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# Serological detection of *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Mycoplasma melleagridis* in free range chickens in Ilorin, Kwara State

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## ABSTRACT

This study was designed to detect the three mycoplasmas from free-range chickens using the diagnostic confirmatory supports from Animal and Plant Health Agency, UK. Out of 602 serum samples screened with rapid serum agglutination test (RSAT), 33 were positive for both *Mycoplasma gallisepticum* (Mg) and *Mycoplasma synoviae* (Ms). The 33 positive sera and 11 cultures with typical fried egg appearance suggesting mycoplasma organism were submitted to the Animal and Plant Health Agency (APHA), UK for confirmation using RSA (different antigen batches) and commercial ELISA tests, based on manufacturers interpretative criteria, specific for each of the three species' antibodies as well as re-cultivation and denaturing gradient gel electrophoresis PCR-DGGE as well as real-time (Mg/Ms combination) on the submitted stored cultures. Rapid serum agglutination test for the three species were used with associated positive control sera, typically showing 2+ or 3+ reactions for the correct antigen, with no obvious reaction for the negative sera. ELISA data revealed 21 of the 33 samples to be positive for Mg, 19 positive for Ms and 14 for both. Two samples were also positive for *Mycoplasma melleagridis* (Mm) by ELISA. The difference between the RSAT and ELISA findings may be associated with the ability of the RSAT to detect antibody response to exposure at an earlier stage than ELISA. Of the six cultures tested, none was positive for Mg or Ms. PCR-DGGE, confirmed the real-time PCR results with many bands. Further serological testing has confirmed the presence of antibody response to Mg and Ms in a high proportion and indicating likely exposure of free- range chickens to these avian pathogens.

**Keywords:** chickens, *Mycoplasma gallisepticum*, *Mycoplasma melleagridis*, *Mycoplasma synoviae*, serological detection.

## INTRODUCTION

Mycoplasmas are bacteria that lack cell wall and belong to the class mollicutes and family mycoplamataceae. Mycoplasmas are widespread in nature and infect a wide range of hosts. Species from the genus Mycoplasma have been isolated (over 110 species) from mammals, birds, reptiles, and fish (Gharibi *et al*., 2018; Petrone-Garcia *et al*., 2020). Avian mycoplasmosis remains one of the major health problems affecting poultry species and limiting their productivity (Gharibi *et al*., 2018). The disease is associated with significant economic loses (Bergeron *et al*., 2021) in terms of reduced weight gain and egg production and increased embryo mortality (Gharibi *et al*., 2018).

Serological methods are widely employed for detecting Mycoplasma species due to their sensitivity, specificity, and ability to detect both active and past infections (Manivannan *et al*., 2024). Common techniques include enzyme-linked immunosorbent assays (ELISA), which detect antibodies against Mycoplasma antigens, providing information on exposure history (Okella, *et al*., 2023). Indirect hemagglutination (IHA) and complement fixation tests (CFT) are also used to identify specific Mycoplasma-associated antibodies. Additionally, immunofluorescence assays (IFA) offer precise visualization of Mycoplasma antigens using fluorescently labeled antibodies, enabling direct detection in clinical samples (Waites *et al*., 2024). Western blot analysis is another method that can identify specific proteins of Mycoplasma species, enhancing diagnostic accuracy (Gomes *et al*., 2024). These serological methods play a critical role in veterinary and medical microbiology, allowing for effective diagnosis and epidemiological studies.

Avian mycoplasmosis is caused by four commonly recognized pathogens namely:*Mycoplasma gallisepticum*, *Mycoplasma synoviae* , Mycoplasma meleagridis and Mycoplasma iowae (More *et al*., 2017). Of these, *Mycoplasma gallisepticum* (Mg)

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and *Mycoplasma synoviae* (Ms) are pathogenic for chickens and turkeys while M. iowae affects primarily turkeys, and M. meleagridis infects turkeys only (Cortés *et al*., 2021). Apart from these four Mycoplasma species, other Mycoplasmas that have been incriminated in Mycoplasma infections in birds include: Mycoplasma anseris (affects geese), M. columbinum (affects pigeons), M. gallinarum, M. gallinaceum, M. lipofaciens and M.pullorum which affects chickens (Grózner *et al*., 2019; Sawicka-Durkalec *et al*., 2021).

