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Development of footrot vaccine best practice

THE UNIVERSITY OF SYDNEY

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Executive Summary

Footrot is a significant economic and animal welfare issue in most countries where sheep are produced. In Australia, footrot is estimated to cost the Australian sheep meat and wool industries approximately $44 million per annum due to production losses and the cost of controlling the disease (Lane et al., 2015). Two forms of the disease are recognised: virulent and benign. The virulent form of the disease has the greatest impact on animal welfare and production and is estimated to cost industry more than $32 million per annum in treatment, prevention and production losses. The essential causative agent, *Dichelobacter nodosus*, possesses fine, filamentous appendages called fimbriae or pili, encoded by the *fimA* gene. Strains of *D. nodosus* can be assigned to 10 serogroups (A to I, and M) owing to variation in the fimbrial protein and the *fimA* gene. Fimbriae are the primary surface antigen of *D. nodosus*, and they are highly immunogenic. Footrot can be treated, controlled and eliminated with vaccine containing *D. nodosus* fimbriae, but vaccination can be challenging: immunity against *D. nodosus* is serogroup-specific, with little or no cross-protection between serogroups, and virulent strains of *D. nodosus* belonging to several serogroups (up to seven) may be present in a flock.

In Australia, targeted (serogroup-specific, flock-specific or outbreak-specific) vaccination has been used to treat, control and eliminate footrot in individual flocks. However, targeted vaccination has some limitations. In large flocks infected with several strains of *D. nodosus*, targeted vaccination can be expensive due to the requirement for multiple vaccines and extensive diagnostic testing. A multivalent footrot vaccine (Footvax®), which targeted nine serogroups (A to I), was previously available in Australia, but provided only limited duration and extent of protection due to antigenic competition. Nevertheless, it was regarded as a valuable tool in areas without distinct seasonal transmission periods as it enabled producers there to gain some control over the disease. Their alternative control method was foot bathing, and in some locations, this had to be done weekly.

The aim of this study was to define best practice for vaccination against footrot in different flocks with different types of footrot. In the first stage of the project, we evaluated four novel multivalent vaccine formulations in an immunological trial at The University of Sydney farms at Camden. The multivalent vaccine formulations contained fimbrial proteins representing six (A, B, C, G, H, I) or nine (A to I) serogroups with different doses. Two two-millilitre doses of each vaccine were administered one month apart to small groups of 1.5-year-old Merino wethers. Commercial bivalent vaccines were included for comparison. The antibody responses stimulated by the commercial bivalent vaccines were greater than those of all the novel multivalent vaccines. However, some novel formulations stimulated antibody responses that were deemed sufficient to provide some control over the disease on-farm. Based on these results we selected the best multivalent formulation containing nine serogroups (A to I) for evaluation in a field trial.

To study the impact of vaccine formulation method on immune response two methods for preparing bivalent vaccines were evaluated. The standard method is to emulsify each fimbrial protein with a mineral oil adjuvant separately, to produce two mono-valent vaccines, and then mix the two mono-valent vaccines together to produce the desired bivalent vaccine formulation. Bivalent vaccines prepared using this standard method probably consist of oil micelles containing either the first or second fimbrial protein, but usually not both unless micelles fuse after mixing. We evaluated an alternative method, in which the two antigens were mixed together, and then emulsified with a mineral oil adjuvant. The resulting bivalent vaccine consisted of oil micelles containing both antigens. This major variation in the formulation of the vaccine likely changes the way in which the fimbrial proteins are first encountered by the immune system. The alternative method of formulation did not stimulate greater antibody levels than the standard method, thus we concluded that there was no need to change the method by which bivalent vaccines are currently produced.

In the immunological trial we also evaluated inter-vaccination intervals of two- and three-months between successive bivalent vaccines. Previous immunological studies have reported that a three-month inter-vaccination interval is sufficient to avoid antigenic competition. A two-month inter-vaccination interval, which is the minimum practical interval, had not been evaluated previously. Antibody levels were similar for sheep that received a second bivalent vaccine after a two- or three-month interval, which suggests that a two-month interval is sufficient to avoid antigenic competition.

Four commercial Merino flocks in Tasmania were enrolled in the field trial. The trial commenced in July 2019 and finished in February 2020. Virulent strains of *D. nodosus* were detected in each flock, with between two and five serogroups per flock. Three-hundred 1.5-year-old Merino ewes from each parent flock were included in the trial, and were allocated to one of three treatment groups (control, bivalent vaccine, multivalent vaccine) using a systematic random approach. Sheep in the control group received two 1 mL doses of vaccine adjuvant only in July and August 2019; they were also foot-bathed in a 10% zinc sulphate solution on a monthly basis for welfare reasons. A priming and a booster dose of each vaccine was given to each sheep in the 2 vaccine groups; the two doses were about one month apart. The schedule for each treatment group on each farm was as follows. Sheep in the bivalent group received two consecutive bivalent vaccines with a three-month inter-vaccination interval; a 1 mL dose of the first bivalent vaccine was administered in July and August 2019, and a 1 mL dose of the second bivalent vaccine was administered in October and November. Sheep in the multivalent group received a 2 mL dose of the multivalent vaccine in July and August. A three-month inter-vaccination interval was used for the bivalent vaccines. We did not use a two-month interval as some of the producers were unable to accommodate the shorter interval due to other management procedures that needed to be performed. The 300 trial sheep on each farm were run together as a single mob, and they were examined at monthly intervals during the trial. Each month the feet of each sheep were examined, and blood was collected from 10 sheep in each treatment group for evaluation of circulating antibody levels.

Antibody levels were significantly higher ($P < 0.05$) for the groups of sheep that received a bivalent vaccine or a multivalent vaccine than for sheep in the control group. In all four flocks, the antibody levels for sheep that received a bivalent vaccine were similar to those in sheep that received a multivalent vaccine for the first month, i.e. until September 2019, at which point the levels were higher in sheep that received a bivalent vaccine. They remained higher in these sheep through to the end of the trial in February 2020.

Control of footrot was achieved in each group of sheep on each farm, that is, the two different vaccine approaches and foot-bathing were equally effective in suppressing footrot. There were significant differences between the three treatment groups at specific time-points; however, these differences did not form any meaningful pattern.

Cure and protection rates were calculated for September and October 2019, one- and two-months after administration of the booster dose of each vaccine, respectively, and at the end of the trial in February 2020. In September and October 2019, the cure rates from monthly foot-bathing or vaccination were satisfactory on Farms 1 and 2 (>70%) but these treatments were less effective on Farms 3 and 4 (cure rates as low as 25.8% for foot bathing and 18.2% for vaccination). By February 2020, satisfactory cure rates from foot bathing and vaccination were seen on all four farms. Improvement rates were always much higher than cure rates, i.e. lesions were suppressed but not eliminated.

The antibody response differed between the first and second bivalent vaccines. Antibody levels for the first vaccine reached the protective threshold of eight one-month after administration of the second dose of vaccine (Month 2) and remained above this threshold through to Month 4. In contrast, antibody levels for the second bivalent vaccine did not reach that threshold.

The extent to which animal welfare increased as a result of vaccination or regular foot-bathing was inferred from the improvement rates calculated for the three groups of sheep on each farm. The improvement rate, defined as a reduction in the total weighted foot score, was higher than for cure rates for sheep that received a vaccine, whether that be bivalent or multivalent, and for sheep that were regularly foot-bathed. On all four farms, the producers commented that they had observed fewer lame sheep in the mob. We were unable to measure differences in wool growth, but on each farm it was observed that the body condition score (BCS) of sheep in the mob had generally improved from an average score of less than 2 at the start of the trial to 3 and above through the trial period due to the reduction in lesion prevalence and severity and incidences of lameness.

Footrot vaccines – Best practice summary

Government policies and program regarding the control of virulent footrot in Australia differ between States. In NSW and Western Australia for example, footrot is a notifiable disease and infected flocks must undergo a disease eradication program approved by a government veterinarian. Producers are free to choose how they will go about eradicating footrot from their flock but must seek advice from their district veterinarian when designing the eradication program. In contrast, it is not listed as a notifiable disease in other states and producers are left to make their own decisions.

There are several methods for controlling footrot: traditional foot-bathing in an antiseptic solution (e.g. 10% zinc sulphate), administration of antibiotics at an individual or flock-level, vaccination, or a combination of these methods. One factor that may influence the choice of control method is the location of the property and whether or not transmission and expression of footrot is seasonal. In regions with seasonal transmission and expression of the disease like in NSW and Western Australia traditional control measures such as foot-bathing may be appropriate. Foot-bathing can provide adequate control over the disease during transmission periods (spring, early summer). Elimination of the disease from a flock can be achieved if the prevalence of disease is reduced to a low level so that in the dry summer months any remaining affected sheep can be culled. In regions without seasonal transmission where the disease occurs throughout the year, foot-bathing may need to be undertaken very frequently to achieve sufficient control of the disease, and elimination of the disease is unlikely with foot-bathing only. In these areas, alternative control measures such as vaccination may be preferable.

Antibiotics can be used to treat individual sheep with chronic and severe lesions that do not respond to foot-bathing, but the use of antibiotics at a flock-level is not often recommended due to global concerns about the contribution of blanket antibiotic use to the development of antimicrobial resistance; this has been identified as a major public health threat.

Footrot vaccines can be used to treat, control, and prevent the disease. Government policies regarding the use of footrot vaccines differs between States. In NSW, approval from the State Chief Veterinary officer is required for the use of footrot vaccines. There are two vaccination strategies: serogroup-specific monovalent or bivalent vaccination (also known as targeted, strain-specific or flock-specific vaccination), or multivalent vaccination.

Multivalent vaccines available contain antigens representing nine of the ten known footrot serogroups (A to I). In most cases, after confirmation of virulent footrot in a flock, further diagnostic testing is not a prerequisite for the use of a multivalent footrot vaccine, except in areas such as Tasmania and Victoria where serogroup M is prevalent. Vaccine is not yet available for serogroup M, therefore initial diagnostic testing is necessary for flocks in these areas to confirm that serogroup M is not present.

Serogroup-specific vaccines are formulated for each flock based on diagnostic test results. In flocks with three or more serogroups, sequential bivalent vaccines must be administered with an appropriate inter-vaccination interval

to avoid antigenic competition. An inter-vaccination interval of two to three-months between sequential bivalent vaccines is suitable. The use of a two-month inter-vaccination interval would enable producers to target multiple strains of *D. nodosus* in the shortest time-frame.

The absolute duration of protection afforded by the new formulation of multivalent vaccine requires further investigation but based on limited data obtained during the clinical trial undertaken in this study, multivalent vaccines probably provide protection for 6-8 weeks. In contrast, serogroup-specific monovalent and bivalent vaccines provide protection for 4-6 months. This means that multivalent vaccine booster doses would need to be given more frequently than boosters of monovalent or bivalent vaccines. For this reason, caution is needed around the usage of multivalent vaccine as a longer-term control measure. Its application may be limited to flocks/locations with prevalence of multiple serogroups and where frequent foot-bathing is being applied to suppress foot lesions due to the lack of a seasonal non-transmission period. Flock-specific monovalent and bivalent vaccines are the best available option in other situations.

Elimination of footrot can be achieved using serogroup-specific vaccination, but is unlikely to be achieved with multivalent vaccines, unless booster doses are given frequently and other control measures such as foot-bathing are used alongside.

The success of serogroup-specific vaccination programs is dependent upon isolation, serogrouping and virulence testing of the infecting *D. nodosus* strain(s). In flocks infected with several different serogroups and depending on the clinical response to the first round of vaccination, diagnostic testing may need to be undertaken after each bivalent vaccination to determine which serogroups should be included in the subsequent bivalent vaccine. In a flock infected with several virulent strains of *D. nodosus*, one or two strains tend to be dominant/most prevalent. These strains tend to be the most virulent strains in the flock and should be the first strains targeted with vaccine. The prevalence of these strains will decline following vaccination, and one or two of the remaining virulent strains may then become the dominant strain(s). It is not always apparent which of the remaining strains are likely to become the dominant strain after the first vaccination, so additional microbiological testing may be required, which has an associated cost.

In summary:

A cost benefit analysis comparing foot bathing with vaccination may be undertaken. It should include consideration of the likely frequency of foot bathing required for effective control. If a producer decides to use vaccination as a control measure, there are several factors that should be considered before deciding whether to use a multivalent vaccine, or serogroup-specific vaccination:

i. The duration of protection required. This depends on the likely transmission period in the area. Bivalent vaccine protects sheep for longer periods.
ii. Whether or not elimination of footrot from the flock is the target. Elimination is possible with bivalent vaccines due to their greater efficacy.
iii. The number of serogroups present in the flock, and if more than two, the possibility and the cost of administering several bivalent vaccines.
iv. The willingness of the producer to invest in further diagnostic testing in the event that several serogroups are present in the flock.
v. The cost of giving frequent booster doses of multivalent vaccine compared to successive bivalent vaccines

Regardless of the type of control measures used, producers should be willing to undertake subsequent foot inspections and culling to remove non-responders from the flock. Producers should also maintain strict biosecurity to prevent the re-introduction of footrot into the flock.

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1. Introduction

Footrot is a highly contagious disease of sheep and other small ruminants (Beveridge, 1941). The disease is a significant economic and animal welfare issue in most countries where sheep are produced, including Australia and the United Kingdom (Lane et al., 2015; Nieuwhof and Bishop, 2005). In Australia, footrot is estimated to cost the Australian sheep meat and wool industries approximately $44 million per annum due to production losses and the cost of treating and controlling the disease (Lane et al., 2015).

The clinical disease is a result of complex interactions between the essential causative agent, *Dichelobacter nodosus*, and the bacterial community of the foot (Egerton et al., 1969; Roberts and Egerton, 1969). Disease severity is determined by the virulence of the infecting *D. nodosus* strain(s) (Stewart et al., 1986), the susceptibility of the host (Emery et al., 1984), and environmental conditions (Graham and Egerton, 1968).

D. nodosus possesses filamentous appendages called fimbriae or pili (Billington et al., 1996). Fimbriae are composed of a repeating subunit protein, encoded by the *fimA* gene (Mattick et al., 1984). Fimbriae are the primary surface (K) antigen of *D. nodosus* and are highly immunogenic (Egerton, 1973). Strains of *D. nodosus* are divisible into 10 immunological distinct serogroups (A to I, and M) (Claxton et al., 1983; Dhungyel et al., 2015) according to sequence variation of the *fimA* gene, and corresponding structural variation of the fimbrial subunit protein (Elleman, 1988).

Virulent footrot can be treated, controlled and eliminated with vaccine containing *D. nodosus* fimbrial protein (Dhungyel et al., 2013; Dhungyel et al., 2008), but vaccination can be challenging. Immunity against *D. nodosus* is serogroup-specific, with little or no cross-protection between serogroups (Egerton, 1974; Elleman et al., 1990; Stewart et al., 1991). Consequently, in an outbreak of footrot the infecting *D. nodosus* strain(s) must be isolated from foot lesion material and serotyped, and the appropriate vaccine(s) formulated (Dhungyel et al., 2013; Dhungyel et al., 2008). There is anecdotal evidence that benign and intermediate footrot can also be controlled using serogroup-specific vaccines, but this has not been investigated.

