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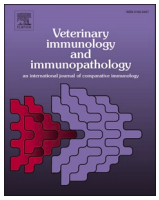

Short communication

A randomised controlled trial of the immunogenicity and safety of a formaldehyde-inactivated *Coxiella burnetii* vaccine in 8-week-old goats

Michael Muleme [a,*], Joanne M. Devlin [a], Angus Campbell [a], Gemma Vincent [b], Paul John Benham [a], Cleide Sprohnle [a], Andrew Stent [a], Alexander Cameron [a], Aminul Islam [b], Stephen Graves [b], Colin Wilks [a], John Stenos [b], Simon M. Firestone [a]

[a] Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, VIC, 3010, Australia
[b] Australian Rickettsial Reference Laboratory, Geelong, VIC, Australia

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ABSTRACT

Coxiella burnetii causes Q fever in individuals exposed to infected ruminants. Vaccination in 3−4-month-old goats, has been reported to result in significantly greater reduction in *C. burnetii* shedding compared to goats vaccinated one month before breeding, the most commonly used strategy of controlling Q fever on infected intensively-managed herds. It is possible that an even greater reduction in the number of animals shedding *C. burnetii* could be achieved if vaccination were administered shortly after protection from colostrum antibodies wanes and animals become susceptible to infection with *C. burnetii*. This study aimed to evaluate the immunogenicity and safety of a formaldehyde-inactivated phase 1 *C. burnetii* vaccine in 8-week-old goats. Two injections, four weeks apart, elicited specific IgM and IgG responses in all vaccinated goats (n = 6), while no antibodies were detected in two control groups (n = 12). Swelling at the site of inoculation was observed in all the vaccinated and in 10/11 of the placebo-treated goats but receded after 3 weeks. Weight change and rectal temperatures were also comparable between vaccinated and control goats. The data indicated that this vaccine could be suitable for immunising 8-week-old goats, although further trials to determine level of protection against challenge are required.

1. Introduction

Coxiella burnetii multiplies to extremely high numbers in ruminant placentas and consequently highly infectious loads may be shed in birth fluids, placenta and foetal membranes, posing a high risk of Q fever infection for susceptible humans (Roest et al., 2011a; Sánchez et al., 2006). Vaccination of ruminants with inactivated phase 1 *C. burnetii* antigen one month before breeding which has been shown to reduce *C. burnetii* shedding, is the most commonly used strategy of controlling *C. burnetii* on infected ruminant herds, as recommended by the manufacturers of the only existing livestock vaccine [Coxevac™, Ceva Sante Animale, France] (Astobiza et al., 2011a; Astobiza et al., 2011b; Eibach et al., 2012; Garcia-Ispierto et al., 2015; Guatteo et al., 2008; Hogerwerf et al., 2011; Piñero et al., 2014; Rousset et al., 2009; Taurel et al., 2014).

In contrast, a study that compared the efficacy of vaccinating 3 to 4-month-old goats to vaccination of goats one month before breeding, reported that those vaccinated at 3 to 4-months of age had a significantly greater reduction in the proportion of shedders and the amount of *C. burnetii* shed per animal compared to goats vaccinated one month before breeding (De Cremoux et al., 2012). The greater reduction in the proportion of shedders in goats vaccinated at 4-months of age than those vaccinated one month before breeding was likely due to the lower proportion of the 3 to 4-month-old animals already infected with *C. burnetii* at the time of vaccination.

Research on an infected intensive dairy enterprise in Victoria, Australia, revealed that snatch-reared goats fed 500 mL of pooled colostrum, started mounting IgM antibodies at around 9 weeks of age, shortly after maternally-derived colostrum antibodies had waned (Muleme et al., 2017a). This demonstrates that kid goats are susceptible and are being exposed to *C. burnetii* before 9 weeks of age (Muleme et al., 2017a). Prevention of infection and elimination of infection from herds, could be achieved if vaccination were administered shortly after protection from colostrum antibodies wanes and before animals lacking protective immunity are exposed to *C. burnetii*.

* Corresponding author.
E-mail address: michael.muleme@unimelb.edu.au (M. Muleme).

