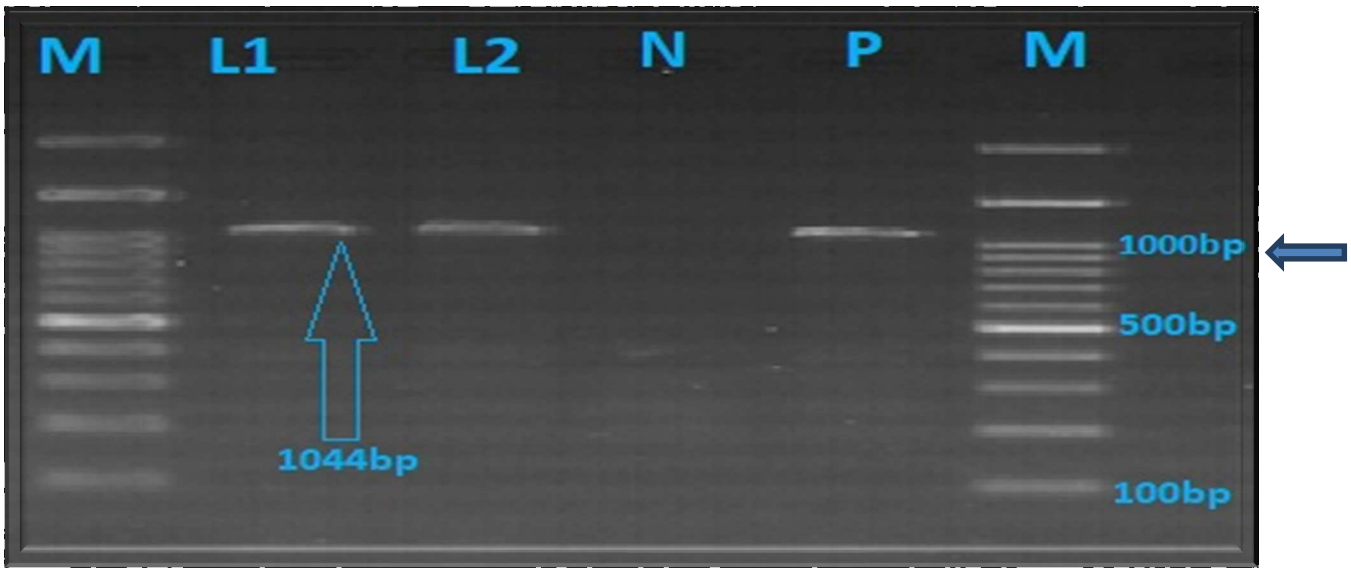


Figure 3: Conventional PCR agarose gel showing amplification of the hyaD-hyaC gene with the CAPA primer, resulting in a 1044 bp band. M: Molecular Ladder Biotechrabbit GmbH 100 bp Berlin, Germany; Lanes L1 and L2 are samples; N: negative control; P: positive control.

4.3. Optimization of antigen coating and serum dilution

The antigen was diluted to concentrations of 0.5 mg/ml, 1 mg/ml, 1.5 mg/ml, and 2 mg/ml, and the positive and negative sera were diluted 1:400, 1:500, 1:600, 1:800 and 1:1000. An indirect ELISA method was applied, and the results were evaluated. Finally, 1.5 mg/ml and 1:600 were considered the best antigen concentration and serum dilution, respectively (Figure 4).