Targeted, flock-specific vaccination has been used to eradicate footrot from Nepal and Bhutan (Egerton et al., 2002; Gurung et al., 2006) and from individual flocks in Australia (Dhungyel et al., 2013; Dhungyel et al., 2008). Where three or more serogroups are detected in a flock, sequential mono- or bivalent vaccines can be administered with an appropriate inter-vaccination interval (Dhungyel et al., 2013; Dhungyel et al., 2008; Dhungyel and Whittington, 2010). Previous field trials have reported the use of a 12-month interval, but immunological trials indicate that a three-month inter-vaccination interval is sufficient to avoid antigenic competition (Dhungyel and Whittington, 2010). This interval has not yet been evaluated in flocks naturally infected with multiple strains of *D. nodosus*, and nor has a two-month inter-vaccination interval, which is probably the minimum practical interval.

The success of targeted vaccination programs is dependent upon isolation, serogrouping and virulence testing of the infecting *D. nodosus* strain(s) (Dhungyel et al., 2013; Dhungyel et al., 2008). Identifying all virulent strains in a flock can be difficult; in Australia, up to seven virulent strains have been identified in a single flock (Dhungyel et al., 2013), and strains can be missed, even with intensive sampling strategies (Hill et al., 2010). Direct PCR testing of foot swabs is more sensitive than culture-dependent serogrouping (McPherson et al., 2018), but does not provide any indication of virulence. Thus, microbiological culture is still necessary to determine which serogroups are virulent and should be targeted with vaccine.

There is a requirement for strict biosecurity following vaccination and other treatment and control measures, to prevent sheep straying from neighbouring properties, and to ensure that sheep brought onto the property are free of *D. nodosus* infection. Otherwise *D. nodosus* may be reintroduced and additional strains of *D. nodosus* may circumvent vaccine immunity.

A commercial multivalent vaccine (Footvax®) containing 10 fimbrial antigens representing nine *D. nodosus* serogroups (A – I, including two serogroup B sub-types) was previously available in Australia, but import permits were ceased due to biosecurity concerns. The cure and protection rates reported for Footvax® vary considerably: a study involving four sheep flocks in New Zealand, each of a different breed, reported a cure rate of 81% to 100%, and a protection rate of 73% to 100% (Liardet et al., 1989). However, studies conducted in Australia under dry conditions reported a cure rate of only 55% in sheep with underrun lesions (Kennedy et al., 1985). The limited and highly variable efficacy of multivalent vaccines like Footvax® is due to the phenomenon of antigenic competition, which reduces the magnitude and duration of the antibody response following vaccination (Hunt et al., 1994).

Despite the limited protection provided by Footvax®, it was regarded as a valuable control tool by producers in areas without a well-defined non-transmission period, i.e. year-round transmission, where traditional control measures like foot-bathing had to be done frequently. The withdrawal of Footvax® from the Australian market left these producers with limited options for controlling the disease; consequently, they enquired about the possibility of developing a new multivalent vaccine to replace Footvax® in the Australian market.

Hypotheses

We hypothesised:

i) A new multivalent vaccine formulation (recombinant antigens in an optimal dose plus modern adjuvant combined in an optimum ratio) might overcome some of the antigenic competition seen with previous multivalent vaccines and lead to immune responses of sufficient magnitude and duration for effective protection against footrot in certain flocks where short-term protection is sufficient. Protection would be deemed to be sufficient where the prevalence and severity of footrot lesions was reduced to the same or a greater degree than the alternative approach of frequent foot-bathing;

ii) For bivalent vaccines, an inter-vaccination interval of two-months may compare favourably with the current three-month inter-vaccination interval;

iii) Even if the multi-valent vaccine was not successful, the benefits of sequential bi-valent vaccine would be seen by producers and veterinary consultants during field trials, leading to uptake.

The results will define best practice for footrot vaccine in different flocks with different types of footrot.

2. Literature review

Multivalent footrot vaccines

Dichelobacter nodosus, the causative agent of ovine footrot, possesses hair-like appendages called fimbriae or pili (Billington et al., 1996). Fimbriae are composed of a repeating subunit protein, encoded by the *fimA* gene (Mattick et al., 1984). Fimbriae are the primary surface (K) antigen of *D. nodosus* and are highly immunogenic (Egerton, 1973). Strains of *D. nodosus* are divisible into 10 immunologically distinct serogroups (A to I, and M) (Claxton et al., 1983; Ghimire et al., 1998) according to sequence variation of the *fimA* gene, and corresponding structural variation of the fimbrial subunit protein (Elleman, 1988).

Sheep can be immunised against *D. nodosus* infection with vaccines containing whole-cell antigens or fimbrial antigen (Every and Skerman, 1982; Stewart et al., 1982). Early footrot vaccines contained fimbrial antigens purified from whole *D. nodosus* cells, but more recent vaccines contain recombinant fimbrial antigens produced with genetically modified strains of *Pseudomonas aeruginosa* (O'Meara et al., 1993). Immunity against *D. nodosus* is serogroup-specific, with little or no cross-protection between serogroups (Stewart et al., 1991). Commercial multivalent vaccines containing nine serogroups (A to I), such as Footvax®, are reported to provide limited protection for up to 10 weeks (Hunt et al., 1994; Lambell, 1986; Raadsma et al., 1994; Schwartzkoff et al., 1993a).

The reduced efficacy of multivalent vaccines is due to the phenomenon of antigenic competition, in which there is diminution or inhibition of the immune due to competition between antigen structurally similar antigens for the same binding sites (Schwartzkoff et al., 1993a; Schwartzkoff et al., 1993b). In contrast, targeted (serogroup-specific) vaccination with mono- or bivalent vaccine can be used to control and treat footrot, whilst avoiding antigenic competition. This approach has been used to eradicate footrot from Nepal and Bhutan (Egerton et al., 2002; Gurung et al., 2006) and from some flocks in Australia (Dhungyel et al., 2013; Dhungyel et al., 2008).

An agglutinating antibody titre in excess of 3000, or 8 on a $\log_2$ scale, is believed to be necessary for protection against virulent footrot caused by the same serogroup as in the vaccine (Egerton et al., 1987). However, these data apply only to serogroup A. Different thresholds for protective antibody titres have been reported across different experiments and vary according to serogroup, the level of challenge and environmental conditions (Raadsma et al., 1994).

Three commercial footrot vaccines were produced by CSIRO in the early 1970's, but they were withdrawn from the market in 1979 as they provided only limited protection. The first experimental multivalent footrot vaccines contained whole *D. nodosus* cells representing five serogroups (Reed et al., 1981). These vaccines were protective against the serogroups included in the vaccine but provided no protection against serogroups not included in the vaccine (Reed et al., 1981). A commercial multivalent footrot vaccine (Footvax®) was first released in Australia containing the eight *D. nodosus* serogroups recognised in Australia at the time (Claxton, 1986b). A ninth serogroup (I) was included in the formulation after its discovery (Claxton, 1986a).

The efficacy of previous commercial multivalent vaccines like Footvax® varies considerably between studies. There are several factors relating to vaccine formulation (number of antigens, dose of each antigen, type of adjuvant), vaccine administration (volume, number of doses, interval between doses, site of administration) and design (sample sizes, type of control) that differ between studies. The type of data collected also differs between studies (number of affected sheep, number of affected feet, total foot score (TFS)). These factors make it difficult to compare results across studies.

Field trials indicate that multivalent vaccines do have a curative and protective effect, but cure and protective rates are typically <100%. A field trial conducted in the Central Valley of California reported a cure rate of 53% for footrot-

affected sheep vaccinated with Footvax® formulation, compared to a cure rate of 19% for unvaccinated footrot-affected sheep (Glenn et al., 1985). The authors report that the vaccine also had a significant protective effect, with a footrot prevalence of 9% in the group of vaccinated sheep, and 53% in the unvaccinated sheep, at the end of the 18-week trial period (Glenn et al., 1985). The same study reported that at the end of the trial, the average body condition score (BCS) for unvaccinated sheep that presented with footrot lesions at the start of the trial (2.48) was significantly lower than that of vaccinated sheep with footrot lesions at the start of the trial (3.09), vaccinated sheep without footrot lesions at the start of the trial (3.27), and unvaccinated sheep without footrot lesions at the start of the trial (3.0). There was no significant difference between the latter three groups. Risk factors related to the development of footrot lesions were also evaluated, including vaccination status and initial footrot lesion status. There was a five-fold reduction in the odds of a vaccinated sheep having foot rot lesions at the end of the trial, irrespective of initial footrot lesion status.

A three-month trial conducted in Idaho to evaluate a nine-serogroup multivalent vaccine formulation reported a significant protective effect (Bulgin et al., 1985). The trial included two groups of sheep with a history of footrot. Group 1 consisted of 318 Suffolk, Panama, and crossbred ewes and rams; 152 sheep received a multivalent footrot vaccine, and 166 served as unvaccinated controls. Group 2 consisted of 1050 Panama-Rambouillet ewes; 550 received a multivalent vaccine, and 500 served as unvaccinated controls. The sheep in Group 2 were also foot-bathed in a 10% copper sulphate solution two-to-three time's per-week. In both groups, the vaccine had a significant protective effect: In Group 1, two-percent of vaccinated sheep and 22.3% of unvaccinated sheep had active footrot lesions (≥score 1) at the end of the trial; In Group 2, 15.6% of vaccinated sheep had active footrot lesions at the end of the trial. In contrast, 51.8% of the unvaccinated control sheep had active footrot lesions at the end of the trial, including 28.5% with underrun lesions (≥score 3).

A field trial conducted in The United States with 233 crossbred ewes from Panama dams sired by Border Leicester, Clun Forest, Dorset, Polypay or Suffolk rams also reported that the eight-serogroup Footvax® formulation vaccine protected sheep from re-infection (Lewis et al., 1989). The sheep were divided evenly into two groups, the first of which received two 1 mL doses of vaccine with a six-week interval. The feet of each ewe were also pared at the start of the trial. The sheep were examined at regular intervals, after which they were walked through a foot-bath with a 10% zinc sulphate solution. The authors reported that for previously uninfected ewes, vaccination provided very little protection from infection; however, vaccinated sheep only had a 3% re-infection rate, and unvaccinated sheep had a re-infection rate of 41% (Lewis et al., 1989). These results demonstrate the efficacy of multivalent vaccines and the limited control offered by conventional treatments like foot-bathing, particularly with regard to chronic, underrun lesions. The authors evaluated circulating antibody titres using a microtitre agglutination test. The average circulating antibody titre (log_2) for vaccinated sheep that were footrot-affected or unaffected at the start of the trial were 8 and 7.1, respectively, three-months after administration. The authors also report that sheep with footrot three-months post-vaccination had a lower average circulating antibody titre (5.8) at the time of inspection than sheep without footrot (7.7), which suggests that the latter titre was sufficient to provide protection against re-infection.

The efficacy of an eight-serogroup (A to H) formulation of Footvax® was evaluated in a field trial in the U.K. involving a flock of 733 sheep of mixed aged, breed, and sex with a history of footrot (Hindmarsh et al., 1989). The sheep were randomly allocated to two groups: 317 sheep received two 1 mL doses of vaccine with an inter-vaccination interval of 28 days; and 422 sheep were unvaccinated control animals. The vaccinated and unvaccinated sheep were run together for the duration of the trial. No other treatments were used until the conclusion of the trial. The feet of each sheep were inspected at days 0 and 28, and at fortnightly intervals for eight weeks thereafter. Further inspections were carried out on 506 sheep at weeks 14, 16, and 20. The initial prevalence of footrot lesions in the vaccinated and unvaccinated ewes was 10.7% and 11.7%, respectively. The prevalence dropped to 3.4% in the

vaccinated ewes by day 28 after the second vaccination, increased to 6.6% at day 84, dropped again to 3.8% at day 112, and remained at this prevalence through to day 140. In contrast, the prevalence of footrot lesions in the unvaccinated ewes increased to 22.3% at day 84, decreased to 17% at day 112, and increased to 21.2% at day 140. There were no footrot lesions in the lambs at the start of the trial. At day 56, the prevalence of footrot lesions was 2.8% and 13.7% in the vaccinated and unvaccinated lambs, respectively. The lambs were moved onto a rape crop in the latter stages of the trial, and the prevalence of footrot increased to 30.6% and 57.3% in the vaccinated and unvaccinated lambs, respectively, by day 140. The protection rate reported for the ewes exceeded 70% at six of seven inspections. The protection rate in the lambs was greater than 75% in the early stages of the trial but fell below 50% in the latter stages of the trial. The increased challenge from the unvaccinated control sheep, coupled with mechanical damage from crop stubble, is likely to have contributed to the increase in lesion prevalence in the lambs.

Field trials conducted in Australia and New Zealand reported conflicting results. A field trial conducted in Victoria demonstrated a moderate protective effect (Lambell, 1986). The trial was conducted in a flock of 300 Merino x Border Leicester ewes; 200 sheep received an eight-serogroup multivalent vaccine with an alum/oil adjuvant (Provac®), 100 sheep acted as unvaccinated controls. At the start of the trial, 0.8% and 0.9% of feet were footrot-affected in the control and vaccinated group, respectively. At day 120, the prevalence of footrot was significantly higher in the unvaccinated group, with 2.1% and 16.8% of feet affected in the vaccinated and control groups, respectively. The authors did not evaluate circulating antibody titres.

A field trial undertaken in New South Wales evaluating a nine-serogroup Footvax® formulation (Kennedy et al., 1985) reported that vaccine efficacy was reduced when administered in a dry, non-transmission period (late spring and summer). The trial included 100 Merino wethers with a history of footrot, most of which had at least one underrun lesion (≥ score 3). Fifty sheep received two 1 mL doses of vaccine with an inter-vaccination interval of seven weeks. The sheep were managed as a single group for the duration of the trial. At week seven (seven weeks after administration of the second vaccine), 46% of sheep and 63% of feet in control group were footrot affected; in contrast, 31% of sheep and 38% of feet affected in vaccinated group were footrot-affected. At week 14, 41% of sheep and 54% of feet in control group were footrot-affected, whereas 19% of sheep and 23% of feet affected in vaccinated group were footrot-affected.

