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Evaluation of the safety and efficacy of the novel *Mycoplasma gallisepticum* vaccine, Vaxsafe MG304, after spray-vaccination of 1-day-old specific pathogen-free chicks

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A R T I C L E I N F O

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A B S T R A C T

Mycoplasma gallisepticum causes chronic respiratory disease in poultry. A novel vaccine, Vaxsafe MG304 (the ts-304 strain), has greater protective efficacy in chickens than the Vaxsafe MG (strain ts-11) vaccine when delivered by eye drop at 3 weeks of age. Applying this vaccine in the hatchery to 1-day-old birds, using mass administration methods, would improve animal welfare and reduce labour costs associated with handling individual birds. This study assessed the protection provided by vaccination with Vaxsafe MG304 after administration to 1-day-old chicks. Chicks were administered a single dose of the vaccine to assess the efficacy of either a high dose ($10^{7.0}$ colour changing units, CCU) or a low dose ($10^{5.7}$ CCU) after eye drop or spray (in water or gel) administration against experimental challenge with virulent *M. gallisepticum* strain Ap3AS at 7 weeks of age. The vaccine was able to colonise the palatine cleft of chicks after vaccination by eye drop (at both doses) or by spray (in water or gel) (at the high dose). The high dose of vaccine, when delivered by eye drop or spray, was shown to be safe and induced a serological response and protective immunity (as measured by tracheal mucosal thickness and air sac lesion scores) against challenge. Vaccination of 1-day-old chicks with Vaxsafe MG304 by eye drop induced protective immunity equivalent to vaccination at 3 weeks of age. Vaxsafe MG304 was also protective when applied by both coarse- and gel spray methods at the higher dose and is therefore a suitable live attenuated vaccine for use in 1-day-old chicks.

1. Introduction

Mycoplasma gallisepticum is an important pathogen of poultry worldwide, causing chronic respiratory disease in chickens and turkeys (Whithear, 1993). Vaccines are widely used to prevent and control infectious diseases in poultry and aid in minimising the need for antimicrobial use in commercial poultry production. Live attenuated vaccines play an important role in reducing the incidence and severity of disease, improving the efficiency of agricultural production. Colonisation of the respiratory tract epithelium by *M. gallisepticum* has been shown to be mediated, in part, by the primary cytadhesin GapA (Goh et al., 1998; Keeler et al., 1996). Previous work in our laboratory discovered two ts-11 variants within the live attenuated Vaxsafe MG (strain ts-11) vaccine, one with an intact and fully functional *gapA* primary cytadhesin gene and a second with a 20 bp sequence duplication that resulted in a frameshift within the *gapA* gene (Kanci et al., 2004). A GapA + clone, strain ts-304, which constitutively expresses a functional *gapA* gene, was derived from the Vaxsafe MG (strain ts-11) vaccine and preliminary characterisation studies showed that it had considerable potential as a vaccine for the control of *M. gallisepticum* in chickens (Shil et al., 2011). Administration of a single dose of the vaccine to 4-week-old specific pathogen-free (SPF) chickens protected birds challenged with the

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M. gallisepticum wild type strain Ap3AS by aerosol at 4 weeks after vaccination, and a dose of $10^{5.5}$ colour changing units (CCU) induced a similar level of protection to that afforded by a 40 fold higher dose of the Vaxsafe MG (ts-11) vaccine (Shil et al., 2011). The ts-304 strain has subsequently been developed into a commercial vaccine, Vaxsafe MG304, which is used in the prevention of mycoplasmosis in turkeys (Kanci et al., 2018). Vaxsafe MG304 has more recently been evaluated for use in chickens and these studies have confirmed that it is a safe and effective vaccine against *M. gallisepticum* in 3 week old chickens (Kanci Condello et al., 2020b). A single dose of the Vaxsafe MG304 vaccine is capable of conferring solid protection against disease induced by experimental aerosol challenge in chickens and significant protection against colonisation by the challenge strain for at least 57 weeks after vaccination (Kanci Condello et al., 2020a). We have also shown that the protective immunity induced by Vaxsafe MG304 before the commencement of antimicrobial treatment (from 6 weeks after vaccination) provided protection against development of air sac lesions for 20 weeks after vaccination (Kanci Condello et al., 2023). Based on these studies, Vaxsafe MG304 was registered in Australia (AVPMA registration number #90403/128769) in March 2023 for use in the control of mycoplasmosis in chickens. However, all the studies conducted to date have administered Vaxsafe MG304 by eye drop at 3 weeks of age and assessed its efficacy by challenge at 7 weeks of age. Eye drop vaccination is considered the most effective means of administering live attenuated vaccines to birds as each bird receives a controlled dose of vaccine and the route induces both systemic and mucosal antigen-specific immune responses (Whithear, 1996). However, it requires handling of every individual chicken in the flock, which is laborious and expensive compared to mass administration methods (Bermudez and Stewart-Brown, 2003). There can also be welfare issues associated with catching and handling birds, so the poultry industry prefers to apply vaccines as early as possible in the production process, optimally, where possible, in the hatchery prior to transportation to the farm. A number of vaccines, including those for *Salmonella typhimurium* (Jia et al., 2020, 2023), coccidiosis (Albanese et al., 2018), Newcastle Disease (Landman et al., 2017) and infectious bronchitis (Jordan, 2017) are now applied in the hatchery using the non-invasive method of spray delivery. There are several live *M. gallisepticum* vaccines registered internationally, including the ts-11, 6/85, F and K strains (Carpenter et al., 1981; Evans and Hafez, 1992; Ferguson-Noel et al., 2012; Ferguson-Noel and Williams, 2015; Whithear et al., 1990a; Whithear et al., 1990b). These vaccines can be delivered using a variety of routes, including eye drop, drinking water and spray (Evans et al., 2013; Leigh et al., 2008a; Whithear, 1996). However, none of these live *M. gallisepticum* vaccines are indicated for use in 1-day-old chickens. Safe and effective vaccination of 1-day-old birds by eye drop would allow vaccinated breeders and layers to be shipped from the hatchery to farms, advancing the onset of protection and reducing on-farm labour costs during the rearing period. Mass administration in the hatchery by either coarse-aerosol spray or gel spray would not only reduce the need for handling long-lived birds during the rearing period, but would also allow vaccination of broiler chickens in high-challenge environments, as vaccination of these short-lived birds at 3 weeks of age by eye drop is neither practical nor beneficial. The objectives of this study were to assess the safety and efficacy of Vaxsafe MG304 when delivered to 1-day-old SPF chicks by eye drop, coarse-aerosol spray and gel spray under controlled conditions, and to compare the protection to that provided by vaccination by eye drop at 3 weeks of age.