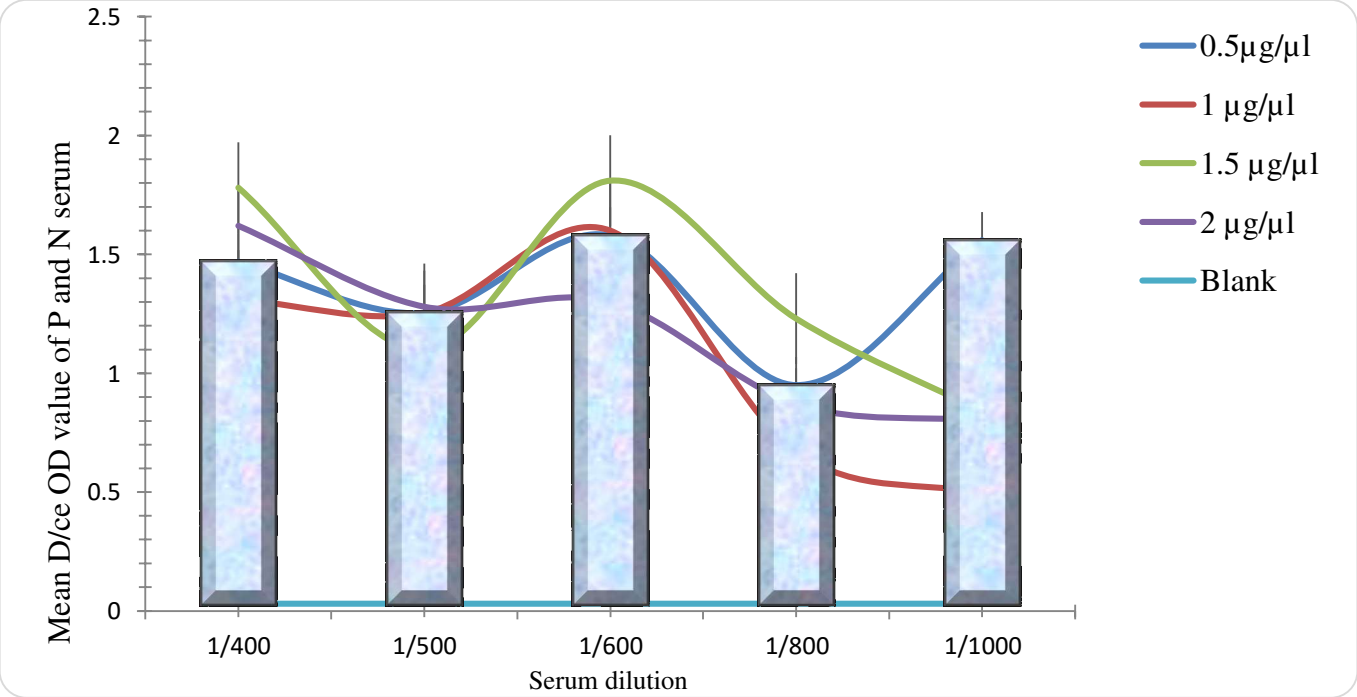


Figure 4: Bar graph showing the optimization of coating antigen and serum dilution. The best antigen and serum dilutions used were 1.5 mg/ml and 1/600, respectively. N: negative serum; P: positive serum; D/ce: diffrence; OD: optical density.

4.4. Optimization of serum dilution and conjugation

Four 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 micro wells and incubated for 30 minutes at 25 °C. The contents were discarded, and the wells were washed three times with washing buffer. The conjugates diluted at 1:2000 and 1: 4000 were prepared, and 100 µL of the diluted conjugate was added to each well and incubated for 30 minutes at 25 °C. After indirect ELISA was performed, dilutions of 1:600 and 1:2000 were selected as the best serum and conjugate dilutions, respectively (Figure 5).

