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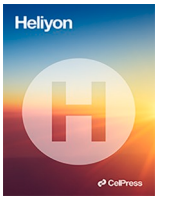

Research article

The safety of overdose and repeat administrations of BCG Danish strain 1331 vaccine in calves and pregnant heifers

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A B S T R A C T

Bovine tuberculosis (bTB), caused by *Mycobacterium bovis* infection, is a zoonotic disease in cattle that represents a significant ongoing challenge to cattle farming productivity and the livelihoods of livestock farmers in the UK. Vaccination of cattle with BCG could directly target the ability of *M. bovis* to proliferate within vaccinates, restricting bTB pathogenesis and onward disease transmission, and represent a step change in the tools available to help control bTB in farmed cattle. A Marketing Authorisation (MA) is required before a cattle BCG vaccine could be sold and supplied as a veterinary medicine within the UK and this requires comprehensive data supporting vaccine quality, efficacy and, most importantly, its safety. We carried out two independent Good Laboratory Practice (GLP) studies in which the safety of BCG vaccination in cattle was stringently tested through overdose and repeat vaccine administrations in young calves and pregnant heifers. Mild and generally short-lived reactions to vaccinations were observed in some animals, most commonly increases in body temperature and swelling at vaccine injection sites, but these did not have a negative impact on the overall health status of vaccinates. BCG was not shed in the saliva, faeces, milk or urine from vaccinated animals and its dissemination was limited to injection site tissues and associated lymph nodes. Overall, young calves and pregnant heifers vaccinated with BCG remained in good general health, and the vaccinated pregnant heifers had normal pregnancies and gave birth to healthy calves. Obtaining a Marketing Authorisation for a cattle BCG vaccine is a critical milestone in the progress towards the eventual use of BCG vaccination in cattle as an additional bTB control tool within the UK; these pivotal GLP vaccine safety studies generated the detailed and essential target animal safety data needed to support this.

1. Introduction

Bovine tuberculosis (bTB) is a chronic disease of cattle primarily caused by the bacterium *Mycobacterium bovis* [1–3]. This bacterium has a diverse host range and although potentially capable of infecting over 85 different species (including farmed animals, household pets and wildlife) cattle are considered its primary or natural host [4,5]. bTB has a detrimental economic impact on cattle and other livestock productivity and trade, and continues to represent an ongoing global zoonotic public health risk alongside forms of human tuberculosis acquired from other animal sources [6–9]. Consequently, the continuing development and application of bTB control measures are essential to maintain and improve global economic, public and animal health [10–13].

In parts of the UK bTB remains an endemic and difficult disease to eradicate despite enhanced national control programmes that have, in recent years, brought about a decline in the incidence and prevalence of infection in cattle herds [14–17]. The deployment of a bTB vaccine for cattle is being considered as an additional disease control tool to supplement existing bTB control measures [17]. BCG vaccination of cattle can have a positive protective effect against bTB and although this may be modest (direct vaccine efficacy meta-analysis estimate of 25%) it would likely mitigate disease transmission to unvaccinated cattle and, in doing so, ease the overall

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bTB disease burden and prevalence at the herd level [12]. Indeed, recently reported results from a natural transmission study in Ethiopia indicated that the positive indirect effects of cattle BCG vaccination on bTB transmission at the group or herd level can be equal to or substantially exceed the observed direct protective effects of vaccination at the individual animal level [18,19]. This recent study reported an overall vaccine efficacy of 89% when both positive direct and indirect protective effects of BCG vaccination in cattle were considered together.

CattleBCG (*Mycobacterium bovis* Bacillus Calmette–Guérin, Danish strain 1331), administered by subcutaneous injection, is the lead candidate bTB vaccine being developed for potential use in cattle in parts of the UK and can significantly reduce bTB disease-associated pathology in cattle experimentally infected with *M. bovis* for up to at least one year after initial vaccination [20,21]. Moreover, the efficacy of BCG vaccination in very young calves [22–24] could help these young animals remain free of bTB as they progress to adult life, or significantly slow disease progression of bTB should they become infected, reducing potential onward disease transmission at an early opportunity [25,26].

A Marketing Authorisation (often referred to as a licence) is required for CattleBCG before it could be supplied for use within the UK. Globally, such licences are issued by independent regulatory authorities (the Veterinary Medicines Directorate in the UK) who assure the safety, quality and efficacy of veterinary medicines before they can be sold and supplied for use. The safety of any potential veterinary vaccine (with respect to the vaccinated animals, the vaccinators, the environment and the food chain) is of the utmost importance during the regulatory assessment of a Marketing Authorisation application as the potential overall benefits of vaccination must be critically assessed against any potential safety risks (benefit-risk assessment) [27–29]. If CattleBCG were to receive a Marketing Authorisation, it could potentially be used in large numbers of cattle as an additional bTB control measure. Eventual deployment of CattleBCG might be expected initially to involve smaller-scale pilot vaccination programmes, perhaps restricted to use in specific bTB epidemiological situations. Data collected during such pilot vaccination deployment programmes would allow a fuller assessment of the beneficial effects of CattleBCG vaccination on bTB incidence and prevalence to be made, which would inform future consideration of the merits of wider-scale vaccine deployment.

As part of the early-phase development of a veterinary vaccine, Good Laboratory Practice (GLP) vaccine safety studies must be carried out in the target animal species and may include single dose, repeat dose and overdose administrations of the veterinary vaccine candidate [30,31]. We have previously reported the results of two independent GLP vaccine safety studies that were carried out in calves and lactating cattle that received a single dose of (at least) the upper limit of the proposed CattleBCG dose range (4×10^6 CFU) [32]. This single dose of vaccine was well tolerated in calves and lactating cattle with only minor local or systemic reactions observed following vaccination. Extending and enhancing the safety profile of CattleBCG, we report here data collected in two independent GLP vaccine overdose safety studies in which calves and pregnant cattle (in each trimester of pregnancy) were vaccinated firstly with an overdose of CattleBCG (nominally ten times the proposed single dose) and secondly with a single dose of CattleBCG seven weeks later. These regulatory vaccine safety studies provide additional supportive data and information on the safety and welfare of cattle vaccinated with CattleBCG; this includes the vaccination of pregnant cattle, a potentially particularly sensitive subpopulation of the target species (cattle, *Bos taurus*) for which data supporting vaccine safety were previously unavailable.

2. Materials and methods

2.1. Regulatory context

These two independent randomised Good Laboratory Practice (GLP) safety studies were designed to collect data on the health and welfare of cattle receiving two separate sequential vaccinations of different doses (an overdose, nominally ten times a single dose, followed seven weeks later by a single dose; see also section 2.5) of CattleBCG (*Mycobacterium bovis* Bacillus Calmette–Guérin, Danish strain 1331). Studies were carried out in calves and pregnant cattle (heifers, at different stages of gestation) as these two categories of cattle represent potentially particularly sensitive subpopulations of the vaccine target species (*Bos taurus*). Sensitive subpopulations are those subgroups of the target species population that might be anticipated to be at a greater risk of experiencing adverse health effects following administration of a veterinary vaccine. The studies were compliant with the Good Laboratory Practice Regulations 1999 and the Good Laboratory Practice (Codification Amendments Etc.) Regulations 2004, and designed in line with EU Commission Directive 2009/9/EC (amending Directive 2001/82/EC of the European Parliament) requirements for immunological veterinary medicinal products and European Pharmacopoeia (EP) Monograph 01/2008/50,206 (5.2.6: Evaluation of safety of veterinary vaccines and immunosera).

2.2. Protection of animals used for experimental or other scientific purposes

All animal work was compliant with the Animals (Scientific Procedures) Act 1986 and carried out under establishment, project and personal UK Government Home Office licences held by APHA, study directors and study investigators.

2.3. Statistical analysis statement

It should be noted that these studies were not designed to test formal hypotheses nor to identify potentially statistically significant differences between treatment groups (vaccinated animals and control animals). These were regulatory vaccine safety studies and, as such, any potential animal health concerns identified in vaccinates would ultimately need careful consideration during subsequent regulatory assessment, regardless of any potential statistical significance identified. For this reason, the safety data collected in these

studies are intentionally reported without statistical inference and restricted to only descriptive statistics summarising the overall characteristics of the data collected, as generally recommended during the laboratory assessment of the safety of investigational veterinary vaccines [33].

2.4. Cattle

2.4.1. Animal sample sizes

The safety studies were designed to detect any potential adverse health effects following the vaccination of cattle with CattleBCG. As these studies were considered exploratory in nature, power analyses were not appropriate to determine the animal group sizes. The number of animals selected for inclusion in each study group was therefore based on relevant regulatory guidance for live veterinary vaccine safety testing, practical and logistical limitations, and expert professional knowledge of and experience in regulatory vaccine safety study design (S. Houghton and H. M Vordermeier). The target group sizes were set at a minimum of four control comparator group animals and 10 vaccine treatment group animals in the calf study and a minimum of six control comparator group animals and six vaccine treatment group animals (in each of three pregnancy trimester groups) in the pregnant cattle study. More detail on the final numbers of animals in each group that completed the studies are provided in sections 2.4.2 and 2.4.3.

