COMPARISON OF SEROLOGIC METHODS FOR THE DETECTION OF MYCOPLASMA GALLISEPTICUM (MG) ANTIBODIES IN BROILERS IN SOUTH LUZON, PHILIPPINES

Sherwin I. Camba[1] and Hope G. Rovira[2]

ABSTRACT

Chronic respiratory disease (CRD), caused by *Mycoplasma gallisepticum* (MG), is one of the important diseases which affects chickens and other fowls. MG antibodies were screened from field cases of CRD in 20 commercial broiler farms in South Luzon, Philippines using the hemagglutination-inhibition test (HI) and enzyme-linked immunosorbent assay (ELISA). The serological results were also compared with the presence of clinical signs (RCS) and lesions (RLS) upon necropsy of submitted birds. Fisher's exact test was used to determine the correlation of RCS and RLS to the results of ELISA and HI tests ($p<0.05$). Among the 57 broiler chickens tested, 8 (14.03%) and 11 (19.2%) were seropositive in the ELISA and HI tests, respectively. The HI test was able to detect more positive reactors than ELISA. Sneezing and air sac lesion were the common signs observed upon necropsy. The correlation of RCS ($p=0.1738$) and RLS ($p=0.8424$) with the HI test titers were not statistically associated. The ELISA results were statistically associated with the RCS ($p=0.0201$) and RLS ($p=0.0357$). This is the first report on the use of the HI test for MG antibody detection in the Philippines which is beneficial to the poultry practitioners and farmers in monitoring MG to control CRD in the field.

Keywords: broilers, HI test, ELISA, CRD, *Mycoplasma gallisepticum*

INTRODUCTION

Mycoplasma, under the class mollicutes, contains both deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) and lack a cell wall (Stipkovits and Kempf, 1996). Several species of mycoplasmas were isolated from domestic avian species (Quinn *et al.*, 2011). Chronic respiratory disease (CRD) has great economic impact in poultry particularly on the decrease of feed conversion (FCR) of broilers and contributes to the increase of medication costs (Mohammed *et al.*, 1987). *Mycoplasma gallisepticum* (MG) is the causative agent of CRD which affects chickens, turkeys, and other fowls. MG can also be spread by horizontal and vertical transmission in poultry farms (Lin and Kleven, 1982). Serologic procedures are useful for flock monitoring and aid in diagnosis when infection is suspected (Ley and Yoder, 1997). Diagnosing CRD in the Philippines has mostly relied on clinical signs manifested by the infected flock, and evaluation of the production parameters (Novilla et al, 1971;

[1]Institute of Animal Science, College of Agriculture and Food Science, University of the Philippines Los Baños, College, Laguna, Philippines; [2]Philippine College of Veterinary Public Health, Quezon City, Philippines (email: sicamba@up.edu.ph)

Watanabe *et al.*,1980). Serological tests such as the enzyme-linked immunosorbent assay (ELISA) and rapid plate agglutination (RPA) tests are usually performed (Greenfield and Bankier, 1972; Kaszanyitzky *et al.*, 1994; Feberwee *et al.*, 2005). Since the time of Novilla (1971), not much had been reported on diagnostic procedures with regards to mycoplasmosis in the Philippines. Due to the high cost of ELISA and low sensitivity of RPA, an alternative serological tool such as hemagglutination-inhibition (HI) test should be explored to detect MG antibodies in suspected flocks. There are also limited data on the prevalence of *Mycoplasma gallisepticum* in commercial broiler farms in the Philippines. Thus, the aim of this study is to determine the seroprevalence of *Mycoplasma gallisepticum* from field cases of CRD using HI test and ELISA. The serological results were also correlated with the respiratory clinical sign (RCS) and lesion (RLS) scores. The findings will provide a better understanding of the prevalence of *Mycoplasma gallisepticum* from CRD cases in commercial broiler farms in South Luzon, Philippines.

MATERIALS AND METHODS

Clinical Signs and Lesions

Based on farm history, fifty-seven (57) live broiler chickens were submitted from twenty (20) commercial broiler farms in Cavite, Laguna, Batangas, Rizal, and Quezon (South Luzon), Philippines. The age of the chickens ranged from 10 to 40 days old. Live birds were brought to the UP College of Veterinary Medicine (UPCVM), Department of Veterinary Paraclinical Sciences (DVPS) for serology and routine necropsy. Serum samples were harvested and stored in a deep freezer (-20 °C) prior to testing. Freezing and thawing was avoided to prevent devaluation of components. All animal procedures have been approved by the Institutional Animal Care and Use Committee of the University of the Philippines College of Veterinary Medicine (2013-16).

