# Comparative Evaluation of the Pathogenicity of *Mycoplasma gallinaceum* in Chickens

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SUMMARY. *Mycoplasma gallinaceum* is not among the most pathogenic mycoplasmas affecting poultry, but its continuous re-isolation from flocks in South Africa displaying typical signs of mycoplasmosis prompted us to revisit its role in respiratory disease. Specific-pathogen-free white leghorn chickens were co-challenged with either *M. gallinaceum* (MGC) and QX-like infectious bronchitis virus (IBV), or the more virulent *Mycoplasm gallisepticum* (MG) and IBV. No clinical signs were observed apart from sneezing in chickens challenged with IBV, MGC + IBV, and MG + IBV. On postmortem examination, one bird each in the MGC + IBV and IBV groups developed peritonitis or airsacculitis, respectively. In the tracheas, the MG + IBV group showed the most severe ciliary damage with a mean ciliostatic score of 32.40 compared to scores of 26.83 and 20.4 for the MGC + IBV and IBV groups, respectively. Corresponding tracheal lesions were recorded. Quantitation of the challenge pathogens by quantitative real-time PCR and real-time reverse transcriptase–PCR determined that MGC was shed in much higher titers from the trachea than MG, when co-infected with IBV. Interestingly, the presence of both MG and MGC appeared to enhance IBV replication in the tracheas of infected chickens, whereas the presence of IBV suppressed MG and MGC proliferation in the trachea. In general, the nonpathogenicity of *M. gallinaceum* in chickens was confirmed, but it was able to aggravate respiratory disease and pathogen proliferation with virulent QX-like IBV.

RESUMEN. Evaluación comparativa de la patogenicidad de *Mycoplasma gallinaceum* en pollos

*Mycoplasma gallinaceum* no se encuentra entre los micoplasmas más patógenos que afectan a la avicultura, pero su continuo aislamiento de las parvadas en Sudáfrica que muestran signos típicos de micoplasmosis condujo a revisar su papel en la enfermedad respiratoria. Pollos Leghorn blancos libres de patógenos específicos se co-desafiaron con *M. gallinaceum* (MGC) y el virus de la bronquitis infecciosa de tipo QX (IBV), o con *Mycoplasma gallisepticum* (MG) más virulento y el virus de la bronquitis infecciosa. No se observaron signos clínicos aparte de los estornudos en pollos desafiados con bronquitis infecciosa, *M. gallinaceum* + bronquitis infecciosa o con *M. gallisepticum* + bronquitis infecciosa. En el examen post mortem, un ave del grupo *M. gallinaceum* + bronquitis infecciosa y del grupo desafiado solo con bronquitis infecciosa desarrollaron peritonitis o aerosaculitis, respectivamente. En las tráqueas, el grupo *M. gallisepticum* + bronquitis infecciosa mostró el daño ciliar más grave con una puntuación media ciliostática de 32.40 en comparación con puntuaciones de 26.83 y de 20.4 para los grupos *M. gallinaceum* + bronquitis infecciosa y el inoculado solo con el virus de la bronquitis, respectivamente. Las lesiones traqueales correspondientes fueron registradas. La cuantificación de los patógenos de desafío mediante PCR cuantitativa en tiempo real y transcripción reversa y PCR en tiempo real determinó que el *M. gallinaceum* se eliminaba en títulos mucho más elevados de la tráquea en comparación con *M. gallisepticum*, cuando coinfectaba con bronquitis infecciosa. Curiosamente, la presencia tanto de *M. gallisepticum* como de *M. gallinaceum* pareció mejorar la replicación del virus de la bronquitis infecciosa en las tráqueas de los pollos infectados, mientras que la presencia del virus de la bronquitis infecciosa suprimió la proliferación de *M. gallisepticum* y *M. gallinaceum* en la tráquea. En general, se confirmó la no patogenicidad de *M. gallinaceum* en pollos, pero fue capaz de agravar la proliferación de patologías y enfermedades respiratorias con el virus de la bronquitis infecciosa virulento tipo QX.

Key words: *Mycoplasma gallisepticum*, *Mycoplasma gallinaceum*, chickens, infectious bronchitis virus, pathogenicity

Abbreviations: BI = bursal index; CFU = colony-forming units; dpc = days postchallenge; MGC = *Mycoplasma gallinaceum*; MG = *Mycoplasma gallisepticum*; IBV = infectious bronchitis virus; MS = *Mycoplasma synoviae*; NCC = nonchallenge control; PBS = phosphate-buffered saline; qPCR = quantitative real-time PCR; RRT-PCR = real-time reverse transcriptase–PCR; SPF = specific-pathogen-free

Avian mycoplasmosis in chickens and turkeys is caused by several pathogenic mycoplasmas that cause significant economic losses. Four avian mycoplasma species are recognized as being pathogenic to poultry, namely *Mycoplasma gallisepticum* (MG), *Mycoplasma synoviae* (MS), *Mycoplasma meleagridis*, and *Mycoplasma iowae*, with MG and MS listed by the World Organization for Animal Health as the two most important species (7,14,19). *Mycoplasma gallinarum*, *Mycoplasma gallinaceum* (MGC), *Mycoplasma pullorum* (in chickens), *Mycoplasma lipofaciens* (in chickens and turkeys), *Mycoplasma anseris* (in geese), and *Ureoplasma* spp. (in chickens and turkeys) are less pathogenic and are not considered to be of much economic significance (19).