2.4.2. Calves

Friesian/Friesian Cross and Holstein/Holstein Cross calves were sourced from UK geographical areas where bTB was not considered endemic and from herds that had had no history of bTB infection in the previous five years. Additionally, the calves' mothers were required to have had a clear bTB skin test (single intradermal comparative cervical tuberculin test; SICCT) in the 12-month period before the calves were recruited to the study. As these calves were younger than 42 days of age at recruitment to the studies bTB skin-testing of these animals was not appropriate, nor would such testing have been informative as the calves would likely have been bTB skin-test negative regardless of their true bTB infection status [25].

Calves were assessed by veterinarians to be in good general health before inclusion in the study and were randomised to either a vaccine treatment group that received BCG vaccinations (eleven calves completed the study) or to a control comparator treatment group to which only vaccine solvent was administered (six animals completed the study). Calves that completed the study were 15–32 days of age (mean: 22; SD: 5) at administration of their first treatment. Vaccine treatment group and control treatment group calves were housed separately from each other (and any other animals) and appropriate biosecurity measures taken to mitigate the risk of any transmission of microorganisms between animal housing enclosures.

2.4.3. Pregnant cattle

Sexually mature, nonpregnant Holstein cattle (heifers) were imported to the UK from Denmark (an Officially Bovine Tuberculosis Free status country, as then defined by European Commission Decision 2003/467/EC) and were additionally confirmed to be bTB skin-test negative (Single Intradermal Tuberculin test; SIT) before importation. These cattle were housed away from any other animals on arrival in the UK, their oestrus cycles synchronised and each then artificially inseminated. Pregnant cattle that completed the study were aged 16.1–20.2 months (mean 18.0; SD 1.4) at the time of artificial insemination and pregnancy was confirmed by transrectal ultrasound examination 33–46 days after artificial insemination. Pregnant cattle were randomly assigned to one of three vaccine treatment groups representing each trimester of pregnancy (20 animals completed the study) or to a single control comparator group in the first trimester of pregnancy (seven animals completed the study) to which only vaccine solvent was administered. For the purpose of this study, the total gestation length was predefined as 285 days from the date of artificial insemination as gestation duration in cattle can be variable and influenced by numerous genetic and environmental factors [34]. This predefined gestation length was divided into three discrete 95-day trimester groups: Trimester Group 1 ($n = 6$), 0–95 days since artificial insemination; Trimester Group 2 ($n = 6$), 96–190 days since artificial insemination; and Trimester Group 3 ($n = 8$), 191–285 days since artificial insemination.

2.4.4. General cattle management

Animals were acclimatised to their new housing enclosures for approximately one week before study treatments were administered. Vaccine treatment group and control treatment group animals (and calves born to these groups) were housed separately from each other (and any other animals) and appropriate biosecurity measures taken to reduce the risk of transfer of microorganisms between animal housing enclosures. Animals were fed a varied diet twice daily based on their nutritional requirements and drinking water was available *ad libitum*. Calves born to the pregnant cattle received a first feed of colostrum from their respective mothers and were then fed calf rearing milk replacer three times a day. Calf rearing nuts and hay were introduced later as food sources in accordance with general calf feeding guidelines. All animals were under veterinary supervision throughout the studies and their general health checked twice daily to monitor for any overt signs of potential health problems, distress or discomfort such as diarrhoea (scour), depressed appetite, weakness, respiratory distress, lethargy, eye/nasal discharge and dull coat, for example.

2.5. Vaccine dose and vaccination

2.5.1. Vaccine and solvent for injection

Lyophilised BCG vaccine (*Mycobacterium bovis* Bacille Calmette-Guérin, Danish 1331 strain) and its solvent for injection were produced to Good Manufacturing Practice (GMP) standards and obtained from the Statens Serum Institute (SSI, Copenhagen, Denmark) at the time of these studies (now produced by AJ Vaccines A/S, Copenhagen, Denmark). The lead BCG vaccine currently

being developed for use in cattle to protect against bTB is referred to as CattleBCG and a single cattle dose of this vaccine would be in the range of 1–4 x 10^6 CFU BCG of this commercially produced strain of BCG Danish 1331. At the time that these studies were carried out consideration was also being given to the potential use of an alternative presentation of therapeutic BCG culture produced by SSI which would have had a marginally higher maximum single dose of 7.2×10^6 CFU BCG. For this reason, a single vaccine dose target of 7.2×10^6 CFU BCG and an overdose vaccine target of 7.2×10^7 CFU BCG (ten times the single dose) were selected for use in these studies. From a regulatory perspective, these vaccine dose targets would also satisfy vaccine safety testing requirements for CattleBCG that has a marginally lower safety testing target requirement of a minimum of 4×10^6 CFU BCG for the single dose (upper limit of the CattleBCG single dose range) and a minimum of 4×10^7 CFU BCG for the overdose (ten times the upper limit of the CattleBCG single dose range).

2.5.2. Vaccine dose preparation

Although the lyophilised BCG and vaccine solvent were commercial products manufactured to GMP standards, with established specifications with respect to CFU BCG per vial, local dose determinations of vials of the same batches of BCG used to prepare the study vaccination doses were also carried out ahead of the study to inform vaccine dose preparation. In summary, sample vials of the lyophilised BCG batches to be used in these studies were each reconstituted in vaccine solvent according to the manufacturer's specifications, serially diluted in Middlebrook 7H9-Tween 80 culture media and the dilution series inoculated onto modified Middlebrook 7H11 agar plates in triplicate. Cultured BCG colonies were enumerated after four to six weeks of incubation at 37 °C and the BCG CFU per vial calculated. The results of these vial dose determinations were used to determine the number of vials of lyophilised BCG vaccine and the volume of vaccine solvent needed to achieve the desired target doses and the vaccination doses were then prepared accordingly. For additional assurance, local determinations of the prepared study vaccination doses were also carried out (as previously described) at the time of their administration. Each single dose vaccine was prepared in a total volume of 0.5 ml for administration and each overdose vaccine in a total volume of 2.0 ml for administration. A larger volume of vaccine solvent was considered necessary to achieve satisfactory resuspension of lyophilised BCG in the overdose vaccine preparations. Control animals received equivalent volumes of vaccine solvent only.

2.5.3. Vaccination administration

Injection sites in the middle third of the left- and right-hand sides of each animal's neck were kept shaved (each area approximately five square centimetres) throughout the studies so that they could be readily identified for subsequent skin thickness measurements (injection site reactions) and the collection of injection site tissue for post-mortem examination at the end of the studies. The prepared BCG vaccine overdoses were administered by subcutaneous injection into the prepared shaved areas in the left-hand side (LHS) of the neck of vaccinates and, seven weeks later, the prepared BCG single vaccine doses administered into the prepared shaved areas on the right-hand side (RHS) of the neck of vaccinates (avoiding the spinous processes on each occasion). Control animals received vaccine solvent only, equivalent in volume to the BCG vaccine administered to vaccinates, to the prepared shaved sites on each side of their necks.

2.6. Animal health and welfare observations

The general behaviour and physical appearance of calves and pregnant cattle were monitored daily and any coughs, breathing abnormalities, inappetence, abnormal demeanour or head lymph node swelling and scored (0: absent, 1: mild, 2: moderate and 3: severe). In the period immediately following vaccine or vaccine solvent administration animal monitoring took place more frequently (five minutes to eight hour intervals). The health and welfare of calves born to pregnant cattle were similarly monitored (three times a day for up to 11 days) and any observed nasal discharge, breathing abnormalities, inappetence, abnormal demeanour or diarrhoea scored. These calves were weighed at birth and again at one week of age. All animals were additionally monitored twice daily for any other indications of possible ill-health such as weakness, lethargy, ocular discharge and dull coat, for example.

2.7. Rectal temperatures and injection-site skin thicknesses

A calibrated digital thermometer was used to measure the rectal body temperatures of study animals the day before and then immediately before the administration of vaccine or vaccine solvent, and then at 4 hours and 8 hours after treatment administration, daily for the next three days and then at weekly intervals thereafter. Skin thickness measurements at the sites of injection were made throughout the study to quantitatively assess any localised injection site swelling (reactions) occurring following subcutaneous administration of study treatments. The thicknesses (pinch skinfold) of the skin at the sites of treatment administration were measured using constant pressure callipers. Calf skin thickness measurements were made immediately before each administration of each treatment, at 4 hours and 8 hours after treatment administration, and then at two-to-five-day sampling point intervals throughout the study. Skin thickness measurements of pregnant cattle were similarly made immediately before each administration of each treatment, then at 4 hours and at one, three, five, seven, ten and fourteen days after treatment administration, and then weekly thereafter.