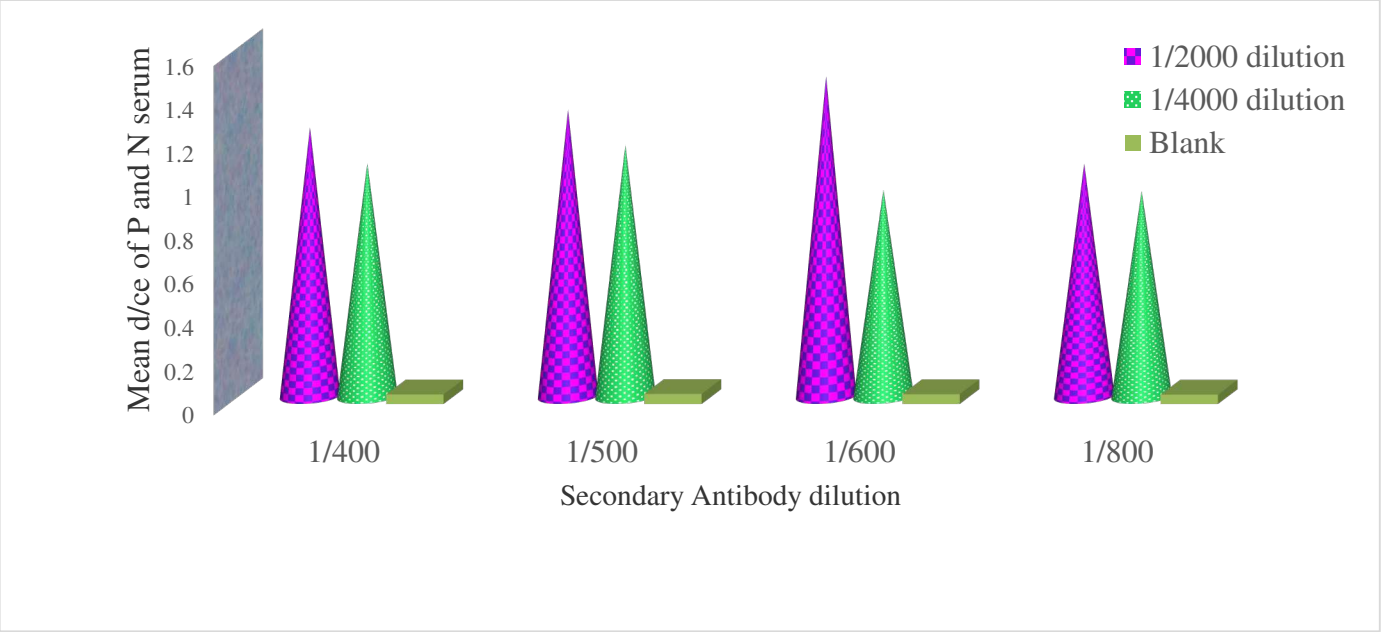


Figure 5: Bar chart illustrating secondary antibody optimization. As shown in Figure 5, the 1/2000 conjugate and 1/600 serum dilutions were selected as the optimal dilutions'/ce: Diffrence; P: positive; N: negative

4.5. Optimization of incubation time and temperature

Following the optimization of the antigen, serum and secondary antibodies, indirect ELISA was carried out. The plates were then incubated at different times and temperatures: 30 minutes at 25 °C, 30 minutes at 37 °C, 1 hr at 25 °C, and 1 hr at 37 °C. Finally, 30 minutes at 25°C was selected as the optimal incubation time and temperature (Figure 6).

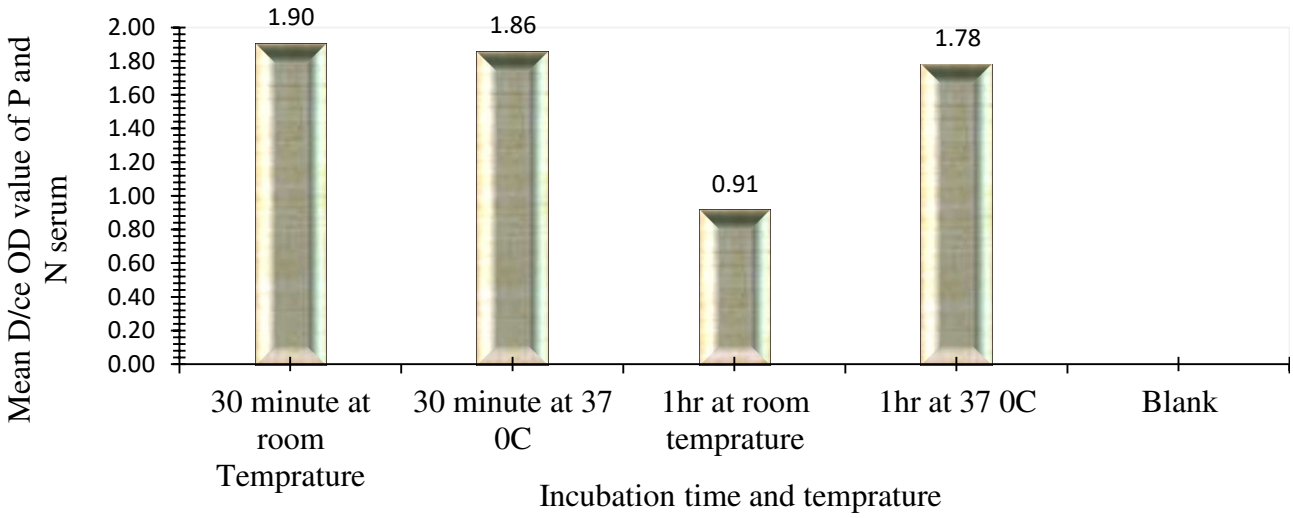


Figure 6: Bar graph displaying incubation time and temperature optimization. D/ce: Diffrence; N: negative; P: positive; ^{0}C; Degree Celsius; hr: hour