As one of the species that are not considered to be of much economic importance, few studies describing the role of MGC in poultry are published. MGC is a fast-growing mycoplasma, frequently isolated as a contaminant during attempts to culture pathogenic mycoplasmas, but it has been isolated from the upper respiratory tract and oviduct of diseased chickens (5,20), and MGC was previously implicated as the cause of avian mycoplasmosis in pheasants and partridges with incidence of respiratory disease (5).

During a survey conducted in South Africa in 2014 and 2015, mycoplasma strains were isolated from chicken flocks displaying typical signs of mycoplasmosis such as rales, coughing, sneezing, and

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nasal discharges, while in some instances, decreased weight gain, feed conversion efficiency, and egg production were also recorded in layers and breeders. It was anticipated that MG or MS would be identified in most circumstances; however, this was not the case. MGC was isolated in 23% of these cases, in most instances as a pure culture (unpubl. data). This raised a question about the potential role that undiagnosed MGC might play in respiratory disease and production losses in South Africa. Mycoplasmas on their own, including MG and MS, generally cause mild or subclinical disease in poultry, but can cause severe disease when they are accompanied by other pathogens that affect the respiratory system such as infectious bronchitis virus (IBV), low pathogenic avian influenza virus, Newcastle disease virus, and *Escherichia coli* (4,12,13,15,18). A recent study described the use of a co-challenge model using virulent MG and QX-like IBV field strains to assess vaccine efficacy (Bwala *et al.*, unpubl.data). We followed a similar approach to comparatively evaluate the pathogenicity of MGC in specific-pathogenic-free (SPF) white leghorn chickens.

## MATERIALS AND METHODS

**Challenge strains.** MGC strain B2096 8B was isolated in September 2014 from 62-wk-old layers displaying typical signs of mycoplasmosis and was confirmed to be a pure culture through next generation sequencing (1). MG strain B2159/13 was isolated in October 2013, and the purity of the culture was similarly confirmed by next generation sequencing (Bwala *et al.*, unpubl. data). Pure cultures of MGC and MG strains were propagated in Hayflick's broth for 2–5 days at 37 C. Serial dilutions from $10^{-1}$ to $10^{-6}$ were made, and 10 μl of each concentration was inoculated onto a nonselective agar medium, with an overnight incubation at 37 C. The number of colonies was counted, and dilutions with colony counts of 20–150 were used to calculate the colony-forming units (CFU) per milliliter by using the formula CFU/ml = (no. of colonies × dilution factor) / volume of culture plate

Titrated aliquots of cultures of both strains, stored at -80 C, were thawed just prior to challenge and diluted in Oculo Nasal diluent (Intervet South Africa (Pty) Ltd) to titers of approximately $5.7 \times 10^{4}$ CFU/ml. Each bird was challenged with 200 μl by eye drop.

The IBV QX-like field strain 3665/11 was isolated in 2011 (10). A titrated aliquot stored at -80 C was thawed and diluted in Oculo Nasal diluent (Intervet) to a challenge dose of $10^{4.0}$ Egg Infective Dose$_{50}$/ml, previously established in a challenge trial to be the optimal dose that causes maximum pathology at 7 days postchallenge (Bwala *et al.*, unpubl.data). Chickens were administered 50 μl by eye drop as a challenge dose.

**Experimental design.** The use of sentient animals for the study was approved by the University of Pretoria's Animal Ethics Committee under project number V083-16. Thirty-six 8-wk-old SPF chickens were obtained from AviFarms (Pty) Ltd., Pretoria. Chickens were housed throughout the period of the experiment in negative pressure isolators in the Biosafety Level 3 facility at the Poultry Research Unit and were provided feed and water *ad libitum*.

Chickens were randomly assigned into six groups of six birds each, designated as follows: nonchallenged control (NCC), MGC, MG, IBV, MGC + IBV (co-challenged), and MG + IBV (co-challenged). Fig. 1 provides an overview of the experimental design. At day 0, chickens in groups MGC and MGC + IBV were challenged with MGC strain B2096 8B, whilst chickens in groups MG and MG + IBV were challenged with MG strain B2159/13. At 7 days post-mycoplasma challenge, chickens in groups IBV, MGC + IBV, and MG + IBV were challenged with IBV strain 3665/11. Chickens were observed twice daily until day 14, and clinical signs were scored according to criteria outlined by Naylor *et al.* (15), with a modification of a scale from 0 to 4. A score
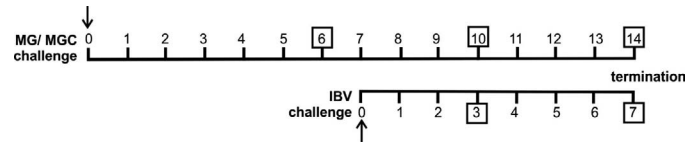