2.8. BCG culture from clinical samples: saliva, faeces, colostrum/milk

Saliva (oral cavity swabbing) and faeces (removed by rectal manipulation, when achievable) samples were collected from vaccinate treatment group calves and pregnant cattle at one, three, five and seven days after each vaccination and then weekly thereafter.

Colostrum/milk samples were collected from vaccinated treatment group pregnant cattle as soon as possible after they had calved, daily for seven days and then twice weekly for a further five weeks. Samples were processed for BCG culture as previously described [32]. Saliva, faeces, colostrum and milk samples were not collected from any control treatment group animals.

2.9. Post-mortem and histopathology examinations, and BCG culture

Animals that completed the calf study were euthanised 16 weeks after administration of their first treatment and those in the vaccine treatment group underwent post-mortem examination. In the pregnant cattle study, control treatment group animals that completed this study were euthanised within 12 days of giving birth and vaccine treatment group animals that completed this study within 11 days of completion of the milk sampling phase of this study (7.5 weeks after giving birth). Those in the vaccine treatment group that completed the study underwent post-mortem examinations which occurred 101–282 days (mean: 196.6; SD: 71.1) after vaccine overdose administration (Trimester Group 1: mean 279.7 days (SD: 3.6); Trimester Group 2: mean 219.7 days (SD: 0.5); Trimester Group 3: mean 116.9 days (SD: 8.8). All calves that completed the pregnant cattle study (i.e., those born to pregnant cattle) were euthanised and underwent post-mortem examination at 7–11 days of age.

Aside from the post-mortem examinations of animals that completed the studies, these examinations were also carried out on some animals that required euthanasia due to ill health issues and had to be withdrawn from the studies before they could complete them: three vaccine treatment group calves enrolled on the calf study, three vaccine treatment group pregnant heifers enrolled on the pregnant cattle study and one enrolled calf born to a control treatment group pregnant heifer in the pregnant cattle study. Additionally, two foetuses that were aborted from two vaccinate treatment group animals in the pregnant cattle study were also subject to detailed post-mortem examinations.

A standard set of tissue samples were collected from vaccination treatment group animals and submitted for histopathology examination: injection site tissue/underlying muscle, muscle adjacent to injection sites, liver, left and right kidney, and left and right prescapular, caudal mediastinal, left bronchial and ileocaecal-mesenteric lymph nodes. In the case of calves born in the pregnant cattle study, the standard set of histopathology examination samples were liver, left and right kidney, ileocaecal-mesenteric and hepatic lymph nodes. Although collected at postmortem, detailed examination of the liver and left and right kidney samples was at the discretion of veterinary pathologists. In summary, samples were collected in 10% neutral-buffered formalin and fixed for at least 7 days before routine processing to paraffin wax. Samples were microtome sectioned (4 μm), stained with either hematoxylin-eosin (H&E) or acid-fast stained (Ziehl-Neelsen/Kinyoun) using automated slide stainers, and examined by a veterinary pathologist for microscopic evidence of bTB lesions, the presence of acid-fast bacilli and any other pathological abnormalities. The standard set of tissue samples collected could be extended during post-mortem examination and additional cell stains applied (Gram-Twort, for example) whenever deemed appropriate or necessary by veterinary pathologists. The discretion afforded to veterinary pathologists, regarding the types and numbers of tissues collected and examined during the postmortem and histopathology study phases, was particularly important when determining the probable cause of death of any vaccinated treatment group animals, and calves born to or aborted from pregnant cattle.

The standard set of tissue samples collected at post-mortem examination from vaccinate treatment group animals for subsequent BCG culture were muscle adjacent to each injection site, left and right pre-scapular lymph nodes, a caudal mediastinal-left bronchial lymph node pool, ileocaecal/mesenteric lymph node, left and right kidney, and liver. The samples collected for BCG culture from calves born in the pregnant cattle study were liver, left and right kidney, ileocaecal-mesenteric and hepatic lymph nodes. Urine samples were also collected at post-mortem examination for BCG culture wherever possible. BCG culture was carried out as previously described [32].

3. Results

3.1. Vaccine doses and vaccination

The vials of lyophilised BCG vaccine used to prepare the calf vaccination doses were determined to contain a mean of 2.63×10^6 CFU BCG (SD 1.36×10^6; range 8.50×10^5–4.42×10^6). The mean doses of the vaccines administered to calves that were prepared using these vials were calculated to be 6.51×10^6 CFU BCG (SD: 1.17×10^6; range 4.50–7.90×10^6) for the single dose and 4.90×10^7 CFU BCG (SD: 1.61×10^7; range 2.22–7.26×10^7) for the overdose. With respect to the pregnant cattle study, the vials of lyophilised BCG vaccine used were determined to contain a mean of 2.99×10^6 CFU BCG (SD 1.19×10^6; range 8.50×10^5–4.42×10^6) and the mean doses of the vaccines prepared from these and administered to pregnant cattle were calculated to be 4.52×10^6 CFU BCG (SD: 8.01×10^5; range: 3.97–5.70×10^6) for the single dose and 4.63×10^7 CFU BCG (SD: 4.64×10^6; range: 4.18–5.28×10^7) for the overdose.

In summary and based on dose determinations of the prepared vaccines administered at the time of the studies, the mean single dose and mean overdose BCG vaccinations administered to calves were equivalent to 1.6 times and 12.3 times the upper limit of the CattleBCG dose range (4×10^6 CFU BCG), respectively. The mean single dose and mean overdose vaccines administered to pregnant cattle were equivalent to 1.1 times and 11.6 times the upper limit of the CattleBCG dose range, respectively.

3.2. Animals that did not complete the studies

All animals enrolled on and randomised to control treatment groups completed the studies. Three animals that were enrolled on the