The number of farms and chicken per province of origin were indicated in Table 1. Most of the 20 farms (18/20) had a conventional housing set up except for 2 in Rizal had tunnel ventilation. In addition to farm history, recent medications were also recorded. All the chickens were subjected to physical examination focusing on the respiratory clinical signs. The following gross respiratory and related system lesions were considered for scoring: 1) nasal discharges, 2) sneezing, 3) tracheal/moist rales, 4) breathing through the partly open beak (gasping), and 5) swollen infraorbital sinus. Then, each bird was humanely sacrificed by cervical dislocation, and disinfected with 70% alcohol and 10% iodine for necropsy. The following gross respiratory and related system lesions were considered for scoring: 1) swollen infraorbital sinus, 2) mucoid trachea or tracheitis, 3) cheesy or cloudy airsacs, 4) pneumonia or any form of damage in lungs, 5) fibrinous or fibrinopurulent pericarditis, and 6) fibrinous or fibrinopurulent perihepatitis. The accumulated respiratory signs and lesions score were collated and then computed as percentages (%).

Serologic Tests

Hemagglutination test was initially performed to adjust the required HA unit for MG in HI test (Crawley, 1960). Test components were prepared and the serum samples were tested and incubated at room temperature for 60 minutes. HI titer was read as the last serum dilution showing inhibition of agglutination (U-botton formation). Complete inhibition of hemagglutination at 1:80 or more dilution of the serum was considered positive for MG,

1:40 dilution was considered suspect or probable and 1:20 or less dilution was considered negative for MG. The commercial antigen *Mycoplasma gallisepticum* A5969 strain was used for the hemagglutination (HA) and hemagglutination-inhibition (HI) tests were obtained from National Veterinary Services Laboratories, APHIS, USDA. The commercial MG-positive control sera in different dilution titers which are low positive 1:40 (lot no. 104-40 9701), medium positive 1:160-1:320 (lot no. 104 M 1002) and high positive 1:640-1:280 (lot no. 104-H 1002) and the commercial MG-negative control serum (Lot no. 119 1601) was also obtained from National Veterinary Services Laboratories, APHIS, USDA.

An indirect commercial MG ELISA kit (ProFLOK®, Lot no. 1104171) by Symbiotics Corporation was used in the study. The kit contained MG (R strain) antigen coated plates. The test plate was read using an ELISA reader (Sunrise™ by Tecan Ltd.) at wavelength of 405-410 nm. Serum samples with an ELISA titer value of ''0'' was presumed negative for MG antibody. ELISA titer range of 149 to 743 are presumed MG antibody probable. MG ELISA titer ranged 744 or greater is considered positive.

The results obtained were analyzed using Fisher's exact test to determine the correlation the respiratory clinical sign (RCS) and lesion scores (RLS) to the results of ELISA and HI tests ($P < 0.05$).

RESULTS AND DISCUSSION

The main objective of this study was to compare two serologic methods, the hemagglutination-inhibition (HI) and enzyme-linked immunosorbent assay (ELISA) tests in determining the presence of *M. gallisepticum* (MG) antibodies in broiler chickens from farms with RCS indicative of CRD. In addition, the results of HI and ELISA were correlated to the RCS and lesions scores obtained. The sample population of broiler chickens came from 20 farms with varying degrees of clinical signs and lesions suggesting CRD.