4.6. Determination of the cutoff value

Twenty (N=20) negative serum samples pre-tested with Fowl cholera were used for determination of the cutoff value. The optical density (OD) values of 20 samples were tested and analyzed via the method described by Kumar and Rao (1991), which involves calculating the mean absorbance of the negative control plus three times STDV [10]. On the basis of the analysis, 0.164, 0.038 and 0.28 are the calculated mean, STDV and cutoff values, respectively (Table 2).

Table 2: Determination of the cutoff value

List of samples	OD value	Mean	STDV	Cut off value
sample 1	0.164			
sample 2	0.161			
sample 3	0.195			
sample 4	0.106			
sample 5	0.199			
sample 6	0.144			
sample 7	0.174			

sample 8	0.177			
sample 9	0.194			
sample 10	0.135	0.164	0.038	0.28
sample 11	0.167			
sample 12	0.18			
sample 13	0.274			
sample 14	0.138			
sample 15	0.115			
sample 16	0.115			
sample 17	0.162			
sample 18	0.164			
sample 19	0.23			
sample 20	0.149			

OD= Optical density, SD= Standard deviation

4.7. Evaluation of test sensitivity and specificity

The method described by Samad et al. (1994) was used for the determination of test sensitivity, and diagnostic specificity. It was determined in comparison with the gold standard. Accordingly, the specificity and sensitivity of the newly developed ELISA kit were 97.5 % and 97.4%, respectively (Fig. 8).

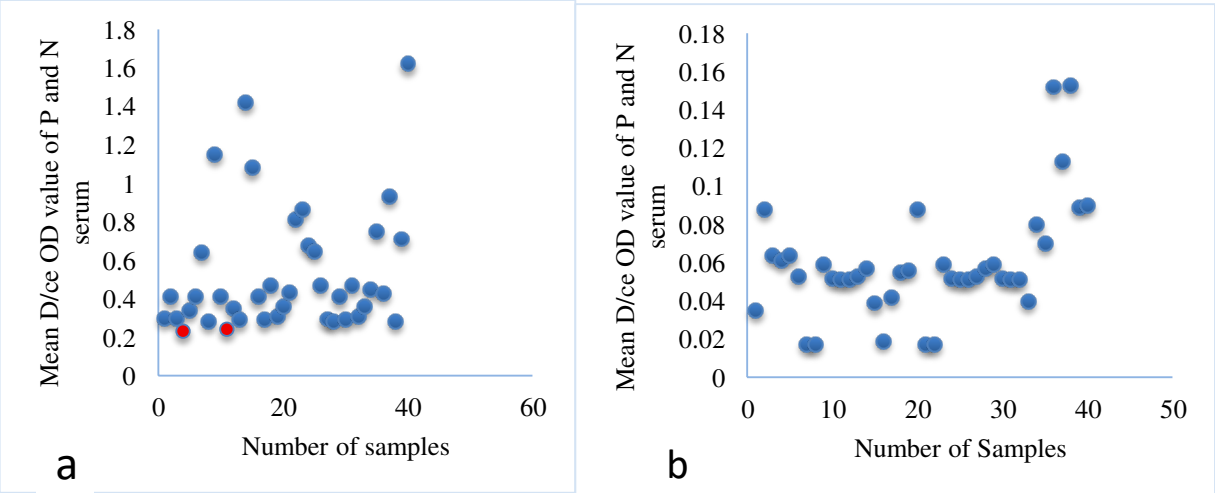


Figure 7: Scatter plot showing the test results of forty (40) positive (a) and forty (40) negative (b) samples, with N=80 samples after being tested by the commercial kit. During the test, two (2)

positive samples tested negative, and all negative samples were tested correctly. D/ce: Diffrence; N: negative; P: positive