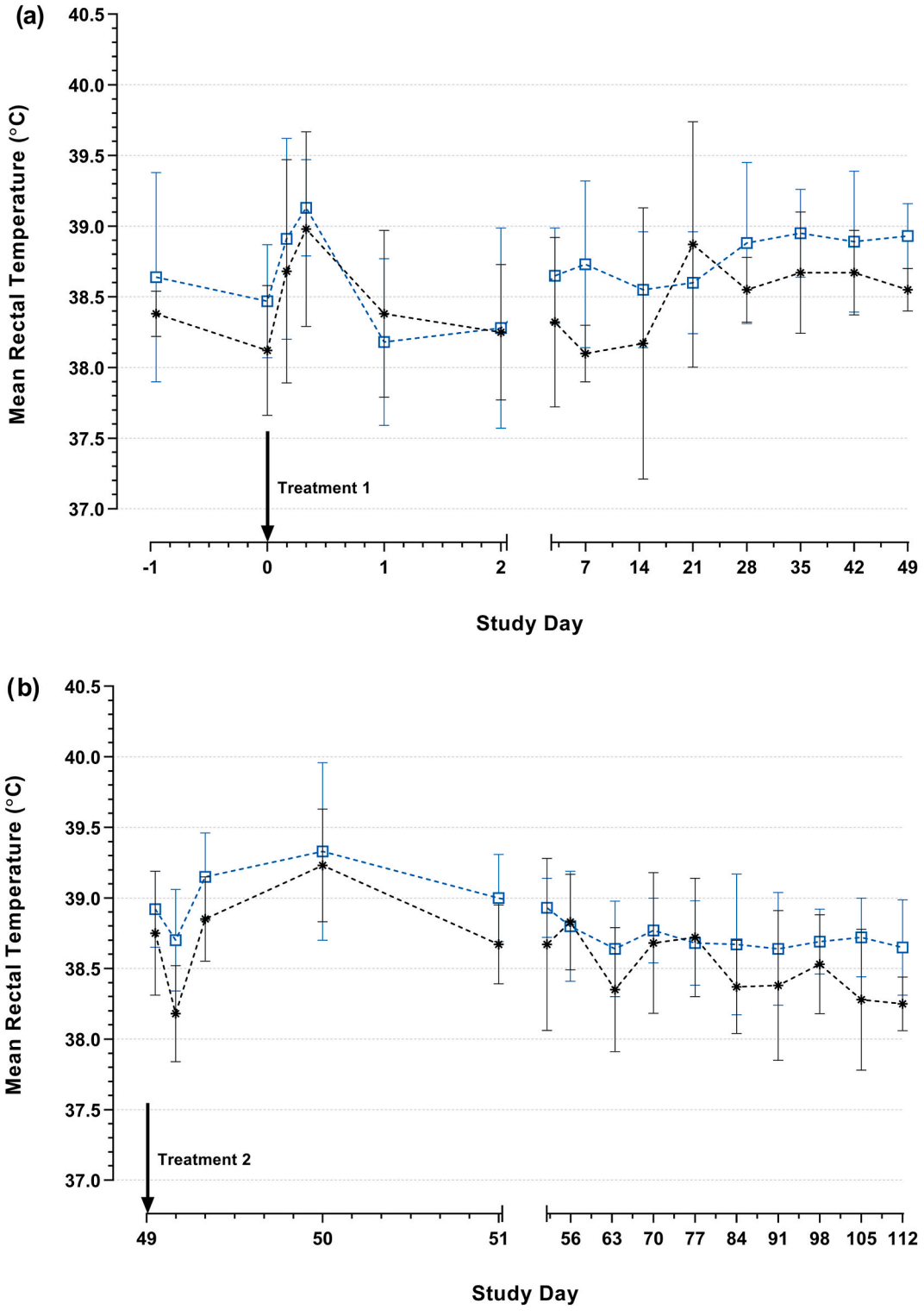

Fig. 1. Mean treatment group rectal temperatures in calves measured at each study time point (SD error bars): * control treatment group; □ vaccine treatment group. The timing of administration of each treatment is indicated by an arrow. **(a) Rectal temperatures after administration of Treatment 1** (control treatment group: vaccine solvent; vaccination treatment group: overdose vaccine) and **(b) Rectal temperatures after administration of Treatment 2** (control treatment group: vaccine solvent; vaccination treatment group: single dose vaccine) seven weeks after administration of Treatment 1.

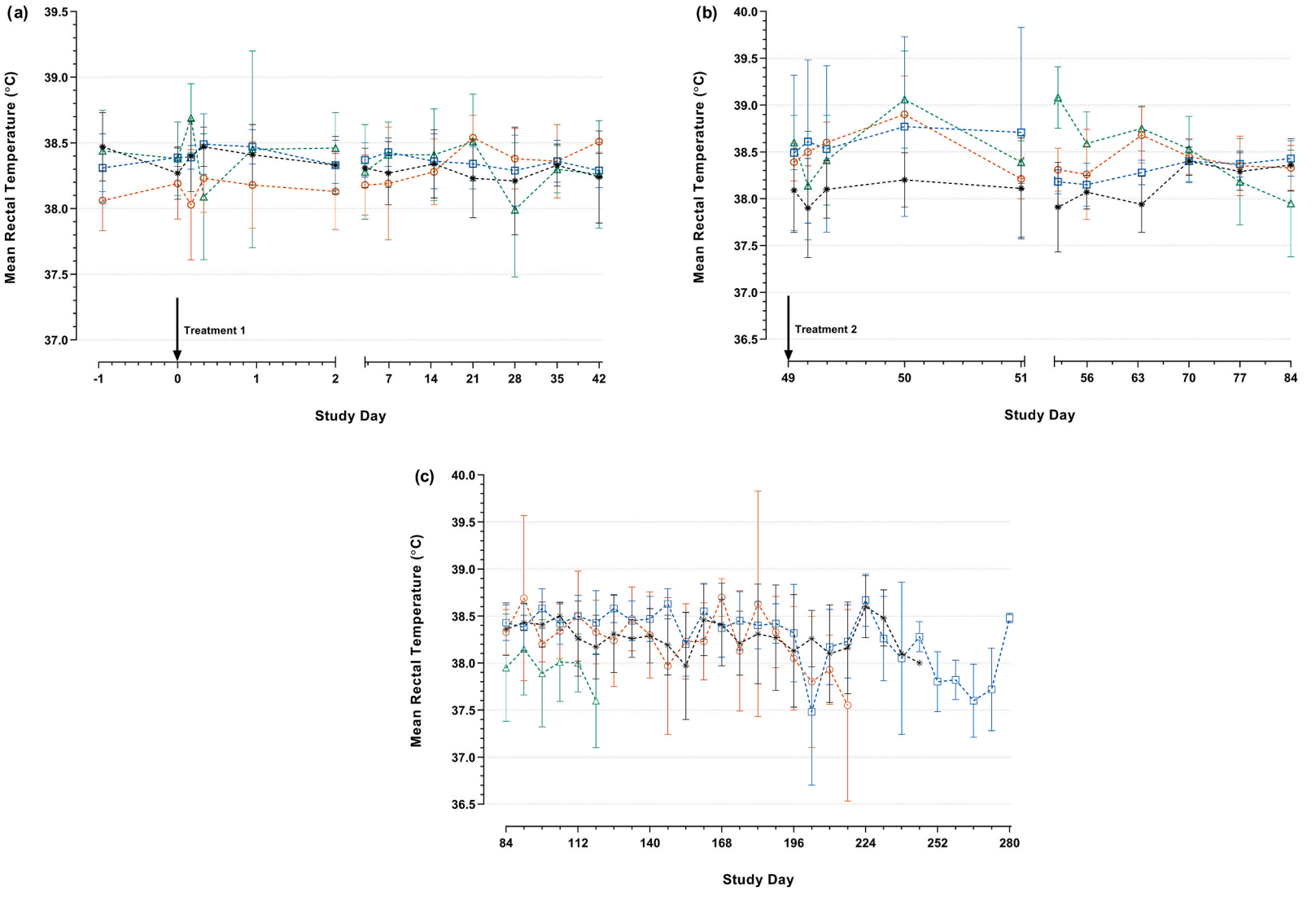

Fig. 2. Mean treatment group rectal temperatures in pregnant cattle measured at each study time point (SD error bars): * control treatment group; □ first pregnancy trimester vaccine treatment group; ○ second pregnancy trimester vaccine treatment group; △ third pregnancy trimester vaccine treatment group. The timing of administration of each treatment is indicated by an arrow. **(a) Rectal temperatures after administration of Treatment 1** (control treatment group: vaccine solvent; vaccination treatment group: overdose vaccine) and **(b) and (c) Rectal temperatures after administration of Treatment 2** (control treatment group: vaccine solvent; vaccination treatment group: single dose vaccine), seven weeks after administration of Treatment 1.

calf study and randomised to the vaccine treatment group and three animals in the pregnant cattle study (one in Trimester Group 1 and two in Trimester Group 2) also randomised to vaccine treatment groups did not complete these studies due to various suspected ill-health issues that did not resolve despite veterinary intervention (example clinical observations: laboured breathing, dull/depressed states, red eyes, difficulty standing after calving etc). The health status of these animals was carefully monitored and veterinary treatment administered when appropriate. Unfortunately, and despite ongoing veterinary care, the condition of these animals continued to deteriorate and it ultimately became necessary to euthanise them on welfare grounds.

In the calf study, two animals were euthanised 12 days after overdose vaccine administration and one animal 5 days after overdose vaccination administration. In the pregnant cattle study one animal was euthanised 52 days after the single dose vaccination, one animal 127 days after the single dose vaccination and one animal 2 days after the single dose vaccination. Detailed post-mortem and histopathology examinations were carried out to identify likely aetiologies for the ill-health issues observed in these animals that led to their euthanasia on welfare grounds. All three calf study animals were found to have had (non-tuberculosis) pneumonia, a common respiratory disease in very young cattle. In the pregnant cattle study, one animal had suppurative placentitis (Gram-negative bacilli or coccobacilli infection), one animal had a diaphragm abscess and one animal was diagnosed to have had pyogranulomatous lymphadenitis, tonsilitis and glossitis (*Actinomyces* spp. or *Staphylococcus* spp. infection suspected). The aetiologies of the ill-health issues observed in these animals were assessed to be unrelated to the vaccination treatments administered.

One female calf born to a control treatment group animal and enrolled on the pregnant cattle study at birth did not complete this study. Following a difficult breach birth, this calf had a weak suckling reflex, was unable to stand within 24 hours and was euthanised on welfare grounds at 1–2 days of age. Subsequent post-mortem examination identified rib injuries in this animal thought to be sustained during the difficult calving and this calf was also observed to have had malformed kidneys. In addition, two pregnant animals in the vaccinate treatment group aborted their foetuses during the pregnant cattle study, one in Trimester Group 2 (male foetus) and one in Trimester Group 3 (female foetus). Detailed post-mortem and histopathology examinations of these foetuses found no evidence of infectious or inflammatory causes for abortion and no aetiologies were suspected.

3.3. Animal health and welfare observations

Veterinary treatment was required at times for various health issues in some animals that completed the studies, four in the control treatment groups and seven in the vaccine treatment groups. These issues included suspected pneumonia and conjunctivitis in calves and pregnant cattle, and suspected mastitis, retained placenta, anaemia, parturient paresis, deformed fetlocks and foot abscess in pregnant cattle. These were assessed, treated and monitored by attending veterinary surgeons and supporting staff during the studies as necessary.