Seroprevalence of *Mycoplasma gallisepticum* using HI and ELISA

The seroprevalence of *Mycoplasma gallisepticum* using HI and ELISA were at 19.2% and 14.03% respectively. The results were comparable with the findings of Novilla *et al.* (1971) where they detected 114 (13%) of 828 serum samples from 37 flocks using rapid serum plate agglutination test. HI and ELISA results are recorded in Tables 1 and 2. The hemagglutination potential of MG antigen used was 1:2560 by hemagglutination (HA) test (Figure 1). The differences in the test results could be attributed to the test format or principle of the two serologic tests. ELISA is a primary binding assay that ideally depends on the antigen-antibody interactions which are measured by combining one of reactants with enzyme indicator (Figure 3). HI test (Figure 2), on the other hand, is a secondary binding assay that measures the level of antibodies in the serum by means of the inhibition of hemagglutination. In addition, the MG strain used in the HI test (A5969 strain) was different from the ELISA assay (R strain) that contributed in the difference in terms of results. Out of 20 farms, the ELISA results revealed that four (20%) farms were seropositive, three probable results of at least one sample while HI test had eight (40%) seropositive results with more probable results (Table 2). These results were in contrast to the reports of Baharsefat and Adler (1965), Ansari *et al.* (1983), Patten *et al.* (1984), Talkington *et al.* (1985), Czifra *et al.* (1993), and Ewing *et al.* (1996) in which ELISA detected more positive reactors than the HI test. Different methods and strains of MG were employed by these authors and the prevalent

strain in their locality may have attributed to differences of results in this study.

Table 1. Seroprevalence of *Mycoplasama gallisepticum* in broiler farms in South Luzon, Philippines

Location	Type of Housing	Number of Farms	Number of Samples	HI test (MG A5969 strain) +/-	ELISA (MG R strain) +/-
Laguna	Conventional	9	29	7	4
Rizal	Conventional (n=5); Tunnel ventilated (n=2)	7	17	0	0
Batangas	Conventional	2	5	4	4
Cavite	Conventional	1	5	0	0
Quezon	Conventional	1	1	0	0
Total		20	57	11 (19.2%)	8 (14.03%)

Table 2. Comparison of HI and ELISA results per farm including clinical signs and lesions.

Farm ID	Age (day)	RCS (%)	RLS (%)	ELISA Results (Positive/Probable/ Negative)	HI Test Result (Positive/Probable/ Negative)
Lb (n=4)	18	0	33.5	(0/1/3)	(0/2/2)
Lc (n=6)	19	0	0	(1/1/4)	(1/2/2)
Ld (n=1)	17	0	33	(1/0/0)	(1/0/0)
Le (n=3)	14-18	13.3	23.3	(2/0/1)	(2/0/1)
Lf (n=2)	15	0	0	(0/0/2)	(0/1/1)
Lg (n=6)	10	0	5.67	(0/1/5)	(1/2/3)
Lh (n=3)	16	0	0	(0/0/3)	(1/1/1)
Li (n=2)	38	20	41.5	(0/0/2)	(1/1/0)
Lj (n=2)	36	20	33	(0/0/2)	(0/1/1)
Rb (n=1)	14	80	66	(0/0/1)	(0/0/1)
Rd (n=3)	40	13.3	0	(0/2/1)	(2/1/0)
Re (n=3)	17	26.71	22.3	(0/0/3)	(0/0/3)
Rf (n=1)	17	40	50	(0/0/1)	(0/1/0)
Rg (n=2)	29	80	16.5	(0/0/2)	(0/2/0)
Rh (n=6)	22-37	80	49.6	(0/0/6)	(0/2/4)
Ri (n=1)	36	20	50	(0/0/1)	(0/0/1)

Table 2. Continuation....

Farm ID	Age (day)	RCS (%)	RLS (%)	ELISA Results (Positive/Probable/ Negative)	HI Test Result (Positive/Probable/ Negative)
Ba (n=1)	21	80	33	(0/0/1)	(0/0/1)
Bb (n=4)	28	40	16.6	(4/0/0)	(4/0/0)
Ca (n=5)	14-19	8	13.6	(0/0/5)	(0/3/2)
Qc (n=1)	2	0	0	(0/0/1)	(0/1/0)
P value for HI test interpretation		0.1738	0.8424	Total (8/5/44)	Total (13/20/23)
P value for ELISA interpretation		0.0201*	0.0357*		

HI Titer: <1/40-Negative, 1/40- Probable, 1/80 above- Positive; ELISA Titer: 0-Negative, 149-743-Probable, 744 or greater-Positive; L: Laguna, R: Rizal, B: Batangas, C: Cavite, Q: Quezon; RCS: Respiratory Clinical Signs RLS: Respiratory Lesions; **P* value of <0.5 indicates association between clinical scores and test interpretation using Fisher's exact test.

Figure 1. Hemagglutination (HA) test result. The end-point was the highest dilution which showed complete hemagglutination (matt formation). Dilution for 1 HA Unit was determined at 1:2560 (White arrow). RBC control (Black arrow) showed the formation of U-botton.

