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Abstract: *Mycoplasma galisepticum* (MG) is one of the most significant pathogens of domestic poultry and turkeys, causing substantial economic losses, especially in the commercial poultry industry, resulting in reduced egg production and the condemnation of carcasses in Ethiopian abattoirs due to the lack of detection methods to design control strategies. The study was aimed to develop an in-house indirect ELISA kit that can detect antibodies against MG in chickens. Coated antigen was purified from a whole culture of *M. galisepticum*, and the protein content was estimated, followed by optimization of the antigen, serum, and conjugate dilutions. The kit was assessed with natural and experimentally prepared serum and compared with a commercial ELISA kit. This in-house indirect ELISA kit had a sensitivity of 93.7% and a specificity of 87.5% and showed no cross-reactivity with positive serum against other avian infections. The current ELISA kit is sensitive and specific and can be used for the detection of antibodies against MG in chickens.

Keywords: antibodies; chicken; detection; in-house indirect ELISA; *M. galisepticum*

1. Introduction

Mycoplasma galisepticum (MG) is a bacterium belonging to the class of Mollicutes and the family Mycoplasmataceae. It is the causative agent of chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys, chickens, game birds, pigeons, and passerine birds of all ages (Hennigana et al, 2011). Likewise, the disease is listed by the World Animal Health Organization (OIE) as one of the most pathogenic avian mycoplasmosis and the main cause of chronic respiratory disease in poultry (OIE, 2008). The infection caused by *M. galisepticum* becomes aggressive when accompanied by other poultry diseases, including Newcastle disease virus (NDV), *Escherichia coli* (*E. coli*), and infectious bronchitis virus (IBV) (Bwala, D.G. 2017, Nakamura et al., 1994, Valks, M. and Burch, D, 2002). The transmission of *M. galisepticum* can occur through direct and indirect, horizontal contact, aerosol transmission via the introduction of contaminated materials, or by people (Bradbury, J.M. et al., 2001a). The disease causes a loss of egg production, a reduction in the hatchability of eggs, and a decline in the quality of meat at slaughter (Stipkovits and Kempf, 1996; Kleven, 1997; Levisohn and Kleven, 2000). In developing countries, the eggs and meat of chickens are the major food components in the daily human diet and sources of income (Gari, 2004).

Despite the significant growth of the poultry industry in Ethiopia, major diseases affecting both commercial and local chickens remain understudied (Jibril et al, 2018). *M. galisepticum* is among the poultry diseases causing considerable economic losses in Ethiopia because of a lack of reliable diagnostic methods, treatment options, and effective control measures. Commercially available diagnostic kits are often expensive and time-consuming and fail to detect experimentally produced

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antibodies against *M. galisepticum* (unpublished). Hereafter, in this study, a homemade indirect ELISA kit that can detect antibodies produced against both natural and experimental infections was developed and evaluated.

2. Materials and Methods

2.1. Reference Serum

Positive serum samples were obtained from naturally infected chickens in the field and screened via a commercial kit, and strongly positive serum was selected and used as a positive control. Serum samples from non-vaccinated control chickens were also screened for antibodies against *M. galisepticum* and used as a negative control.

2.2. Chicken Anti-M. guisepticum Vaccine Serum

Sixty (n=60) local chickens were vaccinated with the *M. galisepticum* vaccine formulated from two different adjuvants: aluminum hydroxide and oil. The chickens received booster doses of the same vaccine, and blood samples were collected at weeks 4, 5, 6, 7 8, and 9 before the initial vaccination and during each subsequent vaccination. The separated serum was compared to a reference serum obtained from chickens with natural *M. gallisepticum* infections.

