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Prevalence of Mycoplasma gallisepticum in Commercial Chickens and Free Flying Birds

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Abstract: *Chronic respiratory disease (CRD) and complicated chronic respiratory disease (CCRD) are caused by Mycoplasma gallisepticum (MG). Infected birds show respiratory and reproductive problems which lead to severe production losses in poultry industry. Mycoplasma gallisepticum (MG) has been isolated in chickens and free flying birds (FFB) in various parts of the world. Therefore the current study was to determine the prevalence of MG infection and also to isolate MG from commercial chickens (broilers, layers and indigenous chicken) and FFB in Selangor. Broiler chickens and FFB at farms showed a high prevalence of MG infection based on serology and DNA detection; however isolation of MG by culture was unsuccessful. Crows from non-farm area did not show any evidence of MG infection. However, birds which are not in close contact with infected birds or farms did not show any evidence of MG infection. This study also shows that only clinically ill infected birds, excreted and spread the organism to other flock or species, as observed in the crows from the infected farm. However, sub-clinically infected birds as in indigenous chickens did not transmit the organism to other chickens or birds. All commercial birds and FFB in farms in this study showed a high evidence of MG infection.*

Keywords: *Mycoplasma gallisepticum, prevalence, free flying birds*

I. Introduction

In Malaysia, poultry industry is the biggest livestock industry. It consists of breeder farms, layers farms and broiler farms. Most of the farms in Malaysia operate under the open house system, which is labour intensive. Breeder and layer farms are slowly converting to the closed house system. Recent studies revealed that there is a high prevalence rate of *Mycoplasma gallisepticum* (MG) infection in poultry farms in Malaysia (Ganapathy et al., 2001; Mutalib et al., 2001). MG is the etiology for chronic respiratory disease (CRD) and complicated chronic respiratory disease (CCRD). These diseases are among the major causes of respiratory and reproductive problems in chickens (Winner et al., 2003; Jordan, 19989; 1990; Yoder, 1991). Infected birds show clinical signs of upper respiratory disease such as sinusitis, coughing, respiratory rales and nasal discharge (Mikaelin et al., 2001). The degree of severity of clinical signs varies between these diseases. Birds with CCRD show more severe clinical signs than birds with CRD. Furthermore, CCRD cause a high mortality, whereas CRD cause a low mortality. Regardless of the types of infection, infected chickens show a reduction in production performance and thus cause considerable economic losses to the poultry industry (Winner et al., 2003; Burhnam et al., 2003; Parker et al., 2003).

MG has also been detected in turkeys, ducks, peacocks, house finches, crows and other free flying birds (Mukarami et al., 2002; Peterson et al., 2002; Mikaelin et al., 2001; Hartup et al., 2000; Connor et al., 1999; Ley et al., 1997; Jessup et al., 1983). In the open house system, birds such as crows, pigeons, swallows and finches, are found in commercial farms premises. A close contact directly or indirectly between these birds and the commercial chickens could not be prevented. Thus both species may play a role as a biological vector for MG. Some species of birds infected with MG, showed similar clinical signs as chickens. However, some birds did not exhibit any clinical sign (Mukarami et al., 2002; Mikaelian et al., 2001; Bozeman et al., 1984; Welchman et al., 2002; Fatunmbi, 1984). Neurological signs were reported in turkey (Chin et al., 1991; Bencina, 2002). There have been no studies on MG infection in free flying birds in poultry farms in Malaysia. Therefore, a study on MG prevalence rate in commercial birds (layer and broiler farms), multiflock farm and free flying birds in the farms were carried out. The isolation of MG from various birds is crucial for future molecular and pathogenicity.

II. Materials and Methods

Study Population

One thousand three hundreds (1,300) birds were sampled from six poultry commercial farms (3 layer and 3 broiler farms), one multiflock backyard farm and FFB in the state of Selangor. The farms were randomly selected within 200 km radius to the Universiti Putra Malaysia campus. Choanal swabs and blood were taken from these birds.

