DISSERTATION

EVALUATION OF AN ALTERNATIVE PRODUCT FOR *BRUCELLA MELITENSIS* VACCINATION IN GOATS

Submitted by

Naseer Abdulaziz N Alnakhli

Department of Clinical Sciences

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Doctoral Committee:

Advisor: Mo Salman
Co-Advisor: Steven Olsen

Brian McCluskey
Josh Daniels

ABSTRACT

EVALUATION OF AN ALTERNATIVE PRODUCT FOR *BRUCELLA MELITENSIS* VACCINATION IN GOATS

Brucellosis is caused by virulent species of Brucella, specifically *B. melitensis*, which is the top worldwide bacterial zoonotic disease, with more than 2.1 million new cases annually. *B. melitensis* is the greatest cause of human brucellosis, primarily transmitted from its preferred host in small ruminants. Addressing the disease in animal hosts is the most efficient way to prevent human brucellosis, as people are essentially dead-end hosts. Vaccination of animal reservoirs is the most cost-effective method to control brucellosis, alongside serologic detection and removal of infected animals. The *B. melitensis* strain Rev1 vaccine has been used to prevent brucellosis in small ruminants since the early 1950s. The vaccine is efficacious but has limitations due to its tendency to cause abortions in pregnant animals, long-term seroconversion in vaccinates that cannot be differentiated from infection with field strains, shedding in milk, and high virulence in humans. This work sought to explore and evaluate an alternative product for *B. melitensis* vaccination in goats. The overall aim of the dissertation is to develop a *B. melitensis* vaccine for use in endemic countries that is efficacious and safe in small ruminants. The first chapter provides an introduction, background, and rationale for developing alternative vaccines in small ruminants. Chapter two summarizes the current literature on *B. melitensis* vaccines and reviews their advantages and disadvantages in order to support the rationale for the need for new or improved small ruminant brucellosis vaccines. Chapter three evaluates the lipopolysaccharide of *B. melitensis* as a potential vaccine in goats in terms of the immunologic responses and protection

against experimental challenge. Chapter four outlines the scientific protocol for conducting a randomized controlled field trial to assess the effectiveness of the *B. melitensis* vaccines in goats. Chapter five is the conclusion of a doctoral dissertation focused on exploring management strategies for controlling and eradicating brucellosis caused by *B. melitensis*, including the use of alternatives to Rev1 vaccines for application in small ruminants. When taken together, implementing improved Brucella vaccines together with effective management strategies for controlling and eradicating brucellosis could significantly reduce the global prevalence of the disease and its associated zoonotic infections.

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CHAPTER 1 – *BRUCELLA MELITENSIS* VACCINES IN SMALL RUMINANTS – INTRODUCTION AND RATIONALE

One Health is a concept that recognizes the interconnection between the health of people, animals, and the environment. *Brucella melitensis* is a worldwide zoonotic disease that causes approximately 2.1 million new human cases of brucellosis annually worldwide. Three *Brucella* species are responsible for most clinical cases in humans: *B. melitensis*, with sheep and goats as its preferential hosts; *B. abortus*, with cattle as its preferential hosts; and *B. suis*, which is most commonly associated with infections in swine [1,2].

B. melitensis is the greatest cause of human brucellosis, primarily transmitted from its preferred host in small ruminants. In the absence of treatment, human brucellosis can be chronic and result in arthritis, abortion, cardiac issues, and sometimes mortality. In addition to high economic costs associated with human disease, brucellosis causes significant losses in livestock by reproductive losses (abortion, weak fetuses, infertility) [3,4]. Human brucellosis is primarily transmitted through contact with contaminated animal products, particularly non-pasteurized dairy products, or through direct exposure to infected animals. Addressing the disease in animal hosts is the most efficient way to prevent human brucellosis, as people are essentially dead-end hosts [5].

The most common vaccination strategy for small ruminants against *B. melitensis* infection is the use of the live *B. melitensis* Rev 1 strain that is typically administered to female animals between 2 and 4 months of age. This vaccine helps control brucellosis in small ruminants in Europe, the Middle East, and Asia, where the disease is endemic. It is important to note that Rev 1 is a human zoonotic and can also cause abortions in pregnant animals and be shed in milk. These

undesirable characteristics influence field use. Additionally, current diagnostic tests do not serologically differentiate between humoral responses induced by Rev1 vaccination and those caused by infection with *B. melitensis* field strains [6,7]. Despite more than six decades of efforts, a more efficacious and/or safer *B. melitensis* vaccine for small ruminants than Rev1 has not been identified.

In Saudi Arabia (KSA), *B. melitensis* is endemic in small ruminants, with disease prevalence estimates expected to be high (>10%). Although veterinary services and economic resources are available to address brucellosis, mass (whole flock) vaccination of all animals is the typical strategy used for disease control. After a reduction in disease prevalence by vaccination, a test-and-removal strategy may be implemented. The Rev 1 vaccine has been used in Saudi Arabia to control *B. melitensis* in small ruminants for many years. However, efforts to control or eradicate brucellosis in small ruminants have been hampered by three factors: 1) no comprehensive surveillance and control system addresses human and animal brucellosis with *B. melitensis* in animal populations, 2) the inability to differentiate between infected and vaccinated animals serologically, and 3) failure to obtain high vaccination coverage within herds due to animal movement or introduction of unvaccinated animals. Therefore, there is a critical need to develop improved *B. melitensis* vaccines with enhanced safety and efficacy characteristics that can also function as DIVA vaccines, thereby enabling the identification of animals infected with field strains of *B. melitensis* for removal. If a new or improved *Brucella* vaccine is developed, addressing the previously mentioned limitations, public health officials can take necessary measures to implement and promote its use for controlling the infection.

This chapter aims to introduce the thesis with a short background of the topic in endemic countries to justify the dissertation topic.

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CHAPTER 2 – *BRUCELLA MELITENSIS* VACCINES: A SYSTEMATIC REVIEW[1]

ABSTRACT

Background: *Brucella melitensis* is recognized as one of the predominant zoonotic pathogens globally. Live-attenuated vaccine Rev 1 is currently the most effective vaccine for controlling *B. melitensis* in small ruminants. While *Brucella* inactivated, nanoparticle, and subunit vaccines are less effective and require multiple doses, live-attenuated vaccines are less expensive and more efficacious. Several drawbacks are associated with the administration of current attenuated *B. melitensis* vaccines, including interference with serological diagnostic tests, inducing abortion in pregnant animals, shedding in milk, and zoonotic infections in humans. In this systematic review, we summarize the current literature (1970–2022) on *B. melitensis* vaccines and review their advantages and disadvantages in order to support the rationale for a need for new or improved small ruminant brucellosis vaccines. Methods: A systematic search was carried out in Web of Science, CAB Abstracts, and PubMed. The original articles describing the *B. melitensis* vaccines were included. Review articles, articles not published in English, articles that did not offer full text, editorials, correspondences, case reports, case series, diagnostic tests, duplicate publications, and other *Brucella* vaccines (e.g., *B. abortus* and *B. suis*) were excluded. Results: Out of 3700 studies, we identified 18 articles that evaluated *B. meltensis* vaccines, including recombinant *B. melitensis* strains (16MΔhfq, 16MΔTcfSR, M5-90ΔmanB, LVM31, M5-90ΔvjbR, 16MΔmucR, ΔznuA, M5-90Δpgm, M5-90ΔwboA), live *B. melitensis* strain (Rev 1), nanoparticle vaccines (*B. melitensis* 16M, *B. melitensis* OMP 31, FliC protein—Mannosylated Chitosan Nanoparticles (FliC and FliC-MCN), *B. melitensis* and *B. abortus* combined, and *B. melitensis*

[1] Naseer, A.; Mo, S.; Olsen, S.C.; McCluskey, B. *Brucella melitensis* Vaccines: A Systematic Review. *Agriculture* **2023**, *13*, 2137. https://doi.org/10.3390/agriculture13112137

16M nanoparticles combined with oligopolysaccharide), subunit vaccines (outer membrane vesicles or outer membrane proteins), and a DNA vaccine based on *B. melitensis* outer membrane proteins (Omp25 and Omp31). The results from these studies revealed that these vaccines can induce humoral and cellular responses and reduce macrophage survival. However, most of these vaccines were evaluated only in murine models, which may not accurately reflect how they work in natural hosts. Conclusions: The high prevalence of *B. melitensis* in humans and animals remains an issue in many parts of the world. Human brucellosis can be prevented by controlling brucellosis in livestock using vaccination and test-and-removal strategies. Prospective vaccines have limitations, including interference with serodiagnostics after vaccination, virulence in humans and animals, the requirement of booster vaccinations, and insufficient efficacy in preventing infection or abortion. Moreover, most of these vaccines have been assessed in mice models, which have failed to predict immunogenicity or efficacy in natural hosts. Because of these limitations and the re-emergence of *B. melitensis* worldwide with a high incidence of human infection, our review suggests a need for additional research into the molecular pathology and immunological properties of *B. melitensis* infection and the identification of protective epitopes or genes that would allow for the development of improved vaccines for small ruminants.

Keywords: Brucellosis; *B. melitensis*; vaccines; Rev 1; small ruminants

2.1. INTRODUCTION

Because of the high number of human cases identified each year, *Brucella melitensis* is one of the most important zoonotic pathogens worldwide [1]. Three biovars of *B. melitensis*, 1, 2, and 3, are known but are not known to differ in virulence [2]. Goats and sheep are the most common natural hosts of *B. melitensis*. The most common route of transmission in natural hosts is most likely through oral or respiratory mucosa. The predilection of *B. melitensis* for colonization of

trophoblasts in the placenta and fetal tissues leads to fetal stress or death and the induction of premature parturition (abortion) [1,2]. Although abortion is the primary clinical sign, it can be minimal in chronically infected herds. In addition to the shedding of high numbers of bacteria after abortion or birth of infected lambs or kids, some animals shed high levels of bacteria in the milk. Bacterial shed after parturition poses a risk for lateral transmission to other animals [2], whereas shedding in milk is a risk for vertical transmission. It is worth noting that contaminated milk can contribute to the human transmission of this infection. Failure to prevent exposure of uninfected animals to those shedding *Brucella* makes control of the disease difficult. Co-housing of ruminant species is a risk factor for brucellosis transmission [3]. Camels are highly susceptible to *B. melitensis* infection and play an important role in its epidemiology in some countries [4]. In other countries, endemic infection has been identified in Alpine ibex (*Capra ibex*) and chamois (*Rupicapra rupicapra*) and has occasionally led to brucellosis spillover into humans and domestic livestock [5].

Since the beginning of the 20th century, researchers have been searching for vaccines to prevent brucellosis in animals and humans [6]. Both live and inactive vaccines have been developed, but live-attenuated vaccines have been found to be better at inducing protective immunity against this intracellular pathogen [7]. Although a variety of killed vaccines have been developed, their success and acceptance have been limited, and none have induced the level of protection of live-attenuated vaccines. Historically, the first *Brucella* vaccine using killed *Brucella* was made in 1906 by Eyre who used it to vaccinate 51 soldiers [7]. Two killed vaccines that had more widespread use were *B. melitensis* H38 and *B. abortus* strain 45/20 with strain 45/20 utilized in cattle and sheep and H38 in cattle [8]. Due to their limitations in protective immunity as

compared to the attenuated strains and their induction of persistent antibody titers, both are no longer utilized under field conditions.

The live vaccine *B. melitensis* Rev 1 (Rev 1) is currently utilized for the control of brucellosis in small ruminants. This strain was developed by Herzberg and Elberg in the mid-1950s and retains common characteristics of *Brucella* but is resistant to streptomycin and susceptible to penicillin G [9]. Subcutaneous or conjunctival immunization with Rev 1 confers protective immunity in small ruminants. However, Rev 1 vaccination stimulates an antibody response that reacts in serological tests, which cannot be differentiated from the humoral responses of infected animals. As *B. melitensis* can infect cattle, limited data have been reported that suggest that vaccination with Rev 1 has some efficacy in controlling infection with this *Brucella* species in cattle [10].

Due to the incubation period and potential latent infections, it is difficult to eradicate brucellosis by using serologic methods to detect and remove infected animals. The inclusion of vaccination programs to improve herd resistance is generally required to control the spread of the disease. To date, Rev 1 is the best available vaccine for the prevention of *B. melitensis* infection in small ruminants [9] and is typically administered to young animals (3–5 months old) at 0.5–2 × 10^9 colony-forming units (CFU) subcutaneously. Although a bacteremia occurs after vaccination, it has been reported that the vaccine strain is cleared in approximately 14 weeks in goats. Although a reduced dose of the Rev 1 vaccine (10^3–10^6 CFU) has been suggested for subcutaneous administration, it has been found to offer limited protection against disease and does not prevent abortions [11,12]. When administered to adult sheep or goats during pregnancy at 10^9 CFU, abortions and shedding of Rev 1 in milk are common [11,12]. This presents a challenge in endemic areas where mass vaccination is necessary for control and pregnant sheep and goats may need to

be vaccinated despite the risk of adverse effects [13]. In addition to the disadvantages listed above, the strain is also virulent to humans, and zoonotic infections have occurred during vaccination from exposure to abortions or consumption of infected milk [14,15].

