GapA⁺ *Mycoplasma gallisepticum* ts-11 has improved vaccine characteristics

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Mycoplasma gallisepticum (MG) is an important poultry pathogen that causes respiratory disease and loss of production worldwide, and is currently controlled with live attenuated vaccines. These vaccines have limitations as they vary in their pathogenicity, the protection afforded and their transmissibility, but have been shown to effectively reduce losses associated with challenge in the field. A live attenuated vaccine, ts-11, has been used for the control of *M. gallisepticum* in several countries. This vaccine is highly dose-dependent and the flock antibody response is weak. GapA is the primary cytoadherence molecule in *M. gallisepticum*, and the absence of GapA expression has been observed in the vast majority of cells in the ts-11 vaccine strain. In this study the immunogenicity of a GapA⁺ *M. gallisepticum* ts-11 vaccine was investigated in specific-pathogen-free chickens. Birds vaccinated with GapA⁺ *M. gallisepticum* ts-11 were protected against clinical signs of disease following challenge with virulent *M. gallisepticum*, and GapA⁺ *M. gallisepticum* ts-11 was shown to be non-pathogenic and more immunogenic at a lower dose than the currently available *M. gallisepticum* ts-11 vaccine. Thus, GapA⁺ *M. gallisepticum* ts-11 appears to have improved potential as a vaccine candidate.

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INTRODUCTION

Mycoplasma gallisepticum (MG) is a major pathogen of gallinaceous and some non-gallinaceous avian species, and is the primary cause of chronic respiratory disease in chickens and of infectious sinusitis in turkeys (Kleven *et al.*, 1998; Stipkovits & Kempf, 1996). It is a highly transmissible organism, and clinical signs of infection include rales, coughing, nasal discharge, sinusitis and air sacculitis, leading to reduced egg production, hatchability, feed conversion efficiency and weight gain, and increased mortality and carcass condemnation (Mohammed *et al.*, 1987). The prevention of exposure of flocks to wild-type challenge by intense biosecurity and biosurveillance via serological monitoring is considered the first choice in control practices (Stipkovits & Kempf, 1996). Vaccination with live attenuated vaccines is an option for controlling *M. gallisepticum* infection when the prevention of exposure is impossible (Whithear, 1996).

The *M. gallisepticum* vaccine strain ts-11 is a temperature-sensitive (ts) mutant generated by chemical mutagenesis of a moderately virulent Australian field isolate (strain 80083), and has a ts phenotype, with normal growth at 33 °C but reduced growth at 39.5 °C. A single dose by eye-drop

application results in colonization of the upper respiratory tract and induces long-term immunity (Whithear *et al.*, 1990a, b). However, this vaccine is highly dose-dependent (Noormohammadi *et al.*, 2002a), and serological monitoring of vaccinated flocks has been difficult because of the low level of antibodies induced by ts-11 in some birds, particularly as detected by the rapid serum agglutination (RSA) test (Noormohammadi *et al.*, 2002b).

Pathogenic mycoplasmas are typically non-invasive organisms and colonize epithelial surfaces of host tissues. Attachment to target cells is mediated by interactions between mycoplasma cytoadhesins and their corresponding host cell receptors (Geary & Gabridge, 1987; Geary *et al.*, 1989). GapA or MGC1, a 105 kDa protein, is thought to be the primary cytoadhesin molecule in *M. gallisepticum* (Goh *et al.*, 1998; Keeler *et al.*, 1996). Recent studies on the molecular mechanisms of cytoadherence have revealed a complex multifactorial cytoadhesion process involving the primary cytoadhesin molecule GapA, and possibly also a number of putative cytoadherence-related molecules. Prolonged colonization and survival of *M. gallisepticum* in the host is thought to require both the cytoadhesin and cytoadherence-related molecules (Papazisi *et al.*, 2000). The cytoadherence-related molecule CrmA, or MGC3, a 116 kDa protein, has significant sequence homology with the *Mycoplasma pneumoniae* ORF 6 gene product, which has been shown to play a role as an accessory protein in

Abbreviations: CCU, colour changing units; HRP, horseradish peroxidase; RSA, rapid serum agglutination; SPF, specific-pathogen-free; ts, temperature-sensitive.

P1-mediated cytodherence (Papazisi *et al.*, 2000; Yoshida *et al.*, 2000). The *crmA* gene is located downstream of the *gapA* gene, as part of the same operon (Papazisi *et al.*, 2000). Both GapA and CrmA are expressed in virulent *M. gallisepticum* strain R-low (Rodriguez & Kleven, 1980), and co-expression of GapA and CrmA is essential for cytodherence and virulence in *M. gallisepticum* (Papazisi *et al.*, 2002). The absence of GapA, but not CrmA, has been observed in the ts-11 vaccine strain (Mudah-Orenstein *et al.*, 2003). Comparative analysis of the DNA sequences of *gapA* from ts-11 and its parent 80083 has revealed a 20 bp reiterated sequence, and the lack of expression of GapA in *M. gallisepticum* ts-11 has been correlated with the frame-shift that results from this sequence duplication. The loss of this DNA repeat and re-expression of GapA in ts-11 reisolates from infected chickens suggest that expression of GapA is necessary for continued colonization (Kanci *et al.*, 2004).

The aim of this work was to study the safety of, and the protective immune response to, GapA⁺ *M. gallisepticum* ts-11. Protection induced by this vaccine strain was assessed by challenging vaccinated specific-pathogen-free (SPF) chickens with the virulent wild-type *M. gallisepticum* strain Ap3AS (Soeripto *et al.*, 1989). Birds vaccinated with GapA⁺ *M. gallisepticum* ts-11 were compared with birds inoculated with medium alone and birds vaccinated with the commercial *M. gallisepticum* ts-11 vaccine. Weight gain, anti-*M. gallisepticum* antibody responses, gross air sac lesions, tracheal histopathology and tracheal B lymphocyte populations were compared.