Although no veterinary treatment was required, all animals (in both vaccinate and control treatment groups) that completed the calf study were scored mild to moderate in relation to some of the clinical observation parameters assessed at times throughout the study; these were predominantly in relation to coughs (occasional/frequent) and demeanour (reduced responsiveness/depressed state, for example). In the pregnant cattle study, one vaccine treatment group animal in Trimester Group 2 was observed to have had a slight cough around the time of administration of the single dose vaccine but this was short-lived and resolved without veterinary intervention; coughing was not observed in any other animals at any other time in this study.

The right-hand side prescapular lymph nodes of ten of the eleven (91%) vaccinate treatment group animals in the calf study were observed to be slightly enlarged for up to 7 days after administration of the single dose vaccine. Similarly, a slight swelling of the head lymph nodes in 5 of the 6 (83%) of the Trimester Group 2 vaccine treatment group animals that completed the pregnant cattle study was observed for up to 7 days after administration of the overdose vaccination but this was not observed at any other time in any other animals that completed this study. The observed slight swelling of the prescapular and head lymph nodes in these vaccinated animals did not appear to negatively impact their overall health status.

Overall, and aside from the veterinary treatments and clinical observations previously mentioned, veterinarians assessed animals that completed the calf study to be generally healthy. This was also the case for animals that completed pregnant cattle study that were additionally assessed to have had normal pregnancies.

3.4. Rectal temperatures

The mean rectal body temperatures of control and vaccine treatment groups in the calf study are shown in Fig. 1 (a and b) and those in the pregnant cattle study in Fig. 2 (a, b and c), with the timing of administration of each treatment indicated by arrows. The period following administration of the first treatments (overdose vaccination in vaccine treatment group animals and vaccine solvent in control treatment group animals) is shown in Figs. 1a and 2a and the period following administration of the second treatments (single dose vaccination in vaccine treatment group animals and vaccine solvent in control treatment group animals) is shown in Fig. 1b for calf study animals and Fig. 2b and c for pregnant cattle study animals.

In the calf study the temperatures of individual animals in the control treatment group remained within the range 36.6–40.0 °C (mean: 38.53; SD 0.53) and those in the vaccinate treatment group within the range 36.3–40.2 °C (mean: 38.76; SD 0.48). In the pregnant cattle study, control treatment group individual animal temperatures remained within the range 37.0–39.0 °C (mean: 38.26; SD 0.36) and those in the vaccinate treatment group within the range 36.1–41.2 °C (mean: 38.34; SD 0.47). With respect to pregnancy trimesters in the vaccinate treatment group, the temperatures of individual animals remained within the following ranges during the cattle study: Trimester Group 1 (first trimester) 36.2–41.2 °C (mean: 38.34; SD: 0.46), Trimester Group 2 (second trimester) 36.1–40.8 °C (mean: 38.31; SD: 0.46) and Trimester Group 3 (third trimester) 36.8–40.2 °C (mean: 38.37; SD: 0.49).

Overall, the mean rectal temperatures fluctuated throughout the studies with substantial variation among animals' temperatures within each treatment group at each study time point. This was not surprising as normal body temperatures for cattle can fall between the ranges of 38.5–39.8 °C for calves and 37.8–39.2 °C for adult cattle. This variation in rectal temperatures is evidenced in the large standard deviation error bars shown in Figs. 1 and 2 making it difficult to identify any clear trends across study measurement time points. Nonetheless, apparent small increases (<1 °C) in the mean rectal temperature of both control and vaccinate treatments groups, relative to mean rectal temperatures immediately before administration of each treatment, in the period immediately following the administration of each treatment are visually discernible in the graphs. These small increases appeared to peak between 8 hours and 3 days after treatment administration. The maximum changes in treatment group mean rectal temperature during the seven-day period following administration of each treatment are provided in Table 1 for information.

3.5. Injection site reactions/skin thickness

The first treatment (either vaccine solvent in control animals or overdose vaccine in vaccinated animals) was administered only to the LHS injection site and the second treatment (either vaccine solvent in control animals or single dose vaccine in vaccinated animals) was administered only to the RHS injection site. The mean injection site skin thickness measurements obtained throughout the studies are shown in Figs. 3 and 4. The mean measurements obtained at each study time point on the neck left-hand side (LHS) injection site are shown in Fig. 3a (calf study) and Fig. 4a (pregnant cattle study) and those for the neck right-hand side (RHS) injection site in Fig. 3b (calf study) and Fig. 4b (pregnant cattle study). The timing of administration of each of these treatments is indicated by arrows on each graph with the injection site to which the treatment was administered shown in parentheses.

LHS injection site skin thickness measurements, remained within the ranges of 2.8–9.4 mm (mean: 5.35; SD: 0.90) for control treatment group calves and 5.2–12.0 mm (mean: 8.38; SD: 1.22) for control treatment group pregnant cattle during the studies. Vaccinate treatment group calves LHS injection site skin thickness measurements remained within the range 3.8–19.6 mm (mean: 8.41; SD: 3.11) and those of pregnant cattle in the vaccinate treatment group within the range 5.2–54.0 mm (mean: 13.40; SD: 7.36). With respect to each trimester of pregnancy in vaccinate treatment group cattle, the LHS injection site skin thickness measurements remained within the following ranges: Trimester Group 1 (first trimester) 5.2–54.0 mm (mean: 13.50; SD: 6.93), Trimester Group 2 (second trimester) 5.2–51.0 (mean: 11.58; SD: 6.23) and Trimester Group 3 (third trimester) 6.0–47.0 mm (mean: 15.88; SD: 8.66).

Similarly, RHS injection site skin thickness measurements remained within the ranges of 3.4–7.0 mm (mean: 4.92; SD: 0.74) for control treatment group calves and 6.0–12.2 mm (mean: 8.95; SD: 1.24) for control treatment group pregnant cattle during the studies. The RHS injection site skin thickness measurements for vaccinate treatment group calves remained within the range 4.0–32.0 mm (mean: 9.38; SD: 5.43) and those of vaccinate treatment group pregnant cattle within the range 5.0–45.0 mm (mean: 13.00; SD: 6.99). With respect to each trimester of pregnancy in vaccinate treatment group cattle, the RHS injection site skin thickness measurements remained within the following ranges: Trimester Group 1 (first trimester) 5.0–37.0 mm (mean: 13.52; SD: 6.29), Trimester Group 2 (second trimester) 5.8–37.0 mm (mean: 11.69; SD: 6.41) and Trimester Group 3 (third trimester) 6.5–45.0 mm (mean: 14.36: SD: 8.77).

As with the rectal temperature measurements and as indicated by the standard deviation error bars shown in Figs. 3 and 4, there was also considerable variation in the injection site skin thickness measurements of animals within treatment groups at each study measurement time point. However, in this instance notable increases in skin thickness measurements were observed in the vaccine treatment group animals immediately following administration of treatments to the LHS injection site (overdose vaccine) and to the RHS injection site (single dose vaccine). The maximum changes in treatment group mean skin thickness (relative to skin thickness measurements made immediately before administration of each treatment) during the six-week period following administration of

Table 1
Maximum increases in treatment group mean rectal temperatures in the seven-day period following administration of Treatment 1 (T1) and of Treatment 2 (T2). T1 = Vaccine solvent administration in control group animals and overdose vaccine administration in vaccine treatment group animals; T2 = Vaccine solvent administration in control group animals and single dose vaccine administration in vaccine treatment group animals. N/A = not applicable, C = Control treatment group, V = Vaccine treatment group. The increases in the treatment group mean rectal temperatures are relative to those obtained immediately before administration of each treatment (T1 LHS only: Study Day 0; T2 RHS only: Study Day 49). The study time points at which the maximum mean temperature increases occurred (during the seven-day period following administration) are indicated in parentheses.