Fig. 1. Experimental design. Tracheal and cloacal swabs (square boxes) were collected from groups challenged with MG or MGC on 6, 10, and 14 dpc. On day 7, some groups were challenged with IBV, and swabs were collected on day 3 (10 dpc MG and MGC groups) and day 7 (14 dpc MG and MGC groups). The trial was terminated on day 14, when postmortem examination and ciliary motility assessments took place. Clinical signs were scored across the 14-day period.

of 0 was assigned to birds with no visible clinical symptoms; a score of 1 = slight change in the habitus, e.g. huddling, sneezing, tracheal rales, coughing; a score of 2 = a clear nasal discharge; a score of 3 = a turbid nasal discharge; a score of 4 = as for 3, but with swollen infra-orbital sinuses or conjunctivitis or both. On days 6, 10, and 14 post-MG or post-MGC challenge (corresponding to days 3 and 7 post-IBV challenge), tracheal and cloacal swabs were collected from each bird into 3 ml of pre-aliquoted phosphate-buffered saline (PBS). Swab samples were stored at -80 C until use. On day 14, all birds were euthanatized and examined for the presence of pathologic lesions. The bursae of Fabricius were removed, weighed, and measured with the aid of a bursal meter and the bursal index (BI) was determined as described elsewhere (3).

**Ciliary motility scoring.** At necropsy on day 14 (14 days post-MG or post-MGC challenge, or 7 days post-IBV challenge, or a combination), tracheas from each chicken were removed after euthanasia and immersed in PBS containing 1% glucose for ciliary motility scoring by using a method adapted from Cook *et al.* (6). Three transverse rings from the upper section, four from the midsection, and three from the lower section were prepared and examined under a low magnification light microscope (10×). Each of the 10 rings was scored as follows: 0 = all cilia beating; 1 = 75% beating; 2 = 50% beating; 3 = 25% beating; and 4 = none beating (100% ciliostasis). This gave a maximum possible score of 40 (4 × 10 rings per bird) if complete ciliostasis was observed. The sum of the scores for each experimental group was divided by the total number of birds per group (6) to calculate the mean ciliostasis score.

**Quantitative real-time PCR (qPCR).** Total nucleic acids were extracted for the three challenge pathogens from 200 μl of swab fluid by using an automated MagNA Pure LC instrument (Roche Diagnostics, Mannheim and Penzberg, Germany). MGC and MG qPCRs were conducted using the VetMax$^{TM}$ Plus qPCR master mix (Applied Biosystems, California), whereas the IBV-QX real-time reverse transcriptase–PCR (RRT-PCR) was conducted using the VetMax$^{TM}$ Plus One-Step RT-PCR kit (Applied Biosystems, California). Taqman$^{®}$ primers and probes used for MGC, MG, and IBV amplification are detailed in Table 1.

MG and MGC genomic DNAs were detected and quantified by a TaqMan PCR assay. Three microliters of the nucleic acid extracts (DNA or RNA) or no template control was used for each RRT-PCR or qPCR reaction. Each 12-μl qPCR reaction mix for MG or MGC contained 5 μl of PCR-grade water, 6 μl of 2× VetMax qPCR Master mix, 0.5 μl each of 10 pmol/μl forward and reverse primer, and 0.15 μl of 5 pmol/μl probe. Cycling parameters were as follows: one cycle of enzyme activation and template denaturation at 95 C for 10 min, followed by amplification of 40 cycles at 95 C for 15 sec and 60 C for 45 sec. IBV RNA genomes were detected and quantified by a TaqMan RRT-PCR assay. Each 12-μl reaction mix contained 6 μl of PCR-grade water, 6 μl of 2× RT-PCR buffer, 0.5 μl RT Enzyme mix, 0.5 μl each of 10 pmol/μl of forward and reverse primer, and 0.15 μl of 5 pmol/μl probe. The cycling parameters included one cycle of reverse transcription at 48 C

Table 1.  Primers and probe used for qPCR.

| Target gene | Sequence (5′→ 3′) |
|---|---|
| MGC-Forward | CGT GCC CCC TTG ATT GGG ATA ACG CTG |
| MGC-Reverse | TAG CTA ATG TTA CGC ACC CCG ATC CCC TTG T |
| MGC probe | TAG CGC TAA TAC CGG ATA CTT AA (FAM) |
| Mg-Forward | CGGTATTAGAACCATGCTTAAC |
| Mg-Reverse | CAACCWGGTGTTTTATCAGACGC |
| B2159 probe | GAGCTATAGCGAAAAGGTCTTTATCA (VIC) |
| IBV S925-Forward | CCAAACACAAACAGCTCAGAGTGG |
| IBV S1144-Reverse | TACCACTAAARACAGATTGCTTRC |
| IBV QX probe | CAGTTTGTGTATAAGGCAAGC (FAM) |

Table 2.  Scoring of clinical signs in chickens.