Administered subcutaneously, Rev 1 vaccine stimulates protective immunity against *B. melitensis* but can elicit long-lasting antibody responses that interfere with serological testing. In contrast, conjunctival administration of the vaccine confers immunity similar to the standard subcutaneous approaches but reduces the magnitude and persistence of serological responses. For this reason, conjunctival vaccination has been utilized in endemic areas, as it has less impact on serologic screening and facilitates control programs. When eradication is the ultimate goal of a control program, conjunctival vaccination of Rev 1 in adult animals is ideal for enhancing herd immunity and preventing *B. melitensis* infections. It has been hypothesized that Rev 1 vaccination may induce a high level of protective immunity for up to 4.5 years, which essentially is lifelong immunity for small ruminants in most production systems. This hypothesis that Rev 1 vaccination induces lifelong immunity has contributed to the belief that the vaccination of young stock is sufficient for adequate control of *B. melitensis* infection in small ruminants. However, this vaccination strategy is tenuous, and even developed countries have failed to control brucellosis in small ruminants with this approach. The failure of vaccination of young stock only could be due to (i) failure to obtain high vaccination coverage in a herd due to animal movement or introduction of unvaccinated animals; (ii) use of poor quality vaccines or products not maintained with appropriate cold chain conditions; and (iii) a possible decrease over time of protective immunity induced by vaccination. Therefore, vaccination of whole flocks is the only viable alternative to control *B. melitensis* infection in small ruminants under extensive control conditions characteristic of many developing countries [16].

To develop promising vaccines against brucellosis, it is critical to produce T helper 1 (Th1)-derived cytokines (interleukin-12, tumor necrosis factor α, interferon gamma γ) associated with cellular immunity and activation of macrophages, dendritic cells, and CD4+ and CD8+ T cells. T helper 2 (Th2) immune responses associated with humoral responses do not appear to have a major role in the clearance of infection [17]. A study has demonstrated that cytokines, such as IL-4 (Th2 cytokines) and IFN-γ (Th1 cytokines), stimulate different IgG subtypes (IgG1 and IgG2 antibodies, respectively) that may be indicative of differences in the immune response [17].

Although a few recombinant *B. melitensis* live-attenuated, subunit, DNA, and nanoparticle vaccines have been reported, none have progressed to commercial products. Moreover, many of these candidates induce adverse effects, can infect people, induce abortion in pregnant sheep and goats, and/or interfere with serological diagnostic tests. Thus, there continues to be a need for an improved *B. melitensis* vaccine and additional pathobiology, immunology, and molecular research is needed to facilitate the identification of protective epitopes and develop safer and more efficacious vaccine candidates. If a new or improved *Brucella* vaccine has been developed, addressing the previously mentioned limitations, public health officials can take necessary measures to implement and promote its use for controlling the infection. The purpose of this systematic review was to conduct a comprehensive search of the literature on available vaccines against *B. melitensis* and characterize their advantages and disadvantages in order to support the rationale for the need of a small ruminant brucellosis vaccine.

2.2. MATERIALS AND METHODS

This study was conducted in accordance with the reporting items (PRISMA) statement for systematic reviews [18]. This systematic review has not been registered in PRISMA.

2.2.1. Search Strategy

A systematic search was carried out in Web of Science, CAB Abstracts, and PubMed for peer-reviewed articles addressing *B. melitensis* vaccines that may be effective to protect against infection of small ruminants.

A comprehensive search was conducted for the following terms in both the title and abstract fields: "*Brucella melitensis* vaccines" OR "*Brucella melitensis* vaccines in sheep" OR "*Brucella melitensis* vaccines in goat" OR "*Brucella melitensis* vaccines in cattle" OR "*Brucella melitensis* vaccines in bovine" OR "Caprine brucellosis vaccines" OR "Ovine brucellosis vaccines" OR "*Brucella melitensis* vaccines in small ruminants" OR "Malta fever vaccines" OR "undulant fever vaccines" OR "O-antigen polysaccharide *Brucella melitensis* vaccines" OR "O-antigen polysaccharide vaccines and *Brucella melitensis*" OR "O-Linked Glycosylation System *Brucella melitensis* vaccines" OR "O-Linked Glycosylation System vaccines and *Brucella melitensis* ".

2.2.2. Study Selection

Research articles examining *B. melitensis* vaccines were included in this study. A date range of publication between 1970 and 2022 was specified and not limited by geographic location. Duplicate articles were removed, and Zotero (www.zotero.org) was used as the reference software. We performed reviews of the titles, abstracts, and full text. We excluded articles describing other *Brucella* vaccines (e.g., *B. abortus* and *B. suis),* review articles, those not published in English, those that did not offer full text, editorials, correspondences, case reports, case series, and diagnostic tests. Since this research was a systematic review of published articles, approval from an ethical review board was not required.

2.2.3. Screening and Data Extraction

The data were organized in Word for the extraction process. The data extraction terms included references, publication year, vaccine name, type of vaccine, advantages, and disadvantages. The findings were included in the results if the reported data indicated that *B. melitensis* vaccines were efficacious in protecting small ruminants from infection or abortion.

2.2.4. Quality Assessment

The quality assessment of the studies was performed using a critical appraisal checklist provided by the Joanna Briggs Institute (JBI) [19].

2.3. RESULTS

Figure 1 displays an overview of the literature search and study selection methodology. A total of 18 publications met the search criteria and were included for analysis.

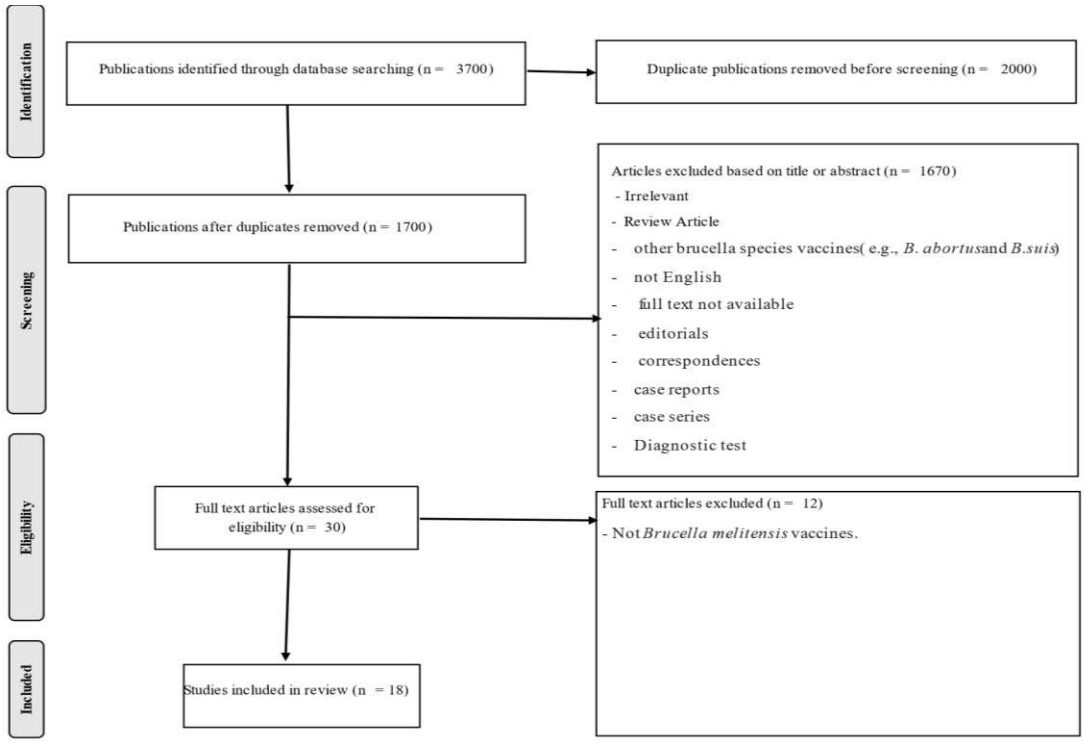

Figure 1. Flow diagram of selection steps after search filtering procedures with Web of Science, CAB Abstracts, and PubMed.

2.3.1. Study Characteristics

The 18 studies described *B. melitensis* vaccines, including recombinant *B. melitensis* strains (16MΔhfq, 16MΔTcfSR, M5-90ΔmanB, LVM31, M5-90ΔvjbR, 16MΔmucR, ΔznuA, M5-90Δpgm, M5-90ΔwboA), live *B. melitensis* strains (Rev 1), nanoparticle vaccines (*B. melitensis* 16M, *B. melitensis* OMP 31, FliC protein—Mannosylated Chitosan Nanoparticles (FliC and FliC-MCN), *B. melitensis* and *B. abortus* combined, and *B. melitensis* 16M nanoparticles combined with oligopolysaccharide), subunit vaccines (outer membrane vesicles or outer membrane proteins), and DNA vaccines based on *B. melitensis* outer membrane proteins (Omp25 and Omp31). A summary of the vaccines described in these 18 studies to address the advantages and disadvantages is shown in Table 1.

Table 1. Characteristics of the included studies.

Name of Vaccine	Type of Vaccine	Animal Models & Ages	Advantages	Disadvantages	Publication Reference Year
B. melitensis 16MΔhfq	Recombinant Vaccine	Six-week-old BALB/c female mice	- Limited persistence in the host. - Reduces survival in macrophages. - Stimulates strong protective immunity in BALB/c mice. - Enhances IgG1 and IgG2a antibodies and IFN-γ and IL-4. - Generates protective immunity that was slightly less than Rev 1 strains. - The possibility of DIVA test.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2013 [20]
B. melitensis 16MΔTcfSR	Recombinant Vaccine	Six-week-old BALB/c female mice	- Induces immune protection against a challenge with *B. melitensis* 16M. - Induces humoral (IgG) and cytokine (IFN-γ) responses. - The possibility of DIVA test. - Provides a similar level of protection of the M5-	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the	2015 [21]

			90 vaccine strain against a *B. melitensis* virulence 16M challenge.	effectiveness of the vaccine in terms of reducing clinical infection.	
B. melitensis M5-90ΔmanB	Recombinant Vaccine	Seven-week-old BALB/c female mice	- Reduces survival in macrophages. - Induces anti-*Brucella*-specific IgG1 and IgG2a subtype responses and IFN-γ and IL-4. - The possibility of DIVA test.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2017 [22]
B. melitensis LVM31 mutant strain	Recombinant Vaccine	Eight-week-old BALB/c female and male mice	- Induces a similar protection that Rev 1 does against the challenge with *B. melitensis* Bm133 virulent strain. - Colonization of bacteria in the spleen is low.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the	2020 [23]

				vaccine in terms of reducing clinical infection.	
B. melitensis M5-90ΔvjbR	Recombinant Vaccine	Six-week-old BALB/c female mice	- Allows for the serological differentiation between infected and vaccinated animals. - Generates high immunity responses, such as IgG, IFN-γ, and IL-4. - Reduces survival in murine macrophages (RAW 264.7) and BALB/c.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2018 [24]
B. melitensis 16MΔmucR	Recombinant Vaccine	Six–eight-week-old BALB/c female mice	- Inducing a protective immunity and reducing the colonization compared to the parental strain. - Decreases the bacterial loads in the splenic, hepatic, and pulmonary at 1 week post-challenge (21 weeks postvaccination) against intraperitoneal *B. melitensis* 16M.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of	2011 [25]

				reducing clinical infection.	
B. melitensis ΔznuA	Recombinant Vaccine	BALB/c female mice	- Laboratory animal models were used to measure levels of protective immunity against nasal challenge with wt *B. melitensis* 16M by inducing both systemic and mucosal Th1 and Th17 cells. - Clear brucella from spleen and lung at approximately 8 weeks after challenge.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2011 [26]
B. melitensis M5-90Δpgm	Recombinant Vaccine	Six-week-old BALB/c female mice	- Induces protective immunity response in BALB/c mice, such as IgG, IFN-γ, and IL-2. - Generates IFN-γ in immunized sheep. - The possibility of DIVA test.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of	2016 [27]

				reducing clinical infection.	
B. melitensis M5-90ΔwboA	Recombinant Vaccine	Six-week-old BALB/c female mice	- Laboratory animal models and sheep were used to measure levels of protective immunity. - Reduces survival in murine macrophages (RAW 264.7) and BALB/c mice. - Inducing an anti-*Brucella*-specific IgG response and (IFN-γ) and (IL-2). - Induces the IFN-γ in the sheep. - The possibility of DIVA test.	- A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2015 [28]
B. melitensis Rev 1	Live *B. melitensis* strains	Goats	- "Proven efficacy in control eradication programs. - Effective against both *B. melitensis* and *B. ovis*. - Safe in males and young replacements. - Single dose affords useful protection for life" [29].	- "Highly abortifacient. - Serological interferences in classic serological tests (RBT, CFT), indirect and competitive ELISAs, fluorescence polarization, and other S-LPS tests. - Virulence for human;	1957[9]

				Streptomycin resistant" [29].	
B. melitensis outer membrane proteins (Omp25 and Omp31)	DNA Vaccine	Seven–eight-week-old BALB/c female mice	- Induces humoral and cellular responses; IgG, Th1 cytokines (IFN-ɣ), and Th2 cytokines (IL-10).	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2018 [30]
B. melitensis 16M	Nanoparticle Vaccine	Six–eight-week-old BALB/c female mice	- Stimulates both cellular and humoral immune responses; IgG and IgA.	- Oral administration would not be able to confer high protection against *B. melitensis* 16M and *B. abortus* 544 in comparison with i.p injection of nano-vaccine. - Immunogenicity and protective against experimental challenge with *B.*	2021 [31]

				melitensis in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	
B. melitensis and *B. abortus* combined	Nanoparticle Vaccine	Four–six-week-old BALB/c female mice	- i.p injection of urease alone and oral administration of both trimethyl chitosan (TMC) nanoparticles formulation of urease (TMC/urease) and urease alone induce a low IgG response. - i.p administration of TMC/urease can generate a high level of IgG. - i.p administration of urease alone and TMC/urease help stimulate a Th1-Th2 immune response. - Oral administration of urease alone and	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2018[32]