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This study was therefore conducted to evaluate the immunogenicity and safety of a formaldehyde-inactivated phase 1 *C. burnetii* vaccine in 8-week-old goats. This is the first of planned studies aimed at investigating the possibility of achieving better *C. burnetii* vaccine effectiveness by vaccinating goats before they get infected. With efforts to import the only available livestock vaccine against *C. burnetii* (Coxevac) into Australia being unsuccessful owing to regulatory concerns (European Medicines Agency, 2010b), an autogenous formaldehyde inactivated vaccine for livestock against *C. burnetii* was developed and tested in 8-week-old goats as a pre-cursor study to planned field trials.

2. Material and methods

2.1. The vaccine and the placebo

A formaldehyde-inactivated vaccine was prepared by culturing, in specific-pathogen-free (SPF) chicken eggs, a *C. burnetii* isolate from an aborted goat foetus from a farm on a large dairy enterprise at the centre of a large Q fever outbreak (Bond et al., 2016). SPF embryonated chicken eggs that were 7 days old were inoculated with 0.1 mL of a *C. burnetii* (Meredith goat strain AuQ60) inoculum into the yolk sac. The eggs were incubated at 40 °C at 50 % humidity and candled every day to confirm embryo viability. Only those eggs in which the embryo had grown were ultimately harvested on day 8 after infection. The yolk-sac membranes and the chorio-allantoic membranes were harvested and pooled while the embryos and remaining yolk were discarded. The pooled membranes were washed three times in Hanks Balanced Salt Solution (HBSS) and then homogenised in a sterile household blender. Blending was done in 12 bursts lasting 10 s, with cooling to room temperature in-between each burst of homogenisation.

Inactivation was achieved by adding homogenised egg membrane suspension (containing *C. burnetii*) to an equal volume of 2% formaldehyde in HBSS, to provide a 1% final formaldehyde concentration. The suspension was continuously agitated with a magnetic spin bar for 24 h at room temperature. The membrane debris was removed with a low speed (500 *g*) centrifugation for 5 min and the supernatant collected and washed three times with HBSS by centrifugation at 18,000 *g* for 30 min to remove formaldehyde. The sediment collected and re-suspended. The final sediment (the vaccine) was shown to be free of both viable *C. burnetii* and residual formaldehyde by inoculation into VERO cell cultures and demonstrating the absence of any cytopathogenic effect after 4 weeks of incubation.

The final suspension was checked for bacterial sterility by inoculation onto Horse Blood Agar and demonstrating no growth after aerobic and anaerobic incubation for 72 h at 37 °C. DNA was extracted from the vaccine using the HiYield Genomic DNA Mini Kit (Real Biotech Corporation) and the *C. burnetii* concentration in the vaccine was then estimated using a quantitative polymerase chain reaction (qPCR) targeting the *com1* single copy gene of *C. burnetii* (Lockhart, 2010).The *C. burnetii* concentration in the vaccine (3.6×10^9/mL) was then adjusted to a concentration of 1.8×10^8*C. burnetii* per mL with HBSS. The protein concentration of the vaccine was measured using a nanodrop at 280 nm.

A placebo was prepared in the same way but with saline inoculated into the embryonic eggs instead of *C. burnetii*. The protein concentration of the placebo was adjusted to 31.9 mg/mL, similar to that of the vaccine (36.4 mg/mL). Both the placebo and the vaccine were stored at 4 °C until they were administered within 4 weeks of preparation.

2.2. Trial goats

The vaccine trial involved three groups of goats: a vaccinated group which received the vaccine; a control group receiving the placebo containing all components of the vaccine except *C. burnetii*; and a no-treatment control group to act as the reference for the vaccine and the placebo groups.

A farm which had repeatedly tested negative for antibodies against *C. burnetii* in bulk milk on both the enzyme-linked immunosorbent assay (ELISA) and the immunofluorescent assay (IFA), and negative for *C. burnetii* DNA on a qPCR targeting the *com1* gene (Muleme et al., 2017b), was identified as a potential source of goats for the trial. This farm is under the same dairy enterprise as the outbreak farm from which the *C. burnetii* vaccine isolate was obtained (Muleme et al., 2017b). Demonstration of freedom from *C. burnetii* infection on the source farm was undertaken by testing 400 blood samples from adult female pregnant goats for IgG and IgM antibodies to *C. burnetii* using a validated IFA (Muleme et al., 2016). The sample size of 400 goats provided 99 % confidence of detecting a $\geq$ 1% prevalence of infection in a 1000-goat herd (Cannon and Roe, 1982) based on published specifications for the IFA of 95 % diagnostic sensitivity and 93 % diagnostic specificity for antibodies to *C. burnetii* in serum (Muleme et al., 2016).