| Group ($n = 6$) | Clinical sign scoring[A] | | | | | No. affected (%) |
|---|---|---|---|---|---|---|
| | 0 | 1 | 2 | 3 | 4 | |
| NCC | 6 | 0 | 0 | 0 | 0 | 0 (0%) |
| MGC | 6 | 0 | 0 | 0 | 0 | 0 (0%) |
| MG | 6 | 0 | 0 | 0 | 0 | 0 (0%) |
| IBV | 4 | 2 | 0 | 0 | 0 | 2 (33.3%) |
| MGC + IBV | 4 | 2 | 0 | 0 | 0 | 2 (33.3%) |
| MG + IBV | 3 | 3 | 0 | 0 | 0 | 3 (50%) |

[A]0 = no signs; 1 = habitual or appearance of the chickens (sneezing, tracheal rales, coughing etc.); 2 = clear nasal exudate, or discharge, or both; 3 = turbid nasal exudate, or discharge, or both; 4 = as for 3, but with swollen infra-orbital sinuses, conjunctivitis, or both.

for 10 min, one cycle of RT inactivation and initial denaturation at 95 C for 10 min, followed by 40 cycles of 95 C for 15 sec and 60 C for 45 sec for annealing and extention. Each assay was done in duplicate and individual standard curves were generated using six 10-fold serial dilutions each of MGC, MG, or IBV of known concentrations. Amplification and data acquisition were carried out using a StepOne Plus real-time PCR system (Life Technologies Ltd., Carlsbad, CA). Data were analyzed using the Student *t*-test, and the probability level for significance was $P < 0.05$.

**Histopathology.** Tracheas sampled at necropsy (preserved in 10% buffered formalin) were routinely processed and stained with hematoxylin and eosin H&E as described by Bancroft *et al.* (2). Cross sections of each trachea were microscopically examined and lesions were scored blind, based on a scale of 0–3 as follows: 0 = no significant change; 0.5 = very small aggregates (1–2 foci) or very slight, diffuse infiltration of lymphocytes; 1 = small aggregates (>2 foci) of lymphocytes or slight thickening of the mucosa due to diffuse lymphocyte infiltration; 2 = moderate thickening of the mucosa due to heterophil and lymphocyte infiltration and edema, accompanied by epithelial degeneration with or without luminal exudation; 3 = extensive thickening due to heterophil and lymphocyte infiltration and edema, with squamous metaplasia or degeneration of the epithelial with luminal exudation (16).

**Statistical analysis.** Statistical analysis of the ciliary motility score, lesion score, and qPCR was performed according to the analysis of variance and *t*-test for comparing means. Significant statistical differences were considered at $P \leq 0.05$.

## RESULTS

**Clinical signs and gross pathology.** Sneezing, which was the only sign observed in this study, was recorded in chickens of the MG + IBV group at day 10 post-mycoplasma challenge and at 3 days post-IBV challenge. Sneezing was also recorded in the MGC + IBV and IBV groups at 12 days post-mycoplasma and 5 days post-IBV challenge. None of the other challenged groups or the NCC group showed any respiratory or other clinical signs (Table 2).

No macroscopic lesions were observed at necropsy in any of the organs of the chickens in the MCG group or the NCC group. The bursae from both groups were also of normal size and index (BI>0.20%) (Table 3). Most chickens in groups MG, IBV, MGC + IBV, and MG + IBV had medium bursal sizes and BI scores (0.18%<BI<0.20%). One bird each in the IBV, MGC + IBV, and MG + IBV groups had small bursae with reduced BI scores (0.15%<BI<0.18%). The IBV-challenged group and the MGC + IBV group displayed severe visible macroscopic lesions (Table 3). In the MGC + IBV group, peritonitis was observed, characterized by

cloudy and thickened abdominal air sac walls and accumulation of serofibrinous exudates, whilst in the IBV group, thoracic airsacculitis was noted.

**Histopathology.** The mean severity scores of tracheal histologic lesions are presented in Fig. 2. The average score of the MG + IBV group was significantly higher ($P < 0.05$) than other groups. The severity of the lesions ranged from small aggregates of lymphocytes to extensive thickening due to lymphoplasmacytic infiltration, with edema and degeneration of epithelia with luminal exudation. Although the severity of the lesions observed in the MG + IBV group was significantly higher than those of the MGC + IBV group, there was no significant difference in the mean lesion score between the IBV group and the MGC + IBV group; however, the severity of lesions in the individual birds was numerically higher in the MGC + IBV group. Birds challenged with MG alone had significantly higher mean lesion scores compared to the group challenged with MGC alone.

**Effects of MG and IBV infection on tracheal cilia.** The ciliostatic effects of the mycoplasmas and IBV on tracheas of the chickens at day 14 (7 days post-IBV challenge) are presented in Fig. 3. The NCC group had complete ciliary activity (0% ciliostasis). Minimal effects on the cilia (a score of 1.5) in the MGC-challenged group were recorded, whilst the MG-challenged group displayed a slightly higher effect on the tracheal cilia (score of 5.91), yet this value remains low. The IBV-infected group had a mean ciliostasis score of 20.4. The aggravating effects of mycoplasma and IBV co-infection on tracheal cilia was evident in the MGC + IBV group with a ciliostatic score of 28.83, and in the MG + IBV group with a ciliostatic score 32.83, out of a maximum 40 points. However, the differences between the IBV-challenged group, the MGC + IBV group, and the MG + IBV group were not statistically significant.

