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Efficiency Comparison of Serum Plate Agglutination Test and Enzyme-Linked Immunosorbent Assay in Diagnosis of *Mycoplasma gallisepticum* in Broiler Chickens

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Abstract: Mycoplasma is one of the smallest free-living Microorganism, it can reproduce independently. *Mycoplasma gallisepticum* (MG) infects chickens causing chronic respiratory disease (CRD) which leads to sever economic losses**,** CRD clinical signs include coughing, rales, sneezing, nasal discharges, dyspnea, and conjunctivitis with a frothy ocular exudate, anorexia, decreased feed conversion, high mortality, medication costs and carcass condemnations. This study was conducted to compare the efficiency of Serum Plate agglutination (SPA) test and Enzyme Linked Immune Sorbent Essay (ELISA) , in the diagnosis of MG. 180 blood samples were collected from broilers (4-6) weeks old who had a CRD symptoms, from 1st /12/2022 to 28th/2/2023. ELISA and SPA test were performed to detect the presence of antibodies against MG in the serum of blood samples. Seroprevalence of MG by SPA test was 75/180(41.6%),by ELISA it was 77/180 (42.7%). The sensitivity and specificity of SPA were (81,81% and 76%) respectively and for ELISA it were (90.9% and 78.4%) respectively. We found both test has a degree of efficiency but false results effect the degree of confidence in them so they use for initial flock screening and their results should be confirmed by Isolation or PCR. SPA test is faster and completely inexpensive wen compare with ELISA and other tests used for MG detection.

Keywords: broiler, *Mycoplasma gallisepticum*, CRD, ELISA, SPA

Introduction

MG belongs to the Mollicutes class (In Latin: Soft skin) and Mycoplasma genus, the smalest self-replicating microorganism, wall-less, genome size about (500-1500) kilobase pairs (kbp), (Razin et al., 1998). Since Mollicutes lack cell walls, it is not affected by cell-wall targeting antibiotics, such as, such as glycopeptides, fosfomycin, and beta-lactam antibiotics (Uphoff and Drexler, 2014)**,** MG is challenging to diagnose even when using an electron microscope because it is polymorphous (Osman et al., 2009)**.** Spread of MG infection may occur horizontally or vertically(Matucci et al., 2020) and can affect all ages of turkeys and chickens, but young are more susceptible (Ali et al., 2015), CRD occurs as a result of infection with MG in chickens and many other types of birds. Clinical symptoms of MG infection include sneezing, rales, nasal discharge, swollen infraorbital sinuses, and coughing. CRD economic losses due to ; reduced egg production, hatchability, feed efficiency; and weight gain, increase in mortality, medical cost and downgraded of bird carcasses, in addition to the costs of control and prevention (Levisohn and Kleven, 2000; Nascimento et al., 2005; De la Cruz, 2020; Qoraa et al., 2023). MG is the most dangerous of Poultry pathogens and has been classified as a must-notifiable pathogen by the International Office of

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Animal Epidemiology (OIE) (Chaidez- Ibarra et al., 2022). The quick and accurate detection of avian pathogenic mycoplasmas can significantly benefit in the early diagnosis and control the disease (Yadav et al., 2022). Mycoplasmas induce conventional symptoms such coughing, nasal discharge, because they infect the respiratory tract, however in many cases it may not cause any clinical signs (Feberwee et al., 2005), because many respiratory infections (such as Escherichia coli, infectious bronchitis, and respiratory form of Newcastle) target the respiratory system and often create identical symptoms to what happens with CRD, thus we cannot depend on clinical symptoms in the diagnosis of MG (Gross, 1990; Bradbury et al., 2001). Therefore, to diagnose MG infection, traditional bacteriological culture, serology, and molecular techniques are typically used (Sprygin et al., 2010; Yadav et al., 2022). Many type of serological tests have been used to detect MG antibodies like serum plate agglutination (SPA) test, hemagglutination inhibition (HI),and enzyme linked immunosorbent assay (ELISA) but sensitivity and specificity were more or less missing in all serological tests (Oie, 2008). SPA test is fast, sensitive and inexpensive, it has been widely used in the initial examination to monitor the infection of flocks with MG (OIE,2008, Arefin et al., 2012).). ELISA was developed for the rapid identification of Mycoplasma gallisepticum , increase test efficiency and improve the specificity and sensitivity of results compared to SPA and HI tests (Oie, 2008; Yadav et al., 2022). Better sensitivity and specificity made possible by PCR allow the detection of MG even in clinical samples taken from asymptomatic animals or those receiving antibiotic treatment (Buim et al., 2009). However, its use in conventional laboratories is limited due to the need for specialized equipment, skilled labor, and the high cost associated with pathogen screening and detection, particularly in developing nations (Ahmed et al., 2015). Isolation is the gold standard in the diagnosis of mycoplasma (Senthilnathan et al., 2015), it is costly, time-consuming, and arduous, and it might be tainted with non-pathogenic mycoplasma and other bacteria. Moreover, mycoplasma frequently does not develop in the laboratory (ABD EL-GHANY, 2008; Siddique et al., 2020; Marouf et al., 2022). This study aim to compare the efficiency of the SPA test and indirect ELISA for the detection of MG in broilers.