			TMC/urease induce Th1-Th17.		
FliC antigen-MCN	Nanoparticle Vaccine	Six–eight-week-old BALB/c female mice	- BALB/c mice were used to evaluate immunogenicity and protective efficacy of vaccine. - Induces specific IgG response (higher IgG2a titers). - Produces a high level of IFN-γ and IL-2 and low-level IL-10 cytokines in immunized mice.	- Induces lower protective immunity than live-attenuated *B. melitensis* Rev 1 and *B. abortus* RB51 vaccines against *B. melitensis* 16M and *B. abortus* 544. - Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2020 [33]
B. melitensis Omp31	Nanoparticle Vaccine	Four–six-week-old BALB/c female mice	- Induces Th1–Th17 immune responses in the oral immunization. - Induces Th1–Th2 immune responses and high IgG titer, IFN-γ,	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking.	2017 [34]

			and IL-12 when i.p immunization with Omp31-IFA (incomplete Freund's adjuvant) and N-trimethyl chitosan/Omp31 (TMC/Omp31). - Administering the vaccine orally induced a higher level of protection compared to the group immunized intraperitoneally due to the Th17 response.	- A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.
B. melitensis 16M combined with oligopolysaccharide	Nanoparticle Vaccine	Six–eight-week-old BALB/c mice	- Stimulates a high level of protection; IgG and IgM and efficient opsonophagocytosis of *Brucella* after challenge with *B. melitensis* strain 16M.	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.
Outer membrane vesicles	Subunit Vaccine	Six–eight-week-old	- Laboratory animal models were used to measure	- Immunogenicity and protective against

		BALB/c female mice	levels of protective immunity. - Increases humoral immune responses (IgG2a and IgG). - Induces cellular immune response (IFN-y, IL-2, and Th1) - Induces protection levels higher than Rev 1 live vaccines.	- experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	
Outer membrane proteins	Subunit Vaccine	Five–six-week-old BALB/c female mice	- Increases humoral immune response (IgG2a and IgG). - Induces cellular immune response (IFN-y, IL-2, IL-4, and Th1).	- Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. - A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.	2020 [37]

Abbreviations used in the table: IFN, Interferon; TNF, Tumor necrosis factor; IL, interleukin; Th, T helper; IgG, Immunoglobulin G; NR, not reported; IFN, interferon gamma γ; IL, interleukin; ORFs, open reading frames; GI-3, genomic island 3; Th1, T-helper 1; SOD, superoxide dismutase; GI3, genomic island; rOmp, outer membrane proteins; CNs, chitosan nanoparticles; W, weeks; i.p, intraperitoneal; OMV, Outer membrane vesicle; CpG ODN, CpG oligodeoxynucleotides; Omp, outer membrane protein (OMP); DIVA, differentiating infected from vaccinated animals; ORFs, open reading frames.

2.3.2. Brucella melitensis Vaccines in Small Ruminants

Of the 18 included publications, nine contained information only relevant to recombinant vaccines, one to a live *B. melitensis* vaccine, five to nanoparticle vaccines, two to subunit vaccines, and one to a DNA vaccine. The recombinant *B. melitensis* 16M hfq (16MΔhfq) strain induced humoral and cellular responses in mice after intraperitoneal vaccination (ip) with increased expression of IgG1, IgG2a, IFN-γ, and IL-4. The recombinant strain did not differ from Rev 1 in the induction of cytokine responses, was cleared at approximately eight weeks post-vaccination (PV), and demonstrated protective immunity that was slightly less than Rev 1 vaccination after experimental challenge with *B. melitensis* 16M at eight weeks PV. [20]. Another study utilized a *B. melitensis* TcfSR promoter (16MΔTcfSR) recombinant strain in which one of the two-component regulatory systems that allow host cells to detect environmental changes and adapt to *Brucella* infection was mutated. The recombinant induced a high level of protective immunity when challenged approximately 1 week after vaccine strain clearance. Vaccination did not induce humoral responses that interfered with serodiagnostic tests [21]. The M5-90ΔwboA recombinant is a reduced virulence, attenuated vaccine that induces reduced inflammatory responses as compared to the parental strain. The reduced virulence and safety of this recombinant were based on the observation that splenomegaly did not occur in a murine host. When compared to the parental strain, comparable protection to the parental strain was observed in mice after experimental challenge approximately 1 week after vaccine strain clearance. Humoral responses in vaccinated sheep and mice allowed for vaccinates to be distinguished from infected animals [28]. Others have demonstrated that a DNA vaccine based on pcDNA3.1, encoding the ORF of *B. melitensis* Omp25 and Opm31 genes, may be a viable vaccine candidate due to induction of humoral (IgG) and cellular (Th1 cytokines IFN-ɣ and Th2 cytokines IL-10) in mice. The DNA

vaccine construct elicited cellular and humoral responses to *B. melitensis* antigens after four inoculations at 1-week intervals [30]. Vaccination of mice with nanoparticle *B. melitensis* and *B. abortus* vaccines conferred less protection than Rev 1 vaccination when experimentally challenged 1 month after the last of three oral vaccinations. Protection was correlated with a mixed Th1-Th17 response [32]. In a different study, the authors demonstrated that a different nanoparticle vaccine (Omp31-loaded N-trimethyl chitosan nanoparticles) induced Th1–Th17 immune responses in mice after three dosages delivered orally or two dosages administered ip. Lower antibody titers were observed in mice orally immunized as compared to ip [34]. When experimentally challenged with *B. melitensis* strain 16M at 1 month after vaccination, oral vaccinates had greater protection than intraperitoneal vaccinates but less protection than Rev 1 vaccinates. A third nanoparticle vaccine (based on poly lactic-co-glycolic acid nanoparticles 50:50 and containing oligopolysaccharide antigens) induced humoral responses in mice that increased after each inoculation. Experimental challenge with *B. melitensis* 2 weeks after the third inoculation demonstrated reduced splenic infection when compared to the control mice [35].

Subunit vaccines offer the advantages of better safety profiles, induction of humoral responses [38], and faster production with reduced costs [39]. Candidate subunit vaccines identified in this search included OMVs vaccines in either Poly (I:C) or 327 CpG + Montanide ISA adjuvant formulations. Humoral responses increased after two vaccine dosages with adjuvanted vaccines demonstrating the greatest responses. Spleenocytes demonstrated greater cytokine responses (IFN-γ and IL-2) in vitro after stimulation with OMV antigens [36]. Another study that used a recombinant protein-based subunit vaccine (Omp10, Omp28, L7/L12 combinations) alone or with Taishan Pinus massoniana pollen polysaccharide adjuvants (TPPPS) demonstrated increased humoral responses after inoculation, but the responses were greater in

mice inoculated with a live *B. melitensis* M5 vaccine. Serum IL-2, IL-4, and IFN-γ were increased in the vaccinated mice. After experimental challenge at 4 weeks after vaccination, the mice inoculated with subunit vaccines containing all antigens and adjuvant had reductions in splenic infection that were similar to but less than the reductions observed in the mice inoculated with the live vaccine [37].

Unfortunately, most *B. melitensis* candidate vaccines have only been evaluated in murine models in which disease pathogenesis can be markedly different from what occurs in ruminant hosts. Murine models are inbred as compared to outbred domestic livestock hosts. There are significant differences in ruminant immunologic responses from those of mice, including the observation that ruminants have a high percentage of circulating T cells expressing γδ markers. Lastly, infection in mice is generally quantified by the evaluation of hepatic and splenic colonization, whereas in ruminant hosts, infection is predominantly within lymphatic tissues. These differences emphasize the need for the evaluation of brucellosis vaccine candidates in the species of interest to address research needs. In addition, some studies in murine models administer experimental challenges prior to the immune system returning to a senescent state, and therefore, colonization data might be influenced by nonspecific immune activation.

2.4. DISCUSSION

Annually, 2.1 million new human cases of brucellosis occur in different parts of the world [40]. Due to its potential to be used as a bioterrorism agent, communicability, pathogenic nature for humans, and clinical effects, *Brucella* has been designated by the Centers for Disease Control and Prevention as a category B agent that should be manipulated under biosafety level III conditions [41]. Human brucellosis can be prevented by controlling the incidence of brucellosis in livestock using vaccination and test-and-removal strategies [42]. Concern has been raised

regarding use of brucellosis as a weapon of bioterrorism in the absence of an adequate human vaccine [41].

The development of a more efficacious and safe vaccine against *B. melitensis* has been a long-term challenge for scientists. Many current licensed brucellosis vaccines have limitations, including virulence in humans and pregnant animals and interference with serodiagnosis [43,44]. For example, Rev. 1, the most commonly used brucellosis vaccine in small ruminants, is a live-attenuated *B. melitensis* strain derived from a virulent *B. melitensis* isolate. The Rev 1 strain became dependent on streptomycin for its growth but lost this growth requirement during subculture while remaining streptomycin resistant [45]. Vaccination with Rev 1 stimulates protective immunity in sheep and goats against *B. melitensis* [9] and also protects rams from *B. ovis*. This vaccine is attenuated compared to the field strains but retains virulence characteristics in animal and human hosts [46]. The Rev 1 vaccine can induce dose-dependent abortions in pregnant sheep and goats [46–48] but is considered nonpathogenic [49] or low pathogenic in rams [50]. The strain is considered smooth, meaning it expresses the O-polysaccharide on its lipopolysaccharide. The O-polysaccharide is an immunodominant humoral antigen utilized in almost all brucellosis serological tests. Limited data suggest that Rev. 1 may be efficacious in protecting cattle against *B. melitensis* [51–53]. The live *B. melitensis* strain 2 is used in China to prevent *Brucella* infections in sheep, goats, cattle, and deer [54,55]. Studies reporting direct comparisons of Rev 1 and other vaccines for immunogenicity and protection against experimental challenge with *B. melitensis* in natural hosts are lacking. The few reports in the literature have failed to find vaccine candidates that have improved efficacy when compared to Rev 1 [56].

Brucella DNA-based vaccines are a type of subunit vaccine, and repeated administration can induce humoral and cellular immune responses [57]. DNA vaccines are plasmids that express

genes encoding specific antigens. The most common genes include L7/L12, BLS, BCSP31, SOD Cu/Zn, Omp16, periplasmic immunogenic protein *(*P39), and BAB1-0278. Adjuvants are not commonly used in DNA vaccines [58]. The DNA vaccine encoding BAB 1-0278 confers protection in mice against *B. abortus* [59]. DNA vaccines containing BAB1 0273 or BAB1 0278 and SOD C induce immune responses in mice but are less protective. DNA vaccines encoding p39 or groEL and other DNA-based vaccine candidates require multiple booster vaccinations but offer only low levels of protection. Therefore, further research in this area is needed [60]. The expression of multiple antigens (Omp16 and L7/L12) in DNA vaccines and expression of cytokines (SOD, including IL-18 or IL-12) can be used to enhance the immune response and efficacy of *Brucella* DNA vaccines [58].

Brucellosis is considered an intracellular pathogen that survives within phagocytes and could be resistant to the bactericidal action of antimicrobial peptides [61]. Researchers reported that the subunit vaccines evaluated were safe and efficacious against brucellosis. These vaccines can be constituted using a recombinant, highly conserved protein that is expressed across the *Brucella* genus. However, the subunit vaccine does not replicate or mimic a natural *Brucella* infection. Thus, they induce lower protective immunity as compared with live-attenuated vaccines [38]. The main challenges for designing an effective subunit vaccine against brucellosis are poor antigenicity, instability, and short half-lives for recombinant subunit antigens [62]. Thus, adjuvants are essential to induce immune responses. Enhancing immunity depends on the type of antigen and adjuvant used in *Brucella* subunit vaccines. Freund's adjuvant, Alum adjuvant, and aluminum hydroxide (the only adjuvant licensed for use in human vaccines) are considered to promote Th2 immune responses, while monophosphoryl lipid A and CpG are believed to generate Th1-type responses, which are considered the type of immunity required for protection against brucellosis.

This study's limitations include the following: (1) scope of literature review: This study exclusively incorporated published papers pertaining to *B. melitensis* vaccines, potentially limiting the breadth of its findings, and (2) language bias: This systematic review includes only articles published in English, which may introduce bias.

When protecting against brucellosis, an ideal vaccine should (i) provide long-term protection against abortion and infection in the majority of animals with only one dose; (ii) prevent colonization and seroconversion with efficacy even when animals are exposed to virulent field strains; (iii) be safe for animals of all ages from young to adult; (iv) not shed the vaccine after inoculation; and (v) be able to differentiate between vaccinated animals and those that are infected [63].

While some scientists argue that the available diagnostic and intervention strategies for brucellosis control are diverse and adequate, the disease's persistent high prevalence in some countries or regions demonstrates a need for additional disease management tools. Cost-effective strategies to combat and prevent the spread of brucellosis worldwide include the use of efficacious vaccines in livestock reservoirs.