Categories of Birds

Three layer farms (A, B and C) and three broiler farms (E, F and G) were sampled. At each sampling, 170 swabs and 170 blood samples were taken. The serum was harvested from the blood samples in 1.5 ml centrifuge tube after being centrifuged at 3,000 rpm for one minute. The layer farms A, B and C practiced open house system. They were located in different districts more than 200 km radius apart. Farm A has a proper management system with strict biosecurity, scheduled medication and vaccination programme. Vaccination of MG F strain intra-muscularly was given at 12 weeks of age. No history of outbreaks of severe disease was recorded. A few birds showed mild respiratory problem. Farm B was poorly managed and it had a history of high mortality and loss of production. During sampling, the birds showed moderate respiratory problems and conjunctivitis with a mortality estimated at 7%. Farm C was also a poorly managed and it had a history of high mortality and loss of production. During sampling, the birds showed mild respiratory problem, and slight conjunctivitis, were undersized and a mortality of about 15%. The broiler farms D, E and F were operating an all-in-all-out system. They were located in different districts more than 200 km radius apart. These farms were small and had a population of less than 25,000 birds each. They were poorly managed and had a history of high mortality. Multi-flock backyard farm was located away from commercial chicken farms. It consisted of indigenous chickens, ducks, turkeys, guinea fowls and peacocks, reared under the same shed. The total population was about 400 birds. Commercial formulated feed and mixed raw feed materials such as corn and maize were given to the birds. Scheduled administration of antibiotics and vitamin were noted in this farm. Birds were apparently healthy at the time of sampling.Two groups of FFB sampled based on their habitats, namely within farm areas (Farm A, B, C, D, E and F) and out-side farm areas (Subang Jaya, Puchong and Serdang). Choanal swabs and blood samples were taken from these birds. The FFB sampled from within farms were sparrows, crows, swallows, finches and pigeons. They were caught by mass net laid around the houses and in between the houses. FFB sampled from outside the farm areas at Subang Jaya, Puchong and Serdang were crows. These crows were shot down by the authorized personnel. Other species of birds were not sampled because permission from the authority could not be obtained.

Analysis of Samples

Blood samples were tested for IgM antibody using the rapid serum agglutination test (RSAT) and the swabs were used for isolation using mycoplasma media inoculation. Polymerase chain reaction (PCR) test was used for detection of MG DNA in the swabs.Rapid Serum Agglutination Test (RSAT) - Fifteen microlitre (µl) serum and 15 µl commercial MG antigen (Intervet ® USA) was mixed on a white tile and slowly rotated for two minutes. The degree of agglutination indicated the degree or level of antibody present in the serum/blood of the chicken according to the score recommended by manufacturer.Culturing by Mycoplasma media (agar and broth) **-** Choanal swabs were plated on mycoplasma agar and later dipped in mycoplasma broth. Mycoplasma plates were kept in a humidified candled jar and later incubated at 37 °C for 21 days. Mycoplasma plates were observed daily by stereomicroscope.Mycoplasma, which grew after 3 days were picked up and re-cultured on a new mycoplasma agar. Cultured mycoplasma broths in 1 ml vials were incubated and observed for changes of colours for a period of 7 days. Broth with cloudy appearance was removed, as contamination has taken place or overgrows with other organisms. Broth with clear orange colour was re-cultured on mycoplasma agar as described above. Confirmation by indirect immuno-fluorescent antibody (IFA) test - The purity and identity of each isolate was confirmed using the method of Rosendal and Black (1972). Mycoplasma cultures were plated onto mycoplasma agar and incubated for three to four days until small discreet colonies appeared. Areas of the agar plate with suitable colonies were first cut into rectangular blocks of approximately 1.0-0.5 cm. The lower right corner was cut off to ensure that the block was placed in the correct orientation, i.e., colony side up. Known positive and negative cultures were included in each IFA test. The blocks were placed on labeled microscope slides and 20 µl of 1:40 dilution of the appropriate rabbit antiserum to mycoplasma species was added. As a further control, a 1:40 dilution of normal rabbit serum was added to duplicate set of blocks. These blocks were then placed in a humid chamber and incubated for 30 minutes at room temperature. Humid chambers consisted of inverted boxes containing a level sponge pre-soaked in warm water. The blocks were then washed by placing each block into an individually labeled test tube containing 10 ml of PBS (pH 7.4), and were placed in a rotating mixer for 10 minutes. The PBS was drained off, replenished and the blocks were washed for another 10 minutes. The blocks were then returned to their original position on the slide and 20 µl of

fluorescent conjugated anti-rabbit IgG (H+L) at an appropriate dilution (1:20-1:100, depending on the batch) was added to each block. The blocks were incubated in the humid chamber for 30 minutes and then washed twice, as above. The blocks were relocated to their original position on the slides and examined under incident UV light (epi-illumination) at 100X magnifications using a fluorescence microscope.

