Experimental Coinfection of Chicken Anemia Virus and Mycoplasma gallisepticum Vaccine Strains in Broiler Chicks

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ABSTRACT

This study aimed at determining the clinical and pathological effects of the coinfection of young SPF chickens with chicken anemia virus (CAV) and *Mycoplasma gallisepticum* (MG) vaccine strains. The clinical signs, gross and microscopic lesions were determined after the experimental coinfection broilers with a CAV genotype 1 vaccine strain given intraperitoneally on the first day of age and a MG F-strain vaccine given intranasally on the 8th day of age. The experimental groups included the negative control (group 1), a group infected with the MG F-strain vaccine (group 2), and a group coinfected with CAV and MG vaccines (group 3). Chicks were examined clinically and *post mortem* at 23 days of age, and gross and microscopic lesions of the trachea, thymus, and air sacs were compared among treatments (Kruskal-Wallis test). Infections were confirmed by PCR for specific genetic fragments of each agent in the target tissues. Mortality was only observed in chicks on group 3, with two deaths and more severe lesions in the trachea, thymus and air sacs compared with groups 1 and 2 ($p<0.01$). Dead chicks presented reduced thymus and spleen size, hemorrhagic trachea with catarrhal exudate and partial obstruction, pericarditis, catarrhal airsacculitis, lungs with liquid and ascites. The surviving chicks in group 3 showed more severe respiratory changes than those in group 2, in addition to thymus and spleen size reduction. Results indicate the adverse effects of the coinfection of young chickens with MG F-strain and CAV genotype 1 vaccines.

INTRODUCTION

Mycoplasma gallisepticum (MG) may cause increased mortality, reduction in weight gain, a higher rate of condemnations in the processing plant due to airsacculitis in broilers, and reduction in egg production in layers, which result in very significant losses to the poultry industry (Evans *et al.,* 2005; Ley, 2003; Lütticke, 1997; Nascimento and Pereira, 2009; Yashpal *et al.*, 2004). However, MG may cause a much more serious disease if associated with other respiratory agents, such as other bacteria and viruses (Yashpal *et al.,* 2004; Couto *et al.*, 2015). The chicken anemia virus (CAV) is an immunosuppressive agent that may predispose chickens to primary and secondary infections, mainly by affecting the bone marrow and thymus, thus compromising the immune response and health of poultry (Brentano, 2009; Toro *et al.*, 2009; Gallardo *et al.*, 2012; Silveira *et al.*, 2013).

Important poultry-producing regions are densely concentrated, and occasionally farms have poor biosecurity. Live vaccines, including those against CAV and MG strains, are commercially available and have been used for the prevention of diseases. This study aimed at evaluating the clinical signs and the gross and microscopic lesions in young chickens

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experimentally coinfected with commercial vaccine strains of MG and CAV. For this purpose, chicks were administered with the F strain of *Mycoplasma gallisepticum* (MG) in coinfection or not with a chicken infectious anemia virus (CAV) genotype 1 vaccine strain.

MATERIALS AND METHODS

Chickens

Certified specific pathogen free (SPF) chicken eggs (VALO, Uberlandia, Brazil) were incubated and hatched (Premium Ecologica, Brazil). Chicks were housed in an experimental facility in an isolated room. No other birds were present at the isolation unit. Fifteen chicks were randomly assigned to three treatments with five chicks in each. The birds received water and feed without any growth promoter or anticoccidial drug *ad libitum*. The experiment employed the adequate numbers of experimental animals for approval by the Ethics Committee (CEUA, UFMG).

CAV

A live attenuated phylogenetic group 1-type vaccine was reconstituted to obtain $10^{2.3}$ tissue culture infective doses 50% ($TCID_{50\%}$) per mL.

Mycoplasma gallisepticum

A live attenuated MG F strain-type vaccine was reconstituted and cultured in Frey's medium (Frey *et al.*, 1968) until obtaining a density of 3×10^5 CFU/0.1 mL.

Experimental protocol

Chicks were administered the MG F-strain vaccine of on day 8, in coinfection or not with a chicken infectious anemia virus (CAV) genotype 1 vaccine strain, given on the first day of age. The experimental groups were housed in cages in an isolation room with controlled air supply, independent water and food supply and restricted access.