2.3. Mycoplasma Gallisepticum Isolation, Identification, and Preparation of the Coated Antigen

A local field strain of *M. galisepticum* was cultured on Frey's medium. Mycoplasma colonies were observed under low magnification on agar plates via a light microscope with reduced light intensity. The isolated field strain revealed fried egg morphology with tiny, smooth colonies and dense elevated centers. The colonies were further allowed to grow on Hyflic medium supplemented with Mycoplasma selective supplement G (OXOID, code SR059C) to prepare a seed bank. Three vials of the seed bank were added to 200 mL of Hyflic medium and incubated at 37 °C with slow agitation. The growth was confirmed by turbidity and pH detection. Once the pH reached 6.5, the sterility and purity were verified. A 3-liter sterile medium was then prepared, inoculated with 20 % inoculum containing 20 % horse serum and incubated at 37 °C. After confirming growth, the culture was inactivated with 0.3% saponin.

2.3.1. Preparation of Bacterial Cell Lysate

The bacterial cells were centrifuged at 10,000 rpm for 20 minutes, and the supernatant was discarded without disturbing the pellet. To lyse bacterial cells, a freeze–thaw cycle was used. The cell suspension was rapidly frozen on dry ice and then thawed at room temperature. The pellets were washed three times with phosphate-buffered saline (PBS) reconstituted with 5 ml of PBS and placed at -21 °C until protein quantification was conducted.

2.3.2. Protein Quantification

The protein concentration was determined via the bicinchoninic acid (BCA) method. A standard curve was created using bovine albumin, $Cuso_4$, distilled water, and bicinconic acid.

2.3.3. DNA Extraction and PCR Detection

DNA was extracted from cultured organisms via a gSYNC™ Geneaid extraction kit (Korea). The sample was centrifuged at 16,000 × g for 2 minutes, the floating material was collected in a novel 1.5 ml tube, and 200 µl of GSB was added to the floating material. The mixture was then spun again for 10 seconds and mixed with 200 µl of absolute ethanol by vortexing. The blend was moved to the GS column and centrifuged at 16,000 × g for 1 min, after which 400 and 600 µl of W1 and W2 buffers were added, respectively, to the GS column with centrifugation, and 100 µl of warmed elution buffer was added to the tubes after thorough dehydration to elute the purified DNA. The resulting product was stored at −20 °C until use. For the amplification of MG DNA via PCR, two pairs of PCR primers

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were used on the basis of the National Veterinary Institute (NVI) molecular biology laboratory protocol. The primers used were MG 14-forw-5 pm/µl 5′GAGCTAA TCTGTAAAGTTGGTC-3′ and MG13-REV-5pm/µl 5′-GCTTCCTTGCGGTTAGCAAC. The primers were synthesized by Bioneer Co., Korea. The PCRs were performed with a total volume of 25 µl, as in the kit, and consisted of 5 µl of extracted DNA (template), 2 µl of each primer, 2 µl of MgCl2, 3 µl of RNA-free water, and 5 µl of prepared Mastermix solution. The positive control in both PCR runs was supplied with a kit. The thermocycler programs are explained in Table 1. Electrophoresis was performed via the use of 10 µl of the amplified DNA in a 2% agarose gel. The bands were distinguished at 245–312 nm through a UV transilluminator (Biometra, Germany).

Table 1. PCR amplification conditions.

	Temperature	Time	Cycle
Initial denaturation	95 °C	5 minutes	
Denaturation	95 °C	30 sec	1 cycle
Annealing	50 °C	30 sec	
Elongation	72 °C	30 sec	
Final elongation	72 °C	7 minutes	1 Cycle