Study	Treatment Group	Pregnancy Trimester	T1 Maximum Mean Increase	T2 Maximum Mean Increase
Calf	C	N/A	+0.87 °C (T1 + 8 Hours)	+0.48 °C (T2 + 1 Day)
Calf	V	N/A	+0.65 °C (T1 + 8 Hours)	+0.41 °C (T2 + 1 Day)
Pregnant Cattle	C	1	+0.20 °C (T1 + 8 Hours)	+0.11 °C (T2 + 1 Day)
Pregnant Cattle	V	1	+0.10 °C (T1 + 8 Hours)	+0.29 °C (T2 + 1 Day)
Pregnant Cattle	V	2	+0.04 °C (T1 + 8 Hours)	+0.51 °C (T2 + 1 day)
Pregnant Cattle	V	3	+0.31 °C (T1 + 4 Hours)	+0.48 °C (T2 + 3 days)

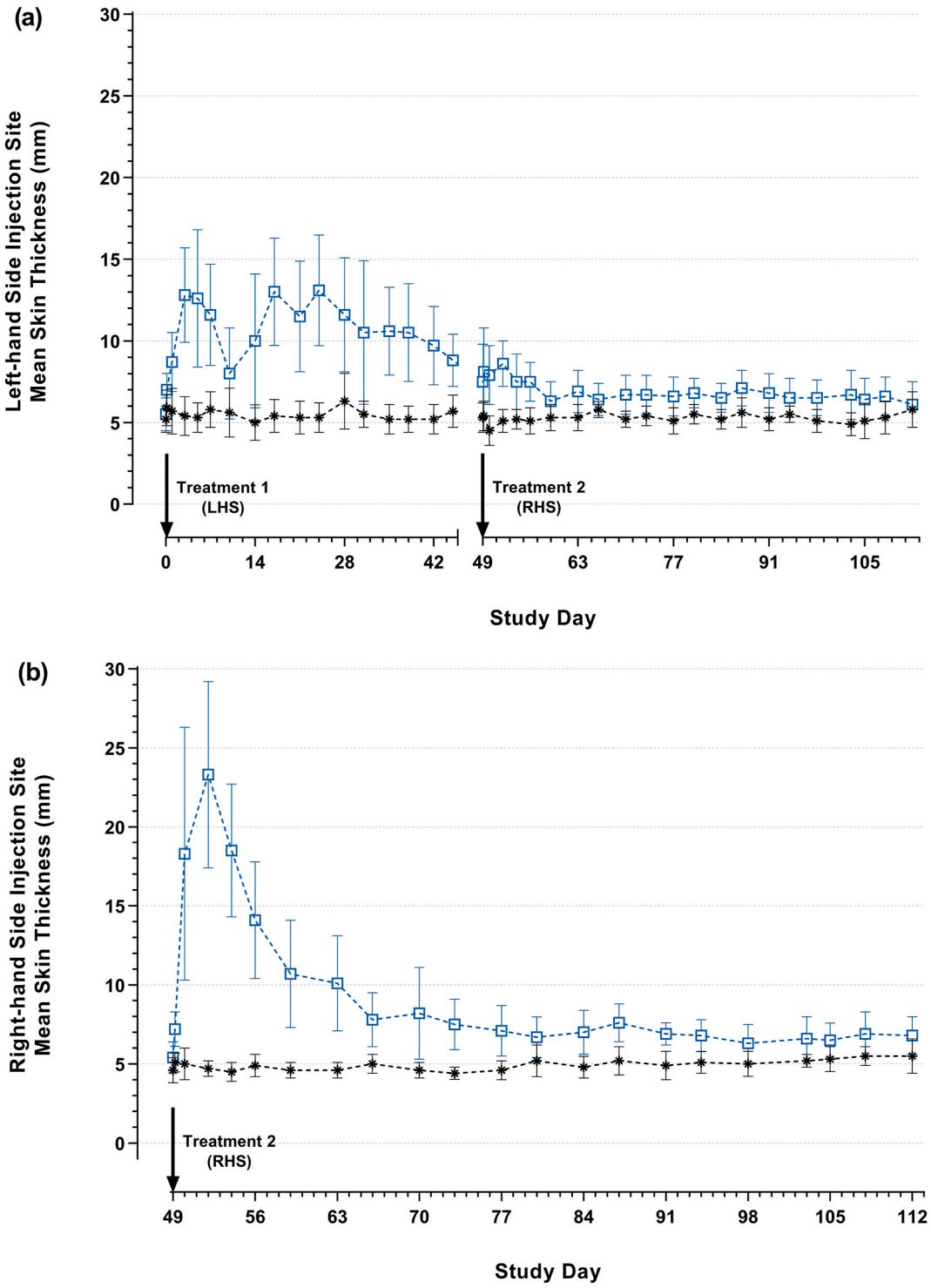

Fig. 3. Mean treatment group injection site skin thickness in calves measured at each study time point (SD error bars): * control treatment group; □ vaccine treatment group. The timing of administration of each treatment is indicated by an arrow. **(a) Left-hand side (LHS) injection site skin thickness** after administration of Treatment 1 (control treatment group: vaccine solvent; vaccination treatment group: overdose vaccination) to the LHS injection site and after administration of Treatment 2 (control treatment group: vaccine solvent; vaccination treatment group: single dose vaccine) to the RHS injection site only. **(b) Right-hand side (RHS) injection site skin thickness** after administration of Treatment 2 to the RHS injection site (control treatment group: vaccine solvent; vaccination treatment group: single dose vaccine).

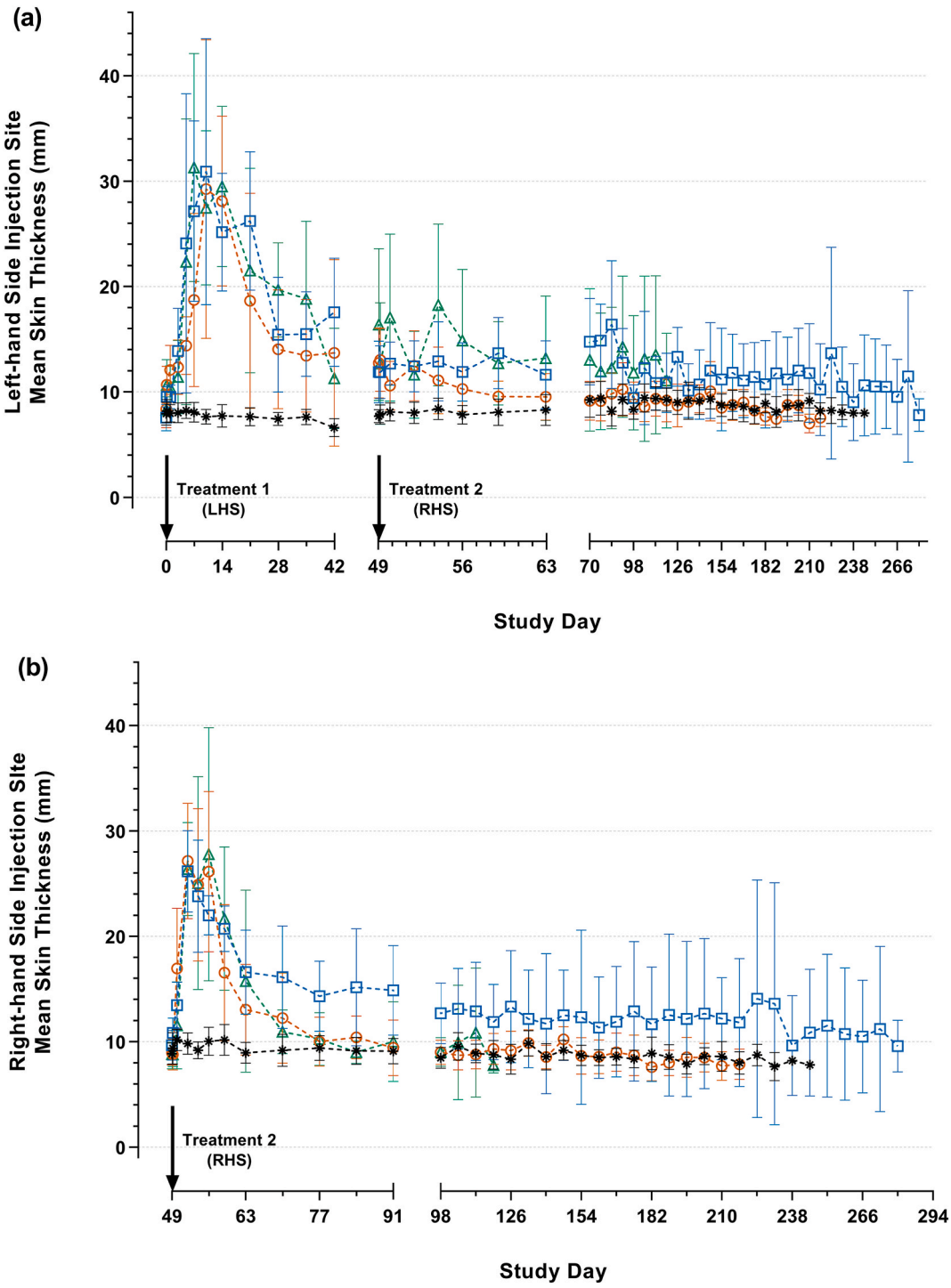

Fig. 4. Mean treatment group injection site skin thickness in pregnant cattle measured at each study time point (SD error bars): * control treatment group; □ first pregnancy trimester vaccine treatment group; ○ second pregnancy trimester vaccine treatment group; △ third pregnancy trimester vaccine treatment group. The timing of administration of each treatment is indicated by an arrow. **(a) Left-hand side (LHS) injection site skin thickness** after administration of Treatment 1 (control treatment group: vaccine solvent; vaccination treatment group: vaccine overdose) to the LHS injection site and after administration of Treatment 2 (control treatment group: vaccine solvent; vaccination treatment group: single dose vaccine) to the RHS injection site only. **(b) Right-hand side (RHS) injection site skin thickness** after administration of Treatment 2 to the RHS injection site (control treatment group: vaccine solvent; vaccination treatment group: single dose vaccine).

each treatment are provided in Table 2 for information.