Field trials in New Zealand report significant cure and protection rates (Liardet et al., 1989). Trial 1 was conducted with a flock of 149 Romney ewes; 72 received two doses of the vaccine with a six-week inter-vaccination interval, and 77 were unvaccinated controls. The duration of the trial was 28 weeks. Trial 2 was conducted in a flock of 200 Corriedale ewes; 100 received two doses of vaccine with an inter-vaccination interval of eight-weeks, and 100 were unvaccinated controls. The duration of the trial was 21 weeks. Trial 3 was conducted in a flock of 300 Corriedale hoggets; 200 sheep received two doses of vaccine with an inter-vaccination interval of 19-weeks, 100 were unvaccinated controls. The duration of the trial was 59 weeks. Trial 4 was conducted in a flock of 198 Penendale ewes; 100 sheep received two doses of vaccine with an inter-vaccination interval of 6 weeks, 98 sheep were unvaccinated controls. The duration of the trial was 14 weeks. In Trial 1, the initial prevalence of footrot was 18.1% and 11.7% in the vaccinated and unvaccinated sheep, respectively. At the completion of the trial, there was no footrot in the vaccinated group. The cure and protection rates were 100%. In Trial 2, the prevalence of footrot was 46% and 53% in the vaccinated and unvaccinated sheep, respectively. At the second inspection, the prevalence of footrot was significantly lower in the vaccinated group (7.1%) than in the control group (42.4%) ($P < 0.001$). At the completion of the trial, the prevalence of footrot in the vaccinated and unvaccinated sheep was 5.2% and 31.3%, respectively. The cure and protection rates in the vaccinated group were 81% and 94%, respectively. In Trial 3, the initial prevalence of footrot was 15% and 17% in the vaccinated and unvaccinated sheep, respectively. At the completion of the trial, the prevalence of footrot in the vaccinated and control sheep was 1.2% and 8.7%,

respectively. Cure and protection rates in the vaccinated sheep were 85% and 86%, respectively. In Trial 4, the initial footrot prevalence in the vaccinated and unvaccinated sheep was 24% and 32.7%, respectively. At the completion of the trial, the footrot prevalence in the vaccinated and unvaccinated sheep was 3% and 19.6% respectively. The cure and protection rates for the vaccinated sheep were 90% and 73%, respectively. The cure rate at week 7 for the vaccinated sheep was 55% - this is lower than reported in other studies.

A field trial undertaken in Central Otago that included five properties (Property I – V) with fine-wool merino and merino-cross ewes reported cure rates of 36% to 67% for vaccinated sheep, but the difference in cure rate between the vaccinated sheep and unvaccinated controls was only significant for one property (Property II: 54% for vaccinated sheep, 18% for unvaccinated sheep) (Mulvaney et al., 1984). Improvement rates of 64% to 89% were reported for vaccinated sheep, but these results were only significant for two properties (Property I: 83% for vaccinated sheep, 65% for unvaccinated sheep; Property II: 89% for vaccinated sheep, 45% for vaccinated sheep). The improvement rate was assessed as (no. sheep improved at final examination/number of sheep affected at start of trial) x 100, with improvement defined as a reduction in the sum of the foot scores.

The efficacy of multivalent vaccines differs according to the length of the inter-vaccination interval between the first and second doses of vaccine, and the time between vaccination and exposure to *D. nodosus*. Multivalent vaccines are most effective when there is a short interval between vaccination and exposure to *D. nodosus* (Raadsma et al., 1994). For example, Lewis et al. (1989) reported that the prevalence of footrot was less in vaccinated ewes that received a second vaccination after a 10-month interval (20%) than in ewes that received a second vaccination after a 12-month interval (26%).

There are several extraneous factors that differ between studies and limit the extent to which meaningful comparisons can be made. The number of *D. nodosus* strains in a flock and their virulence (Egerton et al., 1983; Stewart et al., 1984), environmental conditions (Egerton and Morgan, 1972), sheep breed (Emery et al., 1984; Skerman et al., 1982; Stewart et al., 1985), lesion prevalence at the time of vaccination (Egerton et al., 1983), the interval between vaccination and exposure to *D. nodosus*, and the use of additional treatment and control measures all affect apparent vaccine efficacy.

Hot dry, environmental conditions limit transmission and expression of the disease and result in spontaneous cure (Egerton and Morgan, 1972), which can mask the effect of vaccination. For example, the field trial reported by Mulvaney et al. (1984) was undertaken during an unusually hot, dry spring, which may explain why the authors did not observe a significant cure rate or protection rate on some properties. The apparent curative effect of a multivalent vaccine was less apparent when the study was conducted during a non-transmission period where natural remission was allowed to occur (Egerton and Morgan, 1972; Mulvaney et al., 1984; Kennedy et al., 1985; Glenn et al., 1985).

There is considerable variation in host susceptibility to *D. nodosus* infection at a number of levels. For instance, Egerton and Raadsma (1991) outlined five potential tiers of genetic resistance: (i) variation between breeds; (ii) variation between strains within a breed; (iii) variation between bloodlines within a strain; (iv) variation between sire lines within a bloodline; and (v) variation between individuals. It is widely accepted that Merinos are more susceptible to footrot than British breeds or cross-breeds (Beveridge, 1941; Emery et al., 1984; Skerman, 1982; Stewart et al., 1985; Youatt, 1837), but the reason for this has not been conclusively demonstrated. Following immunisation with a monovalent whole-cell vaccine, Skerman et al. (1982) reported that the antibody response persisted for longer in Romneys (20 weeks) than in Merinos (5 weeks), which suggests that British breeds may be able to mount a more effective humoral immune response

Multivalent vaccines are typically used in conjunction with other control measures, such as foot-bathing and antibiotic therapy. For example, in two of the field trials discussed earlier (Bulgin et al., 1985; Lewis et al., 1989)

antiseptic foot-bathing was used in addition to vaccination. In such cases it is difficult to assess vaccine efficacy, as the additional treatments can mimic the effect of the vaccine.

The literature cited here indicates that multivalent vaccines provide some degree of protection against footrot and support anecdotal evidence that multivalent vaccines can be a valuable tool for the prevention and treatment of footrot. When used in conjunction with other treatments, they can reduce the prevalence of disease to a sufficient level to enable targeted culling. There is evidence that some multivalent formulations stimulate an adequate antibody response and provide an adequate level of protection to enable a meaningful level of control of footrot. However, some formulations fail to stimulate an adequate antibody response and do not provide an adequate level of protection. Further investigation of multivalent vaccine formulations is therefore warranted.

Inter-vaccination intervals between mono- and bivalent vaccines

In flocks infected with multiple virulent *D. nodosus* strains belonging to three or more serogroups, sequential mono- and bivalent vaccines must be administered with an appropriate inter-vaccination interval to avoid antigenic competition. The inter-vaccination interval refers to the time between the first doses of each vaccine. An inter-vaccination interval of 12-months has been evaluated in field trials (Dhungyel et al., 2008; Dhungyel et al., 2013); however, immunological trials indicate that a shorter inter-vaccination interval may be feasible. Dhungyel and Whittington (2010) evaluated inter-vaccination intervals of 12, 9, 6, 3 and 0 months between successive bivalent vaccines and found no significant difference between predicted mean antibody titres for sheep that received a second bivalent vaccine after 12, 9, 6 or 3 months. The predicted mean antibody titre for sheep that received two bivalent vaccines concurrently (i.e. an inter-vaccination interval of 0 months) was significantly lower than in the other groups. An inter-vaccination interval of 2 months, which is the minimum possible interval between two vaccines that are not administered concurrently, was not evaluated in that study. From the farmer's perspective, giving two successive bivalent vaccines with an inter-vaccination interval of two months means that sheep would be mustered and vaccinated four times at monthly intervals: twice for two doses of the first bivalent vaccine and twice for two doses of the second.

3. Project objectives

i. To optimise the formulation and compare the magnitude and duration of immune responses of a novel recombinant multivalent vaccine with bivalent vaccine, in order to optimise the dose of the multivalent vaccine.

ii. Concurrent with objective i, to explore formulation modifications of the bivalent vaccine to confirm that the existing formulation provides maximum antibody levels for the longest duration.

iii. Concurrent with objectives i and ii, to determine whether an inter-vaccination interval of two-months between successive bivalent vaccines overcomes antigenic competition.

iv. To compare the efficacy of a novel recombinant multivalent vaccine with an optimised bivalent vaccine in flocks with two serogroups of virulent *D. nodosus*.

v. Concurrent with iv, to compare the efficacy of a novel recombinant multivalent vaccine with sequentially administered (three-month or shorter inter-vaccination interval) bivalent vaccine in flocks with three-to-six *D. nodosus* serogroups of virulent *D. nodosus*.

vi. Concurrent with objectives iv and v, to demonstrate that vaccination is at least as effective as regular foot-bathing in suppressing the clinical signs of footrot, at no greater cost.

vii. Concurrent with objectives iv, v, and vi, to demonstrate that control of footrot through vaccination results in measurable improvement in wool production and reduces welfare impacts.

viii. Extending the findings from the project to governmental and consultant sheep veterinarians, including presentation at the Australian Sheep Veterinary Society annual meeting in 2019, and delivery of an article for publication in relevant AWI communications.

4. Success in achieving objectives

i. The immunogenicity of four novel multivalent vaccines containing six or nine *D. nodosus* serogroups at low and high doses per serogroup were evaluated in a six-month sheep trial. The immunogenicity of each multivalent vaccine was compared to a commercial bivalent vaccine. One of the novel multivalent vaccines which contained nine serogroups (A – I) stimulated a satisfactory immune response but less than that of the commercial bivalent vaccine. Based on these results it was concluded that this multivalent formulation may be sufficient to provide a level of control over the disease.

ii. Two methods of formulation of vaccines were evaluated in which bivalent vaccines were formulated by emulsifying each fimbrial protein with a mineral oil adjuvant separately or by mixing the two fimbrial proteins prior to emulsification. Thus, the resulting vaccines contained mineral oil micelles containing individual fimbrial proteins or both fimbrial proteins. This is expected to alter the way in which the immune system first encounters the fimbrial proteins. The results of testing these formulations indicated that method of formulation had no effect on the antibody levels stimulated by the vaccines.

iii. The inter-vaccination intervals of two- and three-months between successive bivalent vaccines were evaluated and found that reducing the inter-vaccination interval to two-months had no significant impact on vaccine efficacy. This shorter interval may be more practical for some farmers than the longer interval.

iv. The novel multivalent vaccine was evaluated in four commercial Merino flocks in Tasmania. The flocks were infected with virulent strains of *D. nodosus* belonging to between two-to-five serogroups. The multivalent vaccine was compared to commercial bivalent vaccine(s) and antiseptic foot-bathing in each flock.

v. The commercial bivalent vaccine stimulated higher circulating antibody titres than the novel multivalent vaccine. Overall, the multivalent vaccine had a greater curative effect than the bivalent vaccine. However, the expression of footrot was affected by the hot, dry environmental conditions in Tasmania during the trial period i.e. lesions may have regressed in all sheep due to climatic conditions.

vi. Cure and improvement rates were calculated for September, October and February, 2months, 3 months and 6 months post vaccination using the foot scores recorded at the start of the trial in July as a reference point. There were only a few significant differences between the cure rates for different groups. On Farm 1, the cure rate was significantly higher ($P = 0.036$) for the multivalent vaccine group than for the control group in September. On Farm 3, the cure rate was significantly higher ($P = 0.027$) for the multivalent vaccine group than for the control group in October. On Farm 4, the cure rate was significantly higher for the control group than for the bivalent vaccine group ($P = 0.017$) and the multivalent vaccine group ($P = 0.012$). There were no significant differences in improvement rates between the three groups on any of the four farms at any time-point.

vii. Improvement in animal welfare was inferred from changes in the prevalence and severity of foot lesions for sheep that were vaccinated (bivalent or multivalent vaccine) or foot-bathed regularly on each of the farms. Improvement rates, defined as a reduction in total weighted foot score (TWFS), were higher than cure rates for sheep that received a vaccine, irrespective of formulation, and for sheep that were foot-bathed. The reduction in disease severity resulted in fewer incidences of lameness and improved welfare of the sheep on each farm and the body condition scores of these animals improved from an average of less than 2 to more than 3 for the trial period.

viii. A presentation of this research will be made at the NSW District Veterinarians' Annual Conference 2020 being held in Broken Hill 13th - 15th October, 2020 and also at the Australian Sheep Veterinarians annual conference that has not been scheduled to date and likely to be held in 2021 when COVID-19 restrictions are lifted. AWI will be updated with a new Communication Report once the presentations have been made.

For stakeholder communications – A Footrot Vaccination Fact sheet and a Beyond the Bale article have been drafted and submitted to AWI.

5. Methodology

Objectives i, ii, iii (Immunological trial)

Vaccine formulation and vaccination

Recombinant fimbrial vaccines were formulated specifically for this study by Tréidlia Biovet. Four multivalent vaccine formulations were produced, containing fimbrial proteins representing six or nine *D. nodosus* serogroups, at two concentrations of each fimbrial protein, low or high (Table 1). Two 2 mL doses of each multivalent vaccine were administered with an interval of one-month between doses. The serogroups included in the six-serogroup formulations were the most prevalent serogroups in Tasmania based on diagnostic submissions received by the University of Sydney, Farm Animal Health Infectious Diseases Laboratory, Camden during the two-year period preceding the trial. Serogroup M was not included in either formulation as recombinant fimbrial proteins for this serogroup were not available. Six different bivalent vaccines that differed only in the serogroups included were also formulated, as described in Table 2.

Animals and trial design

Twelve-month old Merino wethers (castrated males) (*n* = 108) were obtained from a flock with no history of footrot, located near Bredbo, New South Wales. The sheep were transported to a secure pasture facility at the University of Sydney at Camden, New South Wales, two weeks prior to the commencement of the trial. To confirm that the sheep were free of *D. nodosus* infection, swabs were collected from the feet of ten randomly selected sheep for microbiological culture and 16S rRNA PCR testing. Two swabs were collected from each of the ten sheep, the first for microbiological culture, and the second for 16S rRNA PCR testing. Each swab was used to sample the entire surface of the interdigital skin of all four feet of each sheep. The sheep were maintained as a single mob for the duration of the trial. The sheep were grazed on pasture consisting of native and introduced species and fed supplementary lucerne hay and barley grain when necessary. Sheep were ear-tagged for individual identification, and randomly assigned to each treatment group using a random number generator in Excel (Microsoft) (Table 2). Power analyses were undertaken with G*Power (Faul et al., 2007) to determine the number of sheep required in each treatment group.

Two methods for preparing bivalent vaccines were evaluated. Using the first method, bivalent vaccines 1 to 4 were formulated by emulsifying each fimbrial protein with a mineral oil adjuvant separately, to produce two mono-valent vaccines, and then mixing the two mono-valent vaccines together to produce the desired bivalent vaccine formulation, as described previously (Mattick et al., 1987). Bivalent vaccines prepared using this standard method probably consist of oil micelles containing either the first or second fimbrial protein, but usually not both unless micelles fuse after mixing. Bivalent vaccine 5 was formulated using an alternative method: the two antigens were mixed together, and then emulsified with a mineral oil adjuvant. The resulting bivalent vaccine consisted of oil micelles containing both antigens. This major variation in the formulation of the vaccine would likely change the way in which the fimbrial proteins are first encountered by the immune system.

Vaccines were administered as outlined in Tables 1 and 2. Group 1 (negative control) received 1 mL primary and booster doses of vaccine adjuvant at Days 0 and 30, respectively. Groups 2 to 5 received a 2 mL primary and booster dose of multivalent vaccine at Days 0 and 30, respectively. Groups 6, 7, and 10 received a 1 mL primary and booster dose of bivalent vaccine at Days 0 and 30, respectively, followed by a 1 mL primary and booster dose of a different bivalent vaccine at Days 60 and 90, respectively, i.e. with an inter-vaccination interval of 2-months. Groups 8 and 9 received a 1 mL primary and booster dose of bivalent vaccine at Days 0 and 30, respectively, followed by a 1 mL primary and booster dose of a different bivalent vaccine at Days 90 and 120, respectively, i.e. with an inter-

vaccination interval of 3-months. Note that the inter-vaccination interval refers to the number of months between primary doses of each bivalent vaccine.