2.4. Optimization of Antigen Coating and Serum Dilution

Following protein estimation, the antigen was diluted to concentrations of 0.5 µg/µl, 0.75 µg/µl, 1 µg/ml, 1.5 µg/µl, and 2 µg/µl and coated onto a 96-well microplate with bicarbonate buffer (pH 9.6) (Sigma–Aldrich). After overnight incubation at +4 °C, the plate was washed with phosphate-buffered saline containing 0.05 % tween 20 (PBST) (Sigma–Aldrich). Nonspecific binding sites were blocked after 5 % skim milk solution was added to each well, and the samples were incubated at 37 °C for 2 hours. Following three washes with PBST, the antigen-coated plate was stored at 4 °C until use. Hyperimmune sera from vaccinated and control chickens were diluted 1:10, 1:50, 1:100, 1:200, and 1:400 in blocking buffer and added to individual wells. After a 30-minute incubation at 37 °C, excess antibodies were removed by washing with PBST. A 100 µL conjugate solution (1:2000) was then added to each well and incubated for 30 minutes at 37 °C, followed by another PBST wash. A TMB substrate solution was added to develop color in positive reactions. The reaction was stopped with 1 N H2SO4, and the optical density (OD) was measured at 450 nm.

2.5. Evaluation of Test Sensitivity and DIAGNOSTIC specificity

The serum samples of naturally infected chickens infected with *M. galisepticum* were obtained from the Addis Ababa University Faculty of Veterinary Medicine. Additionally, positive and negative sera were generated experimentally at the National Veterinary Institute. To evaluate the newly developed in-house indirect ELISA, 100 µL of each serum sample was added to individual wells of a precoated microplate**.** After a 30-minute incubation at 37 °C, excess antibodies were removed by washing with PBST. One hundred microliters of conjugate solution was added to all the wells, which were subsequently incubated for 30 minutes at 37 °C. Following a PBST wash, 100 µL of substrate solution was added, and the mixture was incubated for 30 min at room temperature. Positive reactions resulted in the development of an orange color**.** The reaction was stopped with 1 N H2SO4, and the optical density (OD) was measured at 450 nm.

2.6. Statistical Analysis

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 and ID-vet indirect ELISA are described below.

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Table 2. Determination of test sensitivity and diagnostic specificity.

		The gold standard (ID-vet 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).

2.6. Data Analysis

The data analysis was performed via GraphPad Prism (version 5.01) (GraphPad Software, San Diego, CA, USA), MedCalc (version 10.0.2.0) (MedCalc Software, Mariakerke, Belgium) and Microsoft Excel window 10.

3. Results

3.1. DNA Extraction and PCR Detection

DNA was extracted from cultured organisms via the gSYNC™ Geneaid extraction kit. The PCR time, temperature, and cycle number are described in Table 1. The electrophoretic profile of Mg DNA obtained from culture via PCR revealed 185 bp (Figure 1).