MATERIALS AND METHODS

Ethical approva

The field owners gave their agreement before any handling or sample collection of chickens was done, and extreme precautions were made to avoid any potential problems while closely adhering to the guidelines set forth by the Kirkuk Governorate's Animal Care Committee. in order to prevent the birds from experiencing stress.

Collection and preparation of samples

180 of 9000 (2%) broiler chickens distributed on 3 small fields (60 birds from each field) in the west of Kirkuk Governorate of type Ross 308 were sampled in a non-random way and used in the study. All of the chickens, which ranged in age from 28 to 40 days had respiratory symptoms, and increase of mortality rate. The study was carried out between the beginning of December 2022 and the end of February 2023 . From each bird 3 ml of blood drawn, identification information was recorded on each sample, and blood samples were transferred to the laboratory using an ice box. Blood-containing tubes were kept in the refrigerator at 4°C for five hours. To obtain a clear serum, the serum was decanted into a centrifuge tube and centrifuged at 2500 rpm for five minutes. The serum was then collected in a sterile Eppendorf tube and then kept at 4°C to use in the SPA test, sera if not tested immediately it should be kept at 5°C for less than 3days. The serum was stored at -20 °C until the ELISA test is performed with avoid of repeated freeze-thaw cycles

Serum Plate Agglutination test

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SPA test was done according to manufacturer formations (Lillidale Diagnostics/UK). by adding 30µl of MG antigen to 30µl of blood serum of suspected bird on glass plate, after mixing for two minutes by cycling, and then read the results after 30 second, agglutination (clotting) refer to positive results**.**

Enzyme-Linked Immunosorbent assay

ELISA test was performed according to manufacturer directions (BioChek/Holland). A- The serum samples were diluted by placing 5 microliters of the sample in each well of the dilution plate, then 245 microliters of sample dilution liquid was added to each well, mixing with a pipette, so that the resulting dilution was (1:50).Add to the coated test plate, 90 microliters of sample dilution and then transferred to it 10 microliters of the diluted sample (1:50) for each well according to the order (except for control sample wells) so that the final dilution of serum samples in the test plate was (1:500). Other materials are ready for work, but they should gain room temperature. B- 100 microliters of negative control were added to each of the two wells A1, and B1, and 100 microliters of positive control were added to each of the two wells C1, and D1. After filling the wells, cover plate with lid and incubate at 25°C for half an hour. C- Then the excess materials were emptied from the pits, and a washing buffer (350 microliters per well) was used to wash the pits for 4 consecutive times, to ensure complete removal of moisture, we turned the plate over and hit it on a moisture-absorbing paper or tissue. D- 100 microliters of Conjugate reagent was added to each well of the plate, then the plate was covered and incubated at 25 C for half an hour, then the washing process was repeated as in paragraph C. E- 100 microliters of substrate reagent was added to each well of the plate, then covered and incubated at 25 °C for 15 minutes, then we added to the well 100 microliters of stop solution, after 30 minutes the optical densities (OD) were read by Microplate reader (ELISA reader) at a wavelength of 405 nm connected to a computer containing BioChek ELISA software program for displaying the results. According to manufacturer protocol, if the positive percentage of the sample(S/P) is greater than or equal to (0.5), the sample is considered positive, otherwise, the sample is considered negative, and if the antibody titer is 668 or greater, the result is considerd positive.