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CHAPTER 3 – IMMUNOGENICITY OF A LIPOPOLYSACCHARIDE *BRUCELLA MELITENSIS* VACCINE IN GOATS: AN EXPLORATORY STUDY[2]

ABSTRACT

Background: *Brucella melitensis* is considered one of the most widespread zoonotic pathogens worldwide. Vaccination remains the most cost-effective strategy for controlling *B. melitensis* infection in small ruminants. Methods: In this study, we evaluated the immunologic responses and protection against experimental challenge in 18 goats vaccinated with either lipopolysaccharide (LPS) from *B. melitensis* strain 16M (LPS alone), LPS of *B. melitensis* strain 16M and MONTANIDE™ ISA 61 VG adjuvant (Seppic; 50 Bd national, 92250 La Garenne-Colombes, France) (LPS + ISA 61 VG), or saline as a control. Results: Goats (n = 6) vaccinated with LPS + ISA 61 VG had greater ($p < 0.05$) antibody responses than those that were nonvaccinated. Our data demonstrate that goats vaccinated with LPS + ISA 61 VG exhibited greater lymphocyte proliferative responses ($p < 0.05$) to the LPS antigen than those vaccinated with LPS alone at week 12 after vaccination. However, proliferative responses of peripheral blood mononuclear cell (PBMC) from goats vaccinated with LPS + ISA61VG did not differ ($p > 0.05$) from responses of PBMC from control goats. CD4+, CD8+, and γδ T cells from all vaccinated goats had negligible proliferation and failed to induce antigen-specific IFN-γ production. Control and vaccinated goats did not differ ($p > 0.05$) in their protection against abortion, uterine, fetal, mammary, or maternal infection. Conclusions: Our data suggests that LPS + ISA 61 VG induces a robust humoral response but negligible cellular responses. Our data also suggest that LPS + ISA

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61 VG or LPS alone would not be efficacious for use as a vaccine in goats, but the LPS + ISA 61 VG inoculum may be beneficial as a booster. Additional trials would be necessary to evaluate the vaccine's efficacy as a booster inoculation for small ruminants.

Keywords: brucellosis; LPS vaccine; *Brucella melitensis*; humoral and cellular immunity; experimental challenge; goats.

3.1. INTRODUCTION

Brucellosis is caused by virulent species of *Brucella* spp., specifically *B. abortus*, *B. melitensis*, *and B. suis*, which are the top worldwide bacterial zoonotic diseases, with more than 2.1 million new cases annually [1,2]. *B. melitensis* is the greatest cause of human brucellosis, primarily transmitted from its preferred host in small ruminants. In the absence of treatment, human brucellosis can be chronic and result in arthritis, abortion, cardiac issues, and sometimes mortality [3,4]. In addition to large economic costs associated with human disease, brucellosis causes significant losses in livestock by reproductive losses (abortion, weak fetuses, and infertility). Human brucellosis is primarily transmitted through contact with contaminated animal products, particularly non-pasteurized dairy products, or through direct exposure to infected animals. Addressing the disease in animal hosts, is the most efficient way to prevent human brucellosis, as people are essentially dead-end hosts [5]. Vaccination of animal reservoirs is the most cost-effective basis to control brucellosis [6], alongside serologic detection of antibodies and removal of infected animals. The *B. melitensis* strain Rev1 vaccine has been used to prevent brucellosis in small ruminants since the early 1950s [4,7]. The vaccine is efficacious but has limitations due to its tendency to cause abortions in pregnant animals, long-term seroconversion in vaccinates that cannot be differentiated from infection with field strains, shedding in milk, and

high virulence in humans. Despite more than six decades of efforts, a more efficacious and safer *B. melitensis* vaccine for small ruminants than Rev1 has not been identified.

B. melitensis is classified as a Gram-negative, facultative intracellular bacterium in the Alphaproteobacteria class [8]. Its outer membrane is critical to the infection process, serving as the initial point of interaction between the bacterium and the host [9]. The major constituent, lipopolysaccharide (LPS), consists of three structural domains: (i) lipid A, which anchors the molecule within the outer membrane and contributes to its endotoxic activity; (ii) the inner and outer core oligosaccharides; and (iii) the O-antigen (O-polysaccharide, O-PS), a polymeric sugar chain extending outward from the cell surface. *B. melitensis* is typically found as a smooth LPS (S-LPS) strain that expresses the O-antigen on its LPS. Data suggest that *Brucella* LPS is essential for bacterial invasion, intracellular multiplication, and protecting the bacteria against complement-mediated lysis [10].

The primary innate defense mechanism against *B. melitensis* infection is initiated by the detection of its LPS by pattern recognition receptors (PRRs). These receptors identify specific molecular patterns known as pathogen-associated molecular patterns (PAMPs) [11,12]. When PRRs, such as Toll-like receptors, detect a particular PAMP, they initiate intracellular signaling in antigen-presenting cells, including macrophages and dendritic cells. This signaling cascade leads to an adaptive response that eventually targets infected cells and eliminates the intracellular pathogen [13]. Nevertheless, data indicates that *B. melitensis* infection may evade the innate immune response by diminished PAMP signaling in dendritic cells [13,14].

For protective immunity against brucellosis, it is widely acknowledged that antibody responses generated by vaccination do not correlate with long-term protection in natural hosts [13]. Effective immunity against Brucella infection requires the activation of CD8+ and CD4+ T

lymphocytes. CD4+ T-cells are vital for coordinating the adaptive immune response and can differentiate into T helper (Th1) cells, producing key cytokines like interferon-γ (IFN-γ), interleukin-2 (IL-2), and tumor necrosis factor-α (TNF-α). They also support the growth and maintenance of CD8+ T-cells, which are essential for directly killing infected cells. Both CD4+ and CD8+ T-cells release cytokines that activate macrophages and dendritic cells, enhancing their ability to eliminate Brucella [13,15,16].

Combining the induction of antibodies against the Brucella O-polysaccharide (OPS) with a robust cell-mediated immune response may induce optimal immunity against brucellosis [17]. It has been suggested that this type of immune response may be achieved through the conjugation of Brucella OPS or by combining Brucella OPS with adjuvants or immunogenic Brucella proteins [18]. Current dogma is that both humoral and cellular immune responses are required for protective immunity against *B. melitensis* in small ruminants. In the current study, we tested this hypothesis using vaccines containing purified *B. melitensis* LPS alone or combined with an adjuvant. We hypothesized that *B. melitensis* LPS vaccines would induce robust humoral responses but anticipated less induction of cellular immune responses. Our study's objective was to determine whether immune responses induced by two bioconjugate vaccines would induce humoral and cellular immune responses after vaccination and if they were efficacious in protecting pregnant goats against experimental challenge with virulent *B. melitensis*.

3.2. MATERIALS AND METHODS

3.2.1. Bacterial Cultures

Smooth *B. melitensis* strain 16M was obtained from the culture collection at the National Animal Disease Center in Ames, IA. Frozen stock cultures were propagated on tryptose agar (Difco Laboratories, Detroit, MI, USA) supplemented with 5% bovine serum (TSA) for 72 h at 37

°C with 5% CO_2. The bacteria were then inactivated as previously outlined [19]. Briefly, the cultures were washed with phosphate-buffered saline (PBS) and then suspended in methanol at a ratio of 1:2 (bacterial suspension:methanol). The suspension was incubated at 4 °C for 4 to 5 days. Inactivation was confirmed by plating on Kuzda and Morse (KM) plates. The inactivated cultures were used for subsequent extraction of lipopolysaccharide (LPS). All culture manipulations were conducted in a certified Biosafety Level 3 (BSL-3) cabinet.

3.2.2. Extraction and Quantification of B. melitensis LPS Using the Hot Phenol-Water Microextraction Method

LPS extraction was performed as previously described [20]. The procedure commenced with the collection of the culture through centrifugation, followed by resuspension in distilled deionized water (ddH_2O). An equal volume of 90% phenol was subsequently added to the suspension, which was then shaken vigorously at a temperature range of 65–70 °C. The mixture was then centrifuged to facilitate the separation of the upper aqueous phase from the phenol phase. The aqueous layer was collected, and the phenol phase was re-extracted with ddH_2O. To precipitate the LPS, 5–10 volumes of cold (−20 °C) 95% ethanol were added, followed by incubation at −20 °C overnight. The ethanol was then removed, and the resulting pellet was dissolved in 150 µL of ddH_2O. This precipitation process was repeated once more to enhance LPS purity. The purified LPS was stored at −20 °C until further use.

LPS quantification was performed as previously described [21]. In brief, carbohydrate standards were prepared by diluting a 50:50 stock solution of 0.5 mg/mL sucrose and fructose to produce 1 mL aliquots with final concentrations of 0, 30, 60, 90, and 120 µg/mL. To conduct the assay, 200 µL of each carbohydrate standard, along with a control comprising 200 µL of 50 mM ethylenediaminetetraacetic acid (EDTA) and all sample solutions, were transferred into acid-

washed glass test tubes. Both 200 µL of 5% phenol and 1 mL of 93% sulfuric acid were added to each tube. The reaction produced a yellow color, with the intensity of the color correlating to carbohydrate concentration. All reactions were measured using a spectrophotometer (OD490) (Synergy Neo2, BioTek, Santa Clara, CA, USA), and carbohydrate concentrations (LPS O-antigen) were calculated using a standard curve.

For the assessment of Brucella protein concentration within the inoculum, 1 µg of extracted LPS was treated with 2.5 µg of proteinase K and incubated at 59 °C overnight to digest any protein. The protein quantification within the LPS was carried out using Qubit® Protein Assay Kits (ThermoFisher, New York, NY, USA, Cat. Q33211), in accordance with manufacturer guidelines.

For immunologic assays assessing antibody responses and mononuclear cell proliferation, extracted LPS was utilized as the antigen.

For the experimental challenge, *B. melitensis* strain 16M was cultured on tryptose agar for 72 h at 37 °C. Following incubation, the bacteria were collected from the agar surface using saline aspiration. Strain 16M suspensions were adjusted with a spectrophotometer to roughly 10^8 colony-forming units (CFU) per mL, and concentrations of viable bacteria quantified through standard plate counts.

3.2.3. Emulsification of LPS and Montanide ISA 61 VG

A vaccine formulation containing lipopolysaccharide (LPS) was prepared using ISA 61 VG (SEPPIC) as the adjuvant. Purified Brucella LPS (1.4 mL, 730 µg) was combined with 2.1 mL of ISA 61 VG to achieve a 40:60 (*v*/*v*) antigen-to-adjuvant ratio, following the manufacturer's instructions. Emulsification was performed using a two-way syringe system, consisting of 20 low-

speed cycles (8 s each) followed by 60 high-speed cycles (1 s each). The emulsion was considered stable when droplets floated on the surface and retained their water-in-oil consistency.

3.2.4. Animals and Inoculation

All studies using animals were performed with approval and oversight from the Institutional Animal Care and Use Committee (IACUC) at the National Animal Disease Center. Eighteen adult female goats were obtained from a brucellosis-free herd. Following a two-week acclimatization period, the goats were randomly assigned to three treatments (n = 6/trt). The three treatments were saline (control), 400 µg of LPS alone, and 292 µg of LPS with MONTANIDE™ ISA 61 VG adjuvant. All inoculums were administered subcutaneously in the cervical region. Continuous monitoring was conducted to observe any potential adverse clinical signs in the goats, such as fever, swelling at the injection site or regional lymph nodes, anorexia, lameness, abscess formation at the injection site, and diarrhea.

3.2.5. Post-Vaccination Serologic Responses

Blood samples were obtained by jugular venipuncture and placed into serum separator tubes. After centrifugation, the serum was divided into aliquots and stored at −80 °C until analysis. To evaluate the humoral immune response to LPS, an enzyme-linked immunosorbent assay (ELISA) was conducted to quantify total LPS-specific immunoglobulin G (IgG), following previously described methods [22]. In brief, 96-well flat-bottom plates were coated overnight at 4 °C with 1 µg/well of LPS antigen prepared in carbonate–bicarbonate buffer (0.18 M carbonate, 0.028 M sodium bicarbonate, pH 9.6). Plates were then blocked twice for 15 min with SuperBlock™ (Thermo Fisher Scientific, Waltham, MA, USA). Serum samples diluted 1:800, 1:1600, and 1:3200 were added in triplicate and incubated for 2 h at room temperature (RT). After

four washes with phosphate-buffered saline containing 0.05% Tween 20 (PBST), 100 µL of peroxidase-conjugated AffiniPure Rabbit Anti-Goat IgG (H+L) (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA, USA) diluted 1:5000 was added. Following a 1 h incubation at RT, the plates were washed four times and developed with a TMB substrate kit (Thermo Scientific, Waltham, MA, USA) as per the manufacturer's guidelines. The reaction was terminated by adding 100 µL of 0.18 M sulfuric acid, and optical density (OD) was measured at 450 nm using a microplate reader (Synergy Neo2, BioTek, Santa Clara, CA, USA).

3.2.6. Peripheral Blood Mononuclear Cells and Proliferative Responses

At 12 weeks post-vaccination, jugular blood samples were collected into acid-citrate dextrose tubes, and PBMCs were isolated by density gradient centrifugation [16]. Cell viability was assessed using the trypan blue exclusion method. PBMCs were resuspended in complete RPMI 1640 medium (cRPMI; Gibco Life Technologies, Thermo Fisher Scientific, Waltham, MA, USA) at a final concentration of 1×10^7 cells/mL. Aliquots of 50 µL (5×10^5 cells) were dispensed in duplicate into microtiter plate wells. Each well was filled with either 100 µL of cRPMI medium alone or medium containing LPS at varying concentrations (ranging from 2 µg to 0.125 µg LPS per well, using a 1:2 dilution series). Concanavalin A (ConA; 0.5 µg/well; Sigma, St. Louis, MO, USA) served as a positive control. The plates were incubated for 7 days at 37 °C in a 5% CO2 atmosphere, after which cells were pulsed for 18 h with 1.0 µCi of [^{3}H]-thymidine per well. Radioactive incorporation was quantified following cell harvesting onto glass fiber filters using a liquid scintillation counter (PerkinElmer, Hopkinton, MA, USA) [22].