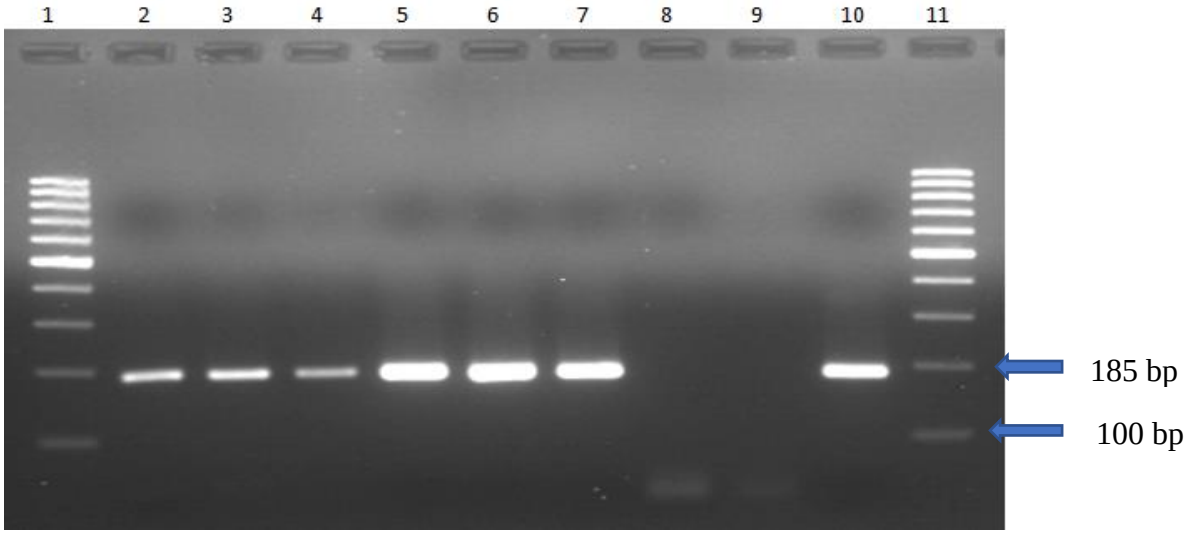

Figure 1. Electrophoresis profile of Mg DNA obtained from culture via PCR (185 bp) on a 2 % agarose gel. Lanes 1 and 11: DNA ladder; Lanes 2, 3, 4, 5, 6 and 7: MG DNA band obtained from culture; Lanes 8 and 9: Negative control; Lane 10: Positive control.

3.2. Optimization of Antigen Coating and Serum Dilution

The antigen was diluted to concentrations of 0.5 µg/µl, 0.75 µg/µl, 1 µg/ml, 1.5 µg/µl, and 2 µg/µl and coated. The serum was diluted 1:10, 1:50, 1:100, 1:200, and 1:400 and incubated with a secondary

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antibody at a dilution of 1:2000, which was optimized earlier. After an indirect ELISA technique was applied, 1.5 µg/µl and 1:10 were selected as the best antigen and serum dilutions respectively.

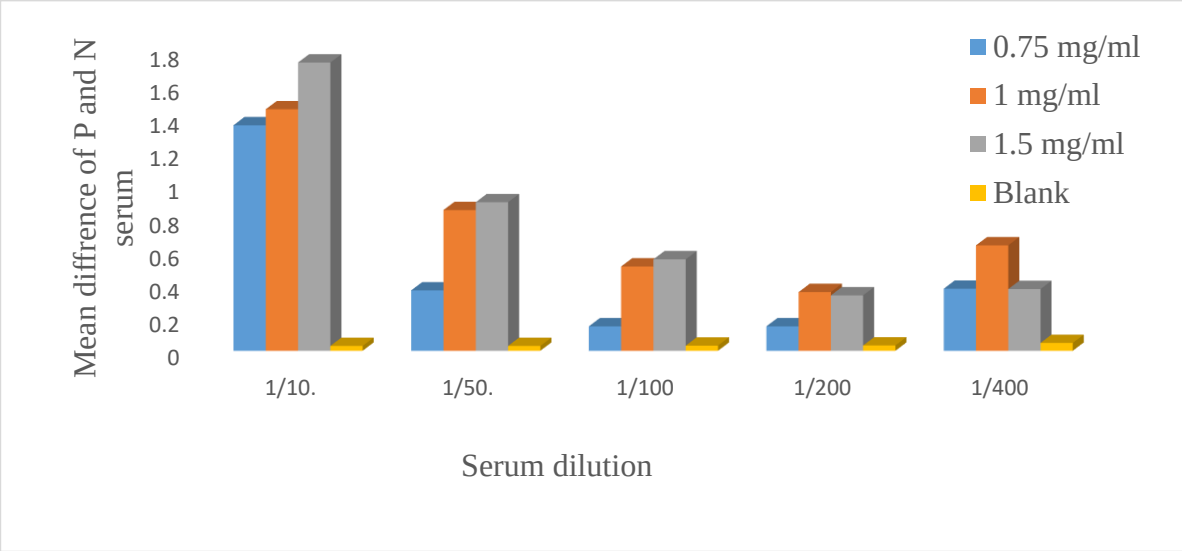

Figure 2. Optimization of the coated antigen and primary antibody dilution. The antigen was diluted to 0.5 µg/µl, 0.75 µg/ml, 1 µg/µl, 1.5 µg/µl and 2 µg/µl, and 100 µl of diluted antigen was dispensed horizontally on the microplate wells. The negative and positive sera were diluted 1:10.1:50, 1:100, 1:200, and 1:400 and added vertically. After the indirect ELISA method was applied, 1.5 µg/µl antigen and a 1:10 serum dilution were selected for the development of the in-house indirect ELISA. P=positive serum, N= negative serum, µg=microgram, µl=microliter.