In order to confirm that adequate antibody levels can be achieved in the field with a three-month inter-vaccination interval (note that this has not been done before), a comparison of antibody levels after administration of the first and second bivalent vaccines, which differed in antigen composition, was undertaken in a field trial on four farms (see below).

Animal ethics

All procedures were approved by The University of Sydney's Animal Ethics Committee (AEC Project no. 2018/1396) (Appendix 7).

Table 1: Vaccination schedule for the sheep trial. The vaccines are described in Table 2.

Group	No. sheep	Vaccine group	Month 0	Month 1	Month 2	Month 3	Month 4
1	9	Control (vaccine adjuvant only)	Vaccinate	Vaccinate	-	-	-
2	11	Multivalent 1	Vaccinate	Vaccinate	-	-	-
3	11	Multivalent 2	Vaccinate	Vaccinate	-	-	-
4	11	Multivalent 3	Vaccinate	Vaccinate	-	-	-
5	11	Multivalent 4	Vaccinate	Vaccinate			
6	11	Bivalent 1 (2-month inter-vaccination interval)	Vaccinate (A + C)	Vaccinate (A + C)	Vaccinate (G + H)	Vaccinate (G + H)	-
7	11	Bivalent 2 (2-month inter-vaccination interval)	Vaccinate (E + G)	Vaccinate (E + G)	Vaccinate (H + I)	Vaccinate (H + I)	-
8	11	Bivalent 3 (3-month inter-vaccination interval)	Vaccinate (A + C)	Vaccinate (A + C)	-	Vaccinate (G + H)	Vaccinate (G + H)
9	11	Bivalent 4 (3-month inter-vaccination interval)	Vaccinate (E + G)	Vaccinate (E + G)	-	Vaccinate (H + I)	Vaccinate (H + I)
10	11	Bivalent 5 (alt.) (2-month inter-vaccination interval)	Vaccinate (A + C)	Vaccinate (A + C)	Vaccinate (G + H)	Vaccinate (G + H)	-

Table 2: Vaccine formulations for the immunological trial.

Group	Vaccine(s)	Antigens (serogroups)	Dose per antigen	Volume per dose
1	Control	Vaccine adjuvant only	n/a	1 mL
2	Multivalent 1	A, B, C, G, H, I	low	2 mL
3	Multivalent 2	A, B, C, G, H, I	high	2 mL
4	Multivalent 3	A, B, C, D, E, F, G, H, I	low	2 mL
5	Multivalent 4	A, B, C, D, E, F, G, H, I	high	2 mL
6	Bivalent 1	A+C, G+H	high	1 mL
7	Bivalent 2	E+G, H+I	high	1 mL
8	Bivalent 3	A+C, G+H	high	1 mL
9	Bivalent 4	E+G, H+I	high	1 mL
10	Bivalent 5*	A+C, G+H	high	1 mL

*Alternative method of formulation

Evaluation of antibody titres

Blood samples were collected from each sheep by jugular venepuncture immediately prior to the administration of the primary and booster doses of each vaccine, and at approximately monthly intervals thereafter. Serum samples were stored at -20°C prior to testing. Antibody responses to each homologous antigen were evaluated using a microtitre agglutination test, as described previously (Raadsma et al., 1995). Each serum sample was diluted 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, 1:10240, 1:20480 and 1:40960. Antibody titres were recorded as the inverse of the highest serum dilution in which there was a visible agglutination reaction.

Statistical analysis

Antibody titres were analysed using a restricted maximum likelihood (REML) analysis in a linear mixed model. Vaccine formulation, time-point and their interactions were included as fixed effects, and sheep was included as a random effect. Variation in antibody response between serogroups was analysed using a separate REML analysis, with serogroup, time-point and their interactions included as fixed effects and sheep included as a random effect. The significance of differences between predicted means was evaluated using least squared difference (LSD), with significance at the 5% level. Residual plots were inspected to confirm that the residuals were normally distributed and were deemed acceptable. Statistical analyses were performed with GenStat 16th Edition (VSN International, Hemel Hempstead, U.K.).

Objectives iv, v, vi, vii (Field trial)

Recruitment of flocks

Foot lesion swabs were collected from seven commercial Merino flocks in Tasmania in April 2019 for the initial microbiological and PCR testing. Four flocks were selected for enrolment in the trial according to the number of virulent *D. nodosus* serogroups detected, the owner/manager's willingness to remain in the trial for a minimum period of six months, and their capacity to meet the requirements of the trial, including sampling and monthly foot-bathing of the control group.

Trial design

Three-hundred 12-month-old Merino ewes from each flock were included in the trial. The trial commenced in July 2020, and the sheep were ear-tagged for individual identification. On each farm, each sheep was allocated to one of three treatment groups (*n* = 100 per group) using a systematic random approach: control, bivalent vaccine and multivalent vaccine. Sheep in the control group were foot-bathed in a 10% zinc sulphate solution on a monthly basis, immediately after each inspection. Sheep in the bivalent group on each farm received two consecutive bivalent vaccines with a three-month inter-vaccination interval; two 1 mL doses of the first bivalent vaccine were administered in July and August, 2019, and two 1 mL doses of the second bivalent vaccine were administered in October and November. Sheep in the multivalent group received two 2 mL doses of multivalent vaccine, in July and August. The trial sheep on each farm were run together as a single mob, and they were examined at monthly intervals during the trial, except for December, when we were unable to visit the properties. At each examination, each foot of each sheep was inspected and scored using a previously described method (Egerton and Roberts, 1971) and transformed using a weighted scoring system (total weighted foot score, TWFS) (Whittington and Nicholls, 1995). At each examination, blood samples (8 to 10 mL) were collected via jugular venepuncture from ten sheep in each treatment group (*n* = 10 per group) for evaluation of blood antibody levels. The same 10 sheep were bled at each time-point. Antibody levels were compared across the three treatment groups. The antibody response of the bivalent group to the first and second bivalent vaccine was also compared across five time-points: Month 0 (pre-vaccination) through to Month 5 (three-months after administration of the second dose of each bivalent vaccine).

Sheep on Farms 1 to 3 were grazed on irrigated pasture for most of the trial but were periodically grazed on crop stubble. The pasture was irrigated every three weeks using a centre-pivot irrigator, with 64 mm of water applied to the paddock each time. The soil remained moist for a period of two-days after irrigation, but by third day a crust had formed that was dry to the touch. Sheep on Farm 4 were grazed on dry-land grasses from the start of the trial in July through to mid-December, at which point they were transferred to an irrigated paddock. The paddock was irrigated twice per week using a centre-pivot irrigator, with 12 mm of water applied to the paddock each time.

Collection of lesion swabs

Foot lesions swabs were collected from sheep with representative active lesions for microbiological culture and direct PCR testing. Swabs for microbiological culture were collected in modified Stuart Transport Medium (STM) (Oxoid). Swabs for direct PCR were collected into a lysis buffer. Specimens were collected from the interdigital skin or the active margin of underrun lesions with a sterile, cotton-tipped swab (CLASSIQswabs; Copan Italia, Brescia, Italy).

Microbiological culture of D. nodosus

Swab samples were transported to the laboratory on ice, and processed immediatley upon receipt. *D. nodosus* was cultured from each swab collected into STM, as described previously (McPherson et al., 2018). Upon receipt at the

laboratory, each foot swab was removed from the STM and streaked onto a 4% hoof agar (HA) plate (Thomas, 1958) in a checkerboard pattern, and incubated in an anaerobic jar with an anaerobic gas pack (GasPak, BD, New Jersey, U.S.A.) and an anaerobic indicator (Oxoid, Hampshire, U.K.) at 37°C for 72 hours (Stewart and Claxton, 1993). Thereafter, individual *D. nodosus* colonies were picked from the primary culture plate using a sterile inoculation loop and sub-cultured onto 2% HA and incubated under the conditions described above. This process was repeated until individual isolates of *D. nodosus* were obtained.

Elastase test

The virulence of each *D. nodosus* isolate was assessed using the elastase test, as described previously (Stewart, 1979). Briefly, each isolate was cultured on one quarter of an elastin agar plate. The plates were incubated for 28 days and inspected at four-day intervals for elastase activity, which was indicate by a zone of clearing around the inoculum. Isolates that were elastase-positive at 12 days or less were deemed virulent; isolates that were positive at 16 days or more, or not at all, were deemed benign. An isolate with known elastase activity (virulent *D. nodosus* type strain A1001; elastase-positive at 4-8 days post-inoculation) was inoculated onto one quarter or each elastin agar plate as a virulent control.

DNA extraction

DNA was extracted from each *D. nodosus* isolate via boiling and centrifugation, as described previously (McPherson et al., 2017). DNA was extracted from each swab collected into lysis buffer using the Wizard Genomic DNA Purification kit (Promega, Madison, U.S.A.), following the protocol for extraction of genomic DNA from Gram-negative bacteria.

Serogrouping

The serogroups of *D. nodosus* that were present in each flock in April 2019, prior to the vaccination phase of the trial, was determined by agglutination tests on *D. nodosus* isolates obtained by culture, and by PCR testing of the same isolates. Direct (culture-independent) PCR testing of DNA extracted from swabs collected into lysis buffer was also undertaken to provide additional information about the serogroups present in each flock. Direct PCR testing was also undertaken in October and November 2019. Culture-based and direct PCR serogrouping were undertaken at the end of the trial in February 2020 to determine which serogroups remained in each flock after the vaccination phase.

Agglutination tests

Each *D. nodosus* isolate was serogrouped using the slide agglutination test as described previously (Stewart and Claxton, 1993). Briefly, each *D. nodosus* isolate was harvested by flooding the surface of the agar plate with 500 µL of sterile phosphate buffered saline PBS pH 7.4 (Astral Scientific, Taren Point, Australia) with 0.5% w/v formalin (Fronine, Riverstone, Australia), scraping the *D. nodosus* colonies from the surface of the agar with a sterile scalpel blade, and collecting the suspended culture into a 1.5 mL screw-cap microcentrifuge tube (SSIBio, Lodi, U.S.A.). Each suspension was mixed for 10 s in a vortex mixer and visually assessed to ensure an even suspension. Antisera, which were prepared in rabbits for each of the 10 *D. nodosus* prototype serogroup antigens as described previously (Claxton et al., 1983) and stored at -20°C, were brought to room temperature. Twenty-microlitres of the harvested *D. nodosus* suspension were mixed with 5 µL of undiluted rabbit antiserum on a clean glass microscope slide. The slide was gently rocked for 10 s and examined. A reaction was regarded as positive when a substantial coarse agglutination reaction was observed within 10 s of the serum and *D. nodosus* suspension being mixed.

Serogroup PCR.

PCR amplification of serogroup-specific regions of the *fimA* gene of *D. nodosus* was undertaken as previously described (Dhungyel et al., 2002). Amplification was performed in a Bio-Rad T100 thermal cycler (Bio-Rad, Gladesville, Australia). DNA extracted from pure cultures of each of the appropriate *D. nodosus* serogroup prototype strains, and sterile nuclease-free water, were included in each run as positive and negative controls, respectively. PCR product was visualised on a 2% agarose gel stained with RedSafe (iNtRON Biotechnology, Gyeonggi-do, South Korea), and viewed under ultraviolet light. A successful PCR run was defined as one in which: (i) there was amplification of the positive controls, indicated by the presence of three amplicons of the appropriate molecular weights on the 2% agarose gel, and (ii) there was no amplification of the negative control. A sample was deemed positive for a *D. nodosus* serogroup when a band of the appropriate size was observed on the gel in the lane corresponding to the sample (Dhungyel et al., 2002).

The results of serogrouping in each flock are shown in Table 3 together with the results of the elastase test conducted on the same isolates. There was incomplete agreement between the agglutination and PCR results however it was clear that there were $\geq$ 2 serogroups of virulent *D. nodosus* present in six of the seven flocks and four or more virulent serogroups on three of the farms. These results were used to make decisions about which flocks to include in the trial and which bivalent vaccine formulations to recommend as described in the sections below.

Table 3: Diagnostic results for the seven commercial Merino flocks screened prior to the commencement of the field trial in April 2019. Highlighted flocks were selected for the field trial.

		Serogroups detected	
Flock	**Location**	**Culture**	**Direct PCR**
A	Bothwell	A*, G*	A, C, G, H
B	Bothwell	A*, C*, E*	C, E, H
C	Flinders Island	A*, B*, I*, G*	A, B, E
D	Flinders Island	C*, H*, I	B, C, E
E	Ouse	A*, B*, H*, I*	A, B, C, E, H, I
F	Cressy	A*, B*, C, D*, E, I*	B, D, H, I
G	Breadalbane	G*	B, E, H

*Confirmed as virulent based on elastase testing

Final flock selection

Four of the seven flocks (Flocks A, B, E, and F) were selected for the field trial based on the number of virulent *D. nodosus* serogroups detected and the willingness of the property owners to participate in the trial (refer to Tables 3 and 4)

Table 4: Vaccine formulations for the four flocks included in the field trial. The letter included in parentheses refers to the flock identifier used during the initial diagnostic phase of the trial (see Table 3).

	Serogroups targeted		
Flock	**Multivalent**	**Bivalent 1**	**Bivalent 2**
1 (A)	A, B, C, D, E, F, G, H, I	A, G	C, H
2 (B)	A, B, C, D, E, F, G, H, I	C, E	A, H
3 (E)	A, B, C, D, E, F, G, H, I	A, B	E, H
4 (F)	A, B, C, D, E, F, G, H, I	D, I	B, H

Vaccine formulation

Flock-specific bivalent and multivalent vaccines were formulated by Tréidlia Biovet (Table 4). The multivalent vaccine contained recombinant fimbrial proteins representing nine (A through to I) *D. nodosus* serogroups. This vaccine formulation was selected for evaluation based on the outcomes of the previous immunological trial conducted at The University of Sydney, Camden (refer to Section 6 of this report). Two bivalent vaccines were formulated for each of the four flocks according the results of the diagnostic testing that was performed prior to the commencement of the trial, and then repeated one-month after the administration of the booster dose of the first vaccine (Table 5).

Evaluation of antibody titres

Blood samples were collected from each sheep by jugular venepuncture immediately prior to the administration of the primary and booster doses of each vaccine, and at approximately monthly intervals thereafter. Antibody responses to each homologous antigen were evaluated using a microtitre agglutination test, as described previously (Raadsma et al., 1995). Each serum sample was diluted 1:20, 1:40, 1:80, 1:160, 1:320, 1:640, 1:1280, 1:2560, 1:5120, 1:10240, 1:20480 and 1:40960. Antibody titres were recorded as the inverse of the highest serum dilution in which there was a visible agglutination reaction.

Animal ethics

All procedures were approved by The University of Sydney's Animal Ethics Committee (AEC Project no. 2019/1532) (Appendix 7).

Environmental data

Temperature and rainfall data were collected from the Bureau of Meteorology (BOM) automatic weather station located nearest to each of the four farms. Environmental data for Farms 1 and 2, which were located close to one another, were collected from the BOM station located at Franklin Street, Bothwell (Station ID: 095001). Environmental data for Farm 3 were collected from the BOM station located at Ouse Fire Station (Station ID: 095048). Environmental data for Farm 4 were collected from the BOM station located at Cressy Research Station (Station ID: 091036).