2. Materials and methods

2.1. Mycoplasma gallisepticum strains

The Vaxsafe MG304 vaccine used in this study was a freeze-dried formulation provided by Bioproperties Pty. Ltd. (Glenorie, NSW, Australia). For vaccination by eye drop, coarse-aerosol spray and gel spray, a vial of the Vaxsafe MG304 vaccine was removed from the freezer and allowed to equilibrate to room temperature for 15 mins before being reconstituted in sterile distilled water to its original fill volume of 4.0 mL (vaccine titre of 6.31 $\times 10^8$ CCU/mL). The vial was gently rocked to ensure all the freeze-dried pellet was dissolved before diluting the vaccine to the final doses prior to inoculation.

For challenge, an ampoule of the virulent *M. gallisepticum* strain Ap3AS was removed from storage at −70°C and thawed at 37°C, inoculated into Mycoplasma broth (MB) (Soeripto et al., 1989) and incubated for 18 h at 37°C until it reached the log phase of growth. Titres were determined using a limiting dilution method and the final titre was expressed in colour changing units (CCU) (Meynell and Meynell, 1970).

2.2. Experimental design

All procedures involving animals were reviewed and approved by the University of Melbourne Animal Ethics Committee under approval number 20075. SPF White Leghorn chicks were hatched from fertile eggs supplied by Australian SPF Services Pty. Ltd. (Woodend, Victoria, Australia) and raised at the Asia-Pacific Centre for Animal Health (APCAH) animal trial facility at The University of Melbourne Veterinary Clinical Centre (Werribee, Victoria, Australia). The chicks were randomly allocated into groups and each group was housed in a separate HEPA-filtered fibreglass isolator unit under negative pressure, with feed and water provided *ad libitum*.

On the day of hatch, ninety-nine SPF chicks were divided into 9 groups: Group 1, negative control (unvaccinated and unchallenged) (n=11); Group 2, challenged only (n=11); Group 3, eye drop vaccinated with $10^{5.7}$ CCU/0.03 mL dose at 1 day of age (n=11); Group 4, eye drop vaccinated with $10^{7.0}$ CCU/0.03 mL dose at 1 day of age (n=11); Group 5, coarse-aerosol spray vaccinated with $10^{5.7}$ CCU/0.20 mL dose at 1 day of age (n=11); Group 6, coarse-aerosol spray vaccinated with $10^{7.0}$ CCU/0.20 mL dose at 1 day of age (n=11); Group 7, gel spray vaccinated with $10^{5.7}$ CCU/0.25 mL dose at 1 day of age (n=11); Group 8, gel spray vaccinated with $10^{7.0}$ CCU/0.25 mL dose at 1 day of age (n=11); and Group 9, vaccinated at 3 weeks of age by eye drop with $10^{5.7}$ CCU/0.03 mL dose (n=11). At 2 weeks of age, all birds were wing tagged with a unique number as a means of identification. At 7 weeks of age, the birds in Groups 2–9 were challenged with the virulent Ap3AS strain by nebulisation of approximately 40 mL of a culture containing 10^8 CCU/mL using compressed air into a purpose-built infection chamber (Kanci et al., 2017). Birds were monitored for clinical signs of respiratory disease associated with infection with *M. gallisepticum* over the duration of the experiment. Two weeks after challenge, all the birds were euthanised by intravenous barbiturate overdose and necropsies were performed.

2.3. Preparation and application of the Vaxsafe MG304 vaccine for eye drop, coarse-aerosol spray and gel spray administration

All vaccinations were conducted within HEPA-filtered fibreglass isolator units. Brilliant blue dye (provided by Bioproperties Pty. Ltd.) was added (1 % v/v for eye drop and coarse-aerosol spray, 0.4 % v/v for gel spray) to the vaccine dilutions to serve as a visual marker of vaccine uptake. Following vaccination, each bird was picked up (for no longer than 15 seconds), and the chick's beak held open to examine for evidence of dye on the tongue. Obvious blue colour of the tongue tip was scored as '+', light staining of just the tip was scored '+/-' and no staining was scored '-'.

For eye drop vaccination, a vial of Vaxsafe MG304 was reconstituted (as described above) before diluting the vaccine to a final dose of $10^{5.7}$ or $10^{7.0}$ CCU/0.03 mL. Each chick was gently held (with its head to one side) and, using a calibrated pipette, 0.03 mL of the vaccine was drawn up and delivered just above the cornea. The chicks were allowed to blink before being released into a holding box within the isolator. Vaccine uptake was evaluated by scoring tongue staining on all chicks 15 min

after vaccination. At 30 min after vaccination all the vaccinated chicks were released.

For coarse-aerosol spray vaccination, a vial of Vaxsafe MG304 was reconstituted in distilled water as described above and diluted to a final dose of $10^{5.7}$ or $10^{7.0}$ CCU/0.20 mL. For application, the 11 chicks were placed into a shallow plastic tub (15 cm×16 cm) within the isolator and gently misted with sterile distilled water to slightly dampen their down feathers. A spray nozzle tip (Spraying Systems Co. Pty. Ltd, Truganina, VIC, Australia) generating droplets of 100–300 μm in diameter was attached to a syringe (containing the vaccine) and held approximately 10 cm directly above the chicks to deliver a spray cone that covered the entire plastic tub. The vaccine was misted evenly over the chicks, so that the fine droplets fell into their eyes, were inhaled and/or fell onto their backs, from where they would be pecked or preened off. Vaccine uptake was evaluated by scoring tongue staining of all chicks 15 min after vaccination. At 30 min after vaccination all the vaccinated chicks were released.

For gel spray vaccination, the gel powder (provided by Bioproperties Pty. Ltd.) was prepared according to the manufacturer's instructions before reconstitution of a vial of the Vaxsafe MG304 vaccine. In brief, 32 g of the gel powder was dissolved in 996 mL of distilled water containing brilliant blue dye (0.4 % v/v). The solution was then thoroughly homogenised using a high-speed handheld emulsifier. The solution was passed through a sieve before being used to dilute the reconstituted vaccine to a final dose of $10^{5.7}$ or $10^{7.0}$ CCU/0.25 mL. The chicks were placed into a shallow plastic tub (16.2 cm×16.2 cm) within the isolator and gently misted with sterile distilled water to slightly dampen their down feathers. A plastic 8-port pipette comb (Merck Pty. Ltd, Bayswater, VIC, Australia) generating droplets of 2–3 mm in diameter was attached to a syringe (containing the vaccine) and held at an angle of approximately 45°, with the comb tips 10 cm above the chicks. The vaccine was dispensed evenly over the chicks, so that the gel droplets fell onto their heads and backs. The chicks were held in the tub within the isolator for approximately 30 mins to encourage preening. Vaccine uptake was evaluated by scoring tongue staining of all chicks at 15 min after vaccination. At 30 min after vaccination all the vaccinated chicks were released into the isolator.

2.4. Serology

Blood samples were collected from birds prior to challenge (7 weeks of age) and prior to necropsy (9 weeks of age). A recombinant protein-based indirect enzyme-linked immunosorbent assay (ELISA) was used for the detection of serum antibodies against *M. gallisepticum* (ID Screen *Mycoplasma gallisepticum* Indirect, Innovative Diagnostics, Grabels, France). Sera were diluted 1/50 and a sample to positive (S/P) ratio of $\geq$ 0.5 was considered positive, according to the manufacturer's instructions.