Proliferative responses of PBMC were also assessed by flow cytometry at week 15, as previously described [16]. PBMCs were stained using a 1:10 dilution of CellTrace Violet (eBioscience, Waltham, MA, USA, Thermo Fisher Scientific) according to the manufacturer's

instructions and resuspended in complete RPMI media. PBMCs were then plated at a concentration of 1×10^6 cells per well in 96-well flat-bottom plates. Wells contained unstimulated (RPMI only), 0.1 μg/well of LPS antigen, ConA, and irradiated *B. melitensis* 16M bacteria (1×10^7 CFU). After incubation for 7 days at 37 °C in a 5% CO_2, cells were treated with either 1× GolgiStop solution (eBioscience, Thermo Fisher Scientific) alone, or Cell Stimulation Cocktail for approximately 16 h prior to harvesting as per manufacturer recommendations. Cells were subsequently processed for flow cytometry staining as previously described methods [16]. After transferring to round-bottom 96-well plates, centrifugation, and 2 washes in Dulbecco's phosphate-buffered saline (DPBS), cells were incubated with a fixable viability dye (Invitrogen EBioscience Fixable Viability Dye eFlour 450, Fisher Scientific) and stained for surface markers. Cell surface markers included: CD4 (clone S-17D, from Washington State University, and an anti-mouse antibody from BD Bioscience, Franklin Lakes, NJ, USA), CD8 (clone St8, from Washington State University, and FITC anti-mouse from BioLegend, San Diego, CA, USA), and γδ T-cells (which recognize the TCR1-N24 δ chain, sourced from Washington State University, along with an anti-mouse antibody, Clone R12-3 (RUO), from BD Bioscience). Cells were fixed and permeabilized using the BD Cytofix/Cytoperm™ kit (BD Bioscience) in accordance with the manufacturer's instructions and then stained with a PE-conjugated anti-bovine IFN-γ antibody (Bio-Rad). The PBMCs were rinsed twice with wash buffer (BD Biosciences) and once with FACS buffer, then finally resuspended in FACS buffer for analysis. Flow cytometry data were collected on a BD FACSymphony™ A5 flow cytometer (BD Bioscience) equipped with DIVA software(v9.3). Data were analyzed using FlowJo® (FlowJo v10.10).

3.2.7. B. melitensis Experimental Challenge

Goats were naturally bred under field conditions, and pregnancy was confirmed using a serological assay that detects pregnancy-specific protein B (Bovine Pregnancy Test, Rapid Visual Kit; IDEXX, Westbrook, ME, USA). At approximately two and a half months of gestation, pregnant goats were transferred to the Agricultural Biosafety Level 3 (AgBSL-3) facility at the National Animal Disease Center (NADC), Ames, Iowa, where they were housed individually for the remainder of the experiment. Each goat was challenged intraconjunctivally with *B. melitensis* strain 16M, receiving 50 µL of inoculum per eye containing approximately 10^7 CFU [23,24]. The concentration of viable bacteria in the inoculum was verified by standard plate count methods. A successful experimental challenge was confirmed by isolating the challenge strain from conjunctival swabs collected five days post-inoculation [25]. The recovered isolates were identified as *Brucella* by means of polymerase chain reaction (PCR) using Brucella-specific primers targeting the omp2a gene [26].

3.2.8. Post-Challenge Serologic Responses

Blood samples were collected through jugular venipuncture prior to and at two weeks following experimental challenge. Additional blood samples were obtained at necropsy following parturition or abortion. Serum was obtained by centrifugation, sterilized through filtration, aliquoted into 1 mL portions, and stored at −70 °C. Post-challenge serologic titers against *B. melitensis* were measured using the ELISA method described above.

3.2.9. Necropsy and Bacterial Culture Procedures

Within 48 h after parturition, or immediately following abortion, goats were euthanized through intravenous injection of sodium pentobarbital. Maternal samples collected during necropsy included milk from two quarters, blood, and various lymph nodes (prescapular, supra-

mammary, internal iliac, hepatic, retropharyngeal, mandibular, bronchial, and parotid). Additional samples included the liver, placentome or caruncle, vaginal swab, spleen, mammary gland tissue from two quarters, and lung. Fetal samples obtained included the gastric contents, lung, liver, blood, bronchial lymph node, rectal swabs, and spleen. Swabs and fluid samples were inoculated directly onto KM media. Tissue samples were weighed, homogenized in 0.15 M NaCl using a tissue grinder, and then plated onto a KM medium. For tissues with high bacterial numbers, homogenates were serially diluted in saline. The level of colonization (colony-forming units (CFU)/g) was determined by standard plate counts. Samples were incubated at 37 °C in a 5% CO_2 atmosphere. *B. melitensis* was identified by colony morphology, growth characteristics, and a Brucella-specific real-time PCR (RT-PCR) assay targeting the outer membrane protein 2 (Table 1). Tissue suspensions were also tested using the Brucella-specific RT-PCR assay.

Table 1. Primers and probes against the *Brucella* outer membrane protein 2 used in real-time polymerase chain reaction assay.

Forward primer	CCCAAGCATTGTCTTCAGCAACAG
Reverse primer	TGG TCT GAA GTA TCA GGC TAC GCA
Probe	56 FAM/ACCTTGGTGTAGGAAACTTCCGGCGT/31ABkFQ
Cycling 30 s 60 °C, 2 min 95 °C, and 40 cycles of 95 °C for 20 s and 60 °C for 1 min	

3.2.10. Definitions

Abortion was defined as the premature expulsion of a nonviable fetus infected with *Brucella* after experimental challenge. The dam or fetus was considered infected if at least one colony of *B. melitensis* was recovered from any tissue collected during necropsy. Mammary infection was confirmed by isolating the 16M challenge strain from mammary gland tissue, milk,

or supramammary lymph nodes. Uterine infection was identified by recovery of the 16M strain from internal iliac lymph nodes, vaginal swabs, or placentomes. Fetal infection was defined by the isolation of the 16M strain from any fetal tissue sample.

3.2.11 Statistical Analysis

Responses from all experimental goats, measured by antibody titer, flow cytometry, [^{3}H]-thymidine incorporation, and tissue colonization, before and after challenge, were considered the primary outcomes for comparison. The clinical outcome, indicated by the occurrence of abortion and 16M infection, was considered the secondary outcome.

Data of the primary outcomes were summarized statistically using means (for normally distributed data) or medians (for non-normally distributed data), along with measures of dispersion (Standard error or range), as appropriate for the tests performed [27].

A one-way ANOVA [28] was used for flow cytometry, [^{3}H]-thymidine, and tissue colonization, and a two-way repeated-measure ANOVA [28] was used for antibody titers, with treatment group and time as the study variables. Prior to applying ANOVA, a normality test (Shapiro–Wilk) [28,29] was performed to confirm the normal distribution of each outcome. If an outcome failed the normality test, the Kruskal–Wallis or Friedman non-parametric test was applied instead.

Chi-square analysis was used to assess the association between experimental groups and the occurrence of abortion and 16M infection. Statistical significance was set at $p < 0.05$. All statistical tests were conducted using SAS v9.4M9 statistical software (SAS Institute, Inc., Cary, NC, USA).

Due to the exploratory nature of this experimental study and the small sample size, statistical testing of the generated data was not intended for population-level inference. Instead, the analyses aimed to compare group differences and to inform sample size estimation for future large-scale field experimental or observational studies.

3.3. RESULTS

The normality test was conducted prior to the analysis. Proliferation, flow cytometry, and colonization data met the normality requirement and were analyzed using one-way ANOVA. However, the serologic data did not meet the normality requirement, and therefore, the Friedman non-parametric test was applied.

3.3.1. LPS Extraction and Quantification

LPS was successfully extracted from *B. melitensis* strain 16M. The extracted LPS product contained 34.7 µg/mL of protein and the concentration of the LPS in our stock was determined to be 280 µg/mL. The average vaccination dosages for treatments two and three were determined to be 400 µg and 292 µg LPS, respectively.

3.3.2. Clinical Signs

In the vaccinated group, two goats were excluded from the experimental challenge, including one diagnosed with lameness and another goat that underwent parturition before challenge. It could not be conclusively determined whether the observed lameness was associated with the vaccination or occurred incidentally, as no other clinical abnormalities were noted. Apart from this isolated case, no local (e.g., injection-site swelling) or systemic adverse events were observed in the remaining vaccinated goats following vaccine administration.

3.3.3. Post-Vaccination Serologic Responses

When compared to non-vaccinated goats, those vaccinated with LPS + ISA61VG demonstrated greater ($p < 0.05$) median IgG responses to the LPS antigen at 1, 2, and 4 weeks. Goats vaccinated with LPS + ISA61VG had greater median titers ($p < 0.05$) only at 4 wks after vaccination when compared to LPS alone. When compared to non-vaccinated goats, goats vaccinated with LPS alone demonstrated a trend for greater median antibody responses to the LPS antigen at week 1 ($p = 0.06$). Goats vaccinated with LPS + ISA61VG- tended ($p = 0.06$) to have greater median antibody responses at 8 and 12 weeks when compared to the other two treatments (Figure 1). Our data suggest that LPS + ISA61VG-vaccinated goats displayed more robust humoral responses against Brucella LPS than those receiving the other two treatments.

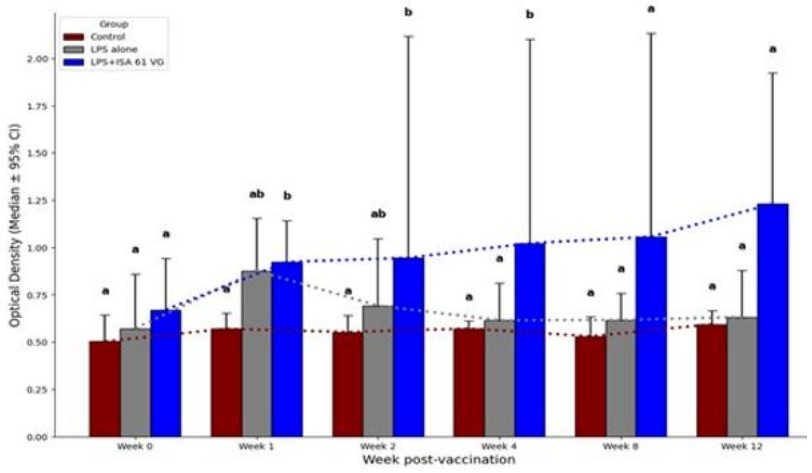


Figure 1. Median IgG serologic responses to LPS antigens of non-vaccinated goats ($n = 6$) and goats vaccinated with either LPS alone ($n = 6$) or LPS + ISA61VG ($n = 6$). Data are presented as a median optical density (OD) ± 95% confidence interval for serum samples diluted 1:1600. Medians bearing different letters (a and b) indicate significant differences ($p < 0.05$) between treatment groups at the same time point.

3.3.4. Post-Vaccination Lymphocyte Proliferation

Goats vaccinated with LPS + ISA61VG had greater mean proliferative responses ($p < 0.05$) to LPS antigens at week 12 after vaccination when compared to responses of goats inoculated with LPS alone. The mean proliferative responses of goats immunized with LPS + ISA61VG did not differ ($p = 0.10$) at this sampling time from mean responses of non-vaccinated goats (Figure 2). These data suggest some antigen-specific proliferative responses were present after LPS + ISA61VG vaccination.

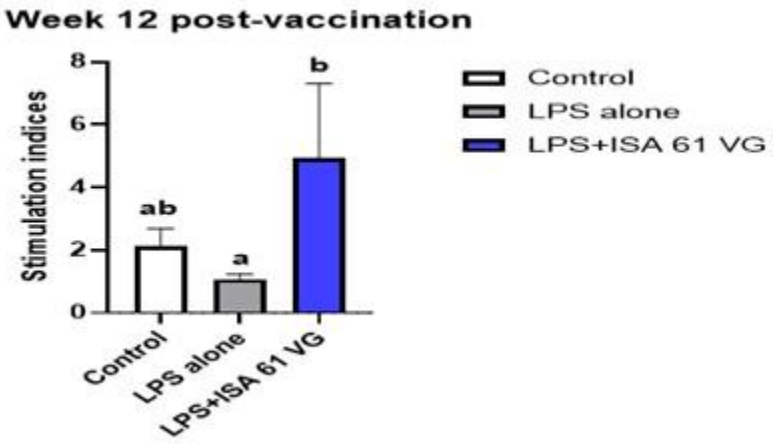


Figure 2. Mean proliferative responses to 0.1 µg LPS by PBMC from non-vaccinated goats ($n = 6$) or goats vaccinated with LPS alone ($n = 6$) or LPS + ISA61VG ($n = 6$). Cells were incubated at 37 °C in 5% CO_2 for 7 days and then pulsed with [^{3}H]-thymidine for 18 h. Data are presented as mean stimulation indices ± SEM, and means labeled with different lowercase letters (a and b) indicate significant differences ($p < 0.05$) comparing treatment groups at the same time point.