3.3. Optimization of Incubation Time and Temperature

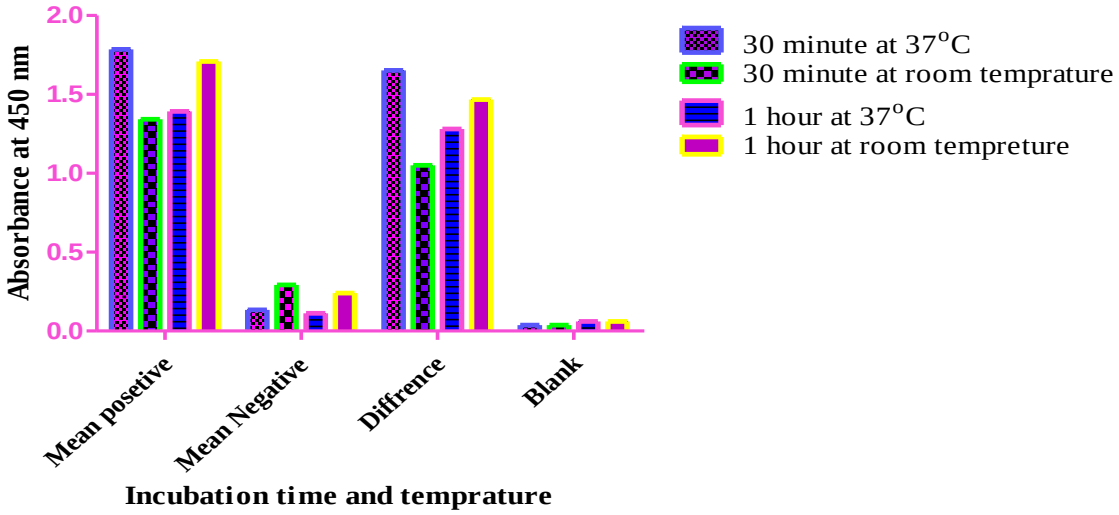

Figure 3. Optimization of the primary antibody incubation time. An in-house indirect ELISA method was used, and the primary antibody incubation times were compared after the samples were incubated for 30 minutes and 1 hr at 37 °C, 30 minutes, and 1 hr at room temperature. As shown in the graph, the best difference OD values of positive and negative sera were observed after an incubation time of 30 minutes at 37 °C, and these values were selected for the development of the current ELISA method.

3.3. Determination of the Cutoff Value

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A cutoff value of 0.59 was established for the In-House Indirect ELISA Kit, which was calculated the method described by Kumar and Rao (1991): mean absorbance of negative controls + 3 standard deviations**.** Serum samples with antibody titers greater than 0.59 were considered positive for *Mycoplasma gallisepticum*, whereas those with values less than 0.59 were deemed negative.

Table 3. Sixteen (N=16) known negative controls were screened, and the means and standard deviations were determined. The cutoff value was calculated as 0.59. Calculation factor: Cutoff value = (mean ± 3 × standard deviation) of the negative control serum.

Table 3. Determination of the cutoff value.

OD values of negative serum	Mean	STDEV	Cutoff value
0.37			
0.24			
0.33			
0.38			
0.39			
0.37			
0.31			
0.37	0.31	0.09	0.59
0.35			
0.23			
0.14			
0.35			
0.32			
0.16			
0.39			
0.21			

3.4. Determination of Test Sensitivity and Diagnostic Specificity

After optimization of the working antigen and serum, 16 known *M. galisepticum*-positive serum samples that were screened from naturally infected chickens and negative control serum samples identified from noninfected chickens were tested via the newly developed ELISA method and a commercial kit (ID vet indirect ELISA kit). The sensitivity and specificity of the current ELISA technique were 93.7 and 87.5, respectively.