2.5. Examination of gross air sac lesions

Gross air sac lesions were scored on a scale of 0–4, as described previously (Kulappu Arachchige et al., 2021).

2.6. Histopathological examination

Sections of the upper trachea (approximately 3 cm distal to the larynx), middle trachea and lower trachea were fixed in 10 % neutral buffered formalin, embedded in wax, and sections then cut and stained with haematoxylin and eosin. The thicknesses of the tracheal mucosae of each bird were calculated by averaging the measurements taken at six points transected by vertical, horizontal and diagonal lines at 400 × magnification using a light microscope with a calibrated eyepiece. The mean mucosal thickness of the trachea of each bird was determined as described previously (Wijesurendra et al., 2015).

2.7. Assessment of colonisation with Vaxsafe MG304 by real-time PCR (RT-PCR)

Palatine cleft swabs (flocked-tipped sterile swab with a plastic applicator; Copan, Murrieta, CA, USA), pre-moistened with phosphate buffered saline (PBS), were collected from birds in Groups 3, 4, 5, 6, 7 and 8 at 2, 4 and 6 weeks of age and from birds in Groups 1, 2 and 9 at 7 weeks of age and kept at 4°C until testing. DNA was extracted from the palatine cleft swabs using the Maxwell RSC Buccal Swab DNA kit (Promega, Auburn, VIC, Australia) and the Maxwell RSC 48 Instrument (Promega, Auburn, VIC, Australia). PCR detection of *M. gallisepticum* DNA was performed as described previously (Callison et al., 2006). The primers used were mglpU26-F (5'-CTAGAGGGTTGGACAGTTATG-3') and mglp164-R (5'-GCTGCACTAAATGATACGTCAAA-3'). The mglp-P Taqman probe used had the sequence 5'-FAM-CAGTCATTAACAACTTACCACCAGAATCTG-BHQ1–3'. Each assay was performed in a total volume of 25 μL containing 12.5 μL QuantiNova Probe PCR 2 × Master Mix (Qiagen, Chadstone, VIC, Australia), 0.2 μM of each primer, 0.2 μM of the probe and 5 μL of template. The assay was incubated in a Rotor-Gene Q thermal cycler (Qiagen, Chadstone, VIC, Australia) at 95°C for 2 min, then through 45 cycles of 95°C for 5 s and 56°C for 10 s. For each assay, the cycle threshold (Ct value) was determined, and any assay that generated a Ct value of $\leq$ 37.8 was considered positive.

2.8. Re-isolation of Mycoplasma gallisepticum at necropsy

Sterile cotton swabs (sterile plain cotton swab with a plastic applicator; Copan, Murrieta, CA, USA), pre-moistened with Mycoplasma broth (MB), were collected from the upper trachea, lower trachea and the left and right abdominal air sacs of all birds at necropsy. Each of the swabs was introduced into a separate 2 mL volume of MB, which was then incubated at 37°C and examined daily for an acidic colour change indicative of mycoplasma growth. DNA was extracted from 0.2 mL samples of each of the broths showing an acidic colour change using the Applied Biosystems MagMAX CORE Nucleic Acid Purification Kit (Thermo Fisher Scientific, Scoresby, VIC, Australia) and the Invitrogen KingFisher Flex Purification System (Thermo Fisher Scientific, Scoresby, VIC, Australia). PCR assays of the extracted DNA to detect and differentiate the *M. gallisepticum* strains were performed as described previously (Garcia et al., 2005; Lysnyansky et al., 2005). The primers used to confirm the presence of *M. gallisepticum* DNA were *mgc2* 2 F (CGCAATTTGGTCCTAATCCCCAACA) and *mgc2* 2 R (TAAACCCACCTCCAGCTTTATTTCC). Each PCR assay was performed in a total volume of 25 μL, containing 0.1 mM dNTPs (Bioline, Alexandria, NSW, Australia), 2.0 mM $MgCl_2$ (Promega, Auburn, VIC, Australia), 100 nM of each primer, 2.5 U of Taq polymerase (5 U/μL) (Promega, Auburn, VIC, Australia) and 5 μL of template. The assay was incubated at 94°C for 3 min, then through 35 cycles of 94°C for 30 s, 58°C for 30 s, and 72°C for 1 min. The expected size of the amplification products for Vaxsafe MG304 and Ap3AS were 290 and 225 base pairs, respectively.

2.9. Statistical analyses

Tests of normality and log-normality were performed on all data, except the air sac lesion scores, before further analyses were performed. The median air sac lesion scores for each group were compared using the Mann-Whitney U test. Normally distributed mean S/P ELISA ratios and normally distributed mean tracheal mucosal thickness scores were compared using one-way ANOVAs. All statistical analyses were conducted using GraphPad Prism version 10 (GraphPad Software, La Jolla, CA, USA).

Table 1
Serum antibodies against *M. gallisepticum* in birds prior to and 2 weeks after challenge.

Group	Treatment	Administration Route	Before challenge		Two weeks after challenge	
			Proportion Positive in MG ELISA	Mean S/P ratio ± SEM	Proportion Positive in MG ELISA	Mean S/P ratio ± SEM
1	Sterile diluent	Eye drop	0/11	0.0 ± 0.0	0/11	0.1 ± 0.0
2	Sterile diluent^	Eye drop	0/11	0.0 ± 0.0	8/11	1.2 ± 0.3
3	MG304 $10^{5.7}$ CCU^	Eye drop	10/11	2.8 ± 0.5*	10/11	2.6 ± 0.4*
4	MG304 $10^{7.0}$ CCU^	Eye drop	10/11	3.3 ± 0.4*	11/11	3.5 ± 0.2*
5	MG304 $10^{5.7}$ CCU^	Coarse-aerosol spray	0/11	0.0 ± 0.0	7/11	1.1 ± 0.4
6	MG304 $10^{7.0}$ CCU^	Coarse-aerosol spray	8/11	2.4 ± 0.6*	10/11	2.5 ± 0.4*
7	MG304 $10^{5.7}$ CCU^,†	Gel spray	1/10	0.1 ± 0.1	8/10	1.8 ± 0.4
8	MG304 $10^{7.0}$ CCU^	Gel spray	8/11	2.2 ± 0.5*	11/11	2.4 ± 0.4*
9	MG304 $10^{5.7}$ CCU^,#	Eye drop	5/11	0.8 ± 0.3	11/11	1.9 ± 0.2

SEM, standard error of the mean; S/P ratio, sample to positive ratio.
^ Challenged with $10^{8.0}$ CCU/mL of wild type strain Ap3AS
# vaccinated at 3 weeks of age
* significantly different from the Challenged only control (Group 2) in the same column ($P < 0.05$)
† one bird died prior to the completion of the study and was omitted from the analyses.