3.3.5. Post-Vaccination T Cell Population Subsets, Proliferation, and IFN-γ Responses

Flow cytometric analysis indicated that the frequencies of CD4+ and CD8+ T cells did not differ (gating scheme for the analysis shown in Supplementary Figure S1) between vaccinated goats at

week 15 (Figure 3A–C). However, goats vaccinated with LPS + ISA61VG demonstrated increased frequencies of γδ T cells when stimulated with Brucella LPS compared to those vaccinated with LPS alone or the control group. Furthermore, goats that received the LPS + ISA61VG exhibited notably higher frequencies of γδ T cells after stimulation with either LPS or killed 16M bacteria when compared to their unstimulated cells (Figure 3C). Overall, the frequencies of CD4+ and CD8+ T cell subsets were stable, with CD4 T cells comprising most of the circulating pool of T cells (Figure 3A).

We also compared frequencies of PBMCs proliferating and producing IFN-γ after stimulation with 0.1 μg LPS, killed 16 M bacteria, or when unstimulated. Our data suggest that CD4+, CD8+, and γδ T cells from vaccinated goats had negligible proliferation and failed to produce IFN-γ (Figures 4 and 5), and responses did not differ ($p > 0.05$) from those of the control treatment. There was a nonsignificant trend for CD8 T cells from LPS alone or LPS + ISA61VG vaccinates to demonstrate increased proliferation after stimulation with Brucella LPS or killed 16M bacteria (Figure 4B). Goats in both vaccination treatments had greater ($p < 0.05$) frequencies of proliferating γδ T cells after stimulation with Brucella LPS or killed 16M bacteria when compared to unstimulated cells (Figure 4C). Goats in both vaccination treatments showed no significant differences ($p > 0.05$) in IFN-γ-producing CD4+, CD8+, and γδ T cells in response to Brucella LPS or killed 16M bacteria compared to unstimulated cells (Figure 5A–C). Our data suggest that most of the cells responsive to Brucella antigens after vaccination were γδ T cells, and CD4+ and CD8+ T cells do not proliferate or produce IFN-γ in response to the vaccination treatments evaluated.

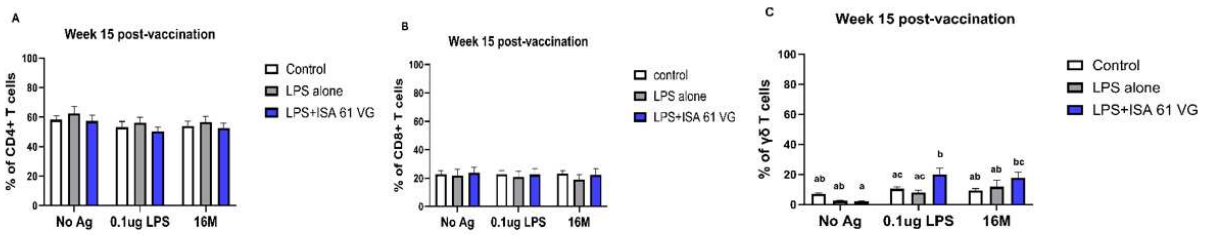


Figure 3. Frequency of CD4+ (**A**), CD8+ (**B**), and γδ (**C**) T cell subsets in PBMC from control (white, $n = 6$), LPS + ISA61VG- (blue, $n = 5$), and LPS-vaccinated (gray, $n = 5$) goats after in vitro incubation for 7 days without stimulation (No Ag) and when incubated with 0.1 μg LPS or killed 16M bacteria. Data are presented as the percentage of CD4+ (**A**), CD8+ (**B**), and γδ (**C**) T cells within the gated cell populations. Means labeled with different lowercase letters (a, b, and c) indicate significant differences ($p < 0.05$).

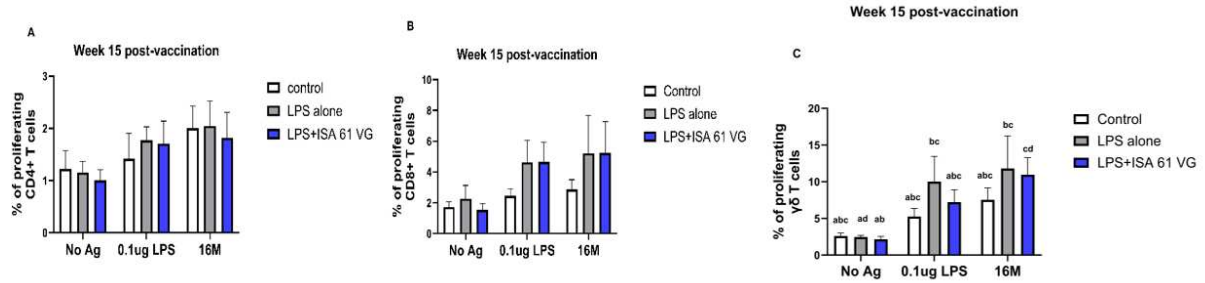


Figure 4. Proliferative responses of CD4+ (**A**), CD8+ (**B**) and γδ T (**C**) cells within PBMC isolated from control (white, $n = 6$), LPS + ISA61VG- (blue, $n = 5$), and LPS-vaccinated (gray, $n = 5$) goats at 15 weeks after vaccination, after in vitro incubation for 7 days without stimulation (No Ag) and when incubated with 0.1 μg LPS or killed 16M bacteria. Cells were analyzed for proliferation using a membrane-based dye. Data are shown as mean frequencies ± SEM, and different lowercase letters (a, b, c, and d) indicate significant differences ($p < 0.05$).

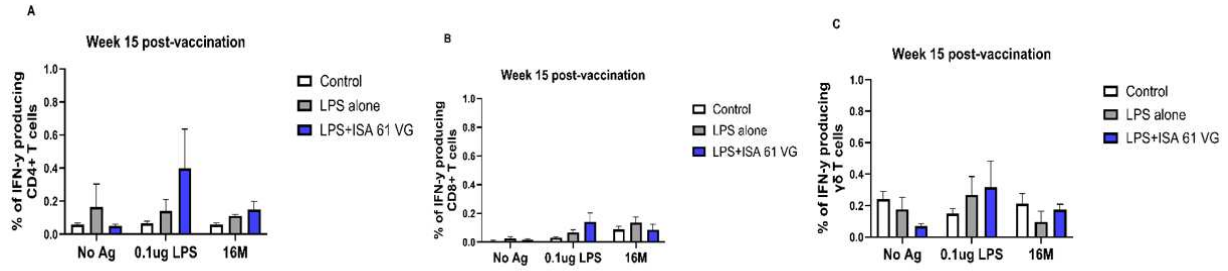


Figure 5. IFN-γ production of CD4+ (**A**), CD8+ (**B**) and γδ T cells (**C**) populations of PBMC from PBMC isolated control (white, $n = 6$), LPS + ISA61VG- (blue, $n = 5$), and LPS-vaccinated (gray $n = 5$) goats at 15 weeks after vaccination, after in vitro incubation for 7 days without stimulation (No Ag) and when incubated with 0.1 μg LPS or killed 16M bacteria. Data are presented as mean frequencies of cells expressing IFN-γ ± SEM.

3.3.6. Post-Challenge Results

Before the challenge and at 2 weeks post challenge, median ELISA titers to killed 16M bacteria did not differ ($p > 0.05$) between the two vaccination treatments and the control group (Figure 6).

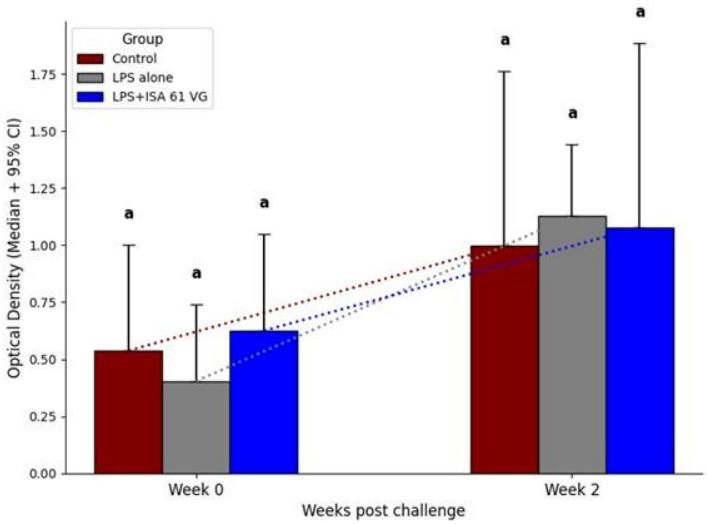


Figure 6. Serologic IgG responses to killed 16M bacteria in an ELISA assay prior to experimental challenge and at 2 weeks after challenge in an ELISA in nonvaccinated goats (burgundy, $n = 6$) and LPS alone (gray, $n = 5$) or LPS + ISA61VG vaccinated goats (blue, $n = 5$). Serum samples were collected at pre- and post-challenge. Data is from samples diluted 1:1600 and presented as median optical density ± 95%CI. Medians with different superscripts (a and b) are different ($p < 0.05$).

All treatments exhibited high rates of abortion and colonization in uterine, mammary, fetal, and maternal tissues (Table 2). In most instances, fetuses were severely autolytic with significant amounts of bacterial contamination, which prevented numeration of Brucella colonization. Control and vaccination treatments did not differ ($p > 0.05$) in the frequency of Brucella colonization in tissues (Table 3) and in milk, blood, or other samples. When evaluated across tissues, the placentome, fetal lung, and fetal liver had the highest levels of Brucella colonization (Table 3).

Table 2. Efficacies of LPS alone or combined with ISA 61 VG vaccination in protecting against experimental challenge at midgestation with 10^7 *B. melitensis* strain 16M. Rate of abortion or infection (% [no. aborted or infected/total]).

Vaccination Group	N	Abortion	Uterine Infection *	Mammary Infection **	Fetal Infection ***	Remaining Maternal Tissues ****
control	6	100 (6/6)	100 (6/6)	100 (6/6)	80 (5/6)	100 (6/6)
LPS alone	5	80 (4/5)	60 (3/5)	100 (5/5)	60 (3/5)	100 (5/5)
LPS + ISA61VG	5	80 (4/5)	80 (4/5)	100 (5/5)	80 (4/5)	100 (5/5)

* Internal iliac lymph node, vaginal swab, and/or placentome; ** supramammary lymph node, milk, and/or mammary gland tissues; *** fetal gastric contents, liver, spleen, lung, rectal swab or bronchial lymph node; **** all maternal tissues not included in uterine infection or mammary infection column.

Table 3. Tissue colonization at parturition after conjunctival infection of control or vaccinated goats with *B. melitensis* strain 16M.

	Culture Positive/Total	Mean CFU/gm ± SEM †
Maternal		
Lung	13/16 (81%) [ab]	2.23 ± 0.44 [a] (*n* = 6)
Liver	13/16 (81%) [ab]	2.03 ± 0.22 [a] (*n* = 7)
Spleen	14/16 (87%) [ab]	2.45 ± 0.44 [a] (*n* = 6)
Bronchial LN	15/16 (93%) [ab]	3.06 ± 0.22 [a] (*n* = 6)
Hepatic LN	16/16 (100%) [ab]	2.83 ± 0.23 [a] (*n* = 11)
Iliac LN	13/16 (81%) [ab]	2.90 ± 0.34 [a] (*n* = 7)
Mandibular LN	16/16 (100%) [ab]	2.64 ± 0.15 [a] (*n* = 11)
Mesenteric LN	12/16 (75%) [ab]	1.85 ± 0.30 [a] (*n* = 7)
Parotid LN ‡	30/32 (93%) [a]	3.18 ± 0.29 [a] (*n* = 12)
Prescapular LN	14/16 (87%) [ab]	2.24 ± 0.26 [a] (*n* = 6)
Retropharyngeal LN ‡	29/32 (90%) [a]	2.71 ± 0.18 [a] (*n* = 12)
Supramammary LN ‡	32/32 (100%) [a]	2.58 ± 0.20 [a] (*n* = 14)
Mammary gland ‡	28/32 (87%) [a]	2.35 ± 0.26 [a] (*n* = 12)
Placentome	15/16 (93%) [ab]	10.33 ± 0.23 [c] (*n* = 10)
Fetal		
Fetal Lung	7/16 (43%) [b]	6.25 ± 2.42 [b] (*n* = 3)
Fetal liver	10/16 (62%) [b]	7.13 ± 2.19 [b] (*n* = 3)
Fetal Spleen	7/16 (43%) [b]	3.93 ± 0.86 [a] (*n* = 2)

† Mean colony-forming units per gram in tissues PCR positive for Brucella and for which individual colonies could be numerated. ‡ Samples evaluated from both right and left sides of the tissue. Means bearing different superscripts are significantly different ($p < 0.05$).

3.4. DISCUSSION

The results of this study indicate that vaccination with LPS alone or combined with ISA 61 VG adjuvant is not efficacious in protecting goats against experimental challenge with virulent *B. melitensis* 16M strain. We attribute this lack of efficacy to a failure to induce cellular immunity, as we failed to find differences between treatments in antigen-specific responses in T cell subsets. As with many facultative intracellular pathogens, cell-mediated immunity is considered crucial in providing long-term protection against Brucella [30]. The OPS of lipopolysaccharide serves as the major immunodominant antigen of *B. melitensis*, and nearly all serological tests for brucellosis are based on measuring antibody responses to this antigen [31]. Since our study utilized non-living,

subunit vaccines, the observed lack of cell-mediated responses could be related to how these vaccines are processed within antigen-presenting cells and presented to T cell populations.