Table 4. The test results of serum collected from naturally or experimentally infected animals (positive serum) and serum collected from noninfected animals (negative serum) are shown. The results revealed that out of the 16 positive and negative samples, the in-house indirect ELISA kit accurately detected both positive and negative sera. However, in the case of ID, vet Indirect ELISA kit out of 16 positive and Negative sera 1 sample was tested incorrectly.

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Table 4. Number of positive and negative chicken serum samples tested by in-house indirect ELISA with an ID-vet indirect ELISA kit.

	Serum collected from naturally or experimentally infected animals (positive serum)			Serum collected from noninfected animals (Negative serum)		
	Positive	Negative	Total	Positive	Negative	Total
In-House indirect ELISA kit	16	0	16	0	16	16
ID vet Indirect ELISA kit	15	1	16	1	15	16

3.5. Evaluation of the Kit Using Experimentally Produced Serum

The locally isolated *M. gallisepticum* was subsequently grown in culture media. The vaccine was prepared using oil and aluminum hydroxide adjuvants and was used to immunize chickens. Sera were collected 4 weeks after immunization, and the antibody response was evaluated with a newly developed ELISA kit.

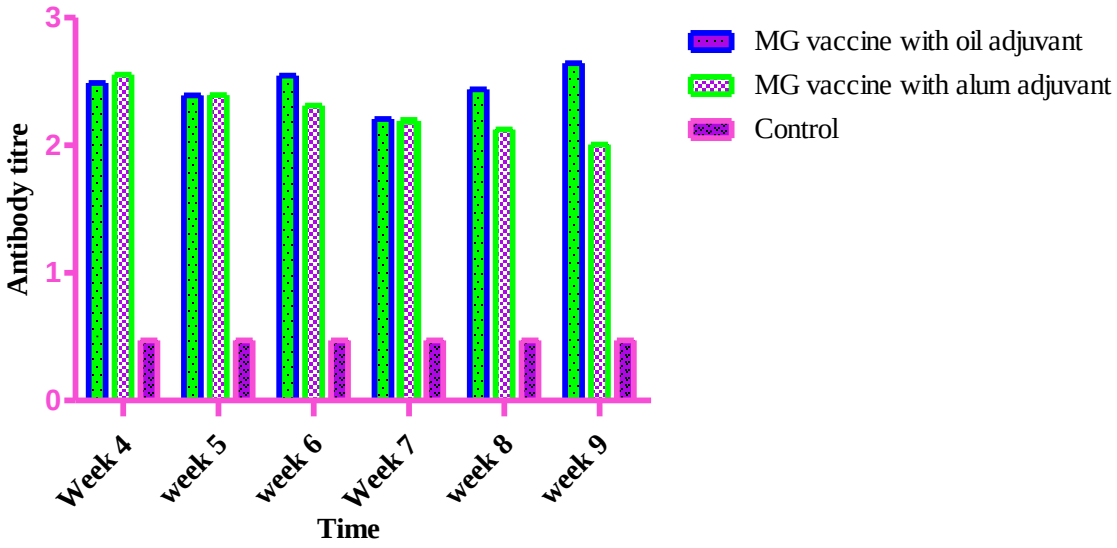

Figure 4. Graphical representation of the mean OD values of the antibody response to *M. galisepticum* vaccines. N=60, every 20 chickens were grouped into three groups and injected. The third group was kept as a negative control without injection. The chickens were bled for six weeks starting from the fourth week, and the mean antibody titers of the immunized and control groups were analyzed via GraphPad Prism 5.

Discussions

The demand for ELISA as a diagnostic method for serologically screening antibodies against *M. galisepticum* in chickens is high. In developing countries, the commercial ELISA kits used for the detection of antibodies against *M. galisepticum* are not adequate and are time-consuming for timely delivery. Furthermore, the sensitivity and specificity of the available kits are low. Accordingly, developing a more appropriate, highly sensitive and specific antibody detection method is compulsory. In the present study, a seemingly easily operational, less costly, more sensitive and specific in-house ELISA technique was developed and evaluated. For this developmental method, the incubation time was optimized to 37 °C for 30 minutes (Figure 1).