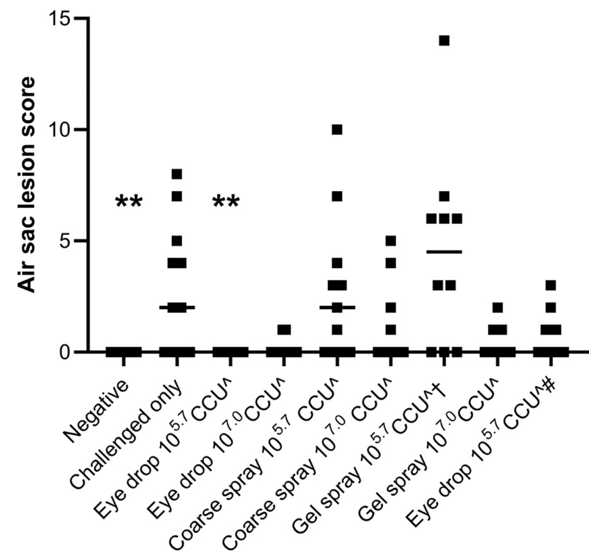

Fig. 1. Median air sac lesion score. ^Challenged with $10^{8.0}$ CCU of wild type strain Ap3AS/mL; #vaccinated at 3 weeks of age; **, $p<0.01$ significantly lower than the Challenged only control; †one bird died prior to the completion of the study and was omitted from the analyses. The horizontal lines indicate the median.

3. Results

3.1. Concentration of vaccine and challenge cultures

The average concentration of the reconstituted Vaxsafe MG304 vaccine was 6.31 $\times 10^8$ CCU/mL and the final doses delivered were confirmed retrospectively for all treatment groups. The average concentration of the Ap3AS challenge culture used for challenge at 7 weeks after vaccination was 2.26 $\times 10^8$ CCU/mL.

3.2. Clinical observations

All chicks appeared healthy at the commencement of the study. No signs of respiratory distress or any other disease were observed throughout the duration of the experiment. One bird vaccinated with $10^{5.7}$ CCU of Vaxsafe MG304 by gel spray died prior to the completion of the study and was omitted from the analyses. A necropsy was performed, and the necropsy findings determined that the cause of death was Ascites Syndrome and not a result of vaccination with Vaxsafe MG304 or challenge with the Ap3AS strain.

3.3. Vaccine up-take by eye drop and spray routes

At 1 day of age, 100 % of chicks vaccinated with $10^{5.7}$ CCU and 82 % of chicks vaccinated with $10^{7.0}$ CCU by the eye drop route, 91 % of chicks vaccinated with $10^{5.7}$ CCU and 100 % of chicks vaccinated with $10^{7.0}$ CCU by coarse-aerosol spray, and 82 % of chicks vaccinated with $10^{5.7}$ CCU and 82 % of chicks vaccinated with $10^{7.0}$ CCU by gel spray had obvious blue coloration of the tongue tip. All of the birds vaccinated at 3 weeks of age with $10^{5.7}$ CCU had obvious blue coloration of the tongue tip.

3.4. Serological analyses

The birds were bled at 7 and 9 weeks after vaccination and the serological response to *M. gallisepticum* was determined by ELISA. No antibodies against *M. gallisepticum* were detected in the serum of any chickens in the negative control group (unvaccinated and unchallenged) at any time point throughout the experiment (Table 1). At 7 weeks after

Table 2
Gross pathology and mean tracheal mucosal thicknesses of birds in each treatment group.

Group	Treatment	Administration Route	Median air sac lesion score (IQR)	Proportion of birds with air sac lesions	Mean tracheal mucosal thickness (μm) ± SEM			
					Upper	Middle	Lower	Average
1	Sterile diluent	Eye drop	0 (0,0)*	0/11*	62.3 ± 2.8*	50.1 ± 2*	46.2 ± 1.6*	52.9 ± 1.8*
2	Sterile diluent^	Eye drop	2 (0,5)	7/11	159.4 ± 36.3	150.4 ± 35	119.3 ± 21.2	143.0 ± 30.1
3	MG304 $10^{5.7}$ CCU^	Eye drop	0 (0,0)*	0/11*	70.9 ± 4.0*	59.3 ± 2.6*	55.4 ± 2.8	62.5 ± 3.0*
4	MG304 $10^{7.0}$ CCU^	Eye drop	0 (0,0)	2/11	60.7 ± 2.0*	53.6 ± 1.3*	48.8 ± 1.1*	54.4 ± 1.0*
5	MG304 $10^{5.7}$ CCU^	Coarse-aerosol spray	2 (0,4)	7/11	234.2 ± 31.4	210.2 ± 31.6	183.3 ± 24.7*	209.3 ± 28.2
6	MG304 $10^{7.0}$ CCU^	Coarse-aerosol spray	0 (0,2)	4/11	92.8 ± 18.6	83.4 ± 19.6	76.8 ± 16.8	84.3 ±18.3
7	MG304 $10^{5.7}$ CCU^†	Gel spray	4.5 (0,6.25)	7/10	245.8 ± 28.2*	222.9 ± 29.9	204.6 ± 25.6*	228.2 ± 24.9*
8	MG304 $10^{7.0}$ CCU^	Gel spray	0 (0,1)	4/11	67.0 ± 3.2*	60.3 ± 2.5*	58.0 ± 4.1	62.2 ± 2.8*
9	MG304 $10^{5.7}$ CCU^#	Eye drop	0 (0,1)	5/11	92.4 ± 24.9	82.7 ± 26.4	49.0 ± 2.1	82.0 ± 24.4

IQR, interquartile range; SEM, standard error of the mean; μm, micrometre
^ Challenged with $10^{8.0}$ CCU/mL of wild type strain Ap3AS
# vaccinated at 3 weeks of age
* significantly different from the Challenged only control (Group 2) in the same column ($P < 0.05$)
† one bird died prior to the completion of the study and was omitted from the analyses.

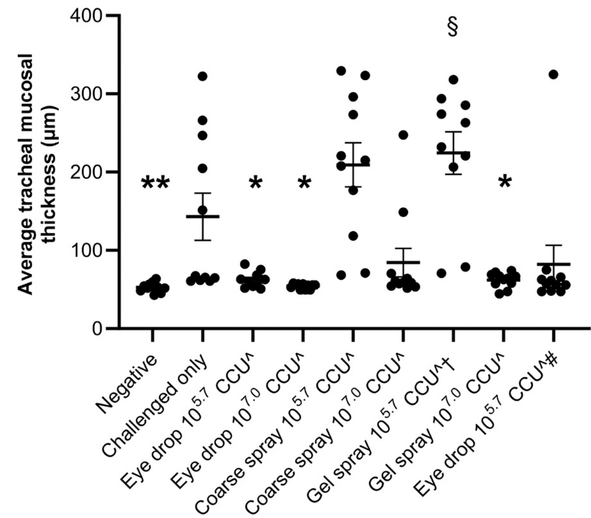

Fig. 2. Average tracheal mucosal thicknesses. ^Challenged with $10^{8.0}$ CCU of wild type strain Ap3AS/mL; #vaccinated at 3 weeks of age; **, p<0.01 and *, p<0.05 significantly lower than the Challenged only control; §, p<0.05 significantly higher than the Challenged only control; †one bird died prior to the completion of the study and was omitted from the analyses. The horizontal lines indicate the mean.