Statistical analysis

Antibody titres were analysed using a restricted maximum likelihood (REML) analysis in a linear mixed model. Pooled agglutination test data from all four farms were analysed together. Vaccine formulation, time-point and their interactions were included as fixed effects, and farm and sheep were included as random effects. The significance of differences between predicted means was evaluated using least squared difference (LSD), with significance at the 5% level. REML analyses were performed with GenStat 16th Edition (VSN International, Hemel Hempstead, U.K.). The predicted mean antibody titres for the first and second bivalent vaccines administered on each property in the field trial were compared using a similar analysis by pooling data from all four farms. Residual plots were inspected to confirm that the residuals were normally distributed. Analyses were performed using untransformed data.

Raw foot score data, which consisted of a score for each foot of each sheep, were analysed with an ordinal logistic regression using a cumulative link function within the ordinal package in R (v3.6.2; R Foundation, Vienna, Austria). Treatment (control, bivalent vaccine, multivalent vaccine), Month, and their interactions were included as fixed effects, and tag number was included as random effect. Data from each farm were analysed separately.

Cure and protection rates were calculated for each group on each farm using a formula modified from Liardet et al. (1989). Cure and protection rates for September, October, and February were calculated, using July as a reference point. The following formulae were used:

Cure rate (%) = (no. sheep cured at finish / no. sheep affected at start) x 100

Improvement rate (%) = (no. sheep improved at finish / no. sheep affected at start) x 100

Where:

Affected = a sheep with a score of 2 or greater for any foot

Cured = a sheep that was affected at the start of the trial in July, but had a foot score of 0 for all four feet at the time-point of interest (September, October 2019, or February 2020)

Improved = a sheep for which the total weighted foot score for all four feet was lower at the time-point of interest (September, October 2019, or February 2020) than for the start of the trial in July

To determine pairwise significance between cure rates and improvement rates, two-sample binomial tests for proportions were conducted in R.

6. Results

Objectives i, ii, iii (Immunological trial)

As expected, vaccinated sheep developed strong antibody responses. The antibody levels for all vaccinated groups of sheep (Groups 2 – 10) were significantly higher ($P < 0.05$) than those of the control group (Group 1), which received a 1 mL dose of oil adjuvant only, at all time-points following administration of the primary dose of vaccine (Figure 1)

In general, sheep vaccinated with bivalent vaccine developed stronger antibody responses than sheep given multivalent vaccine. The antibody levels for all groups of sheep that received a bivalent vaccine (Groups 6 to 11) were significantly higher ($P < 0.05$) at most time-points following administration of the primary dose of vaccine than for those that received a multivalent vaccine (Figure 1). There were some time-points at which there was no significant difference between some multivalent and bivalent groups, but these time points did not form a meaningful pattern (Figure 1).

The dose of fimbrial antigen included in the multivalent vaccine appeared to be important. The predicted mean antibody titres for sheep that received a high dose multivalent fimbrial vaccine (Groups 3 and 5) were significantly higher ($P < 0.05$) than those for sheep that received a low dose multivalent vaccine (Groups 2 and 4) (Figure 1). There was no significant difference in the predicted mean antibody titres between groups of sheep that received a vaccine containing six or nine serogroups ($P < 0.05$) at either dose of fimbrial protein, except for month 6, where the predicted mean antibody titre for Group 4, which received a six serogroup formulation at a low dose per serogroup, was higher than that of Group 5, which received a nine serogroup formulation at a low dose per serogroup (Figure 1).

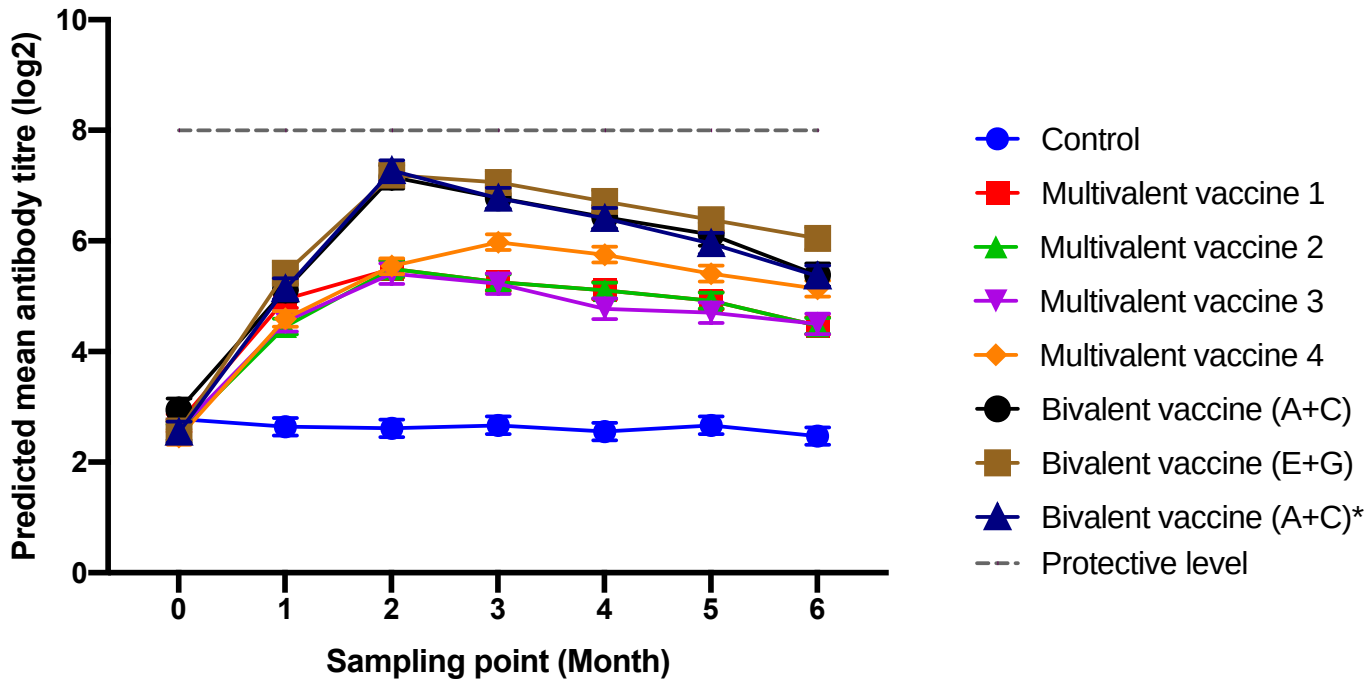

Figure 1: Microplate agglutination test titres. The primary dose of each vaccine was administered at Month 0, and the booster dose of each vaccine administered at Month 1. Data are predicted means and standard errors from the REML model.

Inter-vaccination intervals of two and three months between successive bivalent vaccines were evaluated with a favourable outcome for the shorter interval. Two bivalent vaccine formulations (G+H, H+I) were evaluated to account for variation in antibody responses between different serogroups. There were no significant differences ($P < 0.05$) in predicted mean antibody titres for groups that received a second bivalent vaccine following intervals of two or three months (Figure 2).

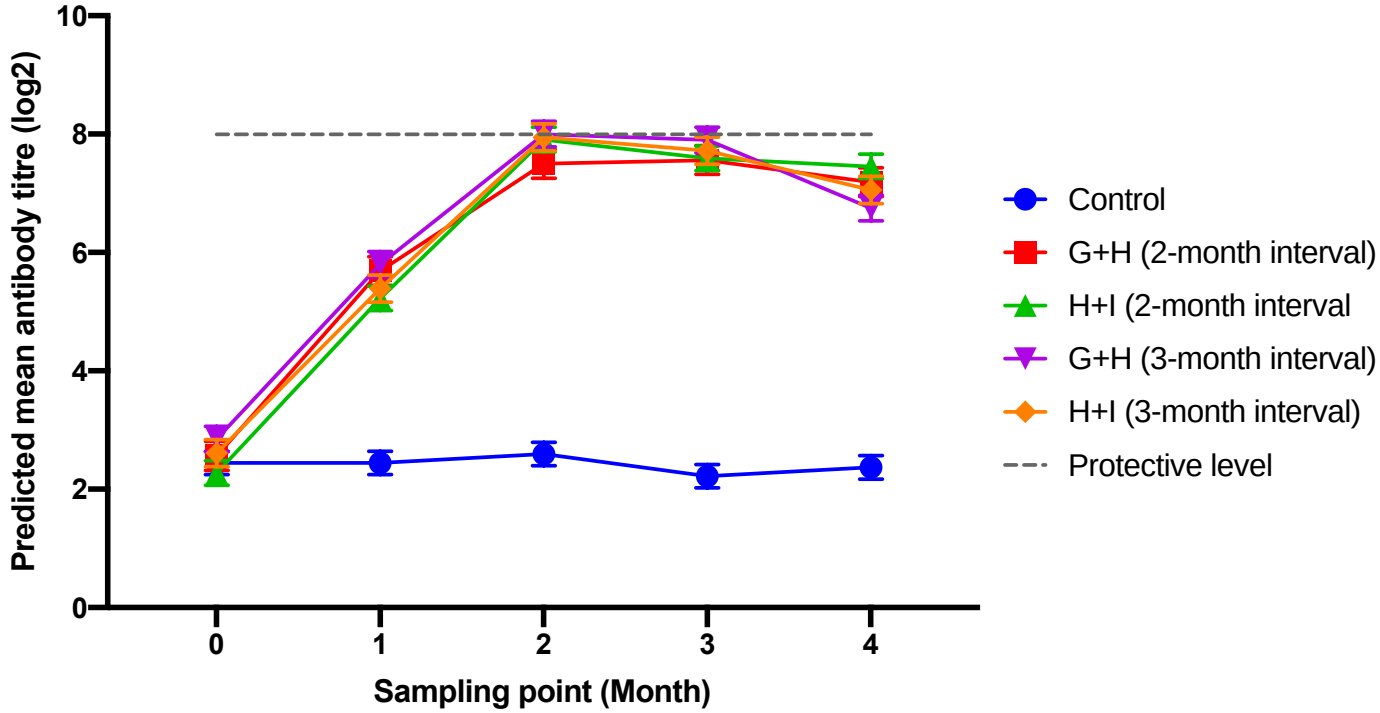

Figure 2: Microplate agglutination test titres for the second bivalent vaccines administered following an inter-vaccination interval of two or three months. The primary dose of each vaccine was administered at time-point 0, and the booster dose of each vaccine administered at time-point 1. Data are predicted means and standard errors from the REML model.

Objectives iv, v, vi, vii (Field trial)

Detection and serogrouping of D. nodosus

Swab samples were collected from one foot each of 20 sheep with active foot lesions in each parent flock in April 2019 for initial serogroup identification, three-months prior to the commencement of the vaccination phase of the trial (Table 3). Two swabs were collected from each foot; the first for microbiological culture, and the second for direct PCR testing. Additional swabs were collected from one foot of 10 randomly selected sheep with active lesions in October and November 2019 for direct PCR testing only. Swabs were collected for microbiological culture from sheep with active lesions in February 2020. The serogroups detected in each flock at each time-point are reported in Table 5.

Table 5: Summary of culture and direct PCR results for the four flocks enrolled in the field trial

		Serogroups detected	
Property	**Sampling date**	**Culture**	**Direct PCR**
1	April, 2019	A*, G*	A, C, G, H
	October, 2019	…	A, C, G, H
	November, 2019	…	A, C, G, H
	February, 2020	A, G*	…
2	April, 2019	A*, C*, E*	C, E, H
	October, 2019	…	C, E
	November, 2019	…	E, H
	February, 2020	E	…
3	April, 2019	A*, B*, H*, I*	A, B, C, E, H, I
	October, 2019	…	A, B, C, E, H, I
	November, 2019	…	A, B, E
	February, 2020	B	…
4	April, 2019	A*, B*, C, D*, E, I*	B, D, H, I
	October, 2019	…	A, B, H, I
	November, 2019	…	B, H
	February, 2020	A, B, M*	…

**D. nodosus* isolates belonging to this serogroup were deemed virulent using the elastase test

Environmental data

Temperature and rainfall data were collected from the BOM weather station nearest to each property. Weather data for Farm 1 and Farm 2 are from the same BOM station as these farms were located close to one another; separate BOM stations were used for the other farms. At each BOM station, rainfall was consistent during the winter months and averaged 44 mm per month for Farms 1 and 2, 64 mm per month for Farm 3 (Figure 4), and 59.5 mm per month for Farm 4 (Figure 5). During winter, the average daily temperatures were typically <10°C. The average daily temperature increased with the advent of spring and summer, and mostly exceeded 10°C from around late September, but rainfall was less consistent or episodic during this period, ranging from 10 to 41 mm per month for Farms 1 and 2, 14 to 50 mm per month for Farm 3, and 9 to 35 mm for Farm 4. Average monthly temperatures were >15°C at all three sites from December through to February.

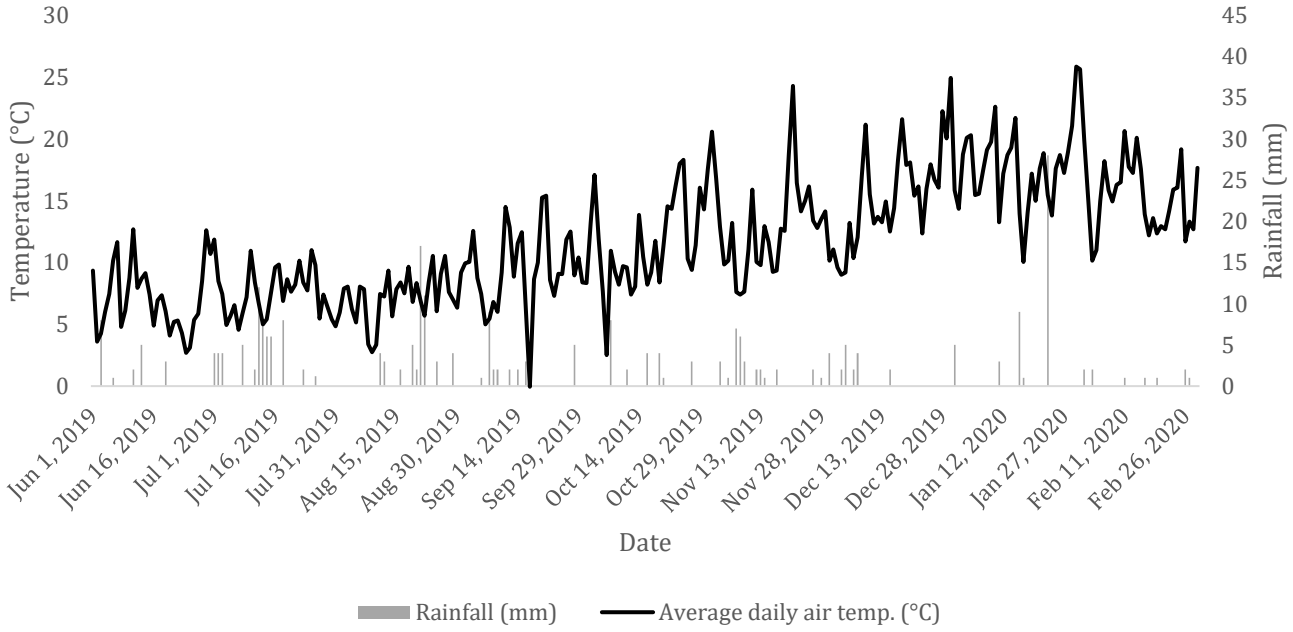

Figure 3: Weather data for Farms 1 and 2. Weather data are reported for June 2019, one-month prior to the vaccination phase of the trial, through to the end of the trial in February, 2020. Weather data were drawn from the BOM weather station located at Franklin Street, Bothwell (Station ID: 095001).