METHODS

Preparation of vaccine and challenge culture. The GapA⁺ *M. gallisepticum* ts-11 strain was isolated from an *M. gallisepticum* ts-11 working seed culture and selected for expression of GapA by plating the culture, picking single colonies and examining them by PCR. The PCR used a pair of PCR primers (Table 1) designed to amplify a 120 bp region in *gapA* that contains a 20 bp duplication, resulting in premature truncation of GapA (Kanci *et al.*, 2004). Clones producing a product of 100 bp (without the 20 bp

duplication) were further screened by immunoblotting using specific antisera to GapA to detect full-length protein (Kanci *et al.*, 2004). Culture of the organism was performed in mycoplasma broth (MB) (7.5 g trypticase peptone, 2.5 g phyton peptone, 0.5 g thiotone peptone, 5 g yeast extract, 0.25 g benzyl penicillin, 5 g NaCl, 0.4 g KCl, 0.35 g MgSO₄·7H₂O, 0.05 g Na₂HPO₄, 0.1 g KH₂PO₄, 1 g glucose, 1.5 ml 1.6% phenol red solution, 100 ml inactivated swine serum, 10 ml 1% NAD solution, 10 ml 0.2% DNA solution, 10 ml yeast autohydrolysate, 1.5 ml 10% thallium acetate solution, 800 ml distilled water, pH adjusted to 8.1). For vaccination, an ampoule of GapA⁺ *M. gallisepticum* ts-11 culture (stored at -80 °C) was thawed at 37 °C, diluted 1:10 in MB and incubated for 24 h at 33 °C. Alternatively, commercially available *M. gallisepticum* ts-11 vaccine (Vaxsafe *M. gallisepticum* ts-11, Bioproperties) was used as recommended by the manufacturer. For challenge, an ampoule of the wild-type *M. gallisepticum* AP3AS culture (stored at -80 °C) was thawed at 37 °C, diluted 1:10 in MB and incubated for 18 h at 37 °C. All experiments were performed under Animal Ethics Committee approval number 06178.

Experimental design. Three groups of four-week-old White Leghorn SPF chickens were housed separately (40 chickens per group) in positive-pressure fibreglass isolators. At day 0, chickens were inoculated by eye-drop with 30 µl MB (group 1), 30 µl Vaxsafe *M. gallisepticum* ts-11 culture (group 2) or 30 µl GapA⁺ *M. gallisepticum* ts-11 vaccine (group 3). Twenty-one days after vaccination, 10 birds from each of the groups were euthanized by exposure to 100% carbon dioxide and necropsied. Fifteen of the remaining birds in each group were challenged by aerosol exposure to Ap3AS and then housed in a single separate isolator. The remaining 15 birds (vaccinated but not challenged) in each group (groups 4, 5 and 6) were kept until day 35 after vaccination. At day 35 (2 weeks after challenge), all birds were euthanized and necropsied.

Collection of samples. Blood samples were collected from all chickens prior to vaccination, challenge and euthanasia. Blood samples were allowed to clot overnight at 4 °C and sera were then collected. The body weight of each bird was measured before immunization and at necropsy.

Air sac lesions were scored grossly for severity on a scale of 0 to 3, as described previously (Nunoya *et al.*, 1987). A cumulative lesion score was then calculated for each bird by adding the scores of six air sacs.

Swabs were taken from the air sacs and upper trachea of each bird. The swabs were used to inoculate mycoplasma agar (MA) plates, which had the same composition as MB except that glucose and phenol red were omitted and the medium was solidified with 1%

Table 1. Primers used in this study

Primer	Sequence (5'–3')	PCR conditions	Expected PCR product size
MG16SrRNAFor	GAGCTAATCTGTAAATTGGTC	94 °C 3 min and 35 cycles of 94 °C 30 s, 55 °C 30 s, 72 °C 60 s	183 bp for <i>M. gallisepticum</i> strains
MG16SrRNARev	GCTTCCTTGCGGTTAGCAAC		
GapAFor	AGATAAATTCACCAAGAAAATAAT	94 °C 5 min and 40 cycles of 94 °C 15 s, 53 °C 30 s, 72 °C 15 s	Ts-11 GapA ⁻ 120 bp
GapARev	TGTTAATCATGTGCTGTCTTT		
WCNewATFor	GGTTTGGGTTGGTGTATAGTTAGTTT	94 °C 1 min and 35 cycles of 94 °C 30 s, 50 °C 30 s, 72 °C 30 s and 1 cycle of 72 °C 1 min	Ts-11 GapA ⁺ 100 bp 229 bp for ts-11 vaccine
WBNewAUREv	TCTTCGAAAACAAAAGGTTTTAC		
			No product for Ap3AS

bacteriological agar (Oxoid), and then placed in 1 ml MB. MA plates were incubated at 33 °C and examined under a binocular dissecting microscope after 7–10 days. MB cultures were incubated at 33 °C and examined for a change in the colour of the indicator.

Sections of the upper, middle and lower trachea were collected from each bird and fixed in 10% neutral buffered formalin for histological examination. For immunohistochemistry, segments of trachea were placed in Tissue-Tek O.C.T. embedding compound (Sakura) and immediately frozen in liquid nitrogen. All frozen tissues were stored at –70 °C until use.