vaccination, 10/11 birds in the day-old eye drop vaccinated groups (both $10^{5.7}$ and $10^{7.0}$ CCU/dose) were positive by ELISA for serum antibodies against *M. gallisepticum* (S/P ≥ 0.5), while 0/11 (in the $10^{5.7}$ CCU dose group) and 8/11 (in the $10^{7.0}$ CCU dose group) birds in the coarse-aerosol spray groups, and 1/10 (in the $10^{5.7}$ CCU dose group) and 8/11 (in the $10^{7.0}$ CCU dose group) birds in the gel spray groups were positive by ELISA (Table 1). Five of 11 birds in Group 9, which were eye drop vaccinated with $10^{5.7}$ CCU/dose at 3 weeks of age, were also positive. The mean S/P ratios of the birds in the challenged only group differed significantly from those of the birds in both the day-old eye drop vaccinated groups ($10^{5.7}$ and $10^{7.0}$ CCU), and those of the birds in the groups vaccinated by coarse-aerosol spray and gel spray with a dose of $10^{7.0}$ CCU. The rates of seropositivity were highest in the groups administered a dose of $10^{7.0}$ CCU, regardless of the route, although the groups vaccinated by the eye drop route had a slightly higher proportion of ELISA positive birds than those vaccinated by coarse-aerosol or gel spray.

At 9 weeks after vaccination, a greater number of birds vaccinated with $10^{5.7}$ CCU by spray (coarse-aerosol or gel) had detectable levels of serum antibodies against *M. gallisepticum*, but the proportion of ELISA positive birds was higher in the birds vaccinated by eye drop (1 day of age or 3 weeks of age) with the same dose. The mean S/P ratios were also significantly higher in the eye drop vaccinated groups ($10^{5.7}$ and $10^{7.0}$ CCU), and the groups vaccinated by coarse-aerosol spray or gel spray with a dose of $10^{7.0}$ CCU, than in the challenged only group (Table 1).

3.5. Pathological analyses

Necropsies were conducted on all the birds at the completion of the study. Their external appearance was assessed, examining them for signs such as changes in feathering, evidence of diarrhoea, level of hydration and any discharges (nasal or ocular), and any observations were recorded. The organs of the cardiovascular, digestive and urogenital systems were then examined, and any findings recorded. To determine whether vaccination with Vaxsafe MG304 by eye drop or spray provided protective immunity, the respiratory tract pathology in the groups vaccinated with Vaxsafe MG304 was compared to that in the challenged only group.