*Mycoplasma gallisepticum* is the most economically significant Mycoplasma pathogen of gallinaceous and certain non-gallinaceous avian species and causes chronic respiratory disease (CRD) in chickens and infectious sinusitis in turkeys (Oman *et al*., 2009). It causes a reduction in weight gain, decrease in feed conversion efficiency and meat quality, increase mortality rate in broilers. Ureaplasma species have also been found to be pathogenic to chickens and turkeys causing air sacculitis (Santos *et al*., 2021).

In poultry production chain, feed, poor hygienic practices and weak biosecurity are the main routes for introducing mycoplasmosis into flocks. Other routes of transmission include litter. Literature on the epidemiology of avian mycoplasmosis in backyard chickens in Africa is scanty, with few reports in Morocco (Fagrach *et al*., 2023) Mozambique (Messa Junior *et al*., 2017) and Ethiopia (Jibril *et al*., 2018). To our knowledge, there are no published reports of this disease in either commercial or backyard chickens in Ilorin, Nigeria. The objective of this study was to assess the presence of avian mycoplasmosis in backyard (local) chickens in Ilorin, Nigeria using serological approach.

## MATERIALS AND METHODS

### SAMPLE AREA

This study was carried out in Ilorin, Kwara State located in latitude 8.9669° N and longitude 4.3874° E. The Mycoplasma diagnosis and research were done at the Department of Veterinary Microbiology, Faculty of Veterinary Medicine, and University of Ilorin. Kwara state. This facility isknown for keeping and rearing of indigenous free-range chicken for sale and for slaughter.

Two markets where indigenous chickens are slaughtered on a large scale were selected. Ojatuntun (New Market) Ilorin (Ilorin West) and Ipata (Old abattoir) market (Ilorin East) were selected for the study. Bothe are located in Ilorin metropolis, North Central Nigeria. Ojatintun is the major abattoir that receives the highest number of local birds in Kwara state for sale and/or slaughter, while Ipata market was chosen as it is next in slaughter size. Birds usually originate from villages within the state and neighbouring states.

### COLLECTION OF SAMPLES

A total of 400 blood samples were collected, 200 samples from each market. Sample size was ascertained using the formula described by Iloh *et al*. (2011), N=z2pq/d2 Where, N=sample size, z=1.96 confidence interval, p=prevalence. With the average prevalence unknown in the selected location p (prevalence) was set at 50%. Using this, the sample size was set at 541. A total of 541 blood samples were collected from free range chickens (501 samples) and wild birds (40 samples) across the three states. In Ilorin, 369 samples were collected. The capture of the wild birds, caging using poultry feeds as baits was used, as the birds are normally seen pecking around the poultry sites. About 1-1.5 ml of blood was collected from wing vein of the birds using a sterile syringe for each bird. The collected blood samples were kept at room temperature for about 1-2 h and then centrifuged at 1500 rpm for 10 min and collected serum were stored at −20°C prior to use as described by Uddinand team (Uddin *et al*., 2016).

### SEROLOGICAL IDENTIFICATION OF MYCOPLASMA SPECIES

Sera samples obtained were screened for Mycoplasma antibodies using rapid Mycoplasma field agglutination plate test supplied by the Office International des Epizooties (OIE) reference laboratory, Pendik, in Istanbul, Turkey. Undiluted sera were heat-inactivated at 560C for 30 minutes on a water bath before testing the samples. Positive samples were re-tested at various dilutions and any sample positive at a 1:4 dilution or higher were regarded as positive.