Identification of *M. gallisepticum*. DNA was extracted from those broths showing a colour change and used as template in PCRs. Each PCR was performed using 1.5 U GoTaq DNA polymerase (Promega) in a 25 µl reaction mix containing 1.5 mM MgCl₂, 200 µM of each dNTP and 1 µM of each primer. The primers used in this study are shown in Table 1. A region of the 16S rRNA gene of *M. gallisepticum* was amplified (García *et al.*, 2005) to confirm the presence of *M. gallisepticum* DNA in the sample. The expected product was 183 bp in size. A fragment of *gapA* was amplified to differentiate between the vaccine strains administered (Kanci *et al.*, 2004). The expected product size for Vaxsafe *M. gallisepticum* ts-11 was 120 bp and for GapA⁺ *M. gallisepticum* ts-11 it was 100 bp. A portion of the *M. gallisepticum* ts-11 *vlhA* gene was amplified using the primer pair WCNewATFor and WBNewAURv to confirm the presence of the vaccine strain and the challenge strain. The expected product size for Vaxsafe *M. gallisepticum* ts-11 and GapA⁺ *M. gallisepticum* ts-11 was 229 bp, but no amplification was expected for Ap3AS. The number of colonies that had grown on plates was counted, or estimated if there was a large number of colonies.

Histopathological examination. Tracheal sections were cut and stained with haematoxylin and eosin. The mean mucosal thickness of the trachea of each bird was determined by measuring the thickness at four points transected by vertical and horizontal lines on the upper, middle and lower sections of the trachea, and the histological lesions in upper, middle and lower sections of trachea were scored for severity on a scale of 0 to 3, as described by Nunoya *et al.* (1987).

Serology

RSA test. The RSA test for antibodies against *M. gallisepticum* was performed with stained agglutination antigen (Intervet), and results were scored on a scale of 0 to 4, as described by Whithear (1993).

***M. gallisepticum* IgG ELISA.** An indirect ELISA was performed using ts-11 *VLhA* as coating antigen, as described previously (Noormohammadi *et al.*, 2002a), with some modifications. Briefly, the optimal antigen concentration and test serum dilution were determined. Individual wells of 96-well flat-bottomed plates (Nunc) were coated with 50 µl antigen diluted in carbonate buffer (0.032 M Na₂CO₃, 0.068 M NaHCO₃, pH 9.6) and the plates were incubated overnight at 4 °C. The wells were washed with avian washing buffer (AWB) (29.22 g NaCl and 0.5 ml Tween-20 in 1 l PBS), followed by a wash with reverse osmosis (RO) water, then incubated for 1.5 h at 37 °C with 150 µl blocking buffer (1% gelatin in carbonate buffer) per well, and washed as above. Test sera were diluted 1:100 in AWB with 1% BSA and added in 50 µl volumes to the wells, and then the plates were incubated for 1 h at 37 °C. The wells were washed with AWB, then 50 µl horseradish peroxidase (HRP)-conjugated rabbit anti-chicken IgG (Chemicon) diluted 1:1000 in AWB with 1% BSA was added to each well, and the plate was incubated for 1 h at 37 °C. The wells were washed as before, and 50 µl 2,2'-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) substrate (KPL) was added to each well. The reactions were stopped after 30 min by addition of 50 µl MilliQ water and the absorbance

was measured at 414 nm. The standard *M. gallisepticum* reactive serum was assayed at a 1:15 dilution and with serial threefold dilutions to 1:32 805.

Analysis of tracheal B lymphocyte populations following vaccination. Tracheal B lymphocyte populations were examined by immunohistochemical staining with a monoclonal antibody against a B cell marker (Bu-1). Frozen 5 µm sections of the upper, middle and lower trachea, collected from each bird 5 weeks after vaccination, were stained as described previously (Gaunson *et al.*, 2000). The monoclonal antibody specific for Bu-1 (mouse anti-chicken Bu-1, Southern Biotechnology) was used at a dilution of 1:100. HRP-conjugated sheep anti-mouse IgG (Chemicon) was used at a dilution of 1:100. Bound antibody was detected with 3,3'-diaminobenzidine (DAB) (Sigma-Aldrich). Cells were counterstained with Mayer's haematoxylin (Sigma-Aldrich). Spleen sections were also stained and used as positive controls. Each tracheal section was examined microscopically, and the numbers of clusters of positively stained cells were recorded (Devlin *et al.*, 2010). The area of stained cells in each of the B cell clusters was analysed in photomicrographs using Image-Pro Plus (Media Cybernetics).

Stability of the ts phenotype of GapA⁺ *M. gallisepticum* ts-11 reisolates. Viable counts of reisolates from GapA⁺ *M. gallisepticum* ts-11-inoculated birds obtained 3 and 5 weeks after vaccination were compared at 33 and 39.5 °C to assess whether passage in the bird had altered the ts phenotype.

Statistical analyses. The mean percentage body weight gains for each group were analysed for differences using a one-way analysis of variance (ANOVA) and Student's *t* test (Minitab v15 for Windows). Median RSA scores, tracheal lesion scores and air sac lesion scores for each experimental group were compared using Mann–Whitney U tests. The proportions of birds with detectable antibody responses and with air sac lesions were compared using Fisher's exact test. Mean mucosal thicknesses in the upper, middle and lower trachea were compared using Student's *t* test and a one-way ANOVA. The differences in absorbance values obtained in the *M. gallisepticum* IgG ELISA for sera from birds in each of the experimental groups were analysed using Student's *t* test. The mean number of clusters of Bu-1-positive cells per field of view and the mean cross-sectional area of B cells in each cluster in the tracheal mucosa were compared between groups using Student's *t* test. A *P* value ≤0.05 was regarded as significant.