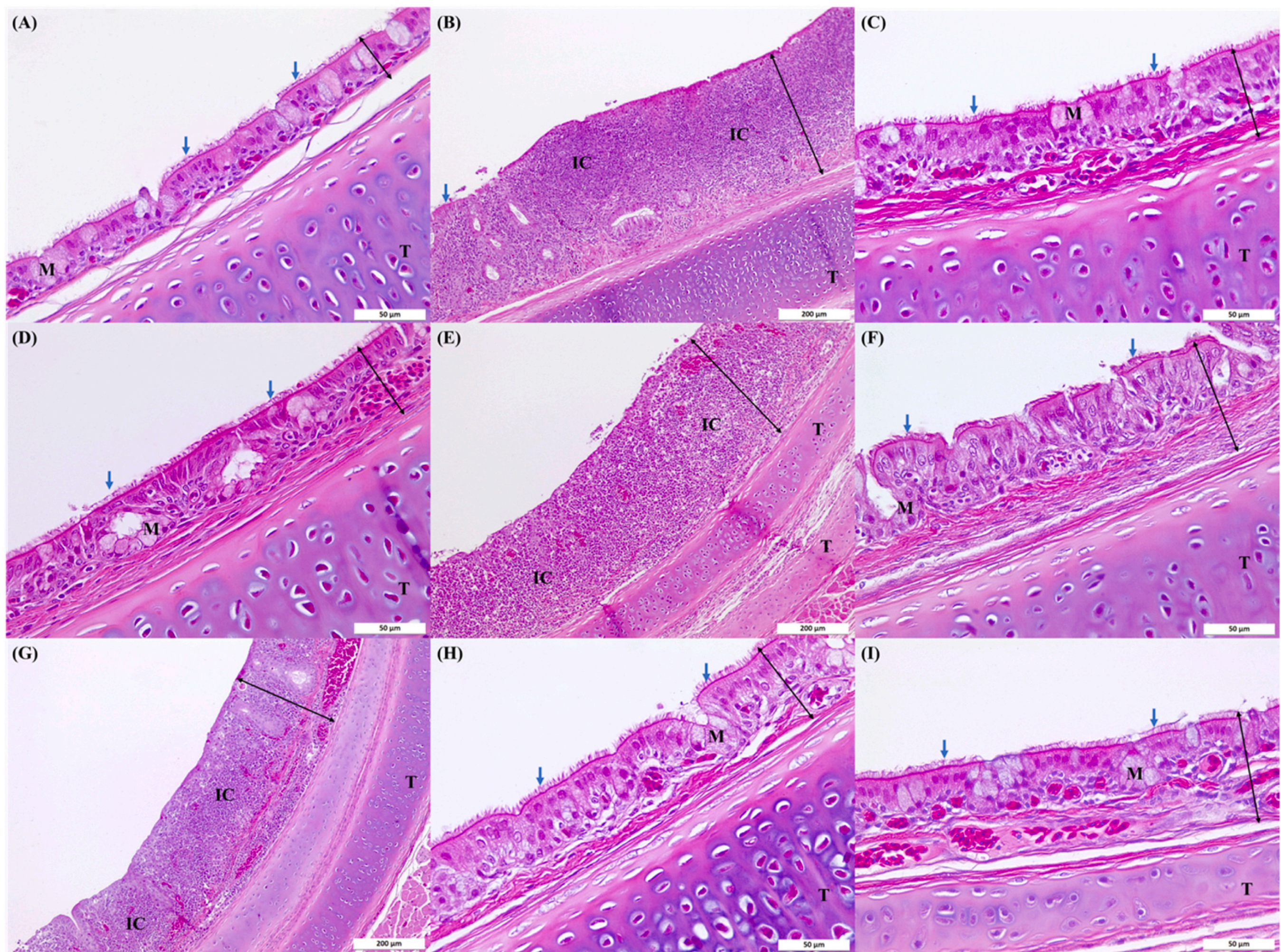

Fig. 3. Photomicrographs of upper tracheal sections from representative birds in each group stained with H & E. (A) Negative control, (B) Challenged only control, (C) Eye drop vaccinated at 1 day of age with $10^{5.7}$ CCU, (D) Eye drop vaccinated at 1 day of age with $10^{7.0}$ CCU, (E) Coarse-aerosol spray vaccinated at 1 day of age with $10^{5.7}$ CCU, (F) Coarse-aerosol spray vaccinated at 1 day of age with $10^{7.0}$ CCU, (G) Gel spray vaccinated at 1 day of age with $10^{5.7}$ CCU, (H) Gel spray vaccinated at 1 day of age with $10^{7.0}$ CCU, (I) Eye drop vaccinated at 3 weeks of age with $10^{5.7}$ CCU. Pseudostratified columnar epithelia, with intact cilia (**blue arrows**) and intraepithelial mucous glands (**M**) were visible in A, C, D, F, H and I. Diffuse infiltration of inflammatory cells (**IC**), thickening of the tracheal mucosae and loss of cilia were observed in B, E and G. **T**, tracheal cartilage; **double headed arrow**, tracheal mucosal thickness. Bar in A, C, D, F, H, and I = 50 μm. Bar in B, E and G = 200 μm.

3.5.1. Air sac lesion scores

In the eye drop vaccinated groups ($10^{5.7}$ and $10^{7.0}$ CCU/dose), and the spray vaccinated groups (both coarse-aerosol and gel; $10^{7.0}$ CCU/dose only), the birds had less severe air sac lesions and lower proportions of birds had air sac lesions than in the challenged only group, and the groups vaccinated by coarse-aerosol or gel spray with $10^{5.7}$ CCU/dose (Fig. 1, Table 2). However, only the birds in the group vaccinated by eye drop at 1 day of age with a dose of $10^{5.7}$ CCU/dose had air sac lesion scores that were significantly less severe than those of birds in the challenged only group (Table 2).

3.5.2. Tracheal mucosal thicknesses

The mean tracheal mucosal thicknesses of the unvaccinated and unchallenged group and the day-old eye drop vaccinated ($10^{7.0}$ CCU/dose) group were significantly lower than those of challenged only group in all three regions of the trachea (Fig. 2, Table 2). Similarly, the mean mucosal thicknesses in the upper and middle regions of the trachea of the day-old eye drop ($10^{5.7}$ CCU/dose) and gel spray ($10^{7.0}$ CCU/dose) vaccinated birds were significantly lower than those of the challenged only birds (Table 2). However, the mean upper and lower tracheal mucosal thicknesses in the gel spray vaccinated ($10^{5.7}$ CCU/dose) birds and the mean lower tracheal thickness of the coarse-aerosol spray vaccinated ($10^{5.7}$ CCU/dose) birds were significantly higher than those of the challenged only birds (Table 2). None of the mean mucosal thicknesses of the different tracheal regions in the 3-week-old eye drop vaccinated ($10^{5.7}$ CCU/dose) or the coarse-aerosol spray vaccinated ($10^{7.0}$ CCU/dose) group 5 were significantly different from those of challenged only group (Table 2). The tracheal mucosae of birds in the challenged only group had prominent lesions along the trachea, with increased thickness, diffuse infiltration of inflammatory cells, deciliation, disintegration of the pseudostratified columnar epithelium and reduction or loss of intraepithelial mucous glands and goblet cells (Fig. 3B). Similar lesions were observed throughout the trachea in the birds vaccinated with the low dose by coarse-aerosol spray (Fig. 3E) or gel spray (Fig. 3G). These lesions were not seen in the birds from the other vaccinated groups and the tracheal mucosae had pseudostratified columnar epithelia with intact cilia, and there were abundant intraepithelial mucous glands and mucus secreting cells (Fig. 3A, C, D, F, H and I).

3.6. Colonisation by Vaxsafe MG304, as determined by RT-PCR

Swabs collected from the palatine cleft of birds in Groups 3, 4, 5, 6, 7 and 8 at 2, 4 and 6 weeks after vaccination, and from birds in Groups 1, 2

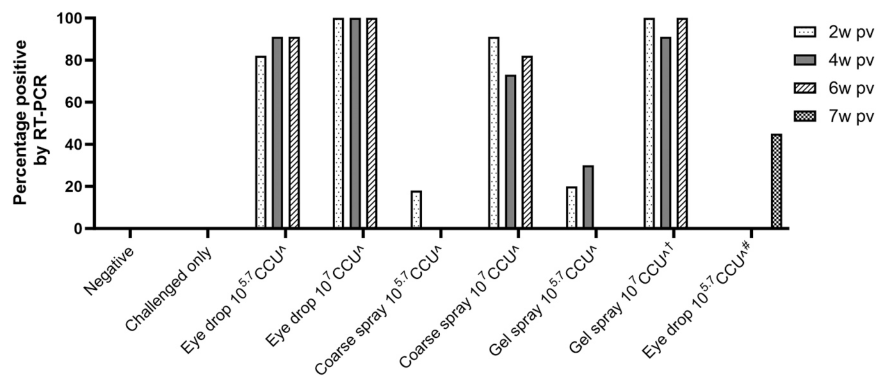

Fig. 4. Colonisation of the Vaxsafe MG ts-304 vaccine in birds at 2, 4, 6 and 7 weeks after vaccination. Number of birds positive (n=11); ^birds were challenged with $10^{8.0}$ CCU/mL of wild type strain Ap3AS; #vaccinated at 3 weeks of age; †one bird in this group died prior to the completion of the study and was omitted from the analyses.

and 9 at 7 weeks after vaccination, were analysed by RT-PCR to assess colonisation by Vaxsafe MG304. Vaxsafe MG304 was detected in most birds vaccinated by eyedrop at 1 day of age with $10^{5.7}$ CCU/dose at 2 (9/11), 4 (10/11) and 6 (10/11) weeks after vaccination. It was also detected in all birds (100 %) vaccinated by eye drop at 1 day of age with $10^{7.0}$ CCU/dose at 2, 4 and 6 weeks after vaccination and in 5/11 birds vaccinated by eye drop at 3 weeks of age with $10^{5.7}$ CCU/dose at 7 weeks after vaccination. The vaccine was only detected in 2/11 birds vaccinated by coarse-aerosol spray with $10^{5.7}$ CCU/dose at 2 weeks after vaccination. However, it was detected in a greater number of birds vaccinated with a dose of $10^{7.0}$ CCU by coarse-aerosol spray at 2 (10/11), 4 (8/11) and 6 (9/11) weeks after vaccination. In the gel spray groups, the vaccine was detected in a smaller proportion of birds in the group vaccinated with a dose of $10^{5.7}$ CCU, and only at 2 (2/11) and 4 (3/11) weeks after vaccination, while in the group vaccinated with a dose of $10^{7.0}$ CCU colonisation was detected in most or all birds at 2 (11/11), 4 (10/11) and 6 (11/11) weeks after vaccination. Vaxsafe MG304 was not detected in any birds in Groups 1 and 2 (unvaccinated unchallenged controls and challenged only) at 7 weeks after vaccination of groups 3 to 8 (Fig. 4).