Day-old SPF chicks were hatched and randomly divided into three groups of five birds each (Table 1). All CAV inoculations were administered intraperitoneally on the first day of age and MG inoculations given intranasally on the eighth day of age. At 23 days of age, all birds were euthanized, submitted to full necropsy, and the liver, spleen, thymus, and trachea were collected for histopathology and thymus and trachea for the investigation of CAV or MG by PCR. Considering that the infection by *Mycoplasma gallisepticum* strains may

be evaluated in the trachea, especially during the acute stage of infection previous to the period needed for an antibody response (typically 21 days) to be detectable in all birds (Levisohn & Dykstra, 1987), the isolation or detection by molecular methods is most successful during the acute stage of infection (Hyman *et al.*, 1989). In addition, the duration of the experiment was reduced to a minimum in view of the risk of field CAV challenge.

Table 1 – Experimental groups.

Group	Treatment	Infection
1	Negative control	None
2	MG-infected	Day 8: Chicks were given the MG F-strain vaccine containing 3×10^{13} CFU/0.1 mL.
3	CAV plus MG	Day zero: At hatching, day-old chicks were given live attenuated genotype 1 CAV vaccine (0.2 mL/$10^{2.3}$ $TCID_{50\%}$) intraperitoneally; Day 8: After CAV at day zero, chicks were given the MG F-strain vaccine containing 3×10^{13} CFU/0.1 mL intranasally.

Lesion score

At necropsy, gross lesions were scored by adapting previously described score systems (Kogut *et al.*, 1994; Leigh *et al.* 2012; Nunoya *et al.*, 1987), with modifications as follows:

- Trachea: 0 - no significant alteration; 1 – mucus; 2 – mucus, thickening and localized hyperemic mucosa; 3 – catarrhal exudate, extensive thickening and generalized hyperemia of the mucosa; 4 – caseous exudate, generalized hyperemia, hemorrhage, partial and/or complete obstruction of the trachea, asphyxiation and death.

- Air sacs: 0 – no alteration; 1 – light opacity and increase in thickness; 2 – moderate increase in thickness and opacity and light accumulation of catarrhal exudate; 3 – severe increase in thickness, blisters and large accumulation of catarrhal exudate (foam); 4 – severe increase in thickness and fibrin deposition (caseous deposition).

- Thymus: 0 – no alteration; 1 – discrete reduction in lobe size; 2 – moderate reduction in lobe size; 3 – severe reduction and absence of a few lobes on both sides of the neck; 4 – complete atrophy of all lobes.

Conventional bacteriology of chicks to investigate the presence of *Escherichia coli* in the liver was performed in MacConkey agar (aerobiosis) at necropsy, in view this is the most common secondary infection after *M. gallisepticum*. *E. coli* infection would

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interfere with the interpretation of *M. gallisepticum* expected lesions.

DNA extraction

Total DNA extraction from tissues (thymus or trachea) was performed using silicon dioxide and sodium iodide (Boom *et al.*, 1990; Vogelstein and Gillespie, 1979). DNA extracts were stored at -20 ° C until use. Qualitative and quantitative DNA analyses were performed determining optical density in a spectrophotometer (NanoVue ®, GE Healthcare, UK) at 260 and 280 nm wavelengths.

Oligonucleotide primers

The two sets of primers used in the CAV PCR were previously described (Marin *et al.*, 2010): CAVMGF 5'CCTGTTCCGACACATTGA3', corresponding to nucleotides 1444 to 1461, and CAVMGR 5'TATGG-CCTCTGCCTGTTA3', corresponding to nucleotides 2119 to 2136. The genomic position of nucleotides in the CAV DNA matched the Cuxhaven-1 strain (Gen-Bank M55918.1) sequence. Primers used for the detection of MG were, as recommended by the *Office International des Epizooties*, MG-14F 5' GAGCTA-ATCTGTAAAGTTGGTC3' and MG-13R 5'GCTTCCTTG-CGGTTAGCAAC3' (OIE, 2008).