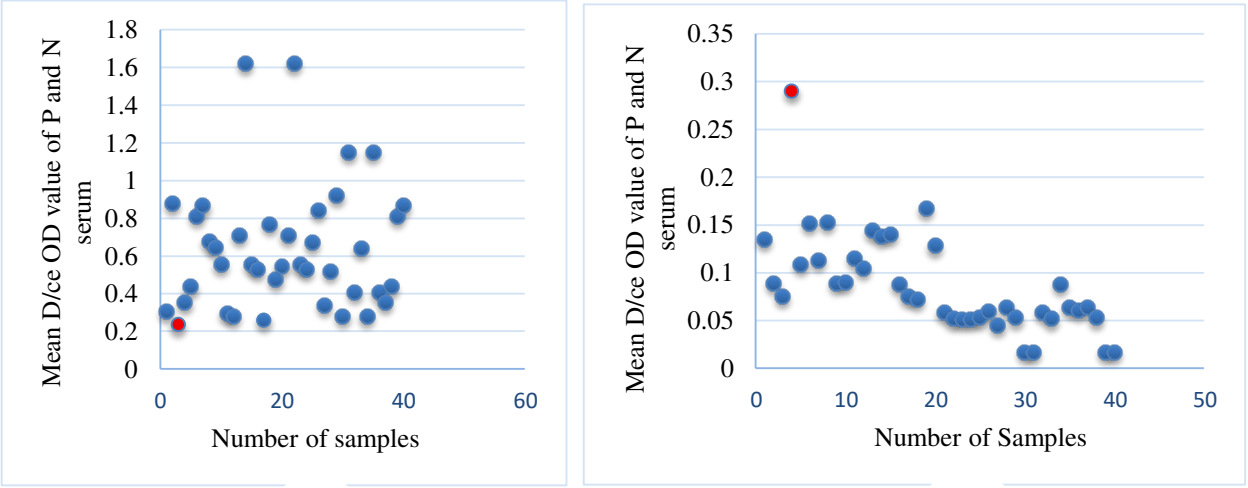


Figure 8: This scatter plot shows the test results of forty (40) positive (a) and forty (40) negative (b) samples, for a total of 80 samples after being tested with the newly developed ELISA kit. During test one, (1) positive samples tested negative, and one, (1) negative sample tested positive. D/ce: Diffrence; N: negative; P: positive

4.8. Validation of the kit

The consistency of the assay was checked by comparing intra- and interassay results after the same samples were run within the wells and between the plates. As indicated in Table 3, 1.67, 8.31, and 3.61 were the % CVs of samples 1, 2 and 3, respectively. The % CV of each sample was within the acceptable range, and the assay was confirmed to be valid (Table 3). Moreover, twenty (20) different samples were also tested on three different plates, and the average % CV of all samples was evaluated; hence, 7.83 was the average % CV for 20 samples (Table 4).

Table 3: Validation of the kit with Intera assay comparison between wells

List of samples	Well 1	Well 2	Well 3	Average	SD	% CV
Sample 1	0.196	0.191	0.19	0.19	0.003215	1.67
Sample 2	0.583	0.686	0.659	0.64	0.053407	8.31
Sample 3	0.556	0.557	0.592	0.57	0.020502	3.61

SD= standard deviation, % CV= percentage of coefficient of variation

Table 4: Validation of the kit with Intera assay comparison between plates

List of Samples	Plate 1	Plate 2	Plate 3	Average	SD	% CV
Sample 1	0.329	0.352	0.35	0.34	0.012741	3.71
Sample 2	0.283	0.301	0.302	0.30	0.010693	3.62
Sample 3	0.082	0.097	0.08	0.09	0.009292	10.76
Sample 4	0.156	0.184	0.155	0.17	0.016462	9.98
Sample 5	0.185	0.206	0.167	0.19	0.019519	10.49
Sample 6	0.285	0.296	0.264	0.28	0.016258	5.77
Sample 7	0.201	0.236	0.187	0.21	0.025239	12.13
Sample 8	0.197	0.2	0.186	0.19	0.007371	3.79
Sample 9	0.11	0.13	0.128	0.12	0.011015	8.98
Sample 10	0.141	0.178	0.175	0.16	0.020551	12.48
Sample 11	0.13	0.155	0.147	0.14	0.012767	8.87
Sample 12	0.185	0.224	0.207	0.21	0.019553	9.52
Sample 13	0.177	0.2	0.221	0.20	0.022008	11.04
Sample 14	0.157	0.178	0.168	0.17	0.010504	6.26
Sample 15	0.185	0.204	0.21	0.20	0.013051	6.54
Sample 16	0.181	0.212	0.203	0.20	0.015948	8.03
Sample 17	0.128	0.137	0.115	0.13	0.01106	8.73
Sample 18	0.143	0.148	0.126	0.14	0.011533	8.30
Sample 19	0.083	0.088	0.091	0.09	0.004041	4.63
Sample 20	0.174	0.165	0.173	0.17	0.004933	2.89
			Average			7.83