For RSAT, the 33 sera samples shipped from Nigeria into the UK were screened for presence of antibodies against M. gallisepticum, M. synoviae and M meleagridisby by RSAT (table v) and ELISA (tablev) test using commercially available kit (IDEXX Laboratories, Inc., Maine, USA). The assays were performed and analysed following manufacturer's instructions. The absorbance was measured at the wavelength of 650 nm using µQuant Microplate reader (Biotech Instruments Inc.). From the absorbance, sample to positive (S/P) ratio and titer were calculated for each sample. Serum samples, with S/P ratio greater than 0.5 or titer greater than 1,076 were considered as positive.

For PCR, using qPCR small aliquot of the 6 samples was tested directly or in indirectly after enrichment in accordance to Eatons' SOP. The broth culture was incubated till mild colour change from red to orange or till 5th day. DNA isolation was carried out from 2 ml of broth culture by phenol chloroform isoamyl alcohol method. DNA concentration was estimated using Genovanano spectrophotometer and approximately 10 ng DNA was used for qPCR test. DNA samples were screened by qPCR for M. gallisepticum using Mg-14F and Mg-13R primers (OIE, 2008) and for M. synoviae using primer pair Ms link-F and Ms cons-R (Jeffery *et al*., 2007). For M. gallisepticum AG lot, 6X151215, M. synoviae AG lot, 6S170427, M. meleagridis AG lot6Z120709. The DNA isolated from commercial attenuated

Mg 6/85 vaccine and M. synoviae isolate MSG510 available at Avian Health Laboratory, Directorate of Poultry Research, Hyderabad, Telangana were used as positive controls for M. gallisepticum and M. synoviae, respectively.

## DATA ANALYSIS

The data collected were analyzed using the chi-square ($\chi^2$) test to evaluate the association between categorical variables. This statistical method was chosen due to its suitability for assessing relationships and independence in frequency data. The chi-square test was performed at a significance level of 0.05, and the results were interpreted to determine the statistical significance of observed differences or associations.

## RESULTS

The detection of *Mycoplasma gallisepticum* and *Mycoplasma synoviae* antibodies using rapid serum agglutinationrevealed 293 of the 500 (58.6%) serum samples were positive to Mg, 443 of the 500 (88.6%) (table iii) serum samples were positive to Ms and 268 of the 500(53.6%) serum samples were positive to both Mg and Ms respectively (Tables I and table IV). The Seroprevalence based on LGAs were as follows; in Ilorin East, 160 of the 250 (64%) serum samples were positive to Mg, 211 of the 250 (84.4%) serum samples were positive to Ms and 140 of the 250 (56%) serum samples were positive to both Mg and Ms. The result of Ilorin West divulged that 133 of the 250 (53.2%) serum samples were positive to Mg, 232 of the 250 (92.8%) serum samples were positive to Ms and 128 of the 250 (51.2%) serum samples were positive to both Mg and Ms. (table i).

There was a significant association (P< 0.05) between seroprevalence of Mg and the LGAs sampled (table ii). The seroprevalence of Ms in the sampled native chickens was found to be higher than Mg and the association was statistically significant (P< 0.05) (table iii). However, exposure to both Mg and Ms prevalence showed that there was no significant association (P>0.05) between the local chickens and the sampled area (table iv and table v).

The PCR bands of the control and test samples are shown in figure I. The bands show the presence of DNA.