PCR amplification

The CAV PCR protocol was described elsewhere (Marín *et al.*, 2010), using a 5 µL aliquot of each total DNA obtained from the thymus. Reactions were performed in 50 µL volumes containing 200 ng of DNA, 1X buffer (200 mM Tris-HCl pH 8.4, 500 mM KCl), 0,4 mM of dNTP, 2 mM of $MgCl_2$, 1 µL of each primer at 10 pmol, 1 UI of Taq Polymerase (Platinum Taq DNA Polymerase, Invitrogen, USA) and 18,2 Mili-Q ultrapure water q.s.p. For the MG PCR protocol, a 5 µL aliquot of each tracheal total DNA sample was employed, using 50 µL reaction volumes containing 35.75 µL Mili-Q water, 10X buffer (200 mM tris-HCl pH 8.4, 500 mM KCl), 1 mM of dNTP, 0.5 µL of each primer at 20 pmol/µL, 0,25 UI of Taq Polymerase and 2 mM of $MgCl_2$.

Histopathology

Tissue samples were processed within 48-hour of fixation, sectioned, and placed in labeled cassettes for histotechnical preparation. Paraffin tissue blocks were cut into 5-µm thickness and sections were placed onto glass slides and stained with hematoxylin and eosin, according to the laboratory routine protocol.

Statistics

Statistical analysis of gross lesions scores of the trachea, air sacs and thymus was performed according to the Kruskal-Wallis test.

RESULTS

No changes were observed regarding clinical signs of birds of all groups. However, two deaths (40%) were observed on day 16 on group 3, sixteen days after the inoculation of CAV and eight days after the inoculation with MG.

Necropsies of chicks of group 3 that died on day 16 revealed atrophic thymus, reduced spleen size (with whitish areas), hemorrhagic trachea with catarrhal exudate that partially obstructed the air passage (score 4), pericarditis, catarrhal airsacculitis, and lungs with liquid and ascites. Twenty-three days after CAV and 15 days after MG infections (23 days of age), significant differences in the analysis of the gross lesions of the trachea ($p<0.01$), air sacs ($p<0.01$) and thymus ($p=0.001$) were observed in group 3 compared with groups 1 and 2 (Figures 1, 2, and 3). The air sacs (Fig. 2) were macroscopically more compromised in chicks of group 3 relative to groups 1 and 2 ($p<0.01$). Severe thymus atrophy was observed 23 days after CAV infection in all birds infected with the CAV vaccine strain (group 3), while the thymuses of chicks in groups 1 and 2 remained unchanged ($p=0,001$) (Fig. 3). In contrast, no significant differences in gross changes were observed when comparing groups 1 and 2. *Escherichia coli* was not isolated in the liver, indicating exclusive *M. gallisepticum* infection.
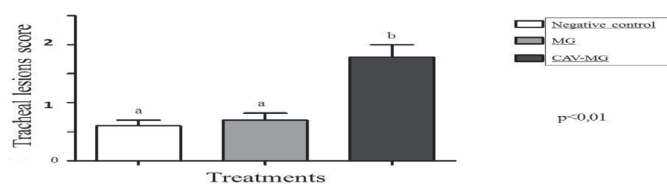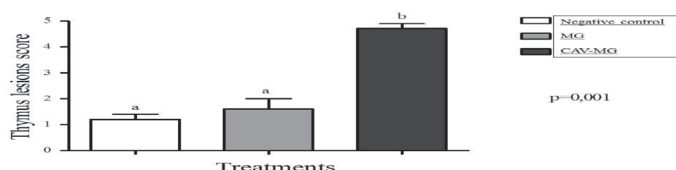

Figure 1 – Gross lesions score on the trachea as observed at necropsy of chicks administered a F-type vaccine strain of *Mycoplasma gallisepticum* (MG) at day 8, in coinfection or not with a chicken infectious anemia virus (CAV) genotype 1 vaccine strain given at first day of age. Chicks were examined at 23 days of age. White bar group 1(negative control); Gray bar MG group 2; Black bar CAV+MG group 3 ($p<0.01$).
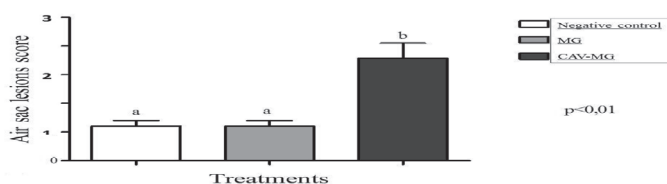

Figure 2 – Gross lesion score of the air sacs, as observed at the necropsy of chicks given CAV at day zero, MG at day 8 and examined at 23 days of age. White bar group 1 (negative control); Gray bar MG group 2; Black bar CAV+MG group 3 ($p<0.01$).