RESULTS

Concentration of *M. gallisepticum* vaccines and challenge

The concentrations of the *M. gallisepticum* strains used for vaccination and challenge were 9.2 × 10⁸ colour changing units (CCU) ml^{−1} at 33 °C for Vaxsafe *M. gallisepticum* ts-11, 9.2 × 10⁶ CCU ml^{−1} at 33 °C for GapA⁺ *M. gallisepticum* ts-11 and 2.2 × 10⁵ CCU ml^{−1} at 37 °C for *M. gallisepticum* Ap3AS.

RSA scores

Following vaccination and challenge, anti-*M. gallisepticum* antibody concentrations were assessed semiquantitatively by RSA. No antibodies against *M. gallisepticum* were detected in the serum of any bird at the time of vaccination. None of the

chickens in the negative control groups (1 and 4) had any detectable antibody against *M. gallisepticum* at the time of necropsy. In group 2, which was vaccinated with Vaxsafe *M. gallisepticum* ts-11, 30 % of the birds were positive (RSA ≥ 1) by 3 weeks and 58 % by 5 weeks after vaccination (group 5). In group 3, which was vaccinated with GapA⁺ *M. gallisepticum* ts-11, 83 % of the birds were positive by 3 weeks and 94 % by 5 weeks after vaccination (group 6). The results are summarized in Table 2.

Gross lesions

After challenge with Ap3AS, gross air sac lesions were most prevalent in birds that had not been vaccinated (group 1). In this group, 10/12 birds had air sac lesions, with total air sac lesion scores ranging from 0.5 to 4.5. Mild air sac lesions were also observed in 4/13 birds in group 2 (vaccinated with Vaxsafe *M. gallisepticum* ts-11) and 2/14 birds in group 3 (vaccinated with GapA⁺ *M. gallisepticum* ts-11). There was no significant difference between the gross air sac lesion scores in the group inoculated with MG medium and the group vaccinated with Vaxsafe *M. gallisepticum* ts-11, but there was a significant difference between the group inoculated with MG medium and the group vaccinated with GapA⁺ *M. gallisepticum* ts-11 ($P=0.03$). There were no lesions in any of the unchallenged birds (groups 4, 5 and 6). The results are summarized in Table 3.

Reisolation of *M. gallisepticum*

M. gallisepticum was frequently isolated from tracheal swabs but not from the air sacs. Broth cultures from each group showing a colour change indicative of mycoplasma growth were confirmed to contain *M. gallisepticum* by PCR.

On day 21, in the unchallenged groups, *M. gallisepticum* was reisolated on MA plates from the tracheas of 3/10 birds in group 8 (Vaxsafe *M. gallisepticum* ts-11-vaccinated) and

4/10 birds in group 9 (GapA⁺ *M. gallisepticum* ts-11-vaccinated), and in broth from 2/10 birds in group 8 and 6/10 birds in group 9.

On day 35, in the unchallenged birds, *M. gallisepticum* was not recovered on MA plates from swabs of the tracheas of any of 14 birds in group 5 (Vaxsafe *M. gallisepticum* ts-11-vaccinated), but was recovered from the tracheas of 4/16 birds in group 6 (GapA⁺ *M. gallisepticum* ts-11-vaccinated), while 2/14 birds in group 5 and 5/16 birds in group 6 yielded positive broth cultures. PCR amplification confirmed that the only mycoplasmas reisolated from groups 5, 6, 8 and 9 were the vaccine strain that had been administered to that group.

After challenge, *M. gallisepticum* was recovered on MA plates from the air sacs of 1/12 birds in group 1 (inoculated with MG medium) and from the tracheas of 9/12 birds. In group 2 (Vaxsafe *M. gallisepticum* ts-11-vaccinated), *M. gallisepticum* was recovered on MA plates from swabs of the tracheas of 7/13 birds and in group 3 (GapA⁺ *M. gallisepticum* ts-11 vaccinated) from 8/14 birds. In broth cultures of groups 1, 2 and 3, only 1/12, 2/13 and 0/14 of the birds, respectively, had concentrations of *M. gallisepticum* detectable by PCR on swabs of their tracheas. PCR using strain-specific primers demonstrated that the only mycoplasmas detected were strain Ap3AS. The results are shown in Table 4.

Weight gains of chickens

There was no significant difference in weight gain between unchallenged birds inoculated with Vaxsafe *M. gallisepticum* ts-11 and GapA⁺ *M. gallisepticum* ts-11 at day 21 (groups 2 and 3) or day 35 (groups 5 and 6) after vaccination. However, at day 35 (day 14 after challenge), the unvaccinated chickens (group 1) had a significantly lower mean weight gain than the chickens in group 2 (Vaxsafe *M. gallisepticum*

Table 2. Rapid serum agglutination test results

Values with the same superscript letter in the same column are not significantly different ($P>0.05$). MG, *M. gallisepticum*.