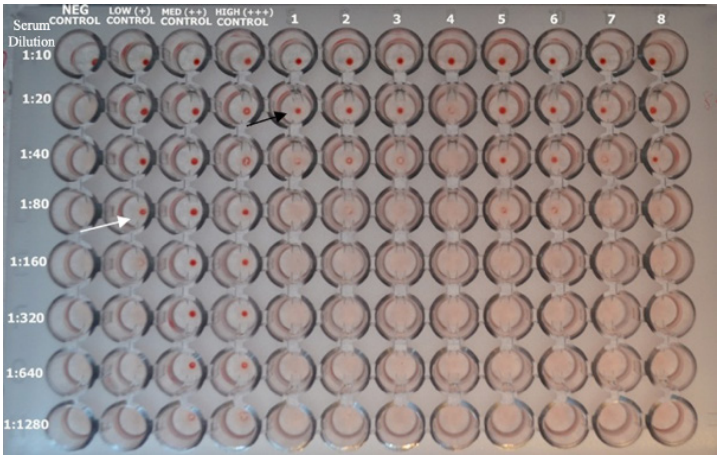

Figure 2. Hemagglutination-Inhibition (HI). On the first row (1:10), serum controls included were negative, low positive (1:80), medium positive (1:640) and high positive (1:1280); followed by test sera from 1 to 8. The end-point (White arrow) was the highest.

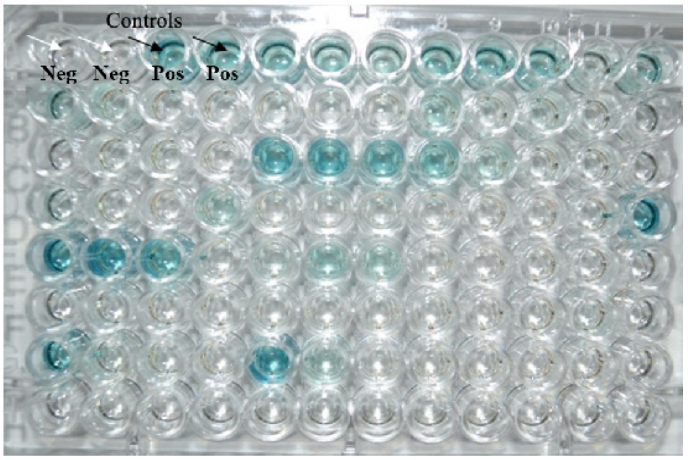

Figure 3. ELISA results. Negative control sera (white arrows) and positive control sera (black arrows) were designated properly from wells 1 to 4. Positive indicator for presence of Mycoplasma gallisepticum antibodies was the development of bluish-green color reaction (black arrow).

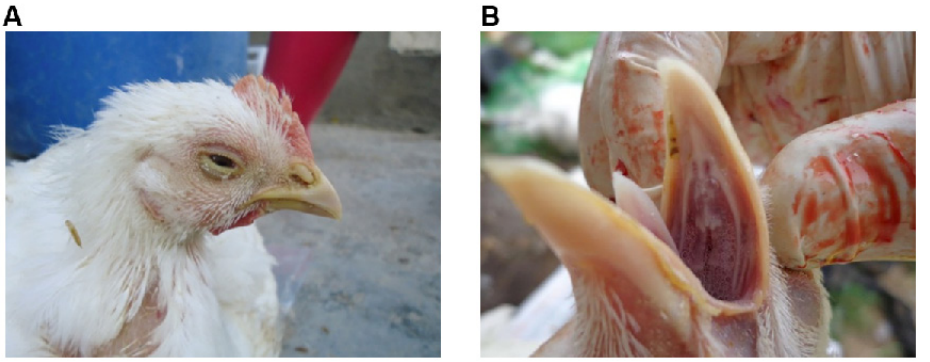

Figure 4. Respiratory clinical signs. (A) Broiler chicken with sign of sneezing (B) Broiler chicken with presence of oronasal exudates.