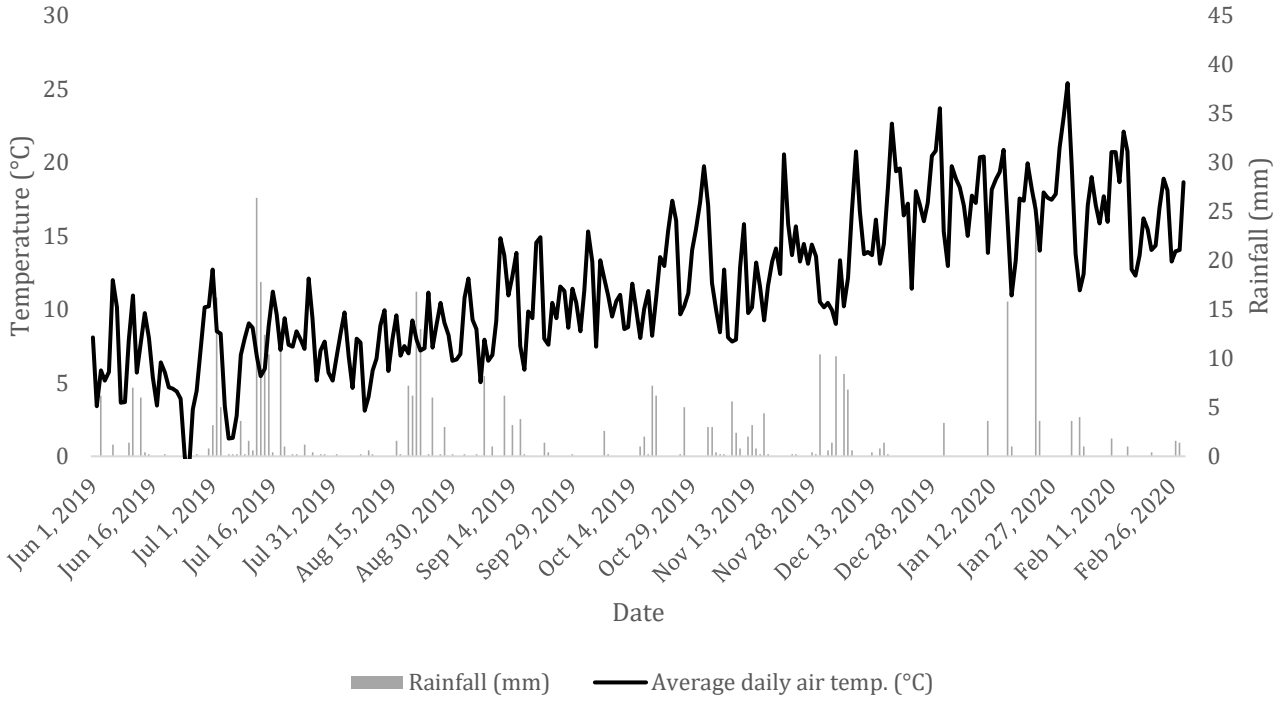

Figure 4: Weather data for Farm 3. Weather data are reported for June 2019, one-month prior to the vaccination phase of the trial, through to the end of the trial in February, 2020. Weather data were drawn from the BOM weather station located at Ouse Fire Station (Station ID: 095048).

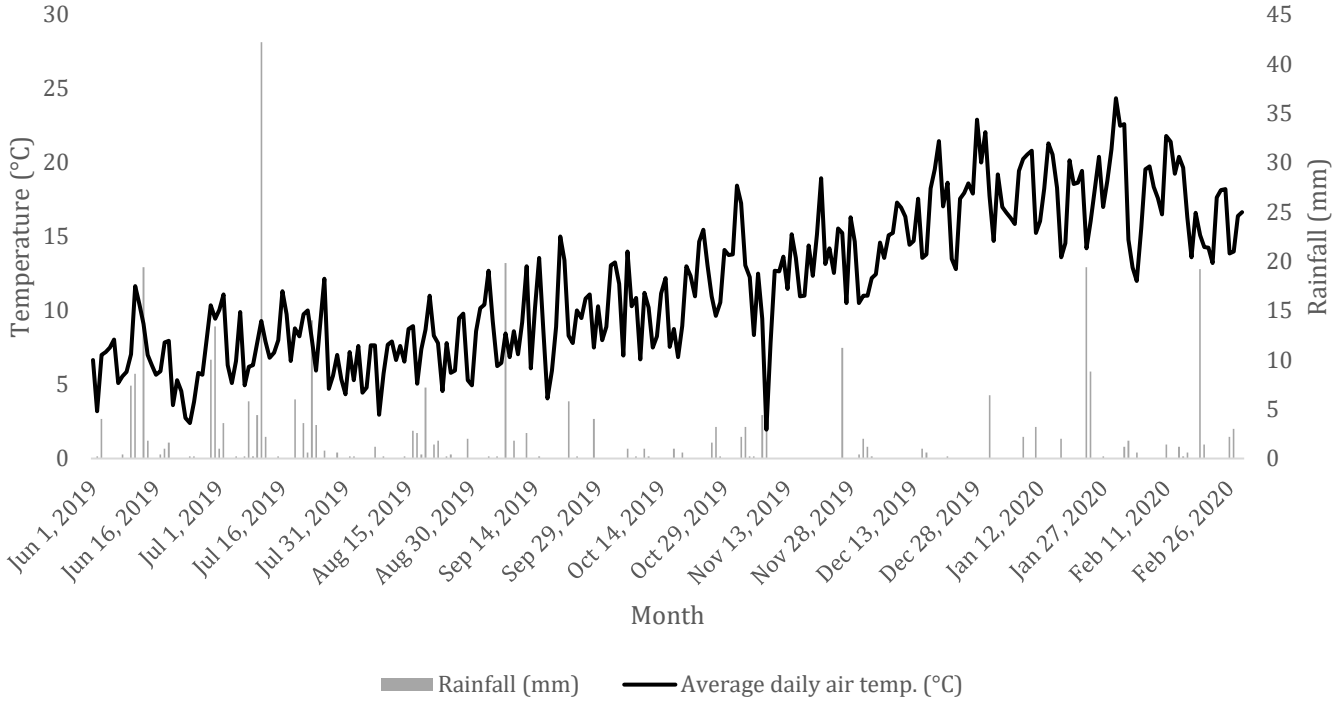

Figure 5: Weather data for Farm 4. Weather data are reported for June, 2019, one-month prior to the vaccination phase of the trial, through to the end of the trial in February, 2020. Weather data were drawn from the BOM weather station located at Cressy Research Station (Station ID: 091306).

Antibody levels

Blood samples were collected on a monthly basis from 10 sheep in each group and the level of circulating antibodies were measured. Antibody levels were significantly higher ($P < 0.05$) in the bivalent groups from September, one-month after administration of the booster dose of each vaccine, through to the end of the trial in February 2020 (Figure 6). Antibody levels in the bivalent group reached the protective threshold of 8 in September and remained above this threshold through to November, before declining in December (Figure 6). Antibody levels for the bivalent group did not reach the protective threshold of 8, but were >7 in September, October, and November 2019 (Figure 6).

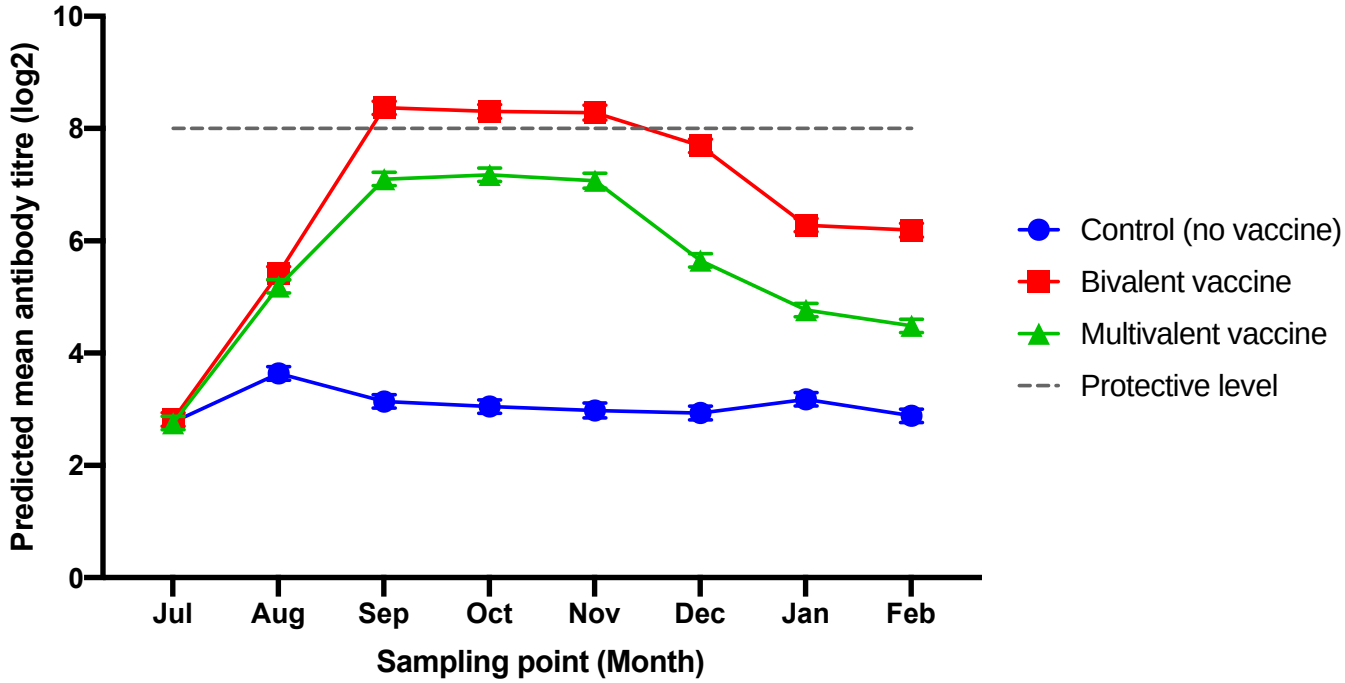

Figure 6: Antibody levels for the control, bivalent vaccine and multivalent vaccine groups. Pooled data from all four farms were analysed. Sheep in the bivalent vaccine and multivalent vaccine groups received two doses of vaccine in July and August, respectively. Sheep in the control group were foot-bathed in a 10% zinc sulphate solution monthly. Data are the predicted means and standard errors from the REML model.

Foot scores

All sheep on each farm were foot-scored monthly during the course of the trial. Sheep at Farm 3 were not foot-scored in December 2019 as we were unable to visit the property that month. The disease was most severe in Flocks 1 and 4, in which >23% and >55% of sheep, respectively, had score 4 lesions (Tables 6 and 9). The prevalence of underrun lesions declined slightly in August 2019 following administration of the first dose of vaccine, but there was a considerable reduction in the number of sheep with underrun lesions in September 2019, one-month after vaccinated sheep received the second dose of vaccine and sheep in the control group had been foot-bathed twice (Tables 6 to 9). On Farms 3 and 4, the number of sheep with score 2 and 3 lesions increased in October 2019, but the number of sheep with score 2 lesions or greater declined in October 2019 on Farms 1 and 2 and remained low through to the end of the trial in February 2020 (Tables 6 to 9). An ordinal logistic regression was conducted separately for each farm to compare foot scores in each group at each time-point. On Farm 1, there was a significant

difference between the three groups in the months of October and November ($P < 0.001$) only. In October, foot scores were significantly higher for sheep in the control group than for sheep in the bivalent vaccine group, with sheep in the control group being 1.69 times more likely to have a higher foot score than sheep that received a bivalent vaccine (OR 1.69; 95% CI 1.13 – 2.54). There was no significant difference between the control group and the multivalent vaccine group, or between the bivalent or multivalent vaccine groups in October. In November, a similar trend was observed, with the sheep in the control group having a significantly higher foot score than sheep that received a bivalent or multivalent vaccine. There was no significant difference between the three groups in any other month. On Farm 2, there was no significant difference between the three groups at any time-point ($P = 0.4167$). On Farm 3, there was a significant difference ($P < 0.001$) between the three groups in the months of August, October and January only. In August and October, foot scores were significantly higher for the sheep in the control group than for those in the groups that received a bivalent vaccine or multivalent vaccine. There was no significant difference in foot scores between sheep that received a bivalent or multivalent vaccine in either of these months. In January, foot scores were significantly higher for sheep in the control group than for sheep that received a bivalent vaccine. There was no significant difference between sheep in the control group and sheep that received a multivalent vaccine, or between the sheep in the bivalent and multivalent groups. There was no significant difference between treatments in any other month. On Farm 4, there was a significant difference between the groups in the months of September and October only. In September, foot scores for sheep in the multivalent vaccine group were significantly higher than for sheep in the bivalent group. There was no significant difference in foot scores between sheep in the control group and sheep in the bivalent vaccine or multivalent vaccine groups in August. In September, foot scores were significantly higher for sheep in the control group than for sheep in multivalent vaccine group. There was no significant difference in foot scores between the control group and the bivalent vaccine groups, or between the bivalent vaccine and multivalent vaccine groups. There were no significant differences in any other months.

In summary, while there were significant differences between the three treatment groups at specific time-points, these differences did not form any meaningful pattern.

Table 6: Summary of foot scores for Farm 1. Foot scores were recorded for all 100 sheep in each group. Data are the percentage of sheep with a maximum foot score of 0 to 4 at each time-point. Sheep in the control group were foot-bathed in a 10% zinc sulphate solution each month and were run together with the vaccinated sheep.

		% Sheep						
Group	**Score**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Jan**	**Feb**
Control (no vaccine)	0	44	41	28	61	68	49	86
	1	14	25	33	27	25	38	7
	2	11	3	8	10	6	10	5
	3	4	6	16	0	0	2	0
	4	27	25	15	2	1	1	2
Bivalent vaccine	0	55	53	40	82	87	60	87
	1	10	19	23	9	9	33	5
	2	10	2	17	6	2	6	3
	3	0	7	10	1	1	0	3
	4	25	19	10	2	1	1	2
Multivalent vaccine	0	50	39	44	81	82	61	91
	1	13	25	31	11	16	34	6
	2	14	4	2	7	2	5	3
	3	0	9	10	1	0	0	0
	4	23	23	13	1	0	0	0

Table 7: Summary of foot scores for Farm 2. Data are the percentage of sheep with a maximum foot score of 0 to 4 at each time-point. Sheep in the control group were foot-bathed in a 10% zinc sulphate solution each month and were run together with the vaccinated sheep. Forty-eight sheep (*n* = 16 sheep per group) were sold by the property owner after the September inspection. Foot score data were recorded for the remaining 84 sheep in each group from October through to February.