Group	Inoculated with:	Challenged with Ap3AS at day 21	Day 14		Day 21*		Day 35	
			Percentage RSA positive (n)†	Median RSA score (range)	Percentage RSA positive (n)†	Median RSA score (range)	Percentage RSA positive (n)†	Median RSA score (range)
1	MG medium	+	0 (37) ^a	—	0 (37) ^a	—	100 (12) ^a	4 (3–4) ^a
2	Vaxsafe MG ts-11	+	14 (37) ^b	0 (0–3) ^a	30 (37) ^b	0 (0–4) ^a	100 (13) ^a	4 (2–4) ^a
3	GapA ⁺ MG ts-11	+	68 (40) ^c	2 (0–4) ^b	83 (40) ^c	3 (0–4) ^b	100 (14) ^a	4 (4) ^a
4	MG medium	—	On day 21, these birds were separated from groups 1, 2 and 3 and kept as unchallenged controls					0 (15) ^b
5	Vaxsafe MG ts-11	—						—
6	GapA ⁺ MG ts-11	—						58 (14) ^c
							94 (16) ^a	2 (0–4) ^b
								3 (0–4) ^a

*On day 21, 10 birds from each of groups 1, 2 and 3 were euthanized and necropsied.

†Number of chickens shown in parentheses. Three birds from groups 1 and 2 died before day 14.

Table 3. Air sac lesion scores

Values with the same superscript letter in the same column are not significantly different ($P>0.05$). MG, *M. gallisepticum*.

Group	Inoculated with:	Challenged with Ap3AS	No. birds	Air sac lesions	
				No. with lesions	Median air sac lesion score (range)
1	MG medium	+	12	10 ^a	1.5 (0–4.5) ^a
2	Vaxsafe MG ts-11	+	13	4 ^b	0 (0–4.0) ^{a,b}
3	GapA ⁺ MG ts-11	+	14	2 ^b	0 (0–2.0) ^b
4	MG medium	–	15	–	–
5	Vaxsafe MG ts-11	–	14	–	–
6	GapA ⁺ MG ts-11	–	16	–	–

ts-11-vaccinated) ($P=0.03$), but not in comparison with the birds in group 3 (GapA⁺ *M. gallisepticum* ts-11-vaccinated), and there was no significant difference in the mean weight gains of the birds in groups 2 and 3.

The mean weight gain of the chickens in group 4 (unvaccinated and unchallenged) was significantly lower than that of the birds in group 5 (Vaxsafe *M. gallisepticum* ts-11-vaccinated, unchallenged) ($P=0.002$) and group 6 (GapA⁺ *M. gallisepticum* ts-11-vaccinated, unchallenged) ($P=0.01$). The results are shown in Table 5.

Tracheal mucosal thickness and lesions

The mean tracheal mucosal thicknesses are shown in Table 6. The mean mucosal thicknesses of the upper, middle and lower tracheas of the birds in group 1 (unvaccinated, challenged) were significantly greater than those of the birds in group 3 (GapA⁺ *M. gallisepticum* ts-11-vaccinated,

challenged) ($P<0.01$). The mean mucosal thickness of the upper trachea of the birds in group 1 was significantly greater than that of the birds in group 2 (Vaxsafe *M. gallisepticum* ts-11-vaccinated, challenged) ($P=0.05$). However, there was no significant difference in mucosal thicknesses between these two groups in the middle and lower sections of the trachea. Mean mucosal thicknesses in all three sections of the trachea were significantly lower in the birds in group 3 than in the birds in group 2 ($P<0.05$). There were no significant differences in the mean mucosal thicknesses of the upper, middle and lower tracheas of the birds in group 3 (GapA⁺ *M. gallisepticum* ts-11-vaccinated, challenged) and group 4 (unvaccinated, unchallenged), group 5 (Vaxsafe *M. gallisepticum* ts-11-vaccinated, unchallenged) and group 6 (GapA⁺ *M. gallisepticum* ts-11-vaccinated, unchallenged).

Tracheal lesions were characterized by the infiltration of inflammatory cells and the formation of follicles in the

Table 4. Reisolation of *M. gallisepticum*

MG, *M. gallisepticum*.

Group	Inoculated with:	Challenge with Ap3AS	Days after vaccination	No. birds	Number of birds positive			
					MA plates*		MB broth†	
					Air sacs	Trachea	Trachea	Size of PCR product (bp)
1	MG medium	+	35	12	1	9	1	183
2	Vaxsafe MG ts-11	+	35	13	0	7	2	183
3	GapA ⁺ MG ts-11	+	35	14	0	8	0	–
4	MG medium	–	35	15	0	0	0	–
5	Vaxsafe MG ts-11	–	35	14	0	0	2	120
6	GapA ⁺ MG ts-11	–	35	16	0	4	5	100
7	MG medium	–	21	10	0	0	0	–
8	Vaxsafe MG ts-11	–	21	10	0	3	2	120
9	GapA ⁺ MG ts-11	–	21	10	0	4	6	100

*Number of birds showing MG colonies growing on an MA plate.

†Number of birds yielding cultures showing a colour change that were *M. gallisepticum*-positive by PCR (PCR was conducted to differentiate the vaccine strains and the challenge strain).

Table 5. Weight gain

Values with the same superscript letter in the same column are not significantly different ($P>0.05$). MG, *M. gallisepticum*.

