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# Safety and efficacy of a *Mycoplasma gallisepticum oppD* knockout mutant as a vaccine candidate

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ABSTRACT

Control of the important poultry pathogen *Mycoplasma gallisepticum* is highly dependent on safe and efficacious attenuated vaccines. In order to assess a novel vaccine candidate we evaluated the safety and efficacy of the *M. gallisepticum* mutant 26-1. The *opp*$D_1$ gene in this mutant has been interrupted by a signature-tagged transposon and previous studies have shown that it can colonise the respiratory tract of chickens without inducing significant disease. The capacity of the *opp*$D_1$ mutant to induce protective immunity in the respiratory tract after vaccination by eye-drop was assessed by challenging vaccinated birds with an aerosol of the virulent *M. gallisepticum* strain Ap3AS. Vaccination with the *opp*$D_1$ mutant was shown to fully protect against the lesions caused by pathogenic *M. gallisepticum* in the air sacs and tracheas. It also protected against the effect of infection on weight gain, and partially protected against colonisation of the trachea by virulent *M. gallisepticum.* These results indicate that a *M. gallisepticum* mutant with the *opp*$D_1$ gene knocked out could be used as a live attenuated vaccine as it is both safe and efficacious when administered by eyedrop to chickens.

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## 1. Introduction

*Mycoplasma gallisepticum* is a major bacterial pathogen of chickens and turkeys throughout the world, and inadequate control contributes significantly to the need for antimicrobial use in the poultry industries. It initially colonises the respiratory tract, where it causes chronic tracheitis and airsacculitis (and sinusitis in turkeys), but may also spread systemically, resulting in arthritis and salpingitis [1,2]. In addition to clinical disease, infection can also result in reduced food conversion efficiency, weight gain and egg production [1,2]. In addition to these production costs, treatment and control programs impose further economic burdens on poultry producers [1].

The most effective way to protect birds from *M. gallisepticum* is vaccination [3]. The commercially available vaccine strain ts-11 has been widely and successfully used in the poultry industry to protect flocks from infection with virulent *M. gallisepticum*. This temperature sensitive strain was developed by chemical mutagenesis, but the mutations responsible for its attenuation have not yet been defined. Eyedrop vaccination with ts-11 provides protection against aerosol challenge, although air sac lesions are occasionally seen after challenge [4], and the protective effect is highly dose sensitive. The attenuated 6/85 vaccine strain has been less thoroughly studied, but in one comparative study induced lower levels of serum agglutination antibodies than ts-11 and afforded a reduced level of protection against challenge [5]. Although the F strain vaccine induces a greater level of protection than ts-11 or 6/85, it is less attenuated, and can induce significant disease if it infects turkeys. Thus, there remains a need for a fully attenuated vaccine that is less dose sensitive than the ts-11 strain.

Several studies have shown that the vaccine efficacy of attenuated strains can be enhanced. The cytadherence protein GapA has been identified as an important factor in attenuated vaccine development. Strain GT5 was generated by inserting the wild-type *gap*A gene into the highly attenuated strain $R_{high}$ using transposon mutagenesis [6]. This complemented strain was then assessed as a modified live Mg vaccine and shown to induce protection against disease caused by inoculation with the virulent Mg strain $R_{low}$

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strain [7]. Similarly, a clone of strain ts-11 that expresses GapA has been shown improved vaccine characteristics compared to the original strain [8].

Several studies have used transposon mutagenesis to identify virulence determinants in *M. gallisepticum*. The genes identified as playing a role in virulence using this approach have included the *gapA* adhesin gene [9–11], *lpd*, which encodes the dihydrolipoamide dehydrogenase [12], *mslA*, which encodes a polynucleotide binding protein [13], *malF*, a predicted ABC sugar transport permease [12], *crmA*, a cytadherence accessory protein [9–11], MGA_0329, which encodes a sialidase [14], *oppD*, a putative oligopeptide transporter gene, and the hypothetical genes MGA_1102, MGA_1079 and MGA_0588 [15]. While these virulence determinants are potential targets for attenuation and thus for generation of defined vaccine candidates, only the *lpd* mutant has been assessed as a potential vaccine candidate [16].

In our previous studies, we showed that chickens exposed to an aerosol of the *opp*$D_1$ mutant 26-1 did develop a detectable systemic anti-mycoplasma antibody response, with very mild airsacculitis and tracheal lesions seen at necropsy 14 days after exposure, even though the mutant was able to colonise and persist in the upper respiratory tract [15]. These findings suggested that this mutant might have potential as an attenuated vaccine. To investigate whether the *opp*$D_1$ mutant 26-1 could induce protective immunity, chickens were administered the *opp*$D_1$ mutant 26-1 by eyedrop and then challenged with the virulent *M. gallisepticum* strain Ap3AS. The development of lesions in the respiratory tract, the colonisation of the respiratory tract by the virulent challenge strain, systemic antibody responses against *M. gallisepticum* and weight gains were compared in birds vaccinated with *opp*$D_1$ mutant 26-1 and unvaccinated birds that had either been challenged with virulent *M. gallisepticum* or left unchallenged.