The minimum required sample size for the vaccination trial was estimated to be 6 animals per treatment group so as to have 80 % power and 95 % confidence of detecting a statistically significant difference in the proportion of goats that seroconvert to *C. burnetii* between the treatment group and the two control groups, using the difference in proportions chi-square test, assuming 75 % of the animals in the treatment group seroconverted 2 weeks after vaccination and one animal seroconverted in each control group.

A total of 36 male kid-goats born on the source farm during the same kidding season were purposively sampled so as to exclude goats with nasal discharges, a rough hair coat and diarrhoea which were evident in some of the kid goats in this kidding season and are reportedly common perinatal illnesses in kid-goats on the farm. The recruited goats were fed colostrum from their mothers for the first day of life as per routine husbandry procedures at the source farm, before being started on a milk replacer diet, still at the source farm. At about 4 weeks of age, the goats were given 1 mL of Tasvax© (Coopers), a vaccine containing 8 different strains of *Clostridium* spp., as per the routine vaccination regime at the source farm. The goats were weaned at 5–6 weeks of age and were shortly after transported to the University of Melbourne animal house in accordance with the Victorian code of transporting animals (Animal Health Australia, 2008).

Acclimatization at The University of Melbourne animal house had been planned to be 7 days but was extended to 11 days after respiratory signs and diarrhoea were observed in some of the goats following arrival and this resulted in 18 goats being excluded from the study (Supplementary file 1).

2.3. Vaccination of the trial goats

Pseudo-random number sequences were generated to allocate groups for the 18 goats that were observed to be clinically normal during the acclimatization week. The part of the neck to which the vaccine or placebo was to be administered was shaved and disinfected and 2 mL of vaccine or placebo was administered subcutaneously in the neck of each of the 12 goats allocated to both the vaccine and placebo groups. The remaining 6 goats were used as no-treatment controls. During the trial, the details of allocation of individual animals were placed on file and blinded from the researchers, including those that did laboratory testing of collected samples. A booster dose (2 mL) of either the vaccine or placebo was administered 3 weeks after the initial dose. The booster of the vaccine was administered at the side opposite to one where the initial dose had been injected.

2.4. Measuring of outcomes of vaccination in goats

Blood samples were collected and complete physical examination including temperature and weight measurement were done on all the goats in the study group at the source farm, during the acclimatization period, at administration of treatments, daily within the first two days and weekly thereafter following administration of the vaccine and the placebo until the end of the study.

Blood was collected into plain Vacutainer tubes and serum was separated and stored at -20 °C until testing. All sera were screened for IgG and IgM antibodies against *C. burnetii* at the 1:160 cut-off dilution using the IFA. Two-fold serial dilutions of the 1:160 diluted sera were also tested for antibodies against *C. burnetii* using the IFA, to obtain endpoint titres as previously described (Muleme et al., 2016).

To evaluate the safety of the vaccine, Kruskal-Wallis test was performed to compare the change in weight in the trial animals following treatment and of rectal temperatures a day after the treatments, to test for associations with treatment group using STATA 13.0 statistical package (StataCorp, 2007). Vaccine safety was also evaluated by comparing the type of reaction and its duration at the injection sites of the vaccine and the placebo to the size of reactions reported in the already licenced European *C. burnetii* vaccine, Coxevac (European Medicines Agency, 2010a, b). The checklist for monitoring reaction at the site of injection of the vaccine and placebo included checking for evidence of swelling, redness, pain, ulceration and discharge.

All the goats were euthanized at the end of the trial by intravenous injection of pentobarbital and a thorough post-mortem examination undertaken on every goat to detect any lesions and compare the distribution of any unexpected findings in vaccinated and control goats. The liver, spleen, kidney, pre-scapular lymph nodes and lungs of all study animals were observed for any gross lesions and sections tested using microbial testing, histological examination, histopathology, immunohistochemistry and qPCR targeting the *com1* gene to rule out the possibility that any abnormalities observed were associated with the vaccine.