**Quantitation of pathogen shedding.** qPCR was used to assess mycoplasma and IBV shedding from the trachea and cloaca over the duration of the experiment. In the MGC-challenged group, mean genome copies in excess of 47 million were detected on tracheal swabs collected at 6 days postchallenge (dpc) (Fig. 4), and this had increased to $1.54 \times 10^8$ genome copies at 10 dpc, before dropping to $1.86 \times 10^5$ genome copies at 14 dpc. In the group challenged with both pathogens (MGC + IBV), similar levels of MGC were detected at 6 dpc at over 44 million mean genome copies (prior to IBV challenge), but this level declined at 10 dpc (3 days post-IBV challenge) to $2.73 \times 10^7$ genome copies before dropping to $6.34 \times 10^4$ genome copies by 14 dpc.

MG was initially detected at much lower levels in the trachea compared to MGC (Fig. 5). At 6 dpc, mean copy numbers in the

Table 3. Gross pathologic observations in chickens.

| Group (n = 6) | BI score | | | Air sac lesions |
| --- | --- | --- | --- | --- |
| | Normal size BI>0.20% | Average size 0.18%<BI<0.20% | Small size 0.15%<BI<0.18% | |
| NCC | 6/6 | — | — | — |
| MGC | 6/6 | — | — | — |
| MG | — | 6/6 | — | — |
| IBV | — | 5/6 | 1/6 | 1/6 (Thoracic airsacculitis) |
| MGC + IBV | — | 5/6 | 1/6 | 1/6 (Peritonitis) |
| MG + IBV | — | 5/6 | 1/6 | — |

MG and MG + IBV groups (pre-IBV challenge) were $7.49 \times 10^6$ and $6.30 \times 10^4$ genome copies, respectively. At 10 dpc, the numbers had increased to $9.01 \times 10^5$ in the MG group, and they continued to increase at day 14 to $4.76 \times 10^6$ genome copies. In the group MG + IBV, numbers increased from $3.07 \times 10^5$ genomes at 10 dpc to $3.46 \times 10^6$ genomes at 14 dpc.

No MG was detected on cloacal swabs in the 14-day trial period (unpubl. data), but in birds challenged with MGC alone, mean genome copies of $2.93 \times 10^4$ and $2.91 \times 10^4$ were detected at 10 and 14 dpc, respectively (Fig. 6). In chickens challenged with both IBV and MG, genome copy numbers of $4.23 \times 10^3$ and $4.28 \times 10^3$ on 6 and 9 dpc, respectively, increased to $3.03 \times 10^4$ by 14 dpc.

For interest, IBVs on the swabs collected at day 10 (3 dpc with IBV) and 14 (7 dpc with IBV) were quantified by RRT-PCR. The highest titers of IBV in the tracheas were detected at 3 dpc (Fig. 7). In the group challenged with IBV alone, $2.81 \times 10^6$ mean genome copies were detected, compared to $1.54 \times 10^7$ genome copies in the MGC + IBV group, and $2.17 \times 10^7$ copies in the MG + IBV group. However, the difference between MG + IBV and MGC + IBV mean genome copies was not statistically significant. By day 7 post-

challenge, titers had dropped dramatically. The IBV shedding patterns from the cloaca were markedly different (Fig. 8). At 3 dpc with IBV, shedding was only detected in the MG + IBV group, at levels of $2.43 \times 10^5$ viral genome copies. By day 7, mean viral genome copies of $2.94 \times 10^5$ were detected in the group that was only challenged with IBV alone, and a similar titer of $2.75 \times 10^5$ mean genome copies were detected on swabs from birds co-infected with MG + IBV, but $1.37 \times 10^6$ viral genome copies were detected in the group co-infected with MGC + IBV.