		% Sheep						
Group	**Score**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Jan**	**Feb**
Control (no vaccine)	0	34	31	22	62	92	58	100
	1	35	42	75	34	8	42	0
	2	18	11	3	3	0	0	0
	3	6	3	0	1	0	0	0
	4	7	13	0	0	0	0	0
Bivalent vaccine	0	23	41	23	86	98	66	99
	1	36	43	76	10	1	33	1
	2	22	12	1	1	0	0	0
	3	11	3	0	2	1	1	0
	4	8	1	0	0	0	0	0
Multivalent vaccine	0	42	43	23	82	90	50	97
	1	26	37	77	15	7	47	1
	2	20	8	0	1	2	2	0
	3	8	9	0	1	1	1	0
	4	4	3	0	1	0	0	2

Table 8: Summary of foot scores for Farm 3. Foot scores were recorded for all 100 sheep in each group. Data are the percentage of sheep with a maximum foot score of 0 to 4 at each time-point. Sheep in the control group were foot-bathed in a 10% zinc sulphate solution each month and were run together with the vaccinated sheep.

		% Sheep					
Group	**Score**	**Jul**	**Aug**	**Sep**	**Oct**	**Jan**	**Feb**
Control (no vaccine)	0	3	4	10	17	62	84
	1	7	9	15	12	13	1
	2	11	10	15	9	2	0
	3	18	27	20	9	3	0
	4	61	50	40	53	20	15
Bivalent vaccine	0	5	13	12	30	53	82
	1	6	5	33	7	17	1
	2	14	16	27	4	3	0
	3	18	43	18	22	1	1
	4	57	23	10	37	26	16
Multivalent vaccine	0	4	7	5	41	60	88
	1	6	9	42	5	18	0
	2	16	10	15	14	1	1
	3	19	46	18	10	1	0
	4	55	28	20	30	20	11

Table 9: Summary of foot scores for Farm 4. Foot scores were recorded for all 100 sheep in each group. Data are the percentage of sheep with a maximum foot score of 0 to 4 at each time-point. Sheep in the control group were foot-bathed in a 10% zinc sulphate solution each month and were run together with the vaccinated sheep.

		% Sheep						
Group	**Score**	**Jul**	**Aug**	**Sep**	**Oct**	**Nov**	**Jan**	**Feb**
Control (no vaccine)	0	59	52	10	33	78	70	90
	1	28	22	79	36	3	13	5
	2	2	13	6	20	5	0	0
	3	6	9	4	10	5	0	0
	4	5	4	1	1	9	17	5
Bivalent vaccine	0	53	56	17	43	68	70	81
	1	36	20	69	16	5	5	12
	2	1	15	6	27	14	1	1
	3	1	5	5	10	6	9	1
	4	9	4	3	4	7	15	5
Multivalent vaccine	0	38	65	12	45	70	67	88
	1	52	15	82	24	2	9	3
	2	4	6	3	20	8	3	0
	3	1	12	1	10	6	2	0
	4	5	2	2	1	14	19	9

Cure and protection rates

Cure and improvement rates were calculated for September and October 2019, one- and two-months after administration of the booster dose of each vaccine, respectively, and at the end of the trial in February 2020 (Table 10). In September and October 2019, the cure rates from monthly foot-bathing or vaccination were satisfactory on Farms 1 and 2 (>70%) but these treatments were less effective on Farms 3 and 4 (cure rates as low as 25.8% for foot bathing and 18.2% for vaccination). By February 2020, satisfactory cure rates from foot bathing and vaccination were seen on all four farms. Improvement rates were always much higher than cure rates, i.e. lesions were suppressed but not eliminated. Cure and improvement rates were calculated for September, October and February using the foot scores recorded at the start of the trial in July as a reference point. There were only a few significant differences between the cure rates for different groups. On Farm 1, the cure rate was significantly higher ($P = 0.036$) for the multivalent vaccine group than for the control group in September. On Farm 3, the cure rate was significantly higher ($P = 0.027$) for the multivalent vaccine group than for the control group in October. On Farm 4, the cure rate was significantly higher for the control group than for the bivalent vaccine group ($P = 0.017$) and the multivalent vaccine group ($P = 0.012$). There were no significant differences in improvement rates between the three groups on any of the four farms at any time-point.

Table 10: Cure and protection rates (%) for Farms 1 to 4. Cure and improvement rates for September 2019, October 2019 and February 2020 were calculated in relation to foot scores recorded before vaccination in July 2019.

		Cure rate (%)			Improvement rate (%)		
Farm	**Group**	**Sep**	**Oct**	**Feb**	**Sep**	**Oct**	**Feb**
1	Control	78.1	70.7	85.4	90.2	90.2	97.6
	Bivalent	85.7	74.3	74.3	97.1	100	100
	Multivalent	94.6	78.4	78.4	91.9	94.6	97.3
2	Control	90.3	93.6	93.6	80.7	67.7	67.7
	Bivalent	97.6	95.1	100	95.1	65.9	70.7
	Multivalent	100	90.6	96.9	77.4	61.3	64.5
3	Control	39.3	25.8	84.3	85.4	75.3	96.6
	Bivalent	42.7	36.0	81.0	88.8	74.2	95.5
	Multivalent	43.3	41.1	86.7	88.9	83.3	98.9
4	Control	69.2	46.2	100	84.6	58.3	100
	Bivalent	54.6	18.2	63.6	81.8	54.6	90.9
	Multivalent	90.0	50.0	60.0	50.0	60.0	70.0

Antibody levels following consecutive bivalent vaccines with a three- month inter-vaccination interval

The efficacy of a three-month intervaccination interval, while confirmed in a research setting, had not been confirmed in a field trial. Therefore, the antibody levels after the first and second bivalent vaccines administered on each property were compared (Figure 7). Data from five time-points were analysed, from immediately prior to the administration of the first dose of each vaccine (Month 0) through to three-months after administration of the second dose of each vaccine (Month 4). In general antibody levels following the second bivalent vaccine reached a peak about a month sooner than those from the first bivalent vaccine (Figure 7). All levels remained elevated at 4 months after vaccination. A log2 antibody level of 8 is considered protective; antibody levels for the first bivalent vaccine crossed this threshold at Month 3 and remained above this threshold through to Month 5, but antibody levels for the second bivalent vaccine did not cross this threshold at any time-point. Predicted mean antibody titres were significantly different between the first and second bivalent vaccines at all five time-points ($P < 0.001$). The pre-vaccination (Month 0) and one month predicted mean antibody titre for Bivalent Vaccine 2 was higher than that of Bivalent Vaccine 1, but after that was lower than that of Bivalent Vaccine 1 (Figure 7).

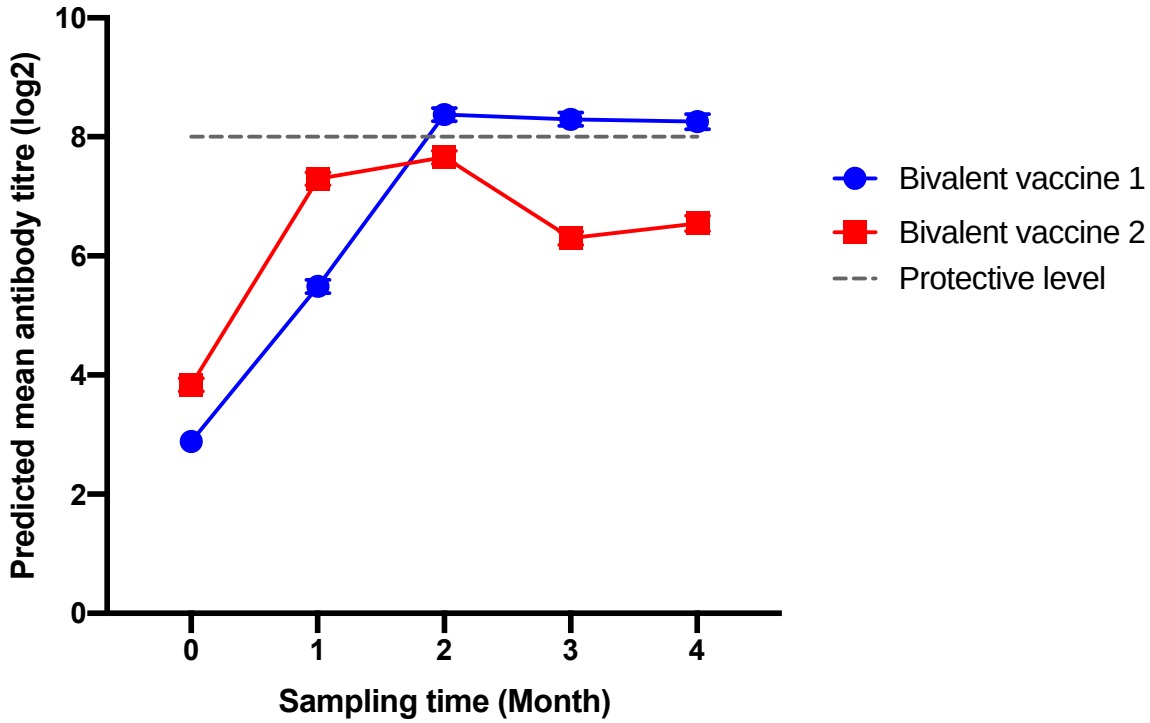

Figure 7: Predicted mean antibody titres (log2) for the first and second bivalent vaccines administered during the trial. Data from all four farms were analysed together. Antibody levels are those that pertain to the specific antigens that were included in each vaccine (see Table 4), pooled across farms.

Animal performance and welfare

The extent to which animal increased as a result of vaccination or regular foot-bathing was inferred from the improvement rate calculated for each group of sheep on each farm. The improvement rate, defined as a reduction in the total weighted foot score, was higher for sheep that received a vaccine, whether that be bivalent or multivalent, than for sheep that were regularly foot-bathed. On all four farms, the producers commented that they had observed fewer lame sheep in all three groups, and that the average body condition score was better than at the start of the trial. We were unable to measure differences in wool growth at the start and end of the trial on each farm, but wool growth is likely to have improved during the trial period due to the reduction in lesion prevalence and severity and incidences of lameness. In our observation body condition scores of the trial sheep were on an average of less than 2 prior to the trial but improved to an average of over 3 and remained so until the end of the trial.

7. Discussion

In Australia, multivalent vaccines are no longer commercially available since Footvax® was withdrawn from the market. Footvax® was reported to provide only limited protection for up to 12 weeks (Hunt et al., 1994), but it was regarded as a valuable control measure by farmers in areas without a defined non-transmission period where traditional control measures such as foot-bathing are ineffective, such as Tasmania and parts of Victoria. This study was prompted by requests from these sheep producers for a new multivalent vaccine to replace Footvax®. Multivalent vaccines can be used to treat and control ovine footrot, but the efficacy of multivalent footrot vaccines varies considerably between studies. Cure rates of 81% to 100% and protection rates of 73% to 100% have been reported (Liardet et al., 1989); however, cure rates can be as low as 55% (Kennedy et al., 1985). Numerous factors including vaccine formulation, sheep breed and environmental conditions are known to have an impact on the efficacy of footrot vaccines, so comparing results across studies is difficult. There is a threshold antibody level for effective protection (see below), so the level of antibodies stimulated by a vaccine and their persistence needs to be evaluated to get a proper picture of the utility of any vaccine for footrot. Bivalent vaccines, which are used commercially in Australia, were also the subject of investigation in this project, because sequential bivalent vaccine scheduling may be able to be improved on farms with problematic, multiple serogroup infections.

Objectives i, ii, iii (Immunological trial)

In the first phase of the study we conducted an immunological trial to evaluate four novel multivalent vaccine formulations containing fimbrial antigens representing six or nine *D. nodosus* serogroups, at both high and low doses. The six-serogroup formulations contained antigens representing the six serogroups (A, B, C, G, H, I) deemed most prevalent in south-eastern Australia based on previous diagnostic submissions received by our laboratory in the two years preceding the trial. The nine-serogroup formulations contained antigens representing serogroups A to I. Serogroup M was not included in any of the formulations as a recombinant fimbrial protein is not yet available for this serogroup, which was detected relatively recently in southeast Australia (Dhungyel et al., 2015).

The antibody titres for high dose vaccines were significantly higher than those of low dose vaccines from one month after administration of the second dose of each vaccine through to the end of the trial, irrespective of the number of serogroups included in the formulation (see Figure 1). This outcome differs somewhat from that of a previous study which reported that the level of circulating antibodies stimulated by a multivalent fimbrial vaccine declines in a linear fashion as the number antigens included in the vaccine formulation increases from one to ten (Raadsma et al., 1994). This suggests that the new formulations used in this study (specifically the adjuvants used) may have overcome to some degree the antigenic competition that was observed in prior studies. In fact, the antibody response stimulated by the high dose vaccine formulations was probably sufficient to be able to provide a meaningful level of control over the disease (Figure 1). The antibody response stimulated by the low dose multivalent vaccine formulations was not satisfactory, and we concluded they did not warrant further evaluation in a field setting. Few studies have evaluated the impact of dose on the efficacy of multivalent footrot vaccines, as most studies have used commercial vaccines like Footvax®. Increasing the dose involves a cost, and the vaccine could become prohibitively expensive if the dose was increased further.

In the immunological trial we also evaluated inter-vaccination intervals of two- and three-months between successive bivalent vaccines. Immunological studies have reported that a three-month inter-vaccination interval is sufficient to avoid antigenic competition (Dhungyel and Whittington, 2010), but a two-month inter-vaccination interval, which is the minimum possible interval, has not been evaluated previously. There was no significant difference ($P > 0.05$) in predicted mean antibody titres between the groups that received a second bivalent vaccine

after two- or three-month intervals, which suggests that a two-month interval could be applied in a commercial setting.

The antibody levels reached in the immunological trial were generally lower than those in the field trial. Only one group that received a bivalent vaccine in the immunological trial reached a titre of 8; others were however >7. None of the remaining multivalent or bivalent vaccines stimulated an antibody response of this magnitude at group level, although the antibody response of some animals did reach this level. It is unclear why the antibody levels in the immunological trial were apparently lower than those in the field trial.

Objectives iv, v, vi, vii (Field trial)

The Multivalent Vaccine 4 (nine serogroups at a high dose) was selected for evaluation in the field trial, based on the outcome of the immunological trial. Four commercial Merino flocks in which we detected between two and four virulent strains of *D. nodosus* were enrolled in the field trial. In each flock, the novel multivalent vaccine was compared to commercial bivalent vaccines and regular foot-bathing in a 10% zinc sulphate solution.