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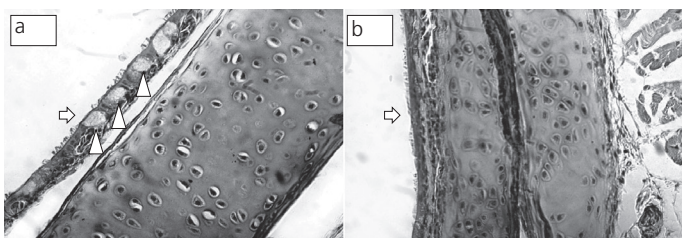and Mycoplasma gallisepticum Vaccine Strains in
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Figure 3 – Gross lesion score of the thymus as observed at the necropsy of chicks given CAV at day zero, MG at day 8 and examined at 23 days of age. White bar group 1 (negative control); Gray bar MG group 2; Black bar CAV-MG group 3 ($p<0.01$).

At histopathology, the tracheas of chicks in group 1 presented normal epithelium, with cilia and goblet cells (Figure 4) and no changes were observed in thymus, liver and spleen. However, in the trachea of chicks in group 2, the epithelium was thinner, with shortened cilia in three birds (3/5); however, the other two birds presented fewer changes and more uniform distribution of the epithelium and normal presence of cilia (Figure 5). Chicks in group 3 showed the most evident changes in the tracheal epithelium, which was thinner, with increased number of goblet cells and sparse to absent cilia cover (Figure 6).
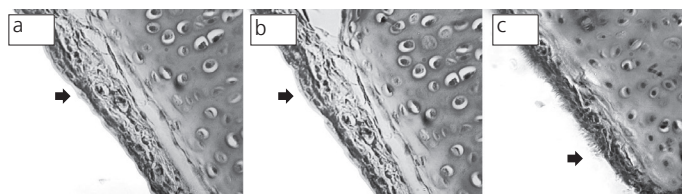
Figure 4 – Tracheas of the negative control chicks (group 1). All birds showed normal epithelium with cilia (black arrows) and goblet cells (H&E).

Figure 5 – Tracheas of the F-type *Mycoplasma gallisepticum* vaccine infected chicks (group 2). The trachea shows moderate changes, absence of cilia (a and b, white arrows) in the affected area and hyperplasia of goblet cells (a, white arrowheads) (H&E).
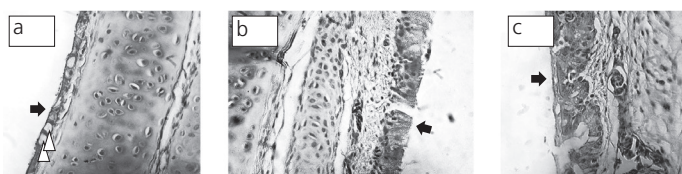
Figure 6 – Trachea of chicken anemia virus - CAV (hatching day) plus F-type *Mycoplasma gallisepticum* vaccine (day 8) infected chicks (group 3). Tracheas with pseudostratified epithelium with reduced thickness, increased number of large goblet cells (a, white arrowheads) and scarce to absent cilia coverage (a, b and c, black arrows).

Regarding the molecular evaluation, CAV DNA was investigated in the thymus and trachea and detected only in the thymus of all chicks in group 3, but in none of the birds in groups 1 and 2. MG DNA was detected in the trachea of chicks in groups 2 and 3. Chicks in group 1 were negative for both MG and CAV DNA.