3.7. Re-isolation of Vaxsafe MG304 and M. gallisepticum strain Ap3AS at necropsy

Swabs were collected from the upper trachea, lower trachea and air sacs (left and right) for re-isolation of *M. gallisepticum* at necropsy. Inoculated broth cultures exhibiting signs of mycoplasma growth were tested using an *mgc2* PCR assay, which distinguishes the Vaxsafe MG304 vaccine strain from the Ap3AS challenge strain by amplicon size. Vaxsafe MG304 was isolated at 9 weeks after vaccination from the upper trachea of 1/11 birds in the groups vaccinated by eye drop at 1 day of age with a dose of $10^{5.7}$ CCU, coarse-aerosol spray with a dose of $10^{7.0}$ CCU and eye drop at 3 weeks of age with a dose of $10^{5.7}$ CCU, in 3/11 birds in the group vaccinated at 1 day of age by eye drop with a dose of $10^{7.0}$ CCU, and in 6/11 birds in the group vaccinated by gel spray with a dose of $10^{7.0}$ CCU. In contrast, Vaxsafe MG304 was not isolated from the lower trachea of any bird in any treatment group, but was isolated from the air sacs of 1/11 birds in each of the groups vaccinated by eye drop at 1 day of age with a dose of $10^{7.0}$ CCU and gel spray with a dose of $10^{7.0}$ CCU/dose (Table 3). The Ap3AS challenge strain was detected in a high proportion of birds in the challenged only group at each of the locations sampled in the respiratory tract. Strain Ap3AS was more commonly isolated from the upper and lower trachea in the groups vaccinated at 1 day of age by eye drop or by coarse-aerosol or gel spray (both $10^{5.7}$ and $10^{7.0}$ CCU/dose groups) than from the air sacs (Table 3).

4. Discussion

This study assessed the protection provided by vaccination of 1-day-old SPF chicks with the Vaxsafe MG304 vaccine against challenge with the wild-type strain, Ap3AS. Vaccine safety and efficacy were compared after administering a low ($10^{5.7}$ CCU) or high ($10^{7.0}$ CCU) dose by eye drop or using two different spray methods. Vaccination at 1-day-old was also compared to eye drop vaccination at 3 weeks of age with $10^{5.7}$ CCU/dose. There was clear evidence that spray vaccination (as a coarse aerosol or in gel) of 1-day-old chicks with a dose of $10^{7.0}$ CCU of the Vaxsafe MG304 vaccine offered significant protection against tracheitis (as measured by tracheal mucosal thickening) and the protection

Table 3
Re-isolation of *M. gallisepticum* at necropsy.

Group	Treatment	Administration Route	No. positive* by culture in MG broth for *M. gallisepticum*					
			Upper Trachea		Lower Trachea		Air Sacs (left and right)	
			Vaxsafe ts-304	Ap3AS	Vaxsafe ts-304	Ap3AS	Vaxsafe ts-304	Ap3AS
1	Sterile diluent	Eye drop	0	0	0	0	0	0
2	Sterile diluent^	Eye drop	0	11	0	9	0	8
3	MG304 $10^{5.7}$ CCU^	Eye drop	1	9	0	7	0	3
4	MG304 $10^{7.0}$ CCU^	Eye drop	3	10	0	7	1	3
5	MG304 $10^{5.7}$ CCU^	Coarse-aerosol spray	0	11	0	11	0	8
6	MG304 $10^{7.0}$ CCU^	Coarse-aerosol spray	1	10	0	10	1	4
7	MG304 $10^{5.7}$ CCU^,†	Gel spray	0	10	0	9	0	8
8	MG304 $10^{7.0}$ CCU^	Gel spray	6	5	0	6	0	2
9	MG304 $10^{5.7}$ CCU^,#	Eye drop	1	7	0	8	0	4

* number of birds yielding a culture showing colour change that were positive by mgc2 PCR (with strain differentiation by PCR product size)
^ Challenged with $10^{8.0}$ CCU/mL of wild type strain Ap3AS
\# vaccinated at 3 weeks of age
† one bird in this group died prior to the completion of the study and was omitted from the analyses.

provided by these spray applications was comparable to vaccination by eye drop at the lower dose.

Live *M. gallisepticum* vaccines can be delivered using a variety of routes, including spray, eye drop and drinking water (Ley and Yoder, 2008), and the site of vaccine deposition has been shown to be an important factor influencing the outcome of vaccination. Spray vaccination is an inexpensive method of vaccine delivery and increasingly the preferred means of mass application for poultry producers, as many birds in large commercial breeding flocks can be vaccinated in poultry farm sheds simultaneously and the close proximity of the birds in these sheds facilitates lateral transfer of the vaccine. As a consequence, a number of studies have been performed to improve vaccine delivery by spray, including determining the optimal solution temperature, standardising delivery parameters such as nozzle type and pressure, and developing improved stabilisers for the live vaccines (Evans et al., 2009; Leigh et al., 2008b; Purswell et al., 2008). On the other hand, eye drop administration is a labour-intensive means of administering live attenuated vaccines, and, because of the need to individually handle each chicken (Bermudez and Stewart-Brown, 2003), imposes additional costs on farmers. However, this cost can be outweighed by the significant increase in vaccination efficacy in flocks vaccinated using this method (Leigh et al., 2018).

In this study, we showed that spray vaccination (either in a coarse-aerosol or gel) resulted in high colonisation rates when delivered at the higher dose of $10^{7.0}$ CCU, but lower rates of colonisation when delivered at the lower dose of $10^{5.7}$ CCU. These results were similar to those obtained in a previous study in which a live vaccine sprayed onto the body of commercial laying hens resulted in lower rates of seroconversion than application by eye drop (Evans et al., 2015). This was attributed, in part, to a portion of the spray-applied vaccine falling into the environment, and onto un-targeted areas of the birds, such as their backs and feathers, rather than being deposited directly onto the mucosal surface of the eyes, inhaled through the nares, or ingested during preening (Cargill, 1999). The lyophilised Vaxsafe MG304 vaccine used in our study was prepared in water or in a gel prior to spray application. The gel is viscous and needs to be completely homogenised prior to the addition of the vaccine, and then gently mixed to ensure a uniform distribution for vaccination. Like spraying in water, gel spraying relies on the droplets landing on the head, face and body of the birds, with subsequent preening resulting in uptake of the vaccine as a result of contact with the eyes, or by ingestion, presumably resulting in colonisation of the palatine cleft during swallowing. The results obtained in the spray vaccination groups in the studies shown here are consistent with previous studies that have reported that vaccine uptake through preening of feathers is less efficient in generating an adequate host immune response than application by eye drop (Leigh et al., 2018). The efficacy of the ts-11 vaccine has been investigated previously after vaccination of chickens at 1, 2, 4 and 6 weeks of age by eye drop (Gaunson et al., 2006) and after vaccination by eye drop or aerosol at 5 weeks of age (Noormohammadi and Whithear, 2019). Other studies have also compared the efficacy of the ts-11, 6/85 and F strain vaccines in chickens vaccinated by eye drop at 10 days of age (Abd-el-Motelib and Kleven, 1993) and the efficacy of vaccination with F strain by eye drop in 1-day-old broiler chicks (Muofaq Khalaf and Jawad Ali, 2023; Rodriguez and Kleven, 1980). The results of the study described here confirmed that chicks administered a live attenuated *M. gallisepticum* vaccine at 1 day of age can respond immunologically to vaccination. In our study, high serum concentrations of IgG against *M. gallisepticum* were detected in the chicks vaccinated with a high dose by spray administration (coarse-aerosol or gel), and in the chicks vaccinated with both low and high doses by eye drop, at 7 weeks after vaccination, indicating that a strong systemic antibody response was induced by Vaxsafe MG304 when it was administered at 1 day of age, similar to the response seen to eye drop vaccination at 3 weeks of age (Kanci Condello et al., 2020b).