## DISCUSSION

White Leghorn SPF chickens were co-challenged with MG and IBV, or MGC and IBV, with relevant controls to assess the potential pathogenicity of MGC as compared with MG. This study recorded sneezing as the only clinical sign, first in the group challenged with MG + IBV and later in the groups challenged with MGC + IBV and IBV alone. Co-infection of MG with other respiratory diseases can cause more severe clinical symptoms (13,14). Previously, signs that included depression, ruffled feathers, inappetence, a depressed appearance, greenish feces, unstable and wobbly gaits leading to recumbency, exaggerated head shaking that mimicked birds with neurologic signs, and swollen eyelids or periocular regions were recorded in chickens challenged with the same MG and IBV strains (Bwala *et al.*, unpubl.data). We consider that the lower challenge doses and mild-challenge method used here probably produced the less-pronounced clinical signs observed in this study.
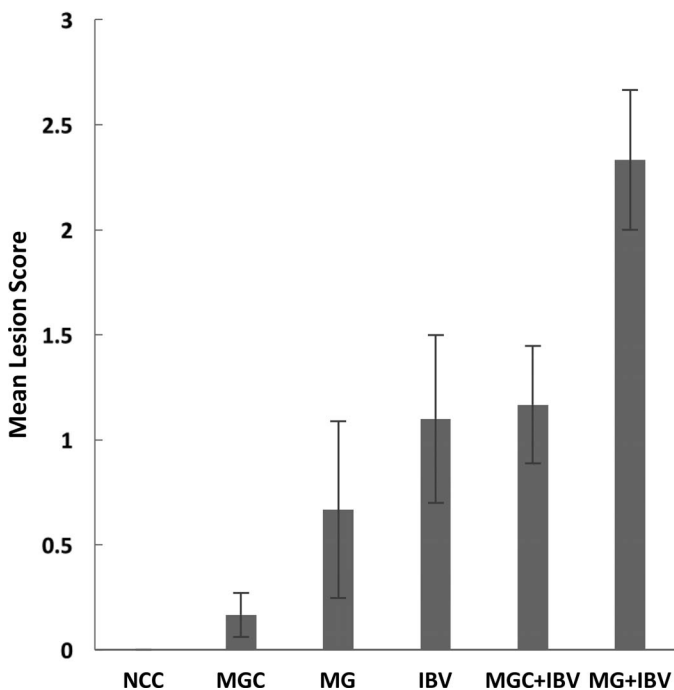


Fig. 2. Mean severity scores of histologic lesions of tracheas in chickens challenged with either of the two mycoplasma species (MG or MGC),or IBV, or both.
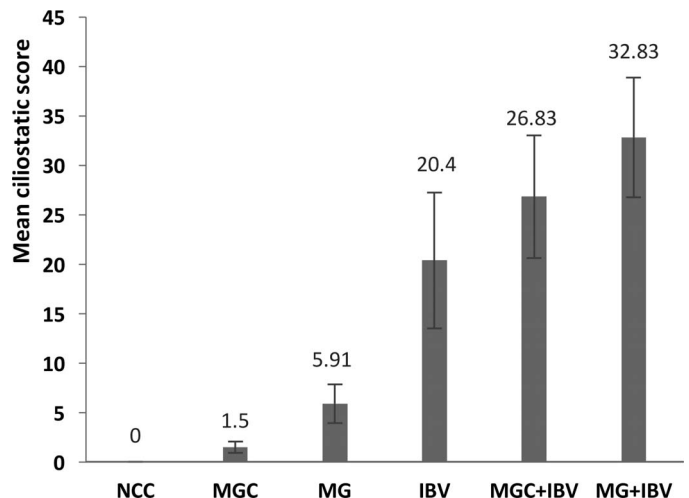


Fig. 3. Tracheal ciliary motility scores scored from 0 (healthy, normal cilia) to 40 (complete ciliary stasis or depletion).
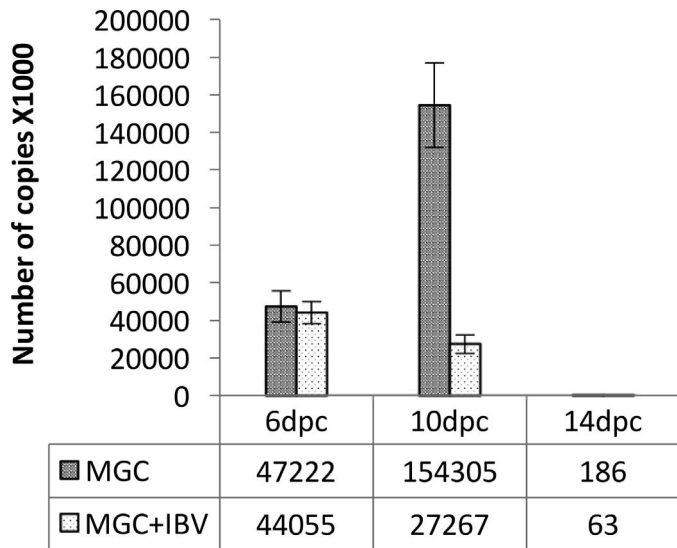
| | 6dpc | 10dpc | 14dpc |
|---|---|---|---|
| ▨ MGC | 47222 | 154305 | 186 |
| ▢ MGC+IBV | 44055 | 27267 | 63 |

Fig. 4. Mean genome copies of MGC detected on tracheal swabs at 6, 10, and 14 dpc in chickens challenged with MGC alone, or MGC and IBV.



| | 6dpc | 10dpc | 14dpc |
|---|---|---|---|
| ▨ MGC | 3 | 29 | 29 |
| ▢ MGC+IBV | 4 | 4 | 30 |

Fig. 6. Mean genome copies of MGC detected on cloacal swabs at 6, 10, and 14 dpc in chickens challenged with MGC alone, or MGC + IBV.

A single bird in the group challenged with IBV alone developed airsaccullitis. No pathology was observed in the group challenged with MG + IBV, but peritonitis of the airsacs was observed in a chicken in the MGC + IBV group. The finding that only a single bird out of each group displayed pathology could be due to the low challenge dose and the intra-ocular challenge route. Although using greater numbers of chickens with higher challenge doses and different inoculation routes may provide stronger evidence, these results suggest that MGC possibly synergized with undiagnosed respiratory pathogens to cause the observed respiratory problems in flocks.