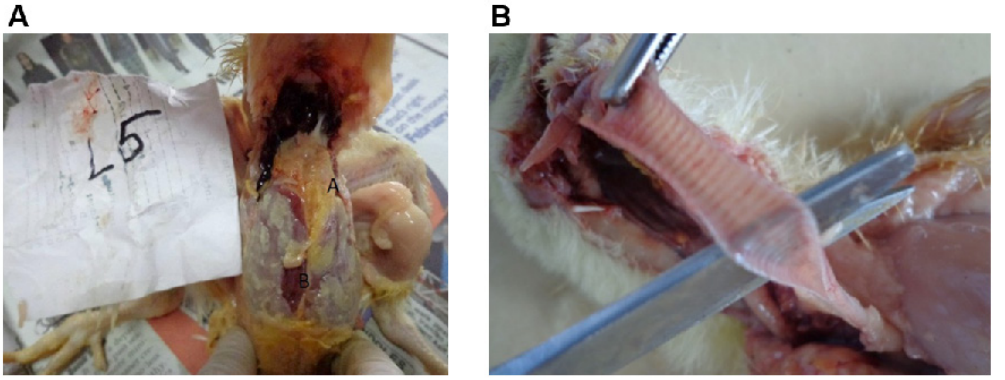

Figure 5. Respiratory lesions. (A) Airssacculitis, fibrinopurulent pericarditis and hepatitis lesions (B) Mild tracheitis with mucoid exudates.

Thirteen (65%) out of 20 farms have manifested signs of CRD based on submitted broiler samples. Sneezing (30.77%) (Figure 3) and nasal discharges (26.15%) (Table 2) were found to be the most common CRD clinical signs (Figure 4a and 4b, respectively). Out of those farms with CRD clinical signs, ELISA detected two (15.3%) positive farms, and one (7.7%) probable farm. On the other hand, using HI test, four out of 13 (30.8%) farms were seropositive, and five (38.5%) were probable to MG. Twenty out of 23 ELISA-negative farm samples (91%) were tested probable using HI test. Only two ELISA probable farms tested positive in HI. Overall, both serologic tests have similar results in terms of seropositive farms.

Seven farms out of 20 farms did not manifest any CRD clinical signs. However, upon necropsy, three birds without CRD signs showed presence of CRD lesions (Figure 5). For bird samples without CRD clinical signs, ELISA detected two seropositive and two probable birds. HI results, on the other hand, have revealed four additional seropositives, and three probable birds. The presence of MG antibodies despite the absence of CRD clinical signs and lesions may be attributed to previous infection or vertical transmission from MG-infected or vaccinated breeders, thus preventing the appearance of lesions. The average age of the birds tested was 18 days (2 to 3 weeks) old. According to Kleven and Levisohn (2000), diagnostically significant titers in the HI test may not be detected until three or more weeks after infection. In addition, MG antibodies arise between the second and third week after MG infection and reach their peak six to eight weeks later (Fahey and Crawley, 1954). For MG screening in broilers, the timing of collection is critical to accuracy of results. Measuring titers of birds younger than 3 or 4 weeks is not recommended because only the maternal antibodies could be present at that time and active antibody will not yet be present (Stanley H. Kleven, personal communication). To improve accuracy, collection of blood sample should be done at the time of slaughter (28 days above) in order to have plenty of time to seroconvert. In addition, young chickens could not develop MG antibodies immediately, especially when there was no obvious disease.

Table 3. Tally of the respiratory clinical sign scores in broilers with CRD per farm sources.

Respiratory signs	**Laguna**	**Rizal**	**Batangas**	**Cavite**	**Quezon**	**Total**
Nasal discharge	4 (66.67%)	13 (28.89%)	1 (8.33%)	0 (0.00%)	0 (0.00%)	17 (26.15%)
Sneezing	0 (0.00%)	13 (28.89%)	5 (41.67%)	2 (100.00%)	0 (0.00%)	20 (30.77%)
Moist rales	1 (16.67%)	11 (24.44%)	1 (8.33%)	0 (0.00%)	0 (0.00%)	13 (20.00%)
Panting/gasping	1 (16.67%)	9 (20.00%)	1 (8.33%)	0 (0.00%)	0 (0.00%)	11 (16.92%)
Swollen infraorbital sinus	0 (0.00%)	0 (0.00%)	4 (33.33%)	0 (0.00%)	0 (0.00%)	4 (6.15%)
Total	6	45	12	2	0	65