2.5. Laboratory materials and methods

The immunohistochemistry assay used was developed specifically for this trial using sera from rabbits vaccinated with the Nine Mile strain of *C. burnetii*, as the primary antibody. The negative control consisted of sera from laboratory rabbits that had not been exposed to *C. burnetii*. Archived placental tissue from a previous goat abortion was used as a positive control tissue. Histology on the placenta revealed multifocal, moderate, placentitis and funisitis with intracellular Macchiavelli-positive organisms consistent with *C. burnetii*; this was further confirmed to be *C. burnetii* through qPCR.

Formaldehyde-fixed tissues from the study group were cut from paraffin blocks and fixed on glass slides. The sections were then deparaffinized, followed by the retrieval of *C. burnetii* antigen using the S1700 target retrieval solution (Dako, Australia), and addition of foetal calf serum as per the University of Melbourne veterinary pathology laboratory inhouse protocols. The primary antibody at a dilution of 1:2500, was then added, followed by the Envision mouse anti-rabbit antibodies as a conjugate (Dako, Australia) before the slides were mounted. See Supplementary file 2 for details.

The DNeasy Blood and Tissue (Qiagen, Australia) DNA extraction protocol was used to extract DNA from samples of ≈25 mg of the lung, kidney, and lymph nodes and ≈10 mg of each spleen. The resultant DNA was then quantified using a qPCR targeting the *com1* gene of *C. burnetii* (Lockhart et al., 2011).

3. Results and discussion

3.1. The vaccine and placebo

The final vaccine suspension had a concentration of 1.8×10^8 *C. burnetii* per mL. The vaccine was free of both viable *C. burnetii* and residual formaldehyde as inoculation into VERO cell cultures resulted in absence of any cytopathogenic effect after 4 weeks of incubation. The vaccine passed the bacterial sterility test as no growth occurred on inoculation of the vaccine onto Horse Blood Agar after aerobic and anaerobic incubation for 72 h at 37 °C.

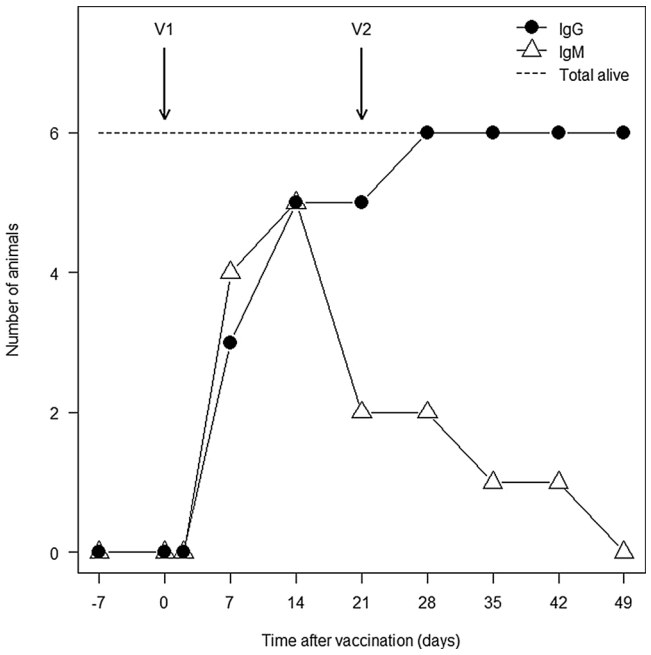

Fig. 1. Number of vaccinated 8-week-old goats producing IgM and IgG antibody-mediated immune responses to a formaldehyde-inactivated *C. burnetii* vaccine at the 1:160 screening dilution. (V1 and V2: time of administration of first and second (booster) vaccine doses, respectively).

3.2. Immunogenicity of the vaccine in goats

The median age of the 18 goats at the time of administration of the first vaccine dose was 58.5 days (interquartile range: 52, 63 days). No statistically significant differences in the age of goats allocated to each treatment group were detected (Kruskal-Wallis statistic with ties = 0.223, degrees of freedom = 2, p = 0.89).

The vaccine was immunogenic in 8-week-old goats as IgM and IgG responses against *C. burnetii* were observed as early as 7 days after the first vaccine was administered (see Fig. 1). At the time of administration of the booster dose (day 21), 5 of the 6 goats in the vaccination treatment group had detectable antibodies from the initial vaccine; all the vaccinated goats had detectable antibodies to *C. burnetii* after the booster vaccination (Fig. 1). Individual and group geometric mean antibody titres of vaccinated goats are presented in Fig. 2. No antibodies against *C. burnetii* were detected in samples from goats in the placebo and the no treatment control groups.