Group	Inoculated with:	Challenge with Ap3AS	Weight (g)	Mean percentage weight gain $\pm$ SD		
			Day 0	Day 21	Day 35	
1	MG medium	+	248.81 $\pm$ 26 ^a	118 $\pm$ 19 ^a	203 $\pm$ 28 ^a	
2	Vaxsafe MG ts-11	+	239.41 $\pm$ 28 ^a	136 $\pm$ 30 ^a	225 $\pm$ 29 ^{b,c}	
3	GapA ⁺ MG ts-11	+	236.73 $\pm$ 26 ^a	127 $\pm$ 35 ^a	218 $\pm$ 29 ^{a,b}	
4	MG medium	—	On day 21, these birds were separated from groups 1, 2 and 3 and kept as unchallenged controls			208 $\pm$ 27 ^{a,b}
5	Vaxsafe MG ts-11	—				240 $\pm$ 22 ^c
6	GapA ⁺ MG ts-11	—				232 $\pm$ 18 ^{b,c}

mucosa. The median tracheal lesion scores are shown in Table 7. The median tracheal lesion scores were significantly higher for the birds in group 1 (unvaccinated, challenged) than for the birds in group 2 (Vaxsafe *M. gallisepticum* ts-11-vaccinated, challenged) ($P<0.005$) and group 3 (GapA⁺ *M. gallisepticum* ts-11-vaccinated, challenged) ($P<0.002$). No significant differences were seen in the middle and lower tracheal lesion scores for birds in groups 2 and 3. Only the upper tracheal lesion scores for the birds in group 2 were significantly greater than those for the birds in group 3 ($P=0.001$). There were no significant differences in the median tracheal lesion scores for the upper, middle and lower tracheas of the birds in group 3 (GapA⁺ *M. gallisepticum* ts-11-vaccinated, challenged) and group 4 (unvaccinated, unchallenged), group 5 (Vaxsafe *M. gallisepticum* ts-11-vaccinated, unchallenged) and group 6 (GapA⁺ *M. gallisepticum* ts-11-vaccinated, unchallenged).

Detection of *M. gallisepticum* specific humoral immunity in chickens

The mean optical density, SD, and the number of positive sera in each group as detected by ts-11 VlhA ELISA are shown in Table 8. No serum from the unvaccinated chickens (group 1) produced an optical density above the cut-off point (mean + 4SD for group 1). There was no

significant difference between the optical densities for group 2 (Vaxsafe *M. gallisepticum* ts-11-vaccinated) and group 3 (GapA⁺ *M. gallisepticum* ts-11-vaccinated) at day 14 after vaccination. The optical densities were significantly higher for group 3 at day 21 ($P=0.001$) and day 35 ($P=0.008$) after vaccination.

Analysis of tracheal B lymphocyte populations following vaccination

B lymphocyte populations in the tracheal mucosae of vaccinated, unchallenged birds were investigated using immunohistochemical staining. Most of the B lymphocytes formed clusters in the mucosa, although some were also observed scattered throughout the mucosa. The analysis of tracheal B lymphocyte populations is shown in Table 9. The size and number of B lymphocyte clusters were compared between groups. No B lymphocytes were detected in the tracheal sections from unvaccinated birds (group 1). Tracheal sections from birds vaccinated with Vaxsafe *M. gallisepticum* ts-11 (group 2) had significantly lower numbers of B lymphocyte clusters than tracheal sections from birds vaccinated with GapA⁺ *M. gallisepticum* ts-11 (group 3) ($P=0.001$). There was no significant difference between the birds in groups 2 and 3 in the mean area of stained cells per cluster or the mean area of stained cells per section. In group 3, aggregations of B lymphocytes

Table 6. Mean tracheal mucosal thicknesses

Values with the same superscript letter in the same column are not significantly different ($P>0.05$). MG, *M. gallisepticum*.

Group	Inoculated with:	No. birds	Challenge with Ap3AS	Mean tracheal mucosal thickness $\pm$ SD (μ m)		
				Upper	Middle	Lower
1	MG medium	12	+	28.98 $\pm$ 21.22 ^a	30.19 $\pm$ 25.83 ^a	33.17 $\pm$ 28.56 ^a
2	Vaxsafe MG ts-11	13	+	15.10 $\pm$ 9.75 ^b	23.35 $\pm$ 25.67 ^a	23.21 $\pm$ 22.52 ^a
3	GapA ⁺ MG ts-11	14	+	9.21 $\pm$ 2.86 ^c	9.65 $\pm$ 2.98 ^b	11.6 $\pm$ 2.86 ^b
4	MG medium	15	—	8.08 $\pm$ 1.82 ^c	8.10 $\pm$ 1.46 ^b	10.57 $\pm$ 3.08 ^b
5	Vaxsafe MG ts-11	14	—	8.37 $\pm$ 1.82 ^c	10.05 $\pm$ 3.02 ^b	10.39 $\pm$ 1.5 ^b
6	GapA ⁺ MG ts-11	16	—	8.55 $\pm$ 1.77 ^c	8.45 $\pm$ 2.52 ^b	11.5 $\pm$ 5.01 ^b

Table 7. Tracheal lesion scores

Values with the same superscript letter in the same column are not significantly different ($P>0.05$). MG, *M. gallisepticum*.

Group	Inoculated with:	No. birds	Challenge with AP3AS	Median tracheal lesion score (range)		
				Upper	Middle	Lower
1	MG medium	12	+	1.5 (0–3) ^a	1.0 (0–3) ^a	1.0 (0–3) ^a
2	Vaxsafe MG ts-11	13	+	1.0 (0–2) ^b	0.25 (0–3) ^b	0.5 (0–3) ^b
3	GapA ⁺ MG ts-11	14	+	0.0 (0–0.5) ^c	0.0 (0–0.5) ^b	0.0 (0–0.5) ^b
4	MG medium	15	–	0.0 (0–0.5) ^c	0.0 (0–0.5) ^b	0.0 (0–1) ^b
5	Vaxsafe MG ts-11	14	–	0.0 (0–0.5) ^c	0.0 (0–0.5) ^b	0.0 (0–1) ^b
6	GapA ⁺ MG ts-11	16	–	0.0 (0–0.5) ^c	0.0 (0–0.5) ^b	0.0 (0–1) ^b

were observed along the upper, middle and lower trachea, whereas in group 2, the B lymphocyte clusters were limited to lower tracheal sections.