Polymerase Chain Reaction (PCR) - Each swab sample was suspended in 200 ul sterile mycoplasma broth in a micro-centrifuge tube. These swabs were vigorously shaken and the fluid from the swabs was squeezed by twisting on the inside of the tubes above the water lines. The suspension was boiled for 10 minutes and then frozen at –20° C for 10 minutes. Two micro-litres (µl) of RNAse was added to each tube. The samples were incubated for 30 minutes at 37°C and centrifuged at 10,000 rpm for 5 minutes. The supernatant was kept frozen at –20 °C until further test was carried out. PCR amplification of mycoplasma DNA was performed as described by Marois (2000) with modification. Mycoplasma DNA was amplified in the PCR mixture containing PCR buffer (10mM Tris-HCl, 50mM KCl, 2.5Mm $MgCl_2$, pH 8.3), a 500 µM concentration of each deoxyribonucleoside triphosphate, 20Pm of each primer MG-14F (5’ GAG CTA ATC TGT AAA GTT GGT 3’) and MG-13R (5’ GCT TCC CCG CGG TTA GCA AC 3’), 1 unit of Taq DNA polymerase and 10 ml of DNA template. The reference strain of MG S6 (provided by University of Liverpool, UK) was used as the positive control, while the distilled water was used as the negative control. The reaction procedure consisted of an initial denatured step at 90°C for 1 minute, followed by 40 cycles of denaturation at 95°C for 15sec, primer annealing at 58°C for 20 seconds and extension at 75°C for 20 sec. The final extension at 75°C for 5 minutes. The amplified products were separated in a 2% agarose gel in the TBE buffer (90mM Tris, 90mM borate, 2.5 mM EDTA, pH 8.0) for 1 hour at a constant voltage of 110 V. The gel was stained in ethidium bromide solution (1 µg/ml) for 20 minutes. Amplified products were visualized and photographed under UV transillumination.

Data Analysis

Differences in the prevalence and isolation of MG detection between farms, or birds categories were evaluated using the Chi-square. Due to the small sample size obtained from FFB and multiflock birds, these groups were not statistically analyzed.

III. Results

Farm A and the multi-flock backyard farm had a good management system whereas the others were poorly managed. Layer farms A, B and C showed 48.82%, 31.76% and 24.70% detection of MG DNA respectively (Table 1). In Farm A, 73.52% of the birds had antibody against MG, followed by Farm C with 43.52% and farm B with 13.53%. Broiler farms D, E and F showed 68.18%, 59.40% and 62.73% detection of MG DNA respectively. Only in Farm D had 40.9% antibody against MG whereas birds in the other two farms birds did not show the presence of antibody against MG. No isolates were obtained from swab samples from these broiler farms where PCR showed positive of MG.There was a significant difference in the rate of MG detection (DNA detection and serology) ($P<0.05$) among the layer farms, whereas among broiler farms, there was significant difference in serology parameter as shown in Table 1. Between the layer farms and broiler farms, there was a significant difference ($P<0.05$) for both parameters (DNA detection and serology). In multi-flock backyard farm, 23.3% of indigenous chickens positive for MG DNA detection and 3.3% showed the presence of antibody against MG. Other in-mate birds, turkeys, ducks and guinea fowls were not positive for both parameters (serology and DNA detection) (Table 1).

Table 1: Isolation of MG organism on mycoplasma media, Percentage of MG antibody and Percentage of MG DNA detection using PCR

Type of birds	No. of samples	% MG DNA detected	% MG antibody	No. MG isolated by culture
LAYER FARMS				
Layer A	170	48.82	73.52	1
Layer B	170	31.76	13.53	10
Layer C	170	24.70	43.52	11
Total	**510**	**35.10**	**43.50**	**22**
BROILER FARMS				
Broiler D	170	68.18	40.91	0
Broiler E	170	59.40	0	0
Broiler F	170	62.73	0	0
TOTAL	**510**	**63.50**	**63.50**	**0**
MULTI FLOCK FARM				
Indigenous chickens	30	23.30	3.30	5
Turkeys	30	0	0	0
Ducks	8	0	0	0
Guinea fowls	8	0	0	0

Layer Farms A, B and C: X^2= 22.95, P=0.0000: Broiler Farms D, E and F: X^2= 2.89, P=0.2352 X^2= 82.47, P = 0.0000; X^2=11.2, P = 0.0000

All the birds captured in the farms showed the presence of MG DNA except for finches. Antibody against MG was negative for finches. Other birds in the farms (sparrows, pigeons and swallows) were serology negative too, whereas 40% of the farm crows had antibody against MG. There was no antibody and MG DNA detected in crows caught in residential areas (Table 2).