## 2. Materials and methods

### 2.1. Bacterial strain and cultural conditions

*M. gallisepticum* (Mg) Ap3AS was originally isolated from the air sacs of a broiler chicken in Australia and has been shown to be highly pathogenic [2]. It was grown in modified Frey's broth (MB) [17] containing 10% swine serum [18] at 37 °C until late logarithmic phase (pH approximately 6.8) [19]. The *opp*$D_1$ mutant 26-1 was grown in MB supplemented with 160 μg gentamicin/ml at 37 °C. The concentration of organisms in the culture, measured as colour changing units per ml (CCU/ml), was determined using a most probable number method. To each well of a sterile 96 well microtitre plate (Nunculon, Nunc), 225 μl of sterile MB containing gentamicin (160 μg/ml) was added. A 25 μl volume of the mutant culture was added to each well of the first column of 8 wells, mixed several times with the MB by pipetting and 25 μl from each well transferred to the next column and mixed with the MB in that well using a fresh set of tips. This sequence of serial 10-fold dilutions was repeated to column 10, from which 25 μl was discarded. Columns 11 and 12 were used as negative controls. The plate was then sealed with a Linbro® plate seal (ICN Biochemicals) and incubated at 37 °C for up to 2 weeks. A drop in the pH of the medium reflected growth of the culture, and was indicated by a colour change from red to yellow of the phenol red indicator in the medium. Column 1 was regarded as a $10^0$ dilution and after correction for the initial dilution of the culture, the numbers of organisms were calculated using most probable number tables [20]. The concentration of the *opp*$D_1$ mutant 26-1 was adjusted to approximately $1 \times 10^7$ CCU/drop (22.5 μl) for vaccination, while the concentration of wild-type Ap3AS was adjusted to approximately $1 \times 10^7$ CCU/ml for challenge.

### 2.2. Experimental design

The use of animals in this study was approved by the University of Melbourne Animal Ethics Committee. A total of eighty 5-week-old specific pathogen free chickens (Australian SPF Services, Woodend, Victoria) were randomly assigned into 4 groups and housed in positive pressure fibreglass isolators. The *opp*$D_1$ mutant 26-1 was administered as a 22.5 μl eye-drop at day 0. Wild-type Ap3AS was administered by aerosol to challenge the birds 2 weeks after immunization [21].

The birds in Group 1 served as the negative controls. These birds were inoculated with MB by eye-drop at day 0 and exposed to an aerosol of MB at day 14. The birds in Group 2 served as the positive controls. They were inoculated with MB by eye-drop at day 0 and exposed to an aerosol of wild-type Ap3AS at day 14. The birds in Group 3 served as the vaccination controls. These birds were inoculated with the *opp*$D_1$ mutant 26-1 by eye-drop at day 0, and exposed to an aerosol of MB at day 14 day. The birds in Group 4 were the vaccinated and challenged group. They were inoculated with the *opp*$D_1$ mutant 26-1 by eye-drop at day 0 and challenged by exposure to an aerosol of wild-type Ap3AS at day 14.

### 2.3. Sample collection from experimental chickens

Blood samples were collected from all chickens prior to vaccination, on the day of challenge and on the day of euthanasia. Sera were then harvested and tested using the rapid serum agglutination (RSA) assay to determine concentrations of anti-mycoplasma antibody [22]. The body weight of each bird was also measured before vaccination, at challenge and at *post mortem*.

At day 28 (14 days after challenge), chickens were euthanized, necropsied, and samples taken for culture of *M. gallisepticum*. The six air sacs were visually assessed for lesions and scored from 0 to 3 [23]. A cumulative lesion score was then calculated for each bird by adding the scores of all six air sacs.

Swabs were taken from the air sacs and tracheas of birds and streaked onto mycoplasma agar (MA; MB with glucose and phenol red omitted and solidified with 1% bacteriological agar) without gentamicin added and then transferred into MB containing no gentamicin to isolate *M. gallisepticum*. The MA plates were incubated at 37 °C for 10 days and the MB cultures were incubated at 37 °C until a colour change was observed. Organisms from broths showing a colour change were pelleted by centrifugation and subjected to PCR amplification to identify ST mutant 26-1 and wild-type Ap3AS.

Samples of the upper, middle and lower regions of the trachea of each bird were collected and fixed in 10% neutral buffered formalin for at least 24 h. Following processing and staining, sections were examined by light microscopy. Lesions were scored for severity on a scale of 0–3 [23]. The mucosal thickness was measured for each tracheal region at 6 different points and the mean thickness was then calculated for each of the three regions.

### 2.4. Re-isolation of M. gallisepticum

The primers used to identify and distinguish the *opp*$D_1$ mutant 26-1 and wild-type Ap3AS are shown in Table 1. A pair of PCR primers that were specific for *opp*$D_1$ mutant 26-1 (IGstmGenmeF3 and STM26-Rev) and the P2/P4 primer pair, which amplified the unique tag region, were used to confirm the re-isolation of *opp*$D_1$ mutant 26-1. The primer pair STM26-genome and STM26-Rev bound either side of the transposon insertion site in the *opp*$D_1$ mutant 26-1 and were used to confirm the re-isolation of wild-type Ap3AS.

The cells from a 200 μl volume of the broth culture were pelleted by centrifugation in a microfuge tube at 16,000 *g* for 5 min

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