S/P = (mean of test sample-mean of negative control)/ (mean of positive control -mean of negative control)

Log10 Titer = 1.1(Log10 S/P)+3.15 ⟶ Antilog = Titer

Interpretation of the results

Equations were used to compute the tests' sensitivity, specificity, and agreement. (Kleven, 1998; Trevethan, 2017)

Sensitivity = True positive/(True positive+ False negative) X 100

Specificity = True negative/(True negative+ False positive) X 100

Prevalence = Positive cases/total population X 100

Agreement = (Positive cases in both tests+ Negative cases in both tests) /total population X 100

RESULTS

Note: *We conducted both the bacterial isolation test and the polymerase chain reaction on the same birds from which we collected the serum samples, but we did not want to mention the two methods here because we wanted to mention them in another research.*

Results of SPA Test

Among one hundred and eighty serum samples, only 75 (41.66%) MG-positive serums (antigen-antibody agglutination, lock at figure 1) were detected. Of the 55 samples that were positive for the isolation of MG, (45) samples gave positive results and (10) gave negative results for SPA test, while the rest (125) samples, which gave negative results for the bacterial isolation test, gave positive

results for SPA test in 30 samples and negative results in 95 samples. The test sensitivity was 81.81%, specificity was 76% and agreement was 77.77%.

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Figure 1. Some results of SPA test

Results of ELISA Test

Among 180 serum samples, a total of 77 (42.7%) MG-positive serums were detected. All positive samples showed an S/P of more than 0.5. Of the 55 samples that were positive for the isolation of MG (50) samples, they gave positive results for ELISA test, while the rest of the samples (125 samples), which gave negative results for the bacterial isolation test, gave positive results for ELISA test in 27 samples and negative results in 98 samples. The test sensitivity was 90.9%, specificity was 78.4% and agreement was 82.22%.

Table(1)

	Prevalence %	Sensitivity %	Specificity%	Agreement%
SPA	**41.66**	**81.81**	**76.00**	**77.77**
ELISA	**42.7**	**90.90**	**78.40**	**82.22**

DISCUSSION

The seroprevalence rate of MG according to SPA in broilers was 41% (75/180). In Pakistan (Jalil and Islam, 2010) indicated that the seroprevalence rate of MG in chickens reached 66% for birds 8–20 weeks old. In Iran (Feizi et al., 2013) recorded a rate of 42%, in Egypt (ABD EL-GHANY, 2008) indicated a seroprevalence rate of 86.67% in four-week-old broilers and 10% in one-day-old chicks when used for the rapid agglutination test. In Turkey, (Ulgen and Kahraman, 1993) recorded a seroprevalence rate of 48.5%, in Saudi Arabia, (Elbehiry et al., 2016) recorded a rate of 46.11%. This difference in proportions may be due to differences in the nature of poultry farming, operational practices, and other biosecurity measures and the stage and severity of infection (Pradhan, 2002; Dulali, 2003). SPA test is characterized by its speed in giving results, and this feature allows us to make the appropriate decision to combat the disease. Its cost is low when compared to other tests, perhaps due to the simplicity of its equipment and materials. Due to its low cost and speed in showing results, it can be used to test large numbers of birds and in large-scale monitoring programs to detect MG. It is a simple test that can be performed in the field without the need for a laboratory. It is considered a qualitative test, not quantitative if it only reveals the presence of antibodies against MG without giving details about the detailed quantity of antibodies in the sample. The agglutination test can also detect MG early, even before symptoms appear, and this is extremely important in providing the opportunity to make appropriate decisions to combat the disease. This test can also be used periodically to detect the effectiveness of the vaccination program against MG in chickens, taking into account the date of vaccination so that false positive results do not occur (Dardeer, 1996; Levisohn and Kleven, 2000; Sharaf, 2004). The weaknesses of the agglutination test are that it is