Table 2: Isolation of MG organism by mycoplasma media and percentage of MG DNA detection using PCR

Type of birds	No. of samples	% MG DNA detected	% MG antibody	No. MG isolated by culture
FREE FLYING BIRDS IN FARMS				
Sparrows	108	16.67	0.0	0
Finches	34	0.00	0.0	0
Crows	5	100.00	40.0	0
Swallows	1	100.00	0.0	0
Pigeons	1	100.00	0.0	0
TOTAL	**149**	**15.00**	**1.3**	**0**
FREE FLYING BIRDS IN RESIDENTIAL AREAS				
Crows	244	0.00	0.0	0

One MG isolate was obtained out of 170 samples from farm A, 10 MG isolates from farm B and 11 MG isolates from farm C. Five MG isolates were obtained from the indigenous chickens reared in a multiflock backyard farm. MG was not isolated from other species of birds even though they had evidence of MG infection based on DNA detection or serology. In general, broilers showed the highest percentage of MG DNA detection (64%), followed by layers (35.1%), free flying birds (15.4%) and multi-flock backyard farm (9%).

IV. Discussion

The prevalence of MG seemed to be significantly higher in the commercial chicken and FFB at the farm areas. Layer and broiler farms showed more than 30% MG infection. Mutalib et al., (2001) and Ganapathy et al., (2001) reported similar findings in commercial birds in Malaysia. However, FFB in urban areas did not show any evidence of MG infection on DNA detection and serology. In multi-flock backyard farm, indigenous chickens showed evidence of MG infection, whereas other in-mates were negative for all parameters. In this study, three different diagnostic tools for detection of MG infection were used. The culture method showed a lower percentage of detection of MG compared to PCR. An earlier study showed that isolation on artificial media gave a low success rate (Fritz et al., 1991). However, isolation on artificial media is important for further studies such as pathogenicity, molecular characterization and antibiotic sensitivity tests. Although MG could be isolated from various sites of the host, choanal and tracheal sites were commonly selected as swab sites by researchers. Other studies showed that choanal swabs had five-time higher chance of isolating MG than tracheal swabs (Tiong, 1978; Simon et al., 1989; Zaini and Badbury, 1986; 1987; Barnton et al., 1984). Choanal site has also been proven a better site for MG isolation, with less contamination compared to tracheal site in live birds (Barnton et al., 1984).

MG is a fastidious organism requiring a long incubation period. Survivability of this organism in the swabs during transportation from the farm to the laboratory is crucial. Various methods were taken to assure the success of isolating this organism. Studies showed that the type of swabs and condition of sampled swabs during transportation influence the recovery of the organism (Zaini and Bradbury, 1995:1996; Branton et al., 1991). Furthermore, swab dipped in mycoplasma broth before sampling had a better isolation results compared to dry swabs, although the material swabbed might not show a significant difference. Sampled swabs should be stored in a cool environment during transportation (Zaini and Bradbury, 1995:1996) to ensure survivability of the organism. In this study, fresh yeast extract was used in the preparation of mycoplasma agar media. Fresh yeast extract provides several amino acids required for the growth of MG. This may have contributed to the success of isolation of MG in this study. Preliminary study showed that by using commercial yeast extract as a replacement of fresh yeast extract, it did not provide a suitable environment for the survivability of field strain MG unless the MG has undergone several passages or another word, media adapted MG. In the present study, antibiotics (penicillin and thallium acetate) were omitted in the cultures during preparation to inhibit the growth of other bacterias such as Proteus spp., and Klebsiella spp.However, due to the high contamination of these bacterias and fungus, have hindered the growth of MG on the media. In addition to that, the presence of fast growing mycoplasmas such as Mycoplasma gallinarum, which grow in less than 2 days after incubation, has hindered late grower mycoplasma such as MG to grow on the culture. Despite using various techniques, the reisolation of MG from field samples was still low. A similar study showed that culture gave a low recovery of about 8.4%, although serologically, it showed clearly MG infection in the population (Firzt et al., 1991; Bradbury et al., 2001: Yagihashi and Tajima, 1986). A similar epidemiological study on Mycoplasma synoviae infection showed that infected birds positive on serology, were negative on culture (Ricardo et al., 1996). Other factors also contributed to the lowering of effectiveness of culturing method in his study. Swabbing chickens at their feeding