Examination of the tracheas provided further evidence of the pathogenicity of MG compared to MGC and the aggravating effects
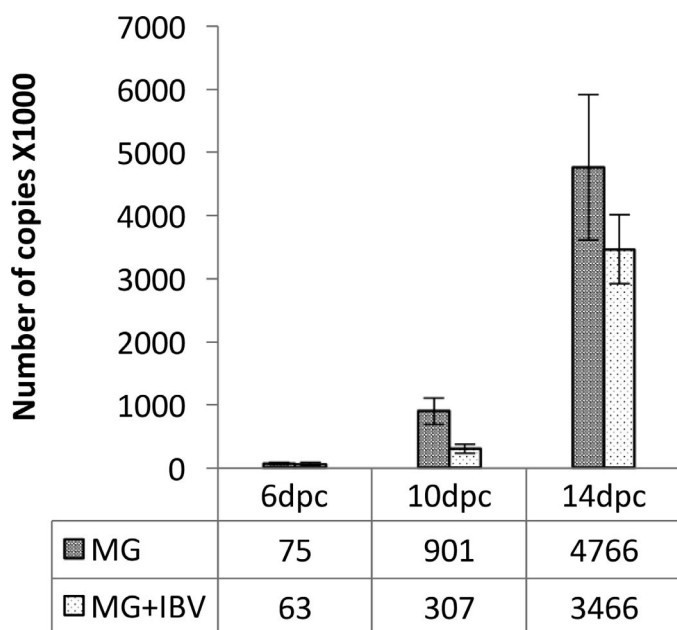


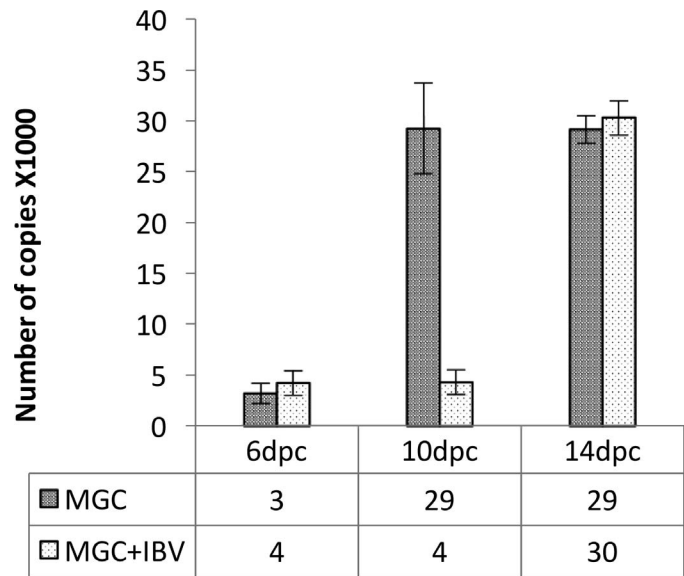| | 6dpc | 10dpc | 14dpc |
|---|---|---|---|
| ▨ MG | 75 | 901 | 4766 |
| ▢ MG+IBV | 63 | 307 | 3466 |

Fig. 5. Mean genome copies of MG detected on tracheal swabs at 6, 10, and 14 dpc in chickens challenged with MG alone, or MG + IBV.

of IBV on mycoplasma infections. Tracheal lesions were more severe in the group co-infected with MG and IBV, but MGC did not appear to cause severe tracheal lesions either on its own or in combination with IBV, compared to the IBV control group. Mycoplasma adhesion to epithelial surfaces of respiratory and urogenital tracts is a requirement for colonization and pathogenesis, and numerous nonpathogenic mycoplasmas appear to lack the ability to adhere to the epithelium (8). The tracheal cilia prevent bacterial colonization through their mucociliary activity and failure to do so presents the first step in the pathogenic process (11). MG is a well-characterized respiratory pathogen with demonstrated negative effects on mucociliary activity in trachea, especially in co-infections with other respiratory pathogens (17). The MG + IBV group had the most severe ciliostasis; although the value was higher than that of the MGC + IBV challenged group and IBV-challenged group, the difference was not statistically significant. A score of above average (20/40) is considered a significant negative effect on the ciliary activity of the chickens according to Cook et al. (6), and the MGC group had a mean score of 26.83. This implies that even though MGC is unable to cause tracheal lesions as severe as those observed with MG, the damage MGC causes to tracheal cilia still predisposes birds to infections with other respiratory pathogens. No data is available describing the cellular attachment abilities of MGC.