DISCUSSION

The severity of coinfection resulted in two deaths in chicks on group 3 (40%). The primary infection with CAV eight days before the MG infection, may have immunocompromised the chicks and allowed a more severe secondary MG infection of the chicks in group 3. The lesions observed, compared with the other two groups (Figures 1, 2 and 3), demonstrate that the coinfection tended to increase the severity of gross lesions and resulted in significantly ($p<0.01$) more severe microscopic lesions of chicks in group 3 (Fig. 1). Immunosuppressive diseases usually negatively affect chickens of the poultry industry by increasing the susceptibility to opportunistic infections by viruses and bacteria (Lutticken, 1997; Balamurugan & Kataria, 2006). Among the known immunosuppressive agents, CAV stands out for its worldwide occurrence, not only in commercial poultry, but also in SPF chickens (Schat, 2003; Balamurugan & Kataria, 2006), backyard chickens (Barrios *et al.*, 2009), as well as contaminates poultry vaccines (Barrios *et al.*, 2012). CAV presents tropism for T-lymphocytes, rendering the cellular immune response ineffective, especially in young chickens, by destroying CD8+ T cells (Adair *et al.*, 1993; Adair, 2000; Brentano, 2009), disabling the protection against intracellular agents. Through a facultative intracellular infection, MG can cause a systemic infection and evade the immune response (Vogl *et al.*, 2008; Santos *et al.*, 2009).

The severe thymus atrophy observed 23 days after CAV vaccine infection indicates a potential risk in case of biosecurity failure when chicks are maintained close to vaccinated breeders. In addition, the proximity of chicks to breeders vaccinated against CAV and MG F-strain may result in infection, resulting in severe immunosuppressive and respiratory disease, and potential increase in mortality.

Gaunson *et al.* (2000) showed that CD8+ T cells response plays an important role in the susceptibility to infection with *Mycoplasma gallisepticum*, as lymphocytes are the main target of CAV (Adair *et al.*, 1993; Adair, 2000; Brentano, 2009). In our experiment, the primary CAV infection on day one may have allowed more severe MG infection, as demonstrated by more severe respiratory lesions in group 3 (Fig. 2).

Although the single MG infection (group 2) did not result in clinical disease, chick performance was not evaluated, and negative effects on these parameters cannot be discarded. Microscopically, rarefaction of

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cilia and altered goblet cells were found, which are microscopic lesions also described in other studies (Leigh *et al.*, 2012; Murakami *et al.*, 2002; Nunoya *et al.*, 1987).

The negative isolation of *E. coli* in liver may be associated with the high quality of the experimental chicks, which were specific-pathogen free, and the experimental housing conditions, which may have reduced spread and challenge. However, natural field conditions, with the possibility of challenge with *E. coli* resulting in a different outcome, possibly with more severe lesions and higher mortality due to the associated colibacillosis.

CAV (Schat, 2003) and MG (Ley, 2003) infections are widely distributed in the poultry industry worldwide, and live vaccines against these pathogens are used in Brazil. This study has shown, through the analysis of gross and microscopic lesions and confirmatory molecular detection, that the coinfection with CAV-MG vaccine strains may have important health consequences, at least in young chickens. Natural coinfection of CAV or MG with other bacteria and viruses in the field are considered common (McNeily *et al.*, 1995; Kleven, 1998; Zanella *et al.*, 2001, Toro *et al.*, 2009). It was previously reported that the MG F-strain vaccine may cause disease in the upper respiratory tract of chickens, which is possibly exacerbated when a concomitant secondary infection is present (Lin & Kleven, 1982; Yoshida *et al.*, 2000). Natural coinfections may occur in the field, under appropriate conditions, with vaccine strains, as well as with wild and more virulent strains, potentially causing more significant productivity losses (Balamurugan & Kataria, 2006; Javed *et al.*, 2005). Vaccines against CAV in breeders and MG in layers are commercially available in Brazil and the impact of the respective diseases may be reduced through vaccination. The correct vaccination of flocks must take into account bird age, vaccine strain, and dose, and should also consider the possibility of accidental coinfection. Vaccination protocols should ideally establish adequate distances among flocks, especially when very young chicks are housed nearby. The results of the present study demonstrate the synergic pathological effects of CAV-MG vaccine strains in coinfection, indicating the need to employ biosecurity strategies for vaccination in order to prevent the spread into susceptible flocks.

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