Our data demonstrate that the LPS + ISA 61 VG vaccine induced stronger humoral responses when compared to the other treatments, but cellular immune responses did not differ from other treatments. It is hypothesized that vaccine strains derived externally and entering the antigen-processing cell through phagocytosis are presented via the Class II MHC (major histocompatibility complex) or exogenous pathway. In contrast, vaccine strains synthesized within the antigen-presenting cell's cytoplasm and transported to the endoplasmic reticulum can be presented through Class I MHC and processed via the endogenous pathway. The intracellular location and/or method of entry of vaccine strains into the cell appears to be critical, as processing via the endogenous pathway tends to evoke a Th1 response associated with cell-mediated immunity. Vaccine strains (such as LPS alone or LPS plus ISA 61 VG in the current study) processed through the exogenous pathway are usually associated with humoral Th2 responses, which do not protect against intracellular pathogens. This may explain why current brucellosis vaccines are almost exclusively composed of live bacteria, whereas vaccinations with killed bacteria typically fail to provide adequate efficacy against the pathological effects of Brucella [32,33].

Cell-mediated immunity plays a vital role in providing protection against brucellosis; however, humoral responses could also contribute to vaccine efficacy. Previous studies have identified humoral mechanisms contributing to protection against *B. abortus* infection, including antibodies targeting Brucella LPS, activation of complement-mediated killing, antibody-dependent cytotoxicity, and enhanced phagocytosis by opsonization [34]. Others have hypothesized that anti-Brucella OPS antibodies, generated either by vaccination or natural

infection, may help to prevent infection by activating complement-mediated bacterial killing mechanisms [35]. Notably, strong antibody responses have been documented in elk vaccinated with the RB51 strain, which have a prolonged bacteremia and lower PBMC proliferative responses after vaccination as compared to responses observed in cattle and bison [36]. Elk also exhibit fewer pathological effects (i.e., abortions) after experimental challenge. The lack of efficacy from vaccines evaluated in the current study suggests that combining the induction of anti-*Brucella* OPS humoral responses using a subunit vaccine with a robust cell-mediated immune response from a live vaccine might offer greater efficacy against brucellosis. Further research is warranted to characterize the efficacy of this combined approach.

Abortion rates were high among all vaccinated goats, which also exhibit significant colonization (CFU/gm) in targeted tissues. Some tissues selected for evaluation were chosen because they serve specific roles: the parotid lymph node represents lymphatic tissues at the site of experimental challenge, the placentome is localized in reproductive tissues where pathologic effects occur, the supramammary lymph node correlates with infection in the mammary gland where shedding may occur in milk, and the prescapular lymph node represents a lymph node not typically associated with Brucella localization but which may be colonized when wide spread in vivo infection occurs. Abortion is the most significant route for brucellosis transmission among ruminants. Higher bacterial counts in the uterine environment are associated with an increased risk of lateral transmission of brucellosis through expelled fluids, placental tissues, or fetal tissues [37]. Therefore, the combination of increased abortions and high reproductive colonization would not reduce disease transmission in our vaccinated goats if similar infections occurred under field conditions.

A glycoconjugate (GC) vaccine, composed of LPS and outer membrane protein (OMP) derived from *B. abortus* S19, has been demonstrated to elicit robust cell-mediated immune responses in both mice and bovine calves [38,39]. Additionally, Mukherjee reported that a 100 μg dose of the S19GC vaccine administered subcutaneously to adult female cattle can provide both therapeutic and prophylactic effects [40]. Similarly, in 1991, Jacques et al. demonstrated the effectiveness of a Brucella O-polysaccharide (PS)-bovine serum albumin (BSA) conjugate vaccine in protecting mice against *B. melitensis* H38 [41]. Our data in goats differ from mice in that vaccination with LPS alone or in combination with ISA 61 VG exhibited less protection against experimental challenge with *B. melitensis* 16M strain.

The current study highlights the critical need to assess potential Brucella vaccine strains in specific targeted species. Regrettably, most candidate vaccines for *B. melitensis* have only been tested in murine models, where disease pathogenesis may significantly differ from that in ruminant hosts. Unlike outbred domestic livestock, murine models are inbred, which can influence observed immune responses. For example, ruminants typically have a higher proportion of circulating T cells expressing γδ markers. Furthermore, while infection in mice is commonly evaluated through colonization in the liver and spleen, in ruminant hosts, the infection primarily localizes within lymphatic tissues [42–44].

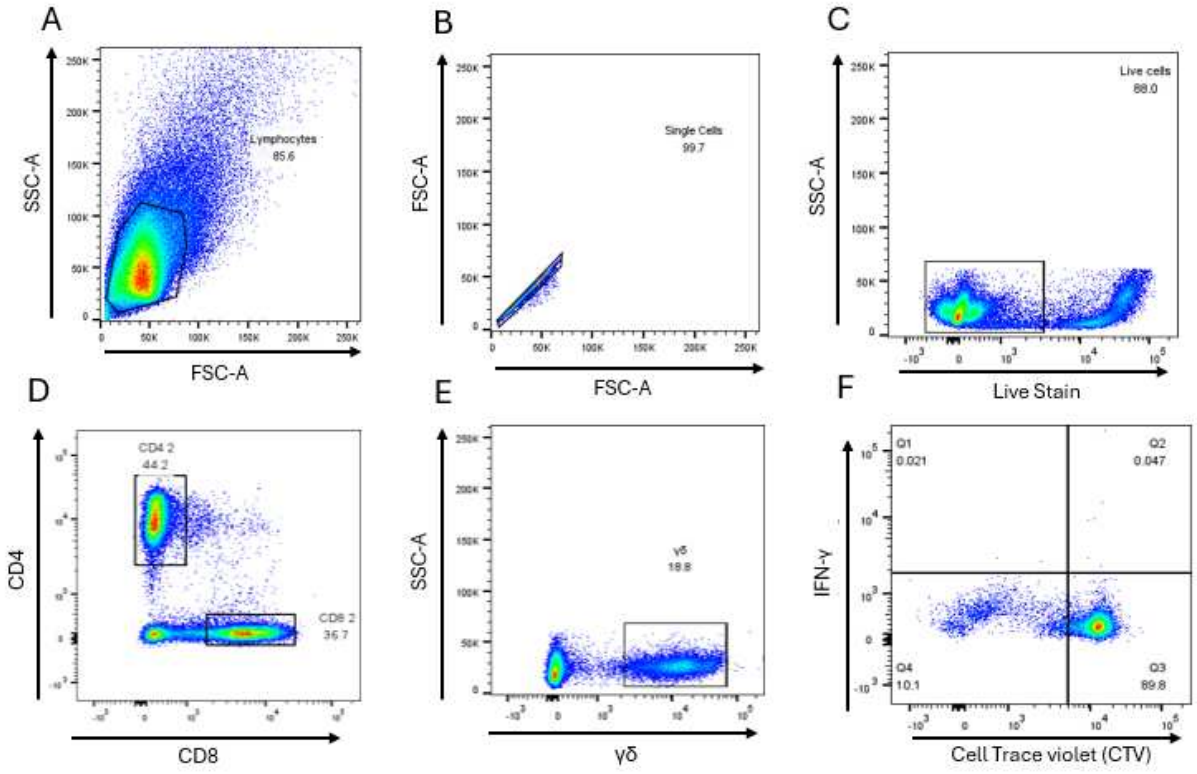
Our data suggest that a single vaccination with LPS + ISA 61 VG or LPS alone does not offer sufficient efficacy in goats. However, the strong humoral responses elicited by LPS + ISA 61 VG could be beneficial for use as a booster vaccine. Although humoral responses (IgG levels) increased, we did not assess the functional properties of these antibodies, including their affinity, subclass distribution, or mucosal IgA responses. It is important to note that our study is limited by the small number of goats involved and may not reflect anamnestic immune responses in goats

that were previously vaccinated or exposed to brucellosis. The current study is also limited by not including the Rev 1 vaccine, which is essential for a favorable control comparison. Furthermore, one limitation of the present study is that the exact antigen specificity of the humoral response remains undetermined. Typically, the effectiveness of brucellosis vaccines in field conditions exceeds that observed in experimental settings, where all animals are pregnant and exposed to a virulent Brucella strain during mid-gestation, the period of highest susceptibility to brucellosis. Until a more effective vaccine is developed, the Rev1 vaccine remains the preferred vaccine to reduce brucellosis in regions where it is endemic in goats.

3.5. CONCLUSIONS

In the present study, we evaluated immune responses and protection against experimental challenge following the vaccination of goats with LPS + ISA 61 VG, LPS alone, or saline as a control. Goats vaccinated with LPS + ISA 61 VG showed significantly higher antibody responses compared to those in the control group. Additionally, antigen-specific proliferative responses were observed in goats vaccinated with LPS + ISA 61 VG compared with those receiving LPS alone or the control. However, CD4+, CD8+, and γδ T cells from vaccinated goats showed minimal proliferation and did not produce IFN-γ. No significant differences were detected in the protection against abortion or in fetal, uterine, mammary, or maternal infections between vaccinated and control groups. Taken together, our findings indicate that a single vaccination with LPS + ISA 61 VG or LPS alone does not confer sufficient protection in goats. Nonetheless, the humoral responses induced by LPS + ISA 61 VG suggest potential value as a booster vaccine. Further studies are warranted to evaluate its efficacy as a booster vaccination in goats.

3.6. SUPPLEMENTARY MATERIALS



Supplementary Figure S1. Gating strategy for flow cytometry analysis. Shown are representative dot plots for lymphocyte gating via forward scatter (FSC-A) vs. side scatter (SSC-A) (A), singlet discrimination (B), gating on live events(C). Live cells were then further gated for CD4, CD8 and γδ expressions (D and E) and CD4, CD8 and γδ T cells were evaluated for IFN-γ vs. Cell Trace violet (CTV), indicative of proliferation.

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CHAPTER 4 – A RANDOMIZED CONTROLLED FIELD TRIAL ASSESSING *BRUCELLA MELITENSIS* VACCINES' EFFICACY IN GOATS: A PROTOCOL FOR IMPLEMENTATION IN ENDEMIC COUNTRIES[3]

ABSTRACT

Brucellosis, caused by the bacterium *Brucella melitensis*, is a highly contagious zoonotic disease that affects livestock and poses significant public health risks in regions where it is endemic. Vaccination is the most cost-effective strategy for controlling brucellosis in small ruminants. The most widely used vaccine to control *B. melitensis* in small ruminants is the live attenuated *B. melitensis* Rev1. While this vaccine is effective, it does have limitations, including residual virulence, the potential for abortion in pregnant animals, and interference with serological diagnosis. This protocol outlines a randomized, controlled field trial to evaluate the effectiveness and immunogenicity of a *B. melitensis* recombinant vaccine compared with the Rev1 strain under natural field conditions in an endemic country. Three goat farms will be randomly assigned to receive either the *B. melitensis* recombinant vaccine, the Rev1 vaccine, or a placebo. Clinical outcomes, including abortion, retained placenta, stillbirths, weak offspring, and mastitis, will be monitored monthly for one year. Humoral and cellular immune responses will be assessed using enzyme-linked immunosorbent assay (ELISA), proliferation assays, and flow cytometry. Data will

[3] Disclaimer: This chapter is formatted for potential publication in a scientific journal. However, because the evaluation of the *B. melitensis* recombinant vaccine under experimental conditions has not yet been conducted as part of this dissertation, and no data are currently available, the chapter will be revised once the study is completed.

be analyzed using chi-square tests, repeated measures ANOVA, and attributable risk calculations to quantify vaccine efficacy. This trial will provide critical insights into the effectiveness of the *B. melitensis* recombinant vaccine under field conditions, informing vaccination strategies for endemic regions.

4.1. INTRODUCTION

Brucellosis is a highly contagious zoonotic disease caused by bacteria of the genus *Brucella* [1]. The World Health Organization (WHO) has classified brucellosis as one of the neglected zoonotic diseases, which are infections that pose ongoing public health threats and contribute to poverty in endemic regions [2]. Among the various *Brucella* species, *B. melitensis* is the most virulent and is the primary causative agent of human brucellosis [3,4]. Each year, approximately 2.1 million new human cases occur worldwide [5]. Although *B. melitensis* primarily affects small ruminants, such as sheep and goats, it can also infect cattle and camels. This has been documented in several Mediterranean and Middle Eastern countries, including Saudi Arabia, Egypt, and Kuwait [6,7].

Transmission of *Brucella* to humans typically occurs through direct contact with infected animals or the consumption of contaminated dairy products. In animals, brucellosis results in severe reproductive disorders, which can include abortion, reduced milk production, stillbirths, retained placenta, weak offspring, and orchitis in males. The bacteria often localize in the mammary glands and supramammary lymph nodes of infected dairy animals, resulting in the continuous shedding of *Brucella* in their milk [8,9]. Controlling brucellosis in animals is crucial for preventing human infections and minimizing the socioeconomic impact of the disease [10].

Vaccination is the most cost-effective method for controlling and eradicating brucellosis in livestock, along with the early detection and removal of infected animals. The *B. melitensis* Rev 1 strain vaccine, developed in the early 1950s, has been widely used to control brucellosis in small ruminants [4,11,12]. Although Rev 1 is efficacious, it has several limitations, including the potential to cause abortion in pregnant animals, its persistence in the mammary glands that can lead to shedding in milk and possible zoonotic transmission, the generation of long-lasting serological responses that cannot be distinguished from those of natural infections. Moreover, Rev 1 displays significant residual virulence in humans, as indicated by cases of brucellosis-like illness following accidental exposure during vaccine handling, including needle-stick injuries, or aerosol generation.