time contributed to high contamination. In crows, most of the cultures were highly contaminated due to contamination of the oral cavity. Choanal swabbing from dead crows with bloody oral cavity gave a high contamination in cultures. Birds such as finches, ducks, guinea fowls, and sparrows had small, shallow and narrow choanal cleft. Swabbing was difficult as even the fine cotton swabs were bigger than the choanal cleft. Thus, these factors may have contributed towards the poor recovery of isolation by mycoplasma agar. Besides that, it required a longer incubation period. Thus, the conventional method had proven to be a poor tool for epidemiological study (Bradbury et al., 2001; Kempf, 1998; Khan et al., 1993).

PCR has shown its ability as an effective tool for epidemiological study. It is able to detect the presence of a minimal amount of MG DNA, even in the presence of other microorganisms. It also only requires 1-2 days to obtain the results. In this study, PCR has shown to have a higher percentage of recovery compared to conventional method (Bradbury et al., 2001; Kempf, 1998; Khan et al., 1993). PCR gave a better detection rate of MG compared with the conventional method, despite these contaminations.The broiler farms showed 1.8 times significantly ($P<0.05$) higher rate in MG DNA detection but produced 0.24 times lesser ($P<0.05$) MG antibody than the layer farms. There was a significant difference ($P<0.05$) among layer farms for both parameters. Factors such as farm management system might have contributed to the difference in prevalence rate of MG infection of the particular farms. In broiler farms D, E and F, MG vaccination was not given to the birds. Birds in farms E and F did not show any evidence of antibody against MG despite a high detection of MG by PCR. These birds might have had immunosuppressive diseases such as infectious bursal disease (IBD), which suppressed the production of antibody. Depletion of bursal cells as a result of possible IBD causes impact to the production of antibody (Murkerje et al., 1990). This may explain the negative detection by serology despite of high detection of MG DNA by PCR. In layer farms A, B and C, the commercial birds showed a high antibody titer against MG by RSAT. MG vaccination using MG F strain was given once to the birds in farm A at age 12 weeks with MG F strain. Antibody level arises due to a field infection or vaccination (Lam et al., 1986; Yagihashi and Tajima, 1986). Most birds in farms A, B and C showed a high agglutination reaction against MG antibody. Farms B and C did not practise any vaccination against MG. The presence of antibody in farms B and C were therefore probably due to MG field infection whereas in farm A it was most probably due to field strain or/and vaccine strain MG F.

Birds with antibodies against MG were shown to harbor MG. Whithear (1990) reported that some birds with antibodies against MG were still susceptible to MG challenge. This is due to the ability of MG to change and vary its protein surface, enabling it to avoid the immune cells. Despite that, MG has the ability to hide in the host's non-phagocytic cells as an intra-cellular pathogen. These may contribute to the failure of eliminating MG despite the presence of antibody against MG (Winner et al., 2001). Only a single MG isolate was recovered by culture method from farm A (from a healthy chicken), even though a high detection rate using PCR was obtained. This single isolate could have originated from either field strain isolate or the vaccine strain MG F used in the farm. A previous field study showed that the vaccine strain MG F was detected in despite of discontinuation of vaccination for several years (Kleven et al., 1984). Several studies have revealed the competition among different strains of MG to dominate in host or in cycle in a farm (Turner and Kleven, 1998; Kleven et al., 1998). MG F field strain was reported to be replaced by vaccine strain ts-11 (Turner and Kleven, 1998) and that MG R strain was replaced by vaccine MG F strain (Kleven et al., 1998). Vaccine strains were able to suppress the field isolates after several vaccinations. As a result of this, only a particular strain would be circulating in a particular system. However, certain vaccine strains had failed to dominate the existing wild-strain in the field. It was reported that ts-11 and MG S6 strains were unable to suppress the MG R strain (Kleven et al., 1998).