Stability of the ts phenotype of GapA⁺ *M. gallisepticum* ts-11 reisolates

The differential viable counts confirmed that GapA⁺ *M. gallisepticum* ts-11 reisolated from vaccinated birds 3 and 5 weeks after inoculation retained the ts phenotype.

DISCUSSION

The failure of *M. gallisepticum* ts-11 to consistently elicit an antibody response poses a problem when assessing the vaccination status of a flock. The dose of vaccine administered can be crucial for successful vaccination with *M. gallisepticum* ts-11. However, the route of administration, host susceptibility, and factors such as the genotype and physiological status of the birds, may also affect the minimal effective dose of the vaccine (Whithear *et al.*, 1990a). The recommended eye-drop dose of *M. gallisepti-*

cum ts-11 is $\geq 10^{7.7}$ CCU (Whithear, 1996), and vaccination with *M. gallisepticum* ts-11 has been recommended only in birds 4 weeks of age and older to ensure that a protective response is elicited against subsequent challenge (Gaunson *et al.*, 2006b). In this experiment, four-week-old birds were used, and the safety and efficacy of GapA⁺ *M. gallisepticum* ts-11 as a vaccine were assessed.

Anti-*M. gallisepticum* antibody, as measured by RSA, was more readily detectable in the group vaccinated with GapA⁺ *M. gallisepticum* ts-11 (group 3), with a greater number of positive birds, than in the group vaccinated with Vaxsafe *M. gallisepticum* ts-11 (group 2) at 2, 3 and 5 weeks (groups 5 and 6) after vaccination. The median RSA score was also significantly higher in the group vaccinated with GapA⁺ *M. gallisepticum* ts-11. The dose of GapA⁺ *M. gallisepticum* ts-11 (9.2×10^6 CCU ml⁻¹) was 100-fold lower than that of Vaxsafe *M. gallisepticum* ts-11 (9.2×10^8 CCU ml⁻¹), but it still elicited a better serum antibody response. GapA⁺ *M. gallisepticum* ts-11 was able to stimulate production of an antibody response detectable by ELISA in birds 2 weeks after vaccination, with the number of positive birds continuing to increase for a further 3 weeks.

Table 8. Detection of antibodies against *M. gallisepticum* by ELISA after vaccination

Values with the same superscript letter in the same column are not significantly different ($P>0.05$). MG, *M. gallisepticum*.

Group	Inoculated with:	No. birds	Optical density in ts-11 VlhA ELISA							
			Day 0		Day 14		Day 21		Day 35	
			Mean $\pm$ SD	No. positive*	Mean $\pm$ SD	No. positive *	Mean $\pm$ SD	No. positive *	Mean $\pm$ SD	No. positive*
1	MG medium	15	0.10 $\pm$ 0.12 ^a	–	0.13 $\pm$ 0.11 ^a	–	0.13 $\pm$ 0.09 ^a	–	0.13 $\pm$ 0.09 ^a	–
2	Vaxsafe MG ts-11	14	0.11 $\pm$ 0.09 ^a	–	0.71 $\pm$ 0.38 ^b	8	0.82 $\pm$ 0.33 ^b	10	1.12 $\pm$ 0.29 ^b	14
3	GapA ⁺ MG ts-11	16	0.10 $\pm$ 0.09 ^a	–	0.76 $\pm$ 0.33 ^b	11	1.42 $\pm$ 0.38 ^c	15	1.54 $\pm$ 0.31 ^c	16

*Number of sera with optical density greater than the cut-off point (mean optical density of group 1 + 4SD).

Table 9. B lymphocyte clusters in the tracheal mucosa after vaccination

Values with the same superscript letter in the same column are not significantly different ($P>0.05$). MG, *M. gallisepticum*.

Group	Inoculated with:	No. birds	Challenge with AP3AS	Mean number of clusters per section $\pm$ SD	Mean area of stained cells per cluster $\pm$ SD (mm ²)	Mean area of stained cells per section $\pm$ SD (mm ²)
1	MG medium	3	—	—	—	—
2	Vaxsafe MG ts-11	7	—	1.14 $\pm$ 0.69 ^a	0.087 $\pm$ 0.10 ^c	0.1 $\pm$ 0.12 ^c
3	GapA ⁺ MG ts-11	7	—	1.85 $\pm$ 0.69 ^b	0.081 $\pm$ 0.06 ^c	0.18 $\pm$ 0.15 ^c

It has been established that the ts-11 strain of *M. gallisepticum* does not colonize the air sacs (Whithear *et al.*, 1990b), so isolation from the air sacs and production of air sac lesions were not expected in unchallenged birds. There were no visible air sac lesions in birds vaccinated with GapA⁺ *M. gallisepticum* ts-11 or Vaxsafe *M. gallisepticum* ts-11 at 3 or 5 weeks after inoculation. This indicates that the GapA⁺ *M. gallisepticum* ts-11 strain has a low level of pathogenicity similar to that of Vaxsafe *M. gallisepticum* ts-11. In challenged birds, minor air sac lesions were observed in GapA⁺ *M. gallisepticum* ts-11-vaccinated birds, but these were less severe than those seen in Vaxsafe *M. gallisepticum* ts-11-vaccinated birds and much less severe than those seen in unvaccinated birds. These data indicate that GapA⁺ *M. gallisepticum* ts-11 may provide protection similar to or better than that provided by Vaxsafe *M. gallisepticum* ts-11.