Previous work in our laboratory has established that the Vaxsafe MG304 vaccine is able to prevent the development of air sac lesions after challenge when birds are vaccinated by eye drop at 3 weeks of age (Kanci Condello et al., 2020b). Here we have shown that chicks administered the Vaxsafe MG304 vaccine by eye drop at 1 day of age (at a higher dose) were protected against disease caused by challenge with an aerosol of the virulent Ap3AS strain, as demonstrated by a reduced incidence and severity of airsacculitis.

Histological examination of the tracheal mucosa has been found to be the most sensitive measure of the chronic respiratory disease caused by virulent *M. gallisepticum* (Kulappu Arachchige et al., 2022). Here we have shown that the mean tracheal mucosal thicknesses of birds vaccinated at 1 day of age by spray with a $10^{7.0}$ dose of Vaxsafe MG 304 in gel or by eye drop with a dose of $10^{5.7}$ or $10^{7.0}$ were significantly less than those of unvaccinated birds after challenge. The birds vaccinated by spray with the lower dose had greater mean mucosal thicknesses than the unvaccinated controls in some regions of the trachea. It is possible that the administration of a lower dose of the vaccine resulted in partial protection and that this influenced the chronology of the pathological response in some regions of the trachea. Subsequent time course studies of the development of tracheal lesions after vaccination with low doses of this vaccine will be needed to further explore this.

The Vaxsafe MG304 vaccine colonised and persisted in the respiratory tract of birds at 2 weeks after vaccination at 1 day of age, with the highest colonisation rates seen in the groups vaccinated with $10^{7.0}$ CCU by coarse-aerosol or gel spray and in the groups vaccinated with $10^{7.0}$ CCU or $10^{5.7}$ CCU by eye drop. The groups that received higher doses had greater detection rates at 2, 4 and 6 weeks after vaccination. The expression of the cytadhesin GapA in Vaxsafe MG304 most probably explains its capacity to bind efficiently to the upper respiratory tract mucosa, as cytadherence to the epithelial surfaces of the trachea has been shown to be a requirement for successful colonisation (Goh et al., 1998). Vaxsafe MG304 retains the temperature sensitive phenotype of its Vaxsafe ts-11 parent (Shil et al., 2011).

Eye drop vaccination at 1 day of age induced a stronger serological response and appeared to provide better protection against challenge with wild-type *M. gallisepticum* than eye drop vaccination at 3 weeks of age, although the difference was not significant. This finding was unexpected, as our previous studies have shown that vaccination with Vaxsafe MG304 by eye drop at 3 weeks of age with a dose of $10^{6.0}$ CCU induces significant levels of protective immunity against challenge (Kanci Condello et al., 2020a, 2023).

The development and maturation of the immune system of chicks after hatching could play an important role in the development of protective immunity against mycoplasmosis. Previous studies in our laboratory have used transcriptomic analyses to help provide a better understanding of the protective immunity afforded by vaccination with Vaxsafe MG304 against chronic infection with *M. gallisepticum* (Kulappu Arachchige et al., 2020a, 2020b). Similar analyses of birds vaccinated at 1 day of age, and comparison of these with our previous studies of birds vaccinated at an older age, may provide further insights into any differences in the protective immune responses induced by vaccination against *M. gallisepticum* at different ages.

Based on the overall findings of our study, administration of the Vaxsafe MG304 live attenuated vaccine by coarse-aerosol or gel spray can be an effective method of delivery. Here we describe the first study investigating a gel-based delivery method for the administration of a live attenuated mycoplasma vaccine. Use of gel spray administration has several advantages, including the capacity to integrate vaccination into an automated process for delivery to day old birds at the hatchery prior to their transport to the farm. Gel spray application of probiotics and live *Eimeria* vaccines has been widely adopted in the poultry industry, so the capacity to deliver Vaxsafe MG304 by this route would enable integration of its use into routine vaccination programs already in place for longer lived birds.

In conclusion, our study has demonstrated that Vaxsafe MG304 is a safe and effective vaccine against disease caused by virulent

M. gallisepticum when administered to 1-day-old chicks and that levels of protection comparable to that provided by eye drop vaccination can be achieved by spray vaccination, albeit at a higher dose.

CRediT authorship contribution statement

Anna Kanci Condello: Conceptualization, Methodology, Investigation, Resources, Writing - Original Draft, Writing - Review & Editing, Visualization, Project administration. **Nadeeka K. Wawegama**: Conceptualization, Methodology, Formal analysis, Investigation, Writing - Review & Editing, Funding acquisition. **Dilhani Ekanayake**: Formal analysis, Investigation, Writing - Review & Editing. **Ling Zhu**: Formal analysis, Investigation, Writing - Review & Editing. **Kelly A. Tivendale**: Investigation, Writing - Review & Editing. **Pollob K. Shil**: Investigation, Resources, Writing - Review & Editing. **June Daly**: Investigation, Resources, Writing - Review & Editing. **Sameera Mohotti**: Formal analysis, Investigation, Writing - Review & Editing. **Philip Todhunter**: Investigation, Resources, Writing - Review & Editing. **Gregory J. Underwood**: Conceptualization, Methodology, Investigation, Writing - Review & Editing, Supervision. **Amir H. Noormohammadi**: Investigation, Writing - Review & Editing, Funding acquisition. **Philip F. Markham**: Conceptualization. **Glenn F. Browning**: Conceptualization, Methodology, Investigation, Writing - Review & Editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

Gregory J. Underwood, Philip Todhunter and Sameera Mohotti are employees of Bioproperties Pty. Ltd. The University of Melbourne licences the *M. gallisepticum* ts-11 and Vaxsafe MG304 vaccines to Bioproperties Pty. Ltd. and, as employees of the university involved in the creation of Vaxsafe MG304, A.KC, P.K.S, P.F.M and G.F.B are entitled to a share of any royalties generated from this license. The other authors have no potential conflicts of interest (financial, professional or personal) related to the research reported here.

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