Farm A, which was supervised by a veterinarian had a proper management system, proper housing system, strict bio-security measures, scheduled antibiotic prophylaxis and vaccination programmes. Proper performance records were practised in this farm. The quality of eggs and the performance of birds were good although they harbored MG organism, despite having a high MG antibody. Burnham et al., (2003) reported that a single infection of MG in the absence of other agents or environmental stressor did not cause a reduction in performance of infected birds. In the presence of external stress factors, severe reduction in poultry production was reported. Vaccination with MG F in MG infected hens at 12 weeks of age had reduced the loss of the production, as reproductive tract of the infected host was not altered (Noormohammadi et al., 2002). Improper operating system with poor bio-security system and farming skills were common in the layer farms (B and C) and broiler farms (D, E and F). Chickens in farm C showed moderate respiratory problems such as watery eyes, nasal discharge and slightly high mortality, estimated at 15%. In farm B, respiratory rales and watery eyes were common in the birds, yet mortality was low (estimated at 7%). Due to unavailable data on egg production from these farms, performance of egg production on these farms B and C could not be evaluated. However, observation on the quality of eggs such as poor shell quality, soft shell etc, may suggest that the reproductive system may have been affected (Burhnam et al., 2002). The broiler farms E and F had a history of an outbreak of immune-suppressed disease suspected to be infectious bursal disease (IBD). The presence of IBD or other

predisposing factors, may have inhibited the production of antibody, resulting in these birds losing their ability to produce specific antibody. Severe respiratory problems such as watery eyes and respiratory rales were seen in these farms, with a high mortality estimated at 20%.The multiflock backyard farm had several species of birds and they were reared under free-range system, thus they had direct contact with each other. MG organisms were detected from healthy indigenous chicken by PCR and serology test. Five isolates were obtained from indigenous chickens. These birds did not exhibit any clinical sign of chronic respiratory disease (CRD) or complicated chronic respiratory disease (CCRD). In Botswana, indigenous chickens showed high evidence of MG antibody by RSAT yet were apparently healthy (Mushi et al., 2003). An earlier similar study showed that chickens, which were clinically ill, transmitted the organism to their fellow mates, such as ducks, geese and peafowls (Bencina et al., 1988). Bencina (1991) reported that transmission of MG infection occur between different species in a multi-flock farm too. The study suggested that MG has failed to be transmitted to other species from these indigenous chickens. This may indicate that healthy birds that harbor MG organism are unable to transmit the organism to the environment, other flock or other species.

All FFB from the infected farms showed the presence of MG except the finches. This might be due to the anatomical structure of the choanal cleft in finches, which were narrow, small and shallow, which might have reduced or prevented the recovery of MG organism. Crows captured from farms B and C had the evidence of MG infection, whereas in the residential area in town, crows showed no evidence of MG infection either by serology or by DNA detection. Crows are intelligent birds. This allowed them to escape from capture by mast net laid in the farms. Hence, only five crows were captured from the two farms. In the farms, crows consumed eggs and carcasses of chickens. MG infected carcasses or eggs may have probably transmitted the infection to these birds. Transmission to other species of FFB may have occurred after several episodes of direct contact with the infected crows, which shared the same habitats. Transmission of infection among different species of free flying birds has been reported in USA (Peterson et al., 2002; Mikaelin et al., 2001; Hartup et al., 1999). Direct contact with exudates from infected chicken through feed or faeces may infect other species of FFB flying birds. Faeces were reported to shed MG too. Re-transmission of these organisms to the commercial chickens from the FFB was possible if close contact between these species of birds had occurred. Bencina (1997) reported control SPF-chickens were infected with MG, which was transmitted by an infected English sparrow. To ensure a high-quality product, diagrams and lettering MUST be either computer-drafted or drawn using India ink.

V. Conclusion

This study revealed high prevalence of MG infection in commercial birds and FFB in farms. Generally, MG infection is common in commercial birds and indigenous chickens, although some of these birds may not show any clinical signs of MG infection. Thus, type of management practiced may determine the severity of MG infection in a farm. FFB exposed to clinically ill commercial birds with MG infection may become infected directly or indirectly. Isolation of MG was achieved in this study.

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