In the six-week period following administration of the overdose vaccination (Study Day 0), the maximum mean increases in skin thickness at the LHS injection site in vaccine treatment group calves occurred at 24 days (+7.60 mm) after treatment administration and in vaccine treatment group pregnant cattle at between 7 and 10 days (+20.88–23.31 mm) after treatment administration. The maximum mean increases in skin thickness at the RHS injection site in the six-week period following administration of the single dose vaccination (Study Day 49) in vaccine treatment group calves occurred at 3 days after treatment administration (+17.91 mm) and in vaccine treatment group pregnant cattle at between 3 and 7 days (+16.51 mm–18.96 mm) after treatment administration. Much smaller mean maximum increases in injection site skin thickness were observed in control treatment groups in the six-week period following vaccine solvent administration to each injection site: LHS injection site - +1.03 mm at 28 days after administration in calves and +0.29 mm at 5 days after administration in pregnant cattle; RHS injection site - +0.67 mm at 38 days after administration in calves and +1.31 mm at 1 day after administration in pregnant cattle. Similarly, small increases in mean maximum LHS injection site skin thicknesses were also noted at the study time point when treatments were administered only to the RHS injection site: control treatment group calves +0.52 mm at 17 days and pregnant cattle +1.73 mm at 28 days after treatment administration; vaccine treatment group calves +1.05 mm at 3 days and pregnant cattle +0.30–4.49 mm at 4 hours to 35 days.

As can be seen in Figs. 3 and 4, the magnitude of the increases in skin thickness measurements in vaccine treatment group animals was most pronounced at the injection sites to which each treatment was administered. These increases typically decreased markedly during the days and weeks following treatment administration although in some cases, and compared to control treatment group animals, small increases in skin thickness in vaccine treatment group animals appeared to persist until the end of the studies.

3.6. BCG culture from clinical and postmortem samples

BCG was not cultured from 582 out of a total of 584 clinical and postmortem samples (see Sections 2.8 and 2.9 for sample details) collected from animals during the calf study, including samples collected from animals that did not complete the study. Only two postmortem examination samples collected from animals that completed the calf study were reported as being BCG-culture positive, the right prescapular lymph node of one vaccine treatment group animal (2 CFU/sample) and the left prescapular lymph node of another vaccine treatment animal (3 CFU/sample). These samples were collected 112 days and 63 days after overdose and single dose vaccine administration, respectively. Similarly, BCG was not cultured from 2252 out of a total of 2254 clinical and postmortem samples (see Sections 2.8 and 2.9) collected during the pregnant cattle study (including those from aborted foetuses). BCG was cultured from the left prescapular lymph node sample of one vaccinate treatment group animal in the first trimester of pregnancy, 51 days after overdose vaccine administration and 2 days after single dose vaccine administration (reported as BCG culture positive only, CFU burden not recorded). This animal did not complete the pregnant cattle study and required euthanasia on welfare grounds following deteriorating ill-health unresponsive to veterinary treatment (suspected *Actinomyces* spp. or *Staphylococcus* spp. infection at post-mortem examination).

3.7. Post-mortem and histopathology examinations

Samples collected from vaccinate treatment group animals, calves born to vaccinated animals and any foetuses aborted from vaccinate treatment group animals in the calf and pregnant cattle studies were examined at postmortem for potential evidence of

Table 2
Maximum increases in treatment group mean injection site skin thickness (injection site swelling) in the six-week period following administration of Treatment 1 (T1) to the left-hand side (LHS) injection site and Treatment 2 (T2) to the right-hand side (RHS) injection site. T1 = Vaccine solvent administration in control group animals and overdose vaccine administration in vaccine treatment group animals; T2 = Vaccine solvent administration in control group animals and single dose vaccine administration in vaccine treatment group animals. N/A = not applicable; C = Control treatment group; V = Vaccine treatment group. The maximum increases are relative to the mean injection site skin thickness measurements of each treatment group obtained immediately before treatments were administered (T1 LHS only: Study Day 0; T2 RHS only: Study Day 49). The study time points at which the maximum increases in treatment group mean skin thickness measurements occurred (during the six-week period following administration) are indicated in parentheses.

Study	Treatment Group	Pregnancy Trimester	LHS Maximum Mean Increase		RHS Maximum Mean Increase
			T1	T2	T2
Calf	C	N/A	+1.03 mm (T1 + 28 Days)	+0.52 mm (T2 + 17 Days)	+0.67 mm (T2 + 38 Days)
Calf	V	N/A	+7.60 mm (T1 + 24 Days)	1.05 mm (T2 + 3 Days)	+17.91 mm (T2 + 3 Days)
Pregnant Cattle	C	1	+0.29 mm (T1 + 5 Days)	+1.73 mm (T2 + 28 Days)	+1.31 mm (T2 + 1 Days)
Pregnant Cattle	V	1	+23.31 mm (T1 + 10 Days)	+4.49 mm (T2 + 35 Days)	+16.51 mm (T2 + 3 Days)
Pregnant Cattle	V	2	+20.88 mm (T1 + 10 Days)	+0.30 mm (T2 + 4 Hours)	+18.20 mm (T2 + 3 Days)
Pregnant Cattle	V	3	+22.99 mm (T1 + 7 Days)	+1.88 mm (T2 + 5 Days)	+18.96 mm (T2 + 7 Days)

macroscopic or microscopic lesions associated with BCG vaccination.

Lymphoid follicle activation (sometimes with congestion) was typically observed in the right and left prescapular, caudal mediastinal, left bronchial and ileocecal-mesenteric lymph node samples collected from vaccine treatment group animals at postmortem. Focal dermatitis, predominantly due to the infiltration of mononuclear cells, was also frequently observed in samples of the injection site tissue/underlying muscle samples from vaccine treatment group animals. Veterinary pathologists reported the follicle activation to be a normal finding in the lymph nodes of farmed animals and the focal dermatitis to be an expected observation in the period following subcutaneous vaccination with BCG. Additionally, in the calf study vaccine treatment group, necrotic foci were observed in the abdominal lymph nodes of one animal, a frequent observation in cattle, and focal areas of atelectasis and bronchus-associated lymphoid tissue (BALT) proliferation in another animal which were assessed to be unrelated to BCG vaccination.

Acid-fast bacilli were found to be present in small numbers, relative to the vaccination doses administered, in three of the eleven vaccinate treatment group animals that completed the calf study and in five of the twenty vaccinate treatment group animals that completed the pregnant cattle study (one animal in the first trimester of pregnancy and four in the third trimester of pregnancy). Where found, acid-fast bacilli were localised only to injection site tissue/underlying muscle samples and were not observed in any other tissue samples collected at post-mortem examination in either the calf or pregnant cattle studies.

3.8. *Calves born in the pregnant cattle study*

A total of 27 calves completed the pregnant cattle study, 7 of these were born to animals in the control treatment group (4 males and 3 females) and 20 to animals in the vaccinate treatment group (12 males and 8 females: Trimester Group 1: 3 males and 3 females; Trimester Group 2: 5 males and 2 females; Trimester Group 3: 4 males and 3 females). One calf (female, born to a Trimester Group 2 vaccinate treatment group animal) was observed to have had laboured breathing that persisted for 3 days after birth (subsequently found to have had congested lungs at post-mortem examination) and another calf (male, born to a control treatment group animal) had an umbilical cord infection; these health issues were assessed to be common occurrences in calves up to eleven days old.