sometimes unable to detect MG during the very early stages of infection when compared to the PCR test, and this leads to false negative results. Also, the agglutination test cannot differentiate between antibodies, whether they are the result of vaccination or the result of natural infection? Which leads to false positive results. It cannot determine the stage of infection, whether it is active or recovering, like other serological tests. The quality of the agglutination test is low, which leads to false positive results that often occur due to the occurrence of cross-reactions with antibodies against other pathogens, such as MS. False positive results may appear in the rapid agglutination test (SPA) due to contamination of the serum or re-freezing it(Kempf et al., 1994; Butcher, 2003). By ELISA we found the seroprevalence rate of MG was 42.7% (77/180), while (Bari and Shareef, 2023) recorded a rate of 52.48% in Dohuk governorate, in Jordan (Gharaibeh and Al Roussan, 2008) that out of 48 fields, 19 gave In the field of positive results (39.58%), in Saudi Arabia (Elbehiry et al., 2016) recorded a rate of 53.88%, in Kuwait (Qasem et al., 2015) recorded a rate of 42%, in Iran (Feizi et al., 2013) recorded a rate of 33%, and in Egypt (Osman et al., 2009) recorded 60%. ELISA was characterized by its speed, sensitivity, specificity, economical, and suitable for the initial examination of large swarms. It is also quantitative, through which the severity of the infection and the efficiency of treatment and vaccine can be determined (El-Ashram et al., 2021). Disadvantages of ELISA were the false positive results that were due either to previous vaccinations, the presence of maternal antibodies, or the occurrence of cross-reactions with antibodies against other types of Mycoplasma, ELISA false negative results occur, in the early stage of infection when the level of antibodies is low. In addition, ELISA sensitivity varies according to the strain (Feberwee et al., 2005; Muhammad et al., 2017; Yadav et al., 2022).

Our findings were different from earlier researchers for several reasons, including the management different and the degree to which it applies biosecurity programs and the provision of favorable environmental factors for birds, such as food, water, air, appropriate antibiotics, and effective vaccination programs, all of which increase bird resistance to disease and slow the rate at which it spreads. The general condition of the birds, birds have healthy conditions are more resistant to diseases and the spread of diseases is difficult among them, but weak birds, diseases spread easily among them and are easily die. Strain type and its capacity to spread the infection. The stage of the illness at the time the samples were taken. The quantity and type of samples, how to handle them, and how to keep them suitable for examination. The test's materials' quality and effectiveness in producing the results. The technique of testing that was used. (Feberwee et al., 2005; Swayne, 2013; Basit et al., 2021, Muhammad et al., 2021; Shiferaw et al., 2022; Raquib et al., 2022).

SPA detects antibodies produced by birds against MG sensitivity 81.8% indicates its ability to identify birds that have been infected with MG and developed an immune response. Specificity 76% suggests a higher of false positives compared to the other methods, this means SPA may identify some birds as positive, in fact, they are not. low agreement of 77.7% with isolation indicates that SPA might not be as concordant with the gold standard(isolation). SPA results influenced by many factors, making it less suitable as a standalone diagnostic method. ELISA sensitivity 91% indicates its ability to identify birds that have been infected with MG and developed an immune response. With a specificity of 78%, ELISA may classify some birds as positive when, in reality, they are not, indicating a larger rate of false positives than the other techniques, low agreement of 82% with isolation indicates that ELISA isn't concordant with the isolation.

CONCLUSION

The two tests we conducted differed in their sensitivity, specificity□□not without questionable results, and each test had its own strengths and weaknesses. ELISA was more sensitive and specific,

SPA was, fast, simple, and more inexpensive. Therefore, we employ the ELISA test when funding and specialized labs are available, and we use the SPA test when none of these factors are present.

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NOVELTY STATEMENT

This study, the first of its kind in Iraq, compares the efficiency of SPAand ELISA for identifying mycoplasma infection in broiler flocks. We also discussed some of the benefits and drawbacks of each test from the perspective of our research in a developing nation.

AUTHORS CONTRIBUTION

All authors collaborated equally in completing this work.

CONFLICT OF INTEREST

The authors have declared no conflict of interest.

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