SD= standard deviation, % CV= percentage of coefficient of variation

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Diagnostic specificity of the in-house I-ELISA

Sera positive for other bacterial and viral diseases, such as NDV, IBDV CBPP and MG, were tested, and no cross-reactivity of the in-house I-ELISA was detected. All of the results presented values beneath the demarcated positivity cutoff values (data not shown). These data showed that the coated fowl cholera antigen has remarkable diagnostic specificity for exclusively detecting antibodies against fowl cholera in chickens.

6. DISCUSSIONS

The enzyme-linked *immunosorbent* assay (ELISA) is a commonly used analytical method that works on the principle that specific antibodies bind with the target antigen in a sample. Earlier studies have shown that the ELISA is a more effective test for detecting antibodies against fowl cholera in avian species [11]. This finding is in agreement with the current study, where we

developed a sensitive, cost-effective, easily operational, in-house indirect ELISA technique that can detect antibodies against FC.

Owing to its specificity for antibody detection, the use of the right coating antigen onto an ELISA micro plate, which is specific to the antibody of interest, is crucial. Related studies revealed that ELISAs that can detect antibodies against FC were developed after coating with whole-cell antigens, sonicated antigens, potassium thiocyanate extracts, lipopolysaccharides, sodium salicylate extracts, and capsular or heat extract antigens of FC [12]. In the present study, we developed an ELISA based on the whole-cell antigen, which is in agreement with the findings of the study described by Afzal et al. (1992). For this ELISA technique, the optimal antigen concentration used to coat the plate was 1.5 mg/ml. However, these findings differ from those of Poolperm et al.(2017), who employed heat extract antigen to coat a plate and reported that a 1 µg/ml concentration was the optimal coating antigen. Another membrane protein-based antigen coating method was also used by Mahboob et al. (2023), who reported that 3.13 µg/µL was the best candidate for determining the antigen concentration. Even when the antigens are similar or the same, the variation in the optimal concentration of a coating antigen for ELISA kit development from country to country and from laboratory to laboratory could be due to several reasons, including purity levels, methods for antigen purification or formulation, plate properties, differences in coating buffer composition and others. In general, the current analysis suggests that most studies have used various coating antigen concentrations and that the optimal coating antigen concentration is not similar to that used in the current study.

The results of the test serum or antibody dilution revealed that a dilution of 1:600 was the optimal condition for the newly developed ELISA. Thus, the results of the present study are relatively in line with the commercial ELISA kit for *P. multocida* used to detect antibodies in the sera of chickens and Turkey (*IDvet ID Screen®, France).* Jung and Rautenschlein (2020) reported that a dilution of 1:500 was the best working test serum dilution during the study of in-house ELISA kit development for the detection of antibodies against *Enterococcus cecorum* in Pekin ducks. The relative similarity of these studies may be due to several interconnected factors, such as similarity in antigen preparations; birds might exhibit similar immune responses to various bacterial infections and cross-reactivity in which some bacterial species might share common antigens or epitopes. This could lead to comparable antibody or test serum dilutions in the assay. Studies

conducted by other researchers [11,13,14] revealed the best serum dilution of 1:100 for the development of an ELISA kit for *P. multocida,* which is not in agreement with the current study. This variation could be due to differences in the antigen concentration used, the source of the serum samples, the variability in the antibody titer, and the various factors that can lead to variations in the optimal serum dilution between the studies.