A mild loss of appetite was observed in twenty-two of the calves for 2–3 days following calving and intermittent mild diarrhoea in fourteen of the calves for up to 7 days following calving. These were assessed to have likely been induced by the stress associated with removal from their mothers and transportation to separate accommodation. The weights of the calves that completed the study were measured at birth and again seven days later; the values obtained are shown in Fig. 5. The mean birth weight of calves born to control

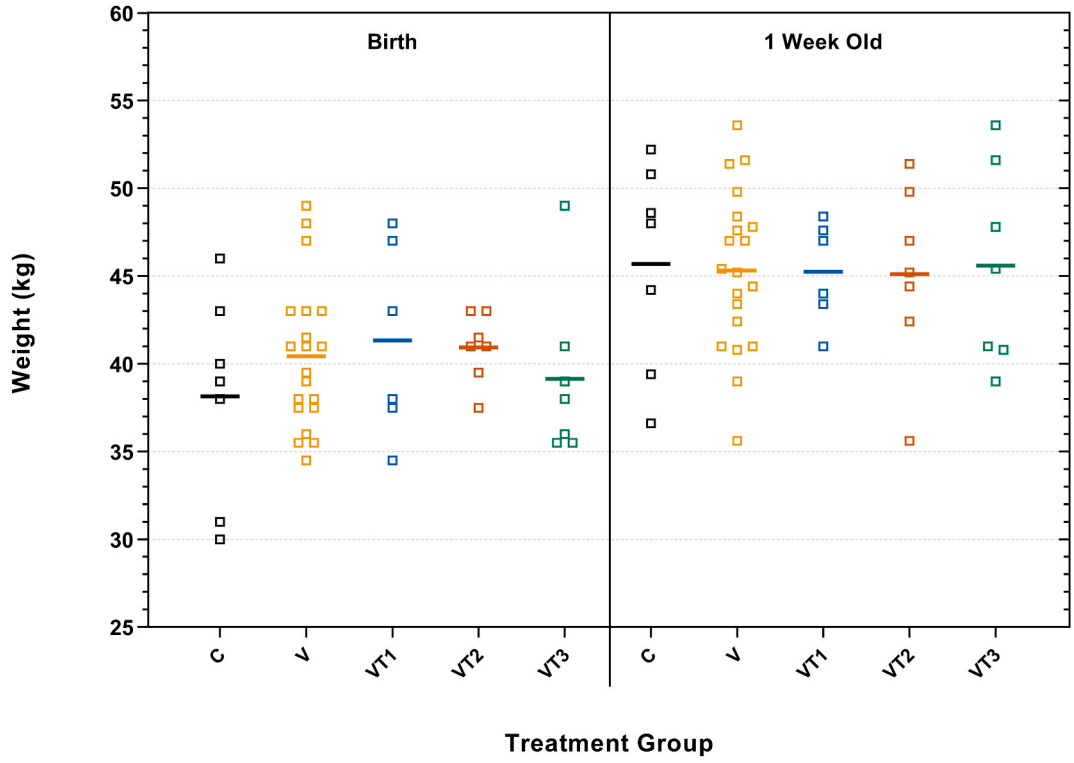

Fig. 5. Body weights of calves that completed the pregnant cattle study. Weights of animals born to pregnant cattle in each treatment group measured at birth and again at seven days of age (mean weights indicated by horizontal bars). Calves were born to control treatment group animals and to vaccinate treatment group animals in each trimester of pregnancy; C: Control treatment group calves; V: Vaccine treatment group calves (all trimesters); VT1: Calves born to Trimester 1 vaccine treatment group cattle; VT2: Calves born to Trimester 2 vaccine treatment group cattle; VT3: Calves born to Trimester 3 vaccine treatment group cattle.

and vaccinate treatment group pregnant cattle were 38.14 kg (SD: 5.87; range: 30.0–46.0) and 40.43 kg (SD: 4.17; range: 34.5–49.0), respectively. Seven days later the mean weights of calves born to control treatment group pregnant cattle were 45.7 kg (SD: 5.9; range 36.6–52.2) and those to vaccinate treatment group pregnant cattle 45.3 kg (SD: 4.6; range: 35.6–53.6).

By seven days old all calves that completed the pregnant cattle study were assessed to be in good overall health and with normal behaviour.

4. Discussion

Vaccination of cattle with BCG could play a key role in the progress towards eradication of bTB by directly targeting the ability of *M. bovis* to proliferate within individual animals but vaccination cannot be introduced to the UK without a Marketing Authorisation. Additionally, cattle vaccinated with BCG would test positive to the primary antemortem diagnostic test for bTB (the single intradermal comparative cervical tuberculin test; SICCT) regardless of their true infection status, although a new companion diagnostic test that can differentiate infected among vaccinated animals (DIVA skin test) has been developed to overcome this issue [35].

Pivotal laboratory GLP vaccine safety studies provide the comprehensive data and information needed for assessment by independent regulatory authorities in support of a Marketing Authorisation application. We have previously reported the results of two BCG vaccine GLP safety studies in calves and lactating cattle that received a single administration of the upper limit of the proposed single dose range (1–4 x 10^6 CFU BCG) of CattleBCG, the lead candidate bTB cattle vaccine being developed for use in the UK [32]. Here we report the results of two further GLP studies in which the safety of CattleBCG vaccine in the target species was assessed following the administration of a vaccine overdose (nominally ten times the proposed single dose) followed by a single dose seven weeks later. Although the nominal tenfold overdose followed by a single dose seven weeks later do not represent the proposed use of CattleBCG (a single dose with annual revaccination), they do serve as a particularly stringent test of vaccine safety in two sensitive subpopulations of the vaccine target species, namely young calves and pregnant cattle.

The overdose and repeat vaccinations with CattleBCG were well tolerated in calves and pregnant cattle and the directors of these studies concluded that no adverse vaccination effects were observed in these animals, nor in calves born to vaccinated pregnant cattle. However, local and systemic reactions were observed in some animals following vaccination but these were frequently mild or transient in nature. The most commonly occurring reactions after vaccination were increases in skin thickness at vaccine injection sites (localised injection site reactions) and small increases in rectal body temperatures, but enlargement/swelling of pre-scapular (calf study) and head lymph nodes (pregnant cattle study) were also observed at times in some vaccinated animals. However, none of these clinical observations appeared to adversely impact the overall health status of vaccinated animals. There was no evidence that BCG was shed in the saliva, faeces, milk/colostrum or urine from vaccinated cattle. BCG was detected at low levels (relative to the vaccination doses administered) at the injection site tissues in a small proportion of vaccinated cattle but further dissemination appeared to be restricted only to the pre-scapular lymph nodes draining these tissues. Aside from veterinary treatment required for health conditions unrelated to vaccination, animals that completed these studies were assessed to be in good general health throughout and, in the case of pregnant cattle, having had normal pregnancies and giving birth to healthy calves. The relatively minor clinical reactions to vaccination observed in these GLP studies were not unexpected and align with the authoritative and comprehensive review carried out by Buddle et al. on the safety of BCG vaccination in animal experimental studies which concluded that this vaccine has been shown to be safe in all animal species in which it has been tested [11].

Together with the GLP vaccine safety studies in cattle that we that we have previously reported [32], the GLP studies presented in this paper contribute to the most comprehensive series of vaccine safety studies conducted in cattle demonstrating the safety of BCG vaccination in the target animal species. These data also provided essential support to an application for a regulatory Animal Test Certificate (ATC) application which was granted and allowed the safety of CattleBCG vaccination to be evaluated in a much larger number of cattle under natural farm conditions. These CattleBCG field trials are underway in several hundred bTB-free cattle from different herds in low bTB risk areas of England and Wales and are expected to provide further data and information, collected under field conditions, supporting the safety of BCG vaccination in cattle [36,37].

5. Conclusion

The results of these GLP studies, stringently testing the safety of BCG vaccination in cattle, demonstrate that CattleBCG is well tolerated in calves and pregnant animals with only mild and generally short-lived local and systemic reactions occurring in vaccinates. There was no evidence of shedding of viable BCG in saliva, faeces, milk/colostrum or urine from vaccinated animals and BCG dissemination appeared to be localised only to vaccination injection site tissues or to lymph nodes draining these tissues following vaccination. Calves vaccinated with CattleBCG remained in overall good general health throughout the studies, as did vaccinated pregnant cattle. The latter were also assessed to have had normal pregnancies and to have given birth to healthy calves. These study data supported regulatory approval for CattleBCG vaccine field trials (conducted to Good Clinical Practice-Veterinary standards) which, in line with regulatory guidance, are needed to generate supplementary vaccine safety data in a much larger number animals under field conditions. The pivotal GLP laboratory studies reported here provide essential vaccine safety data, collected in particularly sensitive subpopulations of the vaccine target animal species, needed to support a Marketing Authorisation application for CattleBCG.

Ethics statement

APHA's Animal Welfare and Ethical Review Body (AWERB) committee reviewed and approved the studies before they were

initiated (reference PPL 70/6414). This paper was prepared with consideration to the ARRIVE Guidelines for reporting animal research [38].

Data availability statement

For commercial reasons, data associated with these studies are confidential and have not been deposited in a publicly available repository.

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CRediT authorship contribution statement

Gareth A. Williams: Writing – review & editing, Writing – original draft, Visualization, Formal analysis. **David Allen:** Writing – review & editing, Validation, Resources, Project administration, Methodology, Investigation, Formal analysis, Data curation. **Jacqueline Brewer:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Francisco J. Salguero:** Writing – review & editing, Validation, Resources, Methodology, Investigation, Formal analysis, Data curation. **Steve Houghton:** Writing – review & editing, Methodology, Conceptualization. **H. Martin Vordermeier:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of generative AI and AI-assisted technologies in the writing process

The authors declare that they did not use generative AI or AI-assisted technologies during the preparation of this paper.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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