Additionally, the IgG antibody titres elicited by the vaccine lasted throughout the 7-week follow-up period. Furthermore, an increase in antibody titres was observed following administration of the booster dose of the vaccine which highlights the importance of a booster dose following the first vaccine dose.

The pattern of antibody response demonstrated in our study is very similar to that reported in another study that evaluated the efficacy of phase 1 *C. burnetii* vaccines in goats (Arricau-Bouvery et al., 2005). The vaccinated goats in the study by Arricau-Bouvery et al. were more protected than unvaccinated goats as only 6/16 vaccinated goats shed *C. burnetii* after challenge compared to 16/16 in the unvaccinated group (Arricau-Bouvery et al., 2005).

Although, antibody titres arising from the vaccination of 8-week-old goats remained above the 160-cut-off value throughout the 7-week monitoring period of this study, the persistence of these antibody titres beyond 7 weeks after vaccination is unknown. Thus, in future field studies, the persistence of antibodies arising from the vaccination of 8-week-old goats needs to be evaluated to determine the frequency of vaccination.

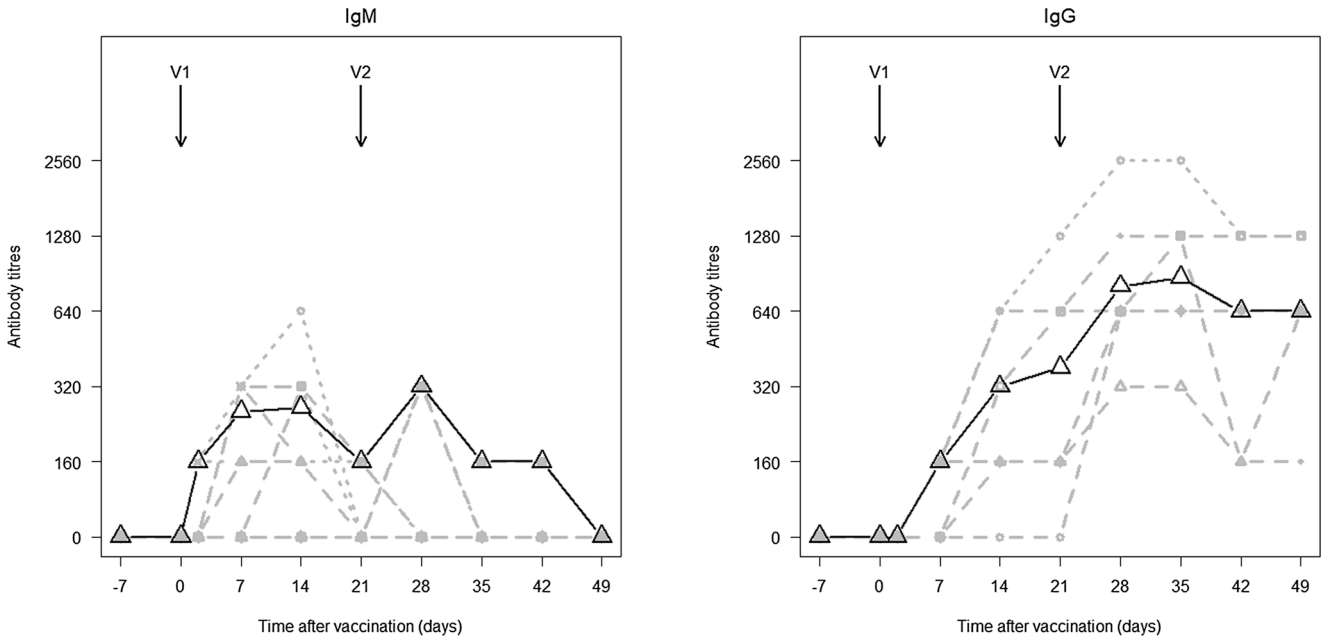

Fig. 2. Individual goat (grey lines) and group (black line) geometric mean antibody titres of 8-week old goats vaccinated with a formaldehyde-inactivated *C. burnetii* vaccine. (V1 and V2: time of administration of first and second (booster) vaccine doses, respectively).

Table 1
Numbers of goats with different types of injection-site reactions after administration of the vaccine and placebo.