Secondary antibody dilutions were performed at 1:2000 and 1:4000 to find the optimal working dilutions. Thus, the ideal working dilution was found to be 1:2000. Similar optimal concentrations of conjugates (1:2000 dilution) were obtained for the assay described in the report of Poolperm et al.(2017). In contrast, studies conducted by Aziz et al.(2022) and Sthitmatee et al.(2023) [15,16] reported 1:1000 and 1:10,000 as the optimal dilutions for in-house ELISA kit development, respectively**.** The difference could be because of variations in the methods used for conjugate and reagent preparation, the optimization procedures used and other factors.

The best incubation temperature and time were obtained at room temperature for 30 minutes. This result was analogous to the results of Jungand Rautenschlein (2020) [17], who reported a suitable incubation temperature of room temperature for 30 minutes.

The cutoff value of the present in-house ELISA was determined as described by Kumar and Rao (1991), using the mean absorbance of the negative control at a wavelength of 450 nm (OD_{450}) plus three times the standard deviation [18]. Accordingly, the cutoff value was 0.28. The serum samples whose OD values were greater than the cutoff value were subsequently interpreted as positive, and those whose values were less than the cutoff value were interpreted as negative. The cutoff value obtained in this study was greater than the value determined in the study carried out by Poolperm et al.(2017) and the cutoff value stated in the commercial ELISA kit for *P. multocida* that was used to detect antibodies in the sera of chickens and Turkey (*IDvet ID Screen®, France)***.** The difference in the cutoff values between the kits may be due to the methods employed to develop the kit and the environment. By comparing commercial (gold standard) and newly developed in-house indirect ELISA kits, the specificity and sensitivity of eighty (80) positive and negative samples were calculated in this study via the methodology outlined by Samad et al. (1994) [19]. The results were 97.5% and 97.4%, respectively.

Limitations of the study

This study was limited to the use of only stored serum in the serum bank of NVI due to time and financial constraints in the collection of large numbers of serum samples from the field for evaluation via the newly developed in-house indirect ELISA kit.

7. CONCLUSIONS

Fowl cholera can lead to significant economic losses for poultry industries due to high mortality rates and reduced productivity. Quick and accurate diagnosis allows for faster interventions, minimizing financial losses. Thus, ELISA is a commonly used serological assay for the detection and screening of antibodies in serum samples. The currently developed ELISA technique has good sensitivity, specificity, and reliability. Additionally, it could be concluded that this technique can be used for the detection of antibodies against Fowl cholera in chickens. Furthermore, this ELISA method can be used as a source of income for NVI through selling and avoids extra costs that are frequently incurred for the purchase of a commercial ELISA kit in Ethiopia.

LIST OF ABBREVATIONS

AGID	Agar Gel Immuno Diffusion
BCA	Bicinconic acid
CBPP	Contagious bovine pleuropneumonia
CV	Coefficient of variation
ELISA	Enzyme-Linked Immune Sorbent Assay
FC	Fowl Cholera
Hr	hour
HRP	Horse Reddish Peroxidase
IBDV	Infectious Bursal Disease Virus

IHA	Indirect hemagglutination assay
LPS	Lipopolysaccharide
MG	*Mycoplasma graisepticum*
N	Negative
NDV	Newcastle disease virus
NVI	National Veterinary Institute
OD	Optical density
OIE	Office of International Des Epizooties
P	Positive
PBS	Phosphate-buffered saline
PBST	Phosphate-buffered saline Tween
PCR	Polymerase Chain Reaction
TA	TryptoseAgar
TB	Tryptose Broth
UV	Ultraviolet
WOAH	World Organization of Animal Health

Acknowledgments

This work was supported by the National Veterinary Institute.

Author contributions

Study concept and design: Takele Tesgera Hurisa and Takele Abayneh; Analysis and interpretation of data: Takele Tesgera Hurisa, Amde Zeleke; Critical revision: Takele Tesgera Hurisa,Takele

Abayneh, Berecha Bayisa, Hika Waktole, Bersissa Kumsa and Hunduma Dinka; Statistical analysis: Takele Tesgera Hurisa, Made Zeleke; Technical and material support: Amde Zeleke, Mirtneh Akalu, Getu Ayele, Kedir Sherefa, Abinet Legesse, Adunya Geresu, Mulatu Mokonnin, Dawit Abdisa, Birhanu Abebe, Warkisa Chala Bulto.

Funding

Not applicable.

Data availability

Yes, the data are 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 that they have no competing interests.

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