**Table II. Chi-square analysis of Mg in indigenous chickens in Ilorin metropolis using RSAT**

| Location | No of sample tested | Seropositive | Seronegative | $\chi 2$ | p-value |
|---|---|---|---|---|---|
| Ilorin East | 250 | 160 | 90 | | |
| Ilorin west | 250 | 133 | 117 | | |
| Total | 500 | 293 | 207 | 6.0 | 0.014* |

**Table III. Chi-square analysis of Ms in local chickens in Ilorin metropolis using RSAT**

| Location | No of sample tested | Seropositive | Seronegative | $\chi 2$ | p-value |
|---|---|---|---|---|---|
| Ilorin East | 250 | 211 | 39 | | |
| Ilorin west | 250 | 232 | 18 | | |
| Total | 500 | 443 | 57 | 8.74 | 0.003* |

**Table IV**. Chi-square analysis of Mg and Ms in local chickens in Ilorin metropolis using RAT

Not significant at p<0.05

| Location | No of sample tested | Seropositive | Seronegative | $\chi 2$ | p-value |
|---|---|---|---|---|---|
| Ilorin East | 250 | 140 | 15 | | |
| Ilorin west | 250 | 128 | 16 | | |
| Total | 500 | 268 | 31 | 0.17 | 0.680* |

**Table I. Seroprevalence of Mg and Ms in indigenous chickens slaughtered in Ilorin metropolis**

| Location | No of sample tested | No of Mg (+) | No of Ms (+) | No of Mg& Ms (+) | No of Mg (-) | No of Ms (-) | No of Mg & Ms (-) | Prevalence (%) | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Mg | Ms | Mg&Ms |
| Ilorin East | 250 | 160 | 211 | 140 | 90 | 39 | 15 | 64 | 84.4 | 56 |
| Ilorin west | 250 | 133 | 232 | 128 | 117 | 18 | 16 | 53.2 | 92.8 | 51.2 |
| Total | 500 | 293 | 443 | 268 | 207 | 57 | 31 | 58.6 | 88.6 | 53.6 |

**Table V. Rapid Serum Agglutination Test (RSAT) and Enzyme Linked Immunosorbent Assay (ELISA)**

| Sample | Rapid Serum Agglutination Test (RSAT) | | | Enzyme Linked Immunosorbent Assay (ELISA) | | |
|---|---|---|---|---|---|---|
| | **Mg** | **Ms** | **MM** | **Mg** | **Ms** | **MM** |
| AS1 | 1 | 0 | 0 | 0 | 1 | 1 |
| AS2 | 1 | 0 | 0 | 1 | 0 | 0 |
| AS3 | 0 | 0 | 0 | 0 | 0 | 0 |
| AS4 | 0 | 1 | 0 | 0 | 1 | 0 |
| **AS5** | 1 | 1 | 0 | 1 | 1 | 0 |
| AS6 | 1 | 1 | 0 | 0 | 0 | 0 |
| AS7 | 0 | 0 | 0 | 0 | 1 | 0 |
| AS8 | 1 | 0 | 0 | 0 | 0 | 0 |
| AS9 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS10 | 1 | 0 | 0 | 1 | 0 | 0 |
| AS11 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS12 | 1 | 0 | 0 | 1 | 0 | 0 |
| AS13 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS14 | 0 | 0 | 0 | 0 | 0 | 0 |
| AS15 | 0 | 0 | 0 | 1 | 1 | 0 |
| AS16 | 1 | 0 | 0 | 1 | 1 | 0 |
| AS17 | 1 | 0 | 0 | 1 | 1 | 0 |
| AS18 | 1 | 0 | 0 | 1 | 1 | 0 |
| AS19 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS20 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS21 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS22 | 1 | 0 | 0 | 1 | 0 | 0 |
| AS23 | 1 | 1 | 1 | 0 | 1 | 0 |
| AS24 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS25 | 1 | 0 | 0 | 0 | 0 | 0 |
| AS26 | 0 | 1 | 0 | 1 | 1 | 0 |
| AS27 | 0 | 1 | 0 | 1 | 1 | 0 |
| AS28 | 1 | 1 | 0 | 1 | 1 | 0 |
| AS29 | 1 | 0 | 0 | 0 | 1 | 0 |
| AS30 | 0 | 0 | 0 | 0 | 0 | 0 |
| AS31 | 0 | 0 | 0 | 0 | 0 | 0 |
| AS32 | 1 | 1 | 0 | 1 | 0 | 1 |
| AS33 | 1 | 1 | 0 | 1 | 1 | 0 |
| **Total** | **24** | **16** | **1** | **21** | **21** | **2** |