Air sac lesions were the most common CRD lesions observed among the submitted bird samples (Table 3). For the comparison of the two serologic tests to the CRD lesions, HI test detected more positive reactors (22.8%) than ELISA (14.03%). Interestingly, three (5.2%) ELISA-negative samples were detected HI positive while 20 (35%) ELISA-negative samples had probable results in the HI test. Previous studies (Lin and Kleven, 1982; Ley, 2003) suggest that respiratory clinical signs and patho-morphological lesions of the respiratory tract were not pathognomonic for MG infection. The presence of clinical signs and lesions could also be due to other respiratory agents such as *E. coli* and other viral pathogens (Vogl *et al.*, 2008). Negative titers despite the presence of clinical signs and lesions could be attributed to other poor environmental conditions particularly the internal temperature/humidity inside the conventional poultry houses. In this study, the presence of high antibody titer in both ELISA and HI still validated the presence of MG infections in broilers with CRD. The clinical and lesion scoring system used in this study can be improved however it was sufficient to assess the state of RCS and RLS (Table 4). The use of a scoring system was mentioned in a study by Bigland and Benson (1968), where the gross air sac lesions were judged according to the severity, number of air sac involved, and thickness. An arbitrary designation of 1, 2 and 3 was used for increasing severity.

Table 4. Tally of the respiratory lesion scores in broilers with CRD per farm sources.

Respiratory signs	Laguna	Rizal	Batangas	Cavite	Quezon	Total
Swollen infraorbital sinus	0 (0.00%)	2 (8.00%)	4 (16.00%)	0 (0.00%)	0 (0.00%)	6 (8.70%)
Mucoid tracheitis	6 (24.00%)	7 (20.59%)	1 (16.67%)	0 (0.00%)	0 (0.00%)	14 (20.29%)
Cheesy/cloudy airsacs	12 (48.00%)	13 (38.24%)	1 (16.67%)	4 (100.00%)	0 (0.00%)	30 (43.48%)
Pneumonia	3 (12.00%)	3 (8.82%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	6 (8.70%)
Fibrinopurulent pericarditis	2 (8.00%)	6 (17.65%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	8 (11.59%)
Fibrinopurulent perihepatitis	2 (8.00%)	3 (8.82%)	0 (0.00%)	0 (0.00%)	0 (0.00%)	5 (7.25%)
Total	25	34	6	4	0	69

This is the first report on the use of the hemagglutination-inhibition (HI) test for *M. gallisepticum* antibody detection in the Philippines. Based on the results of the study, HI test can be used as a primary serological tool in detecting MG antibodies in cases of CRD in poultry. In this study, the correlation of respiratory clinical signs (p=0.1738) and lesions scores (p=0.8424) with HI test titers were not statistically associated using Fisher's exact test. The ELISA results, however, were statistically associated with the respiratory

clinical signs (p=0.0201) and lesions (p=0.0357). Further studies are needed to compare the sensitivity, specificity, and cost efficiency for the HI test in MG screening. It will be a valuable tool in screening vast amount of samples in field MG cases or vaccination trials. In view of this, sampling should be conducted in more regions in the Philippines particularly in the area with high broiler/bird population density. Moreover, the isolation of mycoplasma by culture is highly recommended from CRD field cases to confirm the diagnosis and also to collect and determine the local MG field strain via polymerase chain reaction and sequencing. These field MG isolates can be utilized to produce antigens for HI tests which can provide more sensitive and specific serologic findings. The determination of prevailing local strains is also essential in the understanding of the epidemiology of MG and for the development of autogenous vaccine which is beneficial to the poultry practitioners and farmers. Further studies should also be employed in order to determine the prevalence and distribution of MG in commercial layers, breeder layers or broilers, free-range chickens, and wild birds in the Philippines. In conclusion, the hemagglutination-inhibition test can be utilized as a primary serological tool to detect *Mycoplasma gallisepticum* antibodies in broilers. The seroprevalence of *Mycoplasma gallisepticum* in broiler farms with chronic respiratory disease using HI test and ELISA were at 19.2% and 14.03% respectively. Our findings demonstrated that HI can detect more positive reactors than ELISA in broilers from CRD cases.

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REFERENCES

Ansari AA, Taylor RF and Chang TS. 1983. Application of enzyme-linked immunosorbent assay for detecting antibody to *Mycoplasma gallisepticum* infections in poultry. *Avian Dis* 27(1):21-35.

Baharsefat M and Adler HE. 1965. Comparison of tube agglutination, hemagglutination-inhibition (HI) and antiglobulin titers on serum of chickens and turkeys infected with *Mycoplasma gallisepticum*. *Avian Dis* 9(3):460-464.