Cure and improvements rates were calculated for vaccinated sheep and the control sheep on each farm, and they differed considerably between farms. In September and October 2019, the cure rates from monthly foot-bathing or vaccination were satisfactory on Farms 1 and 2 (>70%) but these treatments were less effective on Farms 3 and 4 (cure rates as low as 25.8% for foot bathing and 18.2% for vaccination). More virulent *D. nodosus* serogroups were detected on Farms 3 and 4, and there was one virulent serogroup we did not target with vaccine during the course of the trial in each flock, which may partly explain the greater severity of disease in these flocks. A virulent serogroup M strain was detected on Farm 4 in February 2020, which was not included in any vaccine formulation because a recombinant fimbrial antigen for this strain is not available. In a previous field trial involving four Merino flocks and one flock of South Suffolk and Southdown sheep in New Zealand, cure rates in sheep vaccinated with Footvax® ranged from 35% to 67% (Mulvaney et al., 1984). The trial was conducted during a dry spring, thus spontaneous resolution may have masked the effect of the vaccine to some extent, although a similar cure rate (55%) was reported in a subsequent field trial for sheep vaccinated with Footvax® under conditions suitable for transmission and expression of the disease (Kennedy et al., 1985). A field trial undertaken in the US also reported a curative effect of 53% for sheep vaccinated with Footvax®, but there was no mention of the breed used (Glenn, 1985). In a subsequent study, cure rates of 81% to 100% were reported for sheep vaccinated with Footvax® (Liardet et al., 1989); however, the trial involved European sheep breeds like the Romney and Corriedale, which are reported to respond better to vaccination than the Merino (Emery et al., 1984).

Cure rates were satisfactory in February 2020 and cure from foot bathing and vaccination were seen on all four farms. The lack of rainfall from September 2019 to February 2020 probably resulted in spontaneous resolution of lesions in all groups, despite sheep on Farms 1 and 2 being grazed on irrigated pasture periodically, and sheep on Farm 4 being placed on irrigated pasture from mid-December through to February. Improvement rates were always much higher than cure rates, i.e. lesions were suppressed but not eliminated.

Clinical response is the primary measure by which footrot vaccines are typically evaluated; consequently, most previous studies have reported clinical observations only, and make no mention of antibody response to multivalent vaccines. In the field trial blood samples were collected from a representative proportion of each group of sheep on a monthly basis and antibody levels were evaluated. Antibody levels were significantly higher for the sheep that received a bivalent vaccine than for the sheep that received a multivalent vaccine in each of the four flocks at every time-point following administration of the second dose of vaccine in August (see Figures 6 to 9). In the bivalent vaccine groups, antibody levels peaked at a log2 mean of approximately 8.4 in September. Antibody levels remained above 8 for the bivalent vaccine groups in October and November, declined to 7.7 in December, and continued to

decline through to the end of the trial in February. In the multivalent vaccine groups, antibody levels peaked at a log2 mean of approximately 7.2 in October, two-months after administration of the second dose of vaccine. Antibody levels in the multivalent vaccine groups remained at approximately 7 in November, declined to 5.7 in December, and continued to decline through to the end of the trial in February. A log2 antibody titre of 8 is generally considered to be protective (Egerton et al., 1987), although different thresholds have been reported across studies and between different serogroups (Thorley and Egerton, 1981; Stewart et al., 1982; Fahey et al., 1983). A previous study reported that the threshold for full protection against infection with *D. nodosus* differs between serogroups (Raadsma et al., 1994). The authors reported that an antibody level of 8 provided full protection against serogroup B, but that an antibody level of 10 was required to provide protection against serogroup A (Raadsma et al., 1994). The authors also noted that an antibody level of 5 provided some protection against serogroup A, and that there was a steep decline in the prevalence of foot lesions as the antibody level increased from 5 to 9. There is also variation between sheep, and there is no agreement on whether group or individual sheep responses need to be given priority, therefore a titre of 8 is merely a rule of thumb. In the field trial, the predicted mean antibody titre for the bivalent vaccine group on each farm exceeded this threshold one-month after administration of the second dose of bivalent vaccine. Titres did not reach this level after the multivalent vaccine, and assuming similar rates of decline for both types of vaccine, this suggests that the duration of protection for the multivalent vaccine would be less than for the bivalent vaccine.

Antibody levels for the first vaccine reached the protective threshold of eight one-month after administration of the second dose of vaccine (Month 2) and remained above this threshold through to Month 4. In contrast, antibody levels for the second bivalent vaccine did not reach that threshold. The sensitivity of the microtitre agglutination test varies between serogroups, and some antigens tend to give lower agglutinating antibody titres than others. This is not thought to correlate with vaccine efficacy.

In the field trial, two bivalent vaccines were administered to the bivalent vaccine group on each farm with a three-month inter-vaccination interval. We were unable to use a two-month interval as some of the producers were unable to accommodate the shorter interval due to other management procedures that needed to be performed. The antibody response differed between the first and second bivalent vaccines.

There are other factors that should be considered when selecting an inter-vaccination interval as part of a targeted vaccination program. In a flock infected with several virulent strains of *D. nodosus*, one or two strains tend to be dominant/most prevalent (Dhungyel et al., 2013). These strains tend to be the most virulent strains in the flock and are the first to be targeted with vaccine (Dhungyel et al., 2013). The prevalence of these strains will decline following vaccination, and one or two of the remaining virulent strains will then become the dominant strain(s). It is not always apparent which of the remaining strains are likely to become the dominant strain after the first vaccination, so additional microbiological testing may be required. Given it can take four or more weeks to isolate, virulence test, and serotype the infecting *D. nodosus* strain(s) in a flock (Stewart and Claxton, 1993), a two-month inter-vaccination interval may not be sufficient to complete the diagnostic work and select the appropriate mono- or bivalent vaccine. This should be considered when selecting an inter-vaccination interval. Recent advances mean that serogrouping can be conducted quickly, directly from foot swabs (McPherson et al., 2018) and in the event that only one or two serogroups are detected by this method, culture is not required, allowing rapid deployment of the next round of bivalent vaccine. The development of a rapid virulence test that could be conducted directly from foot swabs would be a major advance.

The extent to which animal welfare increased as a result of vaccination or regular foot-bathing was inferred from the improvement rates calculated for the three groups of sheep on each farm. The improvement rate, defined as a reduction in the total weighted foot score, were higher than cure rates for sheep that received a vaccine, whether that be bivalent or multivalent, and for sheep that were regularly foot-bathed. On all four farms, the producers

commented that they had observed fewer lame sheep in the mob. We were unable to measure differences in wool growth, but on each farm the producers noted that the body condition score of sheep in the mob had generally improved during the trial period due to a reduction in lesion prevalence and severity, and fewer incidences of lameness.

8. Impact on the industry, now and in five-years' time

The objective of this study was to define best practice for vaccination against ovine footrot in different flocks with different types of footrot. A multivalent vaccine targeting nine *D. nodosus* serogroups (A to I) was developed and the evaluation in field trial demonstrated that this vaccine has a considerable but brief curative and improvement effects, helping to improve the health and welfare of the animals. Through serology it was demonstrated that the bivalent vaccine stimulated a greater immune response and would be likely to have a more long-lasting benefit than multivalent vaccine. For this reason caution is needed around the usage of multivalent vaccine as a longer term control measure. Its application may be limited to flocks/locations with prevalence of multiple serogroups and where frequent foot-bathing is being applied to suppress foot lesions due to the lack of a seasonal non-transmission period. Bivalent vaccine may still be the best option in other situations. It was found that a two-month inter-vaccination interval was sufficient to avoid antigenic competition, which will enhance the delivery of bivalent vaccines and enable producers to target multiple strains of *D. nodosus* in a shorter time frame. Others may find the flexibility of being able to use a two, three or longer inter-vaccination interval to be beneficial.

9. Conclusions and recommendations

The objective of this study was to define best practice for vaccination against ovine footrot.

A new multivalent vaccine formulation targeting nine *D. nodosus* serogroups (A to I) was evaluated and the results demonstrated that this vaccine does provide some control over the disease, comparable to that of commercial sequential bivalent vaccines and foot-bathing. However, it was not feasible to fully evaluate the protective effect of any of these treatments because the disease may have self-cured or not been expressed due to a lack of rainfall and unusually dry conditions in the trial area. It is recommended that a further evaluation of the multivalent vaccine is undertaken when environmental conditions are conducive to transmission and expression of footrot. Regardless, the duration of protection afforded by the multivalent vaccine was almost certainly less than that of bivalent vaccine as antibody titres induced in sheep by the former were lower and less persistent than for bivalent vaccine. This means that multivalent vaccine booster doses would need to be given more often than bivalent vaccine. The absolute duration of protection afforded by multivalent vaccine would need to be determined in field trials.

Different inter-vaccination intervals between the first and second doses of multivalent vaccine have been evaluated in previous studies, as has the impact of annual booster doses of vaccine following the initial two doses. A second dose of the multivalent vaccine was administered at a four-week interval; however, previous studies indicated that a longer interval may be more effective. Administering a third dose of vaccine every 12-months is also reported to stimulate a greater antibody response. A booster dose could be administered to sheep immediately prior to the start of the spring transmission period, which would enable producers to gain some control over the disease during this period. Further investigation may be required to demonstrate the efficacy of other alternate vaccination strategies.

It was not possible to include serogroup M fimbrial antigen in the multivalent vaccine formulation because a recombinant fimbrial protein for this strain is not yet available. Serogroup M has been reported form New Zealand and has been identified from some flocks in Tasmania, including one in this trial, and a flock in Victoria. The inability to target this strain with vaccine can hinder control of the disease in those flocks with mixed serogroup infections. It is recommended that work be undertaken to produce a recombinant *Pseudomonas* strain for serogroup M to enable the production of vaccine for this serogroup. This new antigen could be included in both bivalent and multivalent vaccines.

10. Key messages

- Footrot is a significant economic and animal welfare issue in most countries where sheep are produced.
- The essential causative agent, *Dichelobacter nodosus*, possesses fine, filamentous appendages called fimbriae or pili.
- Strains of *D. nodosus* can be assigned to 10 serogroups (A to I, and M) owing to variation in the fimbriae.
- Fimbriae are the primary surface antigen of *D. nodosus*, and they are highly immunogenic.
- Footrot can be treated, controlled and eliminated with vaccines containing *D. nodosus* fimbriae, but vaccination can be challenging: immunity against *D. nodosus* is serogroup-specific, with little or no cross-protection between serogroups, and virulent strains of *D. nodosus* belonging to several serogroups (up to seven) may be present in a flock.
- In Australia, targeted (serogroup-specific, flock-specific or outbreak-specific) vaccination has been used to treat, control and eliminate footrot in individual flocks. However, targeted vaccination has some limitations. In large flocks infected with several strains of *D. nodosus*, targeted vaccination can be expensive due to the requirement for multiple vaccines and extensive diagnostic testing.
- Targeted vaccines usually contain up to two serogroups of *D. nodosus*.
- The objective of the current study was to define best practice for vaccination against ovine footrot in different flocks with different types of footrot. The study included two experiments, a small-scale trial at the University of Sydney and a field trial in four commercial Merino flocks in Tasmania.
- The small-scale trial at the University of Sydney evaluated inter-vaccination intervals of two- and three-months between successive bivalent vaccines. Previous studies have reported that a three-month inter-vaccination interval is sufficient to avoid antigenic competition. A two-month inter-vaccination interval, which is the minimum practical interval, had not been evaluated previously. Antibody levels were similar for sheep that received a second bivalent vaccine after a two- or three-month interval, which suggests that a two-month interval is sufficient to avoid antigenic competition. This will enhance the delivery of bivalent vaccines and let producers target multiple strains of *D. nodosus* in a shorter time frame. Depending on their management systems, others may find the flexibility of being able to use a two, three or longer inter-vaccination interval to be beneficial.
- Four novel experimental multivalent vaccine formulations, each containing nine *D. nodosus* serogroups (A to I), were also developed and evaluated in the small-scale trial. The trial demonstrated that the multivalent vaccine did not stimulate as large an immune response as the bivalent vaccine, however some novel formulations stimulated immune responses the researchers considered sufficient to provide some control over the disease on-farm.
- Four commercial Merino flocks in Tasmania were enrolled in the field trial. The trial commenced in July 2019 and finished in February 2020. Virulent strains of *D. nodosus* were detected in each flock, with between two and five serogroups per flock.
- In each flock 300 Merino ewes were included in the trial and were allocated to one of three treatment groups: control, bivalent vaccine, multivalent vaccine.
- Sheep in the control group were foot-bathed in a 10% zinc sulphate solution monthly.
- Sheep in the bivalent vaccine group received the first bivalent vaccine in July and August 2019 and the second bivalent vaccine in October and November 2019.
- Sheep in the multivalent vaccine group received the multivalent vaccine in July and August.

- The 300-trial sheep on each farm were run together as a single mob and were examined at monthly intervals during the trial. Each month the feet of each sheep were examined, and blood was collected from 10 sheep in each treatment group for evaluation of circulating antibody levels.
- Antibody levels were higher in sheep that received a bivalent vaccine or a multivalent vaccine than the sheep in the control group.
- In all four flocks, the antibody levels for sheep that received a bivalent vaccine were higher than sheep that received the multivalent vaccine at all timepoints except September 2019, one month after the booster dose of the multivalent vaccine.
- The antibody response differed between the first and second bivalent vaccines. Antibody levels for the first vaccine reached the protective threshold one-month after the second dose of vaccine. In contrast, antibody levels for the second bivalent vaccine did not reach that threshold.
- Despite these differences control of footrot was achieved in each group of sheep on each farm, that is, the two different vaccine approaches and foot-bathing were equally effective in suppressing footrot.
- Cure rates were >90% for three of the four groups that received a multivalent vaccine one-month after the second dose was given; however, cure rates had declined considerably one-month later, which suggests that the multivalent vaccine may only be effective for a limited period.
- The researchers concluded that the greater immune response of the bivalent vaccine suggests that it would be likely to have a more long-lasting benefit than multivalent vaccine.
- A lack of rainfall and unusually dry conditions during the field trial meant that the protective effects of the three treatment groups could not be fully evaluated because the disease may have self-cured or not been expressed.
- Further evaluation of the multivalent vaccine is required under environmental conditions more favourable to disease transmission and/or expression.

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13. Glossary

Footrot – an infectious disease of sheep and other small ruminants characterised by mild to severe inflammation and necrosis of the interdigital skin and separation of the hard wall of the hoof from the underlying dermal tissues (underrunning)

Dichelobacter nodosus – an anaerobic bacterium and parasite of the ruminant hoof; the essential causative agent of ovine footrot

Fimbriae (pili) – fine, hair-like structures on the surface of *D. nodosus*. Fimbriae are a key virulence factor of *D. nodosus*; they enable the bacterium to attach to the epidermis and to secrete protein-degrading enzymes. Fimbriae are the main immunogenic antigens of *D. nodosus*.

fimA – the gene that encodes the fimbrial subunit protein. Multiple subunit proteins are assembled in a chain to form each mature fimbria. Serogroup variation is based on variation in the DNA sequence of this gene.

Antigen – a bacterial protein that stimulates an immune response when encountered by the hosts' immune system

Monovalent – a vaccine targeting one strain of a microorganism

Bivalent – a vaccine targeting two strains of a microorganism

Multivalent – a vaccine targeting three or more strains of a microorganism

Antigenic competition – competition between structurally similar antigens for receptors on immune cells, resulting in a diminution of the immune response

PCR – polymerase chain reaction. A molecular method for making many detectable copies of short fragments of DNA

BCS – body condition score

Inter-vaccination interval – the length of time between the first doses of two consecutive vaccines.