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Butler J et al. (1978) reported that ELISA is a fundamentally sensitive test that possibly overcomes at least some shortages of the remaining tests. In the present study, the *M. galisepticum* antigen was purified from the culture after centrifugation and washed on the basis of the evidence that lysing the mycoplasmas with a high pH buffer may allow for a wider range of antibody recognition in the ELISA (Glenn F et al, 2002). Another report by Nicolet, J et al. (1980) reported that the selective digestion of nucleic acids also resulted in a better-quality product; indeed, it has been hypothesized that the presence of some of these internal cell components may be a cause of nonspecific serological binding. Consequently, after the purification process, the protein content was determined, followed by optimization of the working antigen and serum dilution (Table 1).

The current ELISA method was further evaluated with serum collected from chickens immunized with the *M galisepticum* vaccine using different adjuvants (aluminum hydroxide and oil). Since the antibody response against mycoplasma starts late, blood collection commenced on week 4, continued until week 9, and then was tested. On the basis of these data, the current kit detected negative and positive serum appropriately in both vaccine groups, with a slightly better antibody response against those chickens immunized with the oil adjuvant (Figure 4).

In addition to the ELISA technique, the serum plate agglutination (SPA) test and hemagglutination inhibition (HI) test are the most commonly used assays for the serological detection of avian mycoplasmas (F. D. Talkington et al., 1985). In the report by Valentina A et al. (2022), the SPA test is a rapid and sensitive assay that detects immunoglobulin M (IgM) antibodies. Nevertheless, numerous factors related to the serum or antigen have an abundant effect on the specificity of this test method. Furthermore, the presence of a rheumatoid-like factor in avian serum also results in nonspecific agglutination and thus a lack of specificity (F. D. Talkington et al., 1985). Moreover, some commercial kits fail to detect experimentally produced antibodies. Therefore, the currently developed ELISA method is promising for overcoming the lack of antibody detection methods for *Mycoplasma* gallisepticum in chickens. The performance of this ELISA method was assessed by comparison with that of a commercial kit (ID-vet Indirect ELISA), which revealed a preeminent sensitivity and specificity. The method was well performed by testing positive and negative sera, which were experimentally produced and obtained from naturally infected animals, and this ELISA method will be used for the epidemiological study of *M. galisepticum* in chickens.

Conclusions

From this experimental work, we conclude that an economical and easily operational ELISA method was developed at the National Veterinary Institute and can be used for the serological detection of *Mycoplasma gallisepticum* in naturally and experimentally infected chickens.

Abbreviations

MG= *Mycoplasma* gallpticum, CRD=chronic respiratory disease, OIE= World Animal Health Organization, NDV=Newcastle disease virus, *E. coli*= *Escherichia coli*, IBV= infectious bronchitis virus, PBS=phosphate-buffered saline, BCA= Bicinconic acid, $Cuso_4$=Cupper sulfate, PBST= phosphate-buffered saline Tween 20, TMB=3, 3′, 5, 5′-tetramethylbenzidine, H2SO4= sulfuric acid, OD=optical density, ELISA=enzyme-linked immunosorbent assay, STDEV=standard deviation, SPA= serum plate agglutination, HI= hemagglutination inhibition, IgM= immunoglobulin M,

Ethics statement

Not applicable

Statistical analysis

GraphPad Prism 5 and Microsoft Excell window 10 were used for the statistical analysis.

Declarations

Consent to publish

Not applicable.

Availability of data and materials

Yes, available from the corresponding author upon request

Authors' contributions: TT designed the study, performed the experiment and wrote the manuscript. TA, BB, TS and TT revised the manuscript.AL, KS, GA and AG performed experimental works. WW, WC and DD were supplied materials.

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Competing interests: The authors declare that they have no competing interests.

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