Bigland CH and Benson ML. 1968. *Mycoplasma meleagridis* ("N" strain mycoplasma--PPLO): relationship of air sac lesions and isolations in day old turkeys (*Meleagridis gallopavo*). *Can Vet J* 9(6):138-41.

Crawley JF. 1960. Use of hemagglutination-inhibition test in the control of CRD of chickens. *Ann N Y Acad Sci* 79:562-566.

Czifra G and Sundquist B. 1993. Evaluation of a monoclonal blocking enzyme-linked immunosorbent assay for the detection of *Mycoplasma gallisepticum*-specific antibodies. *Avian Dis* 37(3):680-688.

Ewing ML, Kleven SH and Brown MB. 1996. Comparison of enzyme-linked immunosorbent assay and hemagglutination-inhibition for detection of antibody to *Mycoplasma gallisepticum* in commercial broiler, fair and exhibition, and experimentally infected birds. *Avian Dis* 40(1): 13-22.

Fahey JE and Crawley JF. 1954. Studies on chronic respiratory disease of chickens IV. A

hemagglutination-inhibition diagnostic test. *Can J Comp Med Vet Sci* 18(7):264-272.

Feberwee A, Mekkes DR, de Wit JJ, Hartman EG and Pijpers A. 2005. Comparison of culture, PCR, and different serologic tests for detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* infections. *Avian Dis* 49(2): 260-268.

Greenfield J and Bankier J. 1972. Comparison of hemagglutination-inhibition and serum plate agglutination tests to detect antibodies to *Mycoplasma gallisepticum*. *Can J Comp Med* 36(1):77-78.

Kaszanyitzky E, Czifra G and Stipkovits L. 1994. Detection of *Mycoplasma gallisepticum* in turkey blood samples by ELISA and by the slide agglutination and hemagglutination inhibition tests. *Acta Vet Hung* 42(1): 69-78.

Kleven SH and Levisohn S. 2000. Avian Mycoplasmosis. Revue scientifique et technique International Office of Epizootics. 19: 425-442.

Ley HD. 2003. *Mycoplasma gallisepticum* infection. In: Saif YM, ed. 11th ed. *Diseases of Poultry*. Iowa: Iowa State Press, pp. 722-744.

Lin MY and Kleven SH. 1982. Egg transmission of two strains of *Mycoplasma gallisepticum* in chickens. *Avian Dis* 26(3):487-495.

Mohammed HO, Carpenter TE and Yamamoto R. 1987. Economic Impact of *Mycoplasma gallisepticum* and *M. synoviae* in commercial layer flocks. *Avian Dis* 31(3):477-482.

Novilla MN, Menez CF and Brana EB. 1971. Serologic and cultural studies of chronic respiratory disease (CRD) in the Philippines. *Philipp J Vet Med* 10(1):65-72.

Patten BE, Higgins PA and Whithear KG. 1984. A urease-ELISA for the detection of mycoplasma infections in poultry. *Aust Vet J* 61(5):151-155.

Quinn PJ, Markey BK, Leonard FC, FitzPatrick ES, Fanning S and Hartigan PJ. 2011. *Veterinary Microbiology and Microbial Disease*. 2nd ed. UK: Wiley-Blackwell.

Stipkovits L and Kempf I. 1996. Mycoplasmoses in poultry. *Rev Sci Tech Off Int Epizoot* 15(4): 1495-1525.

Talkington FD, Kleven SH and Brown J. 1985. An enzyme-linked immunosobent assay for the detection of antibodies to *Mycoplasma gallisepticum* in experimentally infected chickens. *Avian Dis* 29(1):53-70.

Vogl G, Plaickner A, Szathmary S, Stipkovits L, Rosengarten R and Szostak MP. 2008. *Mycoplasma gallisepticum* invades chicken erythrocytes during infection. *Infect Immun* 76(1):71-77.

Watanabe N, Nishiwaki M, Ichinoe K, Peneyra J, and Palao M. 1980. Serological survey of avian mycoplasmosis on Palawan Island in the Philippines. *Journal of Japanese Society of Poultry Diseases* 16:119-124.

World Organization for Animal Health. *Manual of diagnostic tests and vaccines for terrestrial animals*. Retrieved on 15 May 2023 from https://www.woah.org/en/what-we-do/standards/codes-and-manuals/terrestrial-manual-online-access/