The ts-11 strain was selected for its ability to multiply in the upper respiratory tract to an extent sufficient to stimulate long-term protective immunity, but not at core body temperature (Whithear *et al.*, 1990b). In this experiment, Vaxsafe *M. gallisepticum* ts-11 was reisolated from the tracheas of 4/24 birds (groups 5 and 8) in broth, whereas GapA⁺ *M. gallisepticum* ts-11 was reisolated from 11/26 birds (groups 6 and 9). Reisolation on agar was only possible for the first 21 days after vaccination of birds with Vaxsafe *M. gallisepticum* ts-11, but GapA⁺ *M. gallisepticum* ts-11 was reisolated from 4/16 birds 35 days after vaccination. These results indicate that GapA⁺ *M. gallisepticum* ts-11 colonized and persisted in the tracheal mucosa better than Vaxsafe *M. gallisepticum* ts-11.

At day 21 after vaccination, the weight gains of all groups were similar, but at day 35 after vaccination, the weight gain of the vaccinated birds was significantly greater than that of the unvaccinated, control group. After challenge with Ap3AS, the weight gain of the Vaxsafe *M. gallisepticum* ts-11-vaccinated birds was significantly greater than that of unvaccinated birds, but was not significantly different from that of the birds vaccinated with GapA⁺ *M. gallisepticum* ts-11. This suggests that with respect to weight gain, vaccination with GapA⁺ *M. gallisepticum* ts-11 provides protection similar to that provided by Vaxsafe *M. gallisepticum* ts-11 against the effects of infection with virulent *M. gallisepticum*.

Histopathological examination of tracheal sections has been used as a measure of protection against challenge with

virulent *M. gallisepticum*. The tracheal mucosal thickness of GapA⁺ *M. gallisepticum* ts-11-vaccinated birds after challenge was similar in the upper, middle and lower sections of the trachea, and significantly less than that of unvaccinated birds at all three sites after challenge. In contrast, there was no significant difference after challenge between the mucosal thickness of birds vaccinated with Vaxsafe *M. gallisepticum* ts-11 and unvaccinated birds in the middle and lower sections of the trachea. This suggests that GapA⁺ *M. gallisepticum* ts-11 can more effectively prevent the damage caused by infection with wild-type *M. gallisepticum* than Vaxsafe *M. gallisepticum* ts-11, particularly in the lower trachea.

Extensive inflammatory cell infiltration was observed in the tracheal mucosa of most of the unvaccinated birds after challenge. Moderate upper tracheal lesions were seen in the birds vaccinated with Vaxsafe *M. gallisepticum* ts-11 following challenge with virulent *M. gallisepticum*, and these were significantly more severe than the upper tracheal lesions seen in the birds vaccinated with GapA⁺ *M. gallisepticum* ts-11 after challenge. In the middle and lower trachea there were some lesions in Vaxsafe *M. gallisepticum* ts-11-vaccinated birds, but the severity of these was not significantly different from those seen in birds vaccinated with GapA⁺ *M. gallisepticum* ts-11 after challenge. This further suggests the greater protective efficacy of vaccination with GapA⁺ *M. gallisepticum* ts-11 against infection with wild-type *M. gallisepticum*.

The ts-11 VlhA ELISA has been shown to be a reliable assay for specific and sensitive detection of serum antibodies against *M. gallisepticum* (Noormohammadi *et al.*, 2002a), and chickens vaccinated with Vaxsafe *M. gallisepticum* ts-11 that have no or low levels of serum antibody detectable by RSA have been shown to still have significant protection against challenge with virulent *M. gallisepticum* (Noormohammadi *et al.*, 2002b). In this study, it was found that the ts-11 VlhA ELISA had a sensitivity superior to that of RSA, particularly in birds vaccinated with Vaxsafe *M. gallisepticum* ts-11. There was only a slight difference in sensitivity between RSA and the ts-11 VlhA ELISA in birds vaccinated with GapA⁺ *M. gallisepticum* ts-11. Thus, the RSA test could be used effectively as an indicator of the flock response to vaccination with GapA⁺ *M. gallisepticum* ts-11.

M. gallisepticum causes a lymphoproliferative response in the tracheal mucosa of infected birds (Gaunson *et al.*,

2000), and this response predominantly consists of proliferating B cells (Gaunson *et al.*, 2006a). Birds vaccinated with GapA⁺ *M. gallisepticum* ts-11 had a significantly greater number of B lymphocyte clusters in their tracheal mucosa than birds vaccinated with Vaxsafe *M. gallisepticum* ts-11. This suggests that greater stimulation of B lymphocytes in the tracheal mucosa and differentiation of these into antibody-producing plasma cells may be responsible for the better serum antibody response in GapA⁺ *M. gallisepticum* ts-11-vaccinated birds and possibly also the better protective immunity seen in these birds.

The viable counts of the reisolates obtained 3 and 5 weeks after inoculation and the absence of air sac lesions at necropsy confirmed that GapA⁺ *M. gallisepticum* ts-11 retained its ts phenotype and did not regain pathogenicity.

The experimental studies described here demonstrated that eye-drop vaccination with GapA⁺ *M. gallisepticum* ts-11 not only induced an antibody response detectable by RSA but also did so at a lower dose than Vaxsafe MG ts-11, and with respect to weight gain protected birds from the effects of infection with virulent *M. gallisepticum*. It also prevented the development of air sac lesions and reduced damage to the trachea. Under well-controlled experimental conditions, the GapA⁺ *M. gallisepticum* ts-11 vaccine outperformed the commercially available Vaxsafe *M. gallisepticum* ts-11 vaccine. Based on these initial findings, GapA⁺ *M. gallisepticum* ts-11 is a promising vaccine candidate for the prevention of avian mycoplasmosis.

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