**Keys: Positive=1; Negative= 0**

**Controls:Rapid Serum Agglutination Test (RSAT)**

- *M.gallisepticum* AG lot 6X151215
- *M.synoviae* AG lot 6S170427
- *M.meleagridis* AG lot 6Z120709

**Enzyme Linked Immunosorbent Assay (ELISA)**

- BioChekMg, MS and MM ELISA

## DISCUSSION

This study observed an overall prevalence of 26.7% for *M. gallisepticum* and 75.3% for *M. synoviae* among free-range chickens. This confirms the endemicity of avian mycoplasma infections within the states surveyed in this study. *M. gallisepticum* and *M. synoviae* are the only two aetiological agents of avian mycoplasma listed by the World Organization for Animal Health (OIE 2007). The enormous economic and veterinary importance of these pathogens indicates the need for concern, intervention and control of the transmission, infection and endemicity of the avian mycoplasma within the study area as recommended previously (Hassan *et al*., 2014, Abbas *et al*., 2018). Abbas *et al*., (2018), reported a similar prevalence (24.8%) of infection for both infections in Pakistan, while Abbas *et al*. (2018) also observed a markedly high prevalence of 46.6% among poultry birds. However, their report on the prevalence of infection among wild birds (27%) is low, compared to the findings of this study. Further low prevalence of avian mycoplasma infection (1.7%), was reported by Michiels *et al.* (2016) among wild birds in Belgium. However, a study from Mozambique, reported prevalence of 48.8% and 84.5% for *M. gallisepticum* and *M. Synoviae*, respectively. The lower prevalence in countries like Belgium and Pakistan could be as a result of the judicious vaccination, appropriate treatment and proper handling of livestock, which has helped to reduce vertical transmission. This is different from what is obtainable in developing countries like Nigeria, where there are limitations to routine vaccination and treatment of birds. Exposure of poultry birds to unvaccinated birds or insufficient spacing and/or isolation, is another factor that is very common in developing countries, which predisposes chickens and other birds to avian mycoplasma infection.

Results from this study showed the absence of *M. gallisepticum* among the wild birds examined. Lack of detection of antibodies to *M. gallisepticum* among wild birds in this study contrasts the findings of Abbas *et al*. (2018) that reported seropositivity among wild birds in Belgium and Pakistan. Sawicka *et al*. (2020) however opined that the prevalence of infection among wild birds is determined by the type of assay used in determining seropositivity, and as such, certain detection methods are more sensitive than others. This may be the case for our study, as we concentrated on sera samples that were positive at a 1:4 dilution rate or higher. Also, another factor that may be responsible for the absence of infection in wild birds could be lack of interaction or absence of cross-transmission between infected birds and the wild birds. The disparity in ecological niche and adaptive environment may have created the gap of interaction between such birds in the study area, thus, minimizing the likelihood of infection.

There are significant implications of *Mycoplasma* in birds. Its presence in culture indicates the ability of the organism to propagate itself intracellularly (Ahmed *et al*., 2015). The ability of *Mycoplasma* to persist as an intracellular parasite, is a major cause of avian mycoplasma diseases. Their persistence in cells is a major protective mechanism used by *Mycoplasma* to evade the immune systems of animals, while having easy access to required nutrient.

The overall seroprevalence of Mg alone in this study was 58.6%. It indicates that Mg is endemic in native chickens in Ilorin. This investigation strongly supports the studies of Mera U. M. and Haruna M. (2005) who recorded 59% of Mg prevalence in local chickens in Sokoto state, Nigeria. The current finding of Mg seroprevalence in local chickens is also in close agreement with the study of Chrysostome *et al.* (1995) with a prevalence of 62% in local chickens in Benin. It corroborates with Sarkar *et al.*, (2005) who reported a prevalence of 58.9% in Bangladesh. However, the Mg prevalence in this study was higher compared to what was obtained by Feizi *et al.*,(2013) who reported a lower Mg prevalence of 33.3% in northwest of Iran and lower than what was observed by Bakren *et al.*, (2021) who recorded 74.3% of Mg seroprevalence in Ibadan.