Intervention	Type of reaction	After first vaccination	After booster dose
Vaccine (n = 6)	Diffuse swelling	2	0
	Lump	1	5
	Small soft swelling	3	1
	No reaction	0	0
Placebo (n = 6)	Diffuse swelling	0	0
	Lump	6	5
	Small soft swelling	0	0
	No reaction	0	1

3.3. Safety of the vaccine in goats

Reactions at the site of injection were observed in all goats that received the vaccine and the placebo. The reactions included well-demarcated and diffuse epithelial and sub-cutaneous swellings and erythema, as described in Table 1. Following administration of the first dose of the vaccine, 3/6 goats had injection-site swellings larger than 4 cm in diameter. In contrast, no swellings larger than 4 cm were observed in goats following the administration of the first dose of the placebo. Three of 6 goats had a swelling larger than 4 cm after the booster dose of the vaccine was administered and 1/6 goats injected with the second placebo dose had a swelling greater than 4 cm in diameter. Erythema at the injection site was observed in 2 of the 6 goats in the vaccine group and in 1 of the goats in the placebo group. Only one goat in each of the vaccine and placebo groups showed erythema at the injection site following administration of the booster dose. No other lesions were observed at the vaccine or placebo injection-site in any of the treated goats.

The erythema and swellings at the vaccination site, including those larger than the 4 cm in diameter, resolved within 3 weeks without any treatment which coincides with what is reported in livestock following vaccination with Coxevac (European Medicines Agency, 2010a). Additionally, the swellings did not affect the physiological activity and feeding behaviour of the goats.

Although not statistically significant, the higher number of vaccinated goats that had lymphoid hyperplasia of the pre-scapular lymph nodes and spleen (Supplementary file 3) might be due to increased recruitment of lymphocytes in response to vaccination. However, lymphoid hyperplasia of lymph nodes and spleen are general reactions and not specific to any antigen and may be influenced by other factors. Therefore assays for detecting specific cell-mediated immunity against *C. burnetii* are required, as cell-mediated immunity has been reported to play an important role in protection against *C. burnetii* (Zhang et al., 2012).

There was no significant difference in rectal temperature (Supplementary file 4) or change in weight (Table 2) among vaccinated, placebo-administered and no treatment control goats. Furthermore, postmortem tissues from both vaccinated and control animals tested negative for *C. burnetii* on immunohistochemistry and *com1* qPCR.

In conclusion, this study provides novel information indicating that a formaldehyde-inactivated vaccine against *C. burnetii* is safe and

Table 2
Comparison of the change in body weight over the 7 days after the first and booster vaccination of 8-week-old goats, by treatment group.

Time	Days after vaccination	Median (Range) kilograms			P-value*
		Vaccine (n = 6)	Placebo (n = 6)	No treatment (n = 6)	
First vaccination	7	−0.3 (-1.2, 0.7)	−0.3 (-0.6, 0.4)	0.2 (-0.6, 1.0)	0.603
	14	0.6 (-1.4, 1.5)	0.8 (0, 1.0)	0.9 (-1.0, 1.8)	0.327
	21	2.3 (0.7, 3.8)	2.7 (1.0, 2.8)	1.9 (1.0, 3.0)	0.574
Booster vaccination	7	−0.8 (-1.3, -0.4)	−0.6 (-1.9, 0.4)	0 (-1.2, 0.8)	0.102
	14	1.5 (-0.3, 1.9)	0.7 (-0.4, 1.8)	1.2 (0.7, 2.1)	0.407
	21	0.8 (-0.1, 3.1)	1.6 (-5.1, 5.3)	2.4 (-0.5, 3.8)	0.622

* P-values estimated using the Kruskal-Wallis test to compare vaccinated, placebo and no treatment control groups.

immunogenic in 8-week old goats, one month earlier than vaccination at 3-month of age reported in previous trials of the Coxevac livestock vaccine (De Cremoux et al., 2012; European Medicines Agency, 2010b, 2014).

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Ethics approval

The culturing of *C. burnetii* in SPF eggs was approved by the Animal Care & Ethics Committee of the Australian Rickettsial Reference Laboratory, approval number ACEC/11. All procedures involving goats were undertaken in accordance with ethics application (ID 1413432) approved by The University of Melbourne Animal Ethics Committee.

Declaration of Competing Interest

The authors have no conflict of interest to declare.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2021.110253.

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