qPCR determined that MGC was shed in much higher titers from the trachea than MG when birds were co-infected with IBV. We also determined that MGC-infected birds start shedding the microorganism sooner than those infected with MG, as peak MGC titers were detected at 9 dpc but peak MG titers were only detected at 14 dpc. IBV co-infection had a dramatic effect on the proliferation and shedding of MGC, whereas its effect on MG proliferation was less pronounced. The incongruent timing of shedding in vivo remains consistent with in vitro laboratory studies that established MGC as a much faster growing species than MG (5,9). Drastically less MGC was shed from the cloaca, but as many as 29,000 microorganisms were detected up to 14 dpc. Interestingly, the shedding of MGC from the cloaca was suppressed or delayed in chickens challenged
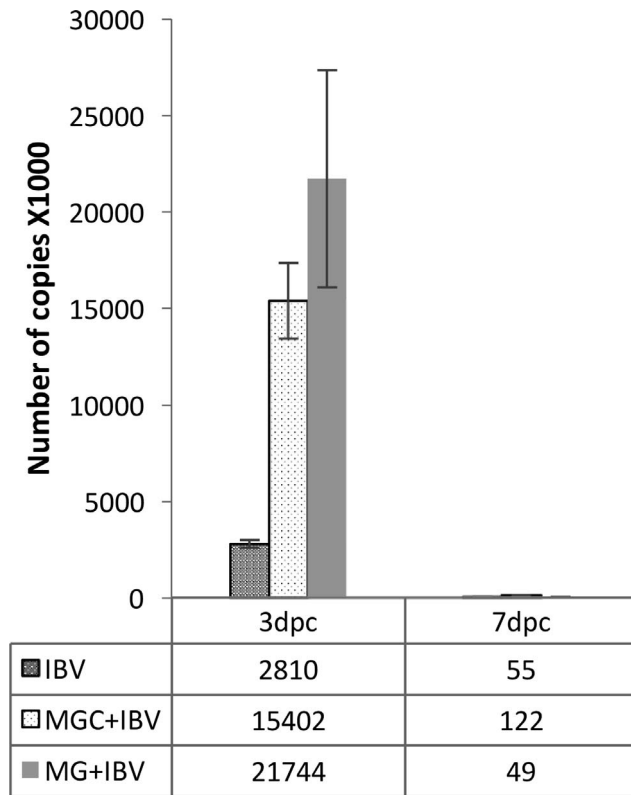
Fig. 7. Mean genome copies of IBV detected on tracheal swabs at 3 and 7 dpc in chickens challenged with IBV alone, IBV + MGC, or IBV + MG. Day 3 and 7 corresponds to 10 and 14 dpc, respectively, with mycoplasmas.
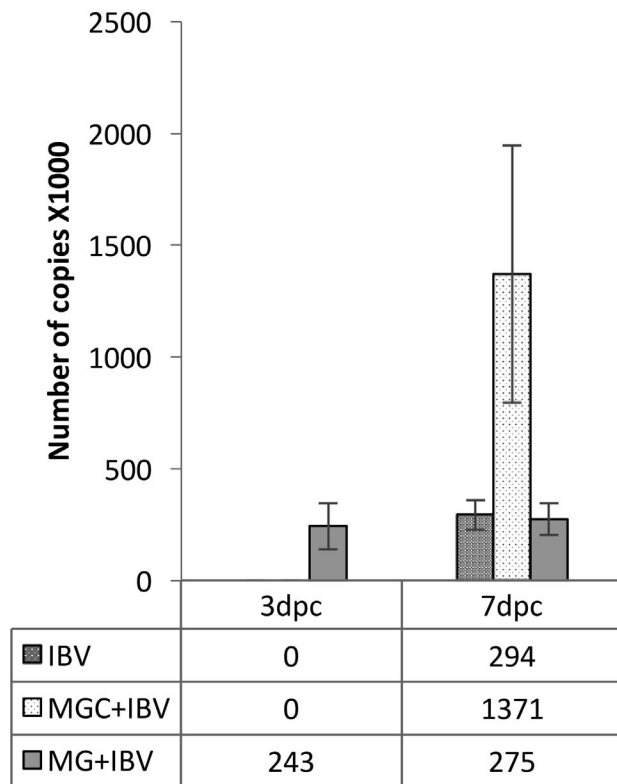
| | 3dpc | 7dpc |
|---|---|---|
| IBV | 2810 | 55 |
| MGC+IBV | 15402 | 122 |
| MG+IBV | 21744 | 49 |



Fig. 8. Mean genome copies of IBV detected on cloacal swabs at 3 and 8 dpc in chickens challenged with IBV alone, IBV + MGC, or IBV + MG. Day 3 and 7 corresponds to 10 and 14 dpc with mycoplasmas, respectively.

| | 3dpc | 7dpc |
|---|---|---|
| IBV | 0 | 294 |
| MGC+IBV | 0 | 1371 |
| MG+IBV | 243 | 275 |

with both MGC and IBV. Although no cloacal shedding of MG was detected, it is possible that this may have been detected if the study had continued beyond 14 days.

The effects of the mycoplasmas on IBV replication were equally interesting. Both MG and MGC appeared to enhance IBV replication in the tracheas at 3 dpc, but MG more so. Whereas the effects of MG on cloacal shedding were comparable to the IBV-infected control group, the presence of MGC significantly increased the amount of IBV shed from the cloaca at 7 dpc. In conclusion, the results demonstrate that MGC on its own is nonpathogenic in chickens, but that it synergistically enhances the pathogenicity of other respiratory pathogens in co-infections. No vaccines against MGC are currently available.

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