This study established that avian mycoplasmosis due to *Mycoplasma synoviae* and *Mycoplasma meleagridis*, is prevalent among indigenous chickens within Ilorin metropolis. The prevalence can be credited to scavenging style of feeding coupled with nutritionseral deficiency, lack of vaccination, absence of Mycoplasma control in local poultry production system and gathering of indigenous chickens at different marketplaces may increase the risk of exposure to *Mycoplasma gallisepticum* and *Mycoplasma synoviae*. The research also revealed that there was a significant association

between Mg infection, Ms infection and the LGAs sampled. However, there was no significant association between the local chickens that were infected with both Mg and Ms at a time or at one or the other of their existence and the study area.

Thus far, BioChek *Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Mycoplasma mealiagridis* ELISA revealed a specificity ratio of 0.5 positive. Charles Rivers RASt for

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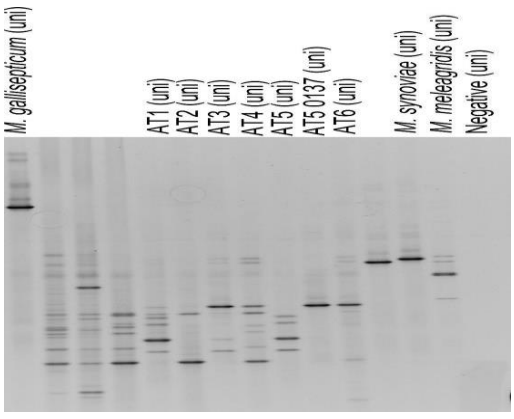


**Figure I. PCR result with bands**

*Mycoplasma gallisepticum*, *Mycoplasma synoviae* and *Mycoplasma mealiagridis* (batches given) were used with associated positive control serum showing 3+ reactions for the correct antigen, with no reaction for the negative sera. The majority of the samples tested gave reaction to *M. gallisepticum* and *M. synoviae*. Known cross reactivity between *M. gallisepticum* and *M. synoviae* via RASt were observed. Direct PCR, PCR-DGGE- the APHA domiciled technique, and streamlined real-time PCR molecular assays is the next phase to conclude this study.

## CONCLUSION

On the basis of the findings and conclusion of this research, the following are recommended: (i) rapid serum agglutination test should only be employed as a screening tool to diagnose avian mycoplasmosis; positive results should be confirmed by culture and isolation or molecular characterisation, (ii) although the indigenous chickens appeared apparently healthy but they could serve as a source of infection for commercial chickens. This calls for a drastic *Mycoplasma* control policy in order to eradicate or minimize *Mycoplasma* infection to the barest minimum in the local poultry production system, (iii) this seroprevalence investigation revealed that *Mycoplasma gallisepticum* and *Mycoplasma synoviae* are widely spread in native chickens but losses due to *Mycoplasma gallisepticum* and *Mycoplasma synoviae* were not evaluated. Hence, further research should assess the consequence of Mg and Ms infection in local poultry industry so that one of aims of local poultry production system (alleviation of poverty) is not defeated. (iv) Strict biosecurity measures should be enhanced in commercial layer farms that are located in a village where large population of local chickens are raised, (v) a nationwide, comprehensive research on the prevalence and characteristics of Mg and Mg should be carried out to ascertain the contemporary status of the diseases in Nigeria, and (vi) a streamlined real-time PCR molecular assays is highly recommended for the next phase to conclude this study.

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## CONFLICTS OF INTEREST

The authors declare no any conflict of interest.

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