These limitations underscore the urgent need for improved *B. melitensis* vaccines that are both safe and effective. To address these challenges, we pursued two vaccine development approaches. The first approach involved evaluating lipopolysaccharide (LPS) derived from *B. melitensis* strain 16M. We characterized the immunologic responses and protection against experimental challenge in goats vaccinated with either LPS from *B. melitensis* strain 16M (LPS alone), LPS from *B. melitensis* strain 16M formulated with MONTANIDE™ ISA 61 VG adjuvant (LPS + ISA 61 VG), or saline as a control. Goats vaccinated with LPS + ISA 61 VG exhibited significantly higher antibody responses compared to nonvaccinated controls. Our data demonstrate that goats vaccinated with LPS + ISA 61 VG exhibited greater lymphocyte proliferative responses to the LPS antigen than those vaccinated with LPS alone at week 12 after vaccination. However, the proliferative responses of peripheral blood mononuclear cells (PBMCs) from goats vaccinated with LPS + ISA 61 VG did not differ significantly from those of control goats. $CD4^+$, $CD8^+$, and γδ T cells from all vaccinated animals exhibited negligible proliferation and failed to induce

antigen-specific IFN-γ production. Control and vaccinated goats had a high abortion, uterine, fetal, mammary, or maternal infection. Our data suggests that LPS + ISA 61 VG induces a robust humoral response but negligible cellular responses. Our data also suggest that LPS + ISA 61 VG or LPS alone would not be efficacious for use as a vaccine in goats, but the LPS + ISA 61 VG inoculum may be beneficial as a booster. Based on these findings, the combination of increased abortions and high reproductive colonization would likely not reduce disease transmission under field conditions in vaccinated goats [13]. Consequently, we pursued an alternative approach to developing a *B. melitensis* vaccine.

The second approach focused on developing recombinant *B. melitensis* vaccine strains. Using Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology, we developed *B.* melitensis recombinant strains with genomic modifications replicating those in the *B. abortus* RB51 vaccine strain. The RB51 strain is classified as a rough vaccine as it doesn't express the immunodominant O side-chain on the LPS surface. Data indicate that the immunogenicity of RB51 in cattle is not solely based on the mutation in the wboA gene, which confers its rough phenotype. Instead, available data suggest RB51's immunogenic and attenuation properties are a result of combining the rough phenotype with other genetic modifications that we have identified [14]. The RB51 strain exhibits a variety of mutations, many of which are intergenic or synonymous, suggesting that they are unlikely to impact protein structure. However, specific mutations in genes such as wboA, rpoB, CapA, NarJ, HlyD, glutathione S-transferase, and endoribonuclease L-PSP have been identified as promising targets for the development of recombinant strains [14]. Replicating these targeted genomic mutations in *B. melitensis* may provide insight into new and promising vaccine candidates. Therefore, the *B. melitensis* recombinant strains were engineered by deleting the relevant genes, producing strains deficient in

the LPS O-side chain of the *Brucella* cell wall and thereby conferring potential DIVA properties. These candidate vaccines are evaluated for safety, stability, immunogenicity, and efficacy in goats under controlled experimental conditions. The outcomes of these studies inform the selection of the most promising candidates for subsequent evaluation under natural field conditions.

This protocol is part of the doctoral research of Naseer Alnakhli, a Ph.D. candidate at Colorado State University. It aims to assess the effectiveness of the *B. melitensis* recombinant vaccine strain, which possesses DIVA properties, against *B. melitensis* infection in goats in field conditions.

4.2. STATEMENT OF OBJECTIVE AND HYPOTHESIS

The objective of this study is to evaluate the effectiveness of vaccination with *B. melitensis* recombinant strain against *B. melitensis* in goats in a field setting as compared to the conventional Rev 1 strain.

Null hypothesis: Rev1 and the *B. melitensis* recombinant strain have an equal protective immunity *against B. melitensis* field infection in goats, in terms of reducing signs of clinical infection (abortion, retained placenta, stillbirths, birth of weak offspring, and mastitis) and inducing humoral and cellular responses.

Alternative hypothesis: The *B. melitensis* recombinant strain has a better protective immunity than Rev1 in terms of reducing signs of clinical infection (abortion, retained placenta, stillbirths, birth of weak offspring, and mastitis) and inducing humoral and cellular responses.

Primary Outcome: The *B. melitensis* recombinant strain generates effective humoral and cellular responses against *B. melitensis* in goats.

Secondary Outcome: The *B. melitensis* recombinant strain decreases the incidence of clinical signs associated with *B. melitensis,* such as abortion, retained placenta, stillbirths, weak offspring, and mastitis in goats.

4.3. STUDY POPULATION AND DESIGN

The study is conducted as a blinded, randomized field trial in accordance with the CONSORT guidelines [15]. It is undertaken in endemic regions where the prevalence of *B. melitensis* infection is expected to exceed 10%. A scientifically based seroprevalence survey should be conducted before commencing the field trial to ensure a high prevalence of *B. melitensis* in the livestock population (see Chapter 5 for further details). Prior to study initiation, all participating investigators should complete training in Good Clinical Practice (GCP) for Veterinary Clinical Trials. Details regarding the training framework and resources are available through the Clinical and Translational Science Award One Health Alliance (CTS-A One Health Alliance).

Animals are obtained from endemic regions and screened using serological assays (Rose Bengal Plate and Enzyme-Linked Immunosorbent Assay), Polymerase Chain Reaction (PCR), and culture to confirm seropositivity and history of *Brucella* infection. Three livestock farms are selected. Each selected farm should house goats, including both sexually mature and non-mature females that are not pregnant at the time of enrollment. The animals on each farm should be divided into three treatment/vaccine groups, as outlined in Table 1. These groups are randomly assigned to receive either the *B. melitensis* recombinant strain, the Rev1 strain, or a placebo (control), following a randomized complete block design. Animals are randomly assigned to treatment groups by numbering each goat sequentially and then allocating them to groups according to a computer-generated random sequence at enrollment. In this design, animals within each block

(e.g., a farm) are relatively uniform, and all treatments are randomly assigned within each block to control for variation caused by environmental or management factors, such as feeding practices.

Following treatment administration, all vaccinated and placebo animals in each group should be naturally bred under field conditions. Pregnancy is confirmed using a serologic test that detects pregnancy-specific protein B (Bovine Pregnancy test (Rapid Visual Pregnancy Test Kit, IDEXX, Westbrook, ME).

The criteria for including animals in the study consist of several vital factors. First, it is imperative to have adequate animal-handling facilities and trained personnel to facilitate all necessary sampling during the trial. This study should enroll unvaccinated animals. To ensure the specificity of the outcomes, goats should be tested for other bacterial and protozoan agents known to cause abortion, including *Campylobacter fetus subsp. fetus*, *Chlamydophila abortus*, *Toxoplasma gondii*, and *Coxiella burnetii*. Only those farms that allow for monthly visits and intensive monitoring by the study investigators and veterinary staff should be considered. Within these farms, female animals that are mature, but not pregnant at the time of enrollment, should be included in the study. The exclusion criteria include pregnant animals and those previously vaccinated against brucellosis.

Online sample size calculators, such as those available in the OpenEpi menu, can help estimate the appropriate sample size. For this trial, we assume a statistical power of 80% (0.8) and a significance level of 0.05. Additionally, the ratio of vaccinated two groups to non-vaccinated farms in the groups be set at 1:1. We estimate that the prevalence of brucellosis in unvaccinated animals on farms is 40%, and we anticipate a 50% reduction in disease prevalence as a result of vaccination.

Table 1 Allocation of Goats by Age and Vaccine Group Across Farms in Randomized Controlled Field Trial Assessing Vaccines' Effectiveness

Animal type	vaccine	Farm 1		Farm 2		Farm 3	
Goats	Recombinant strain	non-sexually females (under 6 months old)	10	non-sexually females (under 6 months old)	10	non-sexually females (under 6 months old)	10
		sexually mature females (over 6 months old)	10	sexually mature females (over 6 months old)	10	sexually mature females (over 6 months old)	10
	Rev1 strain	non-sexually females (under 6 months old)	10	non-sexually females (under 6 months old)	10	non-sexually females (under 6 months old)	10
		sexually mature females (over 6 months old)	10	sexually mature females (over 6 months old)	10	sexually mature females (over 6 months old)	10
	placebo	non-sexually females (under 6 months old)	10	non-sexually females (under 6 months old)	10	non-sexually females (under 6 months old)	10
		sexually mature females (over 6 months old)	10	sexually mature females (over 6 months old)	10	sexually mature females (over 6 months old)	10

4.4. INOCULATION

Vaccines should be administered subcutaneously to goats according to their designated groups. To ensure accurate administration, each animal is individually identified with durable ear tags bearing unique identification numbers. Redcap, a secure web application for building and managing online databases, should be used (Table 2).

Vaccine containers should be labeled with the corresponding animal identification numbers. Vaccinated animals should be temporarily placed in designated pens or stalls that are easily identifiable. Vaccines are administered individually to animals within each allocated group,

with all inoculations given subcutaneously in the cervical region. The dosage for the Rev1 strain is 1–2 × 10^9 CFU, while the *B. melitensis* recombinant strain is administered at a dosage of 1 × 10^{10} CFU. Continuous monitoring should be conducted to record any potential adverse clinical signs after vaccination. It should ensure that each animal's identification matches the vaccine label before administration. All vaccination events, including date, time, vaccine type, dosage, and any special instructions, should be meticulously recorded (Table 2).

Table 2 Animal Enrollment, Vaccination, and Monthly Clinical Monitoring Form for Brucella Vaccine Field Trial in Goats.

Animal Enrollment
Owner's Name
Farm Address
Contact Information
Animal ID / Ear Tag
Farm ID
Species: Goat
Breed
Age
Sex
Brucellosis free (yes/No)
Sexual maturity (Yes/No)
Pregnancy status (Must be negative)
Screened for other abortive pathogens (Campylobacter, Chlamydia, Toxo, Coxiella): Results
Inclusion eligibility: ✓/X
Date of enrollment
Vaccination Administration and Randomization, and Group Allocation
Farm ID
Animal ID
Vaccine type (New / Rev1 / Placebo)
Date/time of administration
Date of allocation
Site of injection
Dosage administered

Adverse events post-vaccination
Initials of the vaccinator
Physical Exam Form for *Brucella Melitensis* in Goats
General Health and Behavior
Behavior: [Normal/Lethargic/Aggressive/Other]
Appetite: [Normal/Decreased/Increased]
Body Condition: [Score 1-5]
Vital Signs
Temperature (°F/°C)
Occurrence and timing of reproductive system signs
Udder Swelling (mastitis)/Discharge/Milk Appearance
Abortion: [Yes/No]
Retained placenta: [Yes/No]
Stillbirths: [Yes/No]
Birth of weak offspring: [Yes/No]
Musculoskeletal System signs
Gait: [Normal/Lameness/Other]
Joint Swelling/Pain: [Location/Severity]
Ocular Examination signs
Discharge: [Yes/No]
Conjunctiva: [Normal/Reddened/Other]
Other Observations/Comments

4.5. CHARACTERIZE THE IMMUNOGENICITY OF *BRUCELLA MELITENSIS* RECOMBINANT STRAIN AND COMPARE IT TO THE IMMUNE RESPONSES OF REV1 VACCINATED ANIMALS

Blood is obtained by jugular venipuncture at 0, 8, 16, 23, 23, 31, 39, and 47 weeks post inoculation and placed into serum separator tubes. After centrifugation, the serum is collected, aliquoted, and stored at -80°C for future analysis. To evaluate humoral responses to Brucella antigens, an enzyme-linked immunosorbent assay (ELISA) should be conducted to quantify total immunoglobulin G (IgG) specific to the Brucella antigen, as previously described [16]. All wells of a 96-well flat-bottom plate should be coated with *Brucella* antigen in a carbonate-bicarbonate

buffer (0.18 M carbonate, 0.028 M sodium bicarbonate, pH 9.6) and incubated overnight at 4°C. Plates are blocked twice (SuperBlock,™ Thermo Fisher Scientific, Waltham, MA) for 15 minutes. After dilution to 1:800, 1:1600, and 1:3200, serum samples are added to plates in triplicate. After incubation for 2 hours at RT, and 4 washes (phosphate buffered saline with 0.05% Tween 20) 100 ul of the secondary antibody at a 1:5000 dilution is added to all wells (Peroxidase-conjugated AffiniPure Rabbit Anti-Goat IgG (H+L), Jackson ImmunoResearch Laboratories, Inc). After 1 hr incubation at RT, plates are washed 4 times and then developed using a commercial substrate kit (TMB, Thermo Scientific) in accordance with the manufacturer's recommendations. The reaction is stopped by the addition of 100 μL of 0.18 M sulfuric acid, and the optical density of each well is measured at 450 nm using a microtiter plate reader (Synergy Neo2, Biotek).

In accordance with previous studies, whole blood is collected via venipuncture of the jugular vein and placed into tubes containing 2x ACD to prevent clotting. Peripheral Blood Mononuclear Cells (PBMCs) are isolated via density centrifugation using Ficoll as described previously. Viable PMBCs are determined by trypan blue exclusion and resuspended to a concentration of 10^7 per ml. In early sampling times, PBMC should be plated at 10^6 per well onto 96 flat-bottom plates, and left unstimulated, stimulated with killed *B. melitensis* antigen, or stimulated with Concanavalin A (ConA; 0.5 μg/well; Sigma). After incubation at 37° C and 5% CO_2 for 7 days, cells are pulsed for 18 hr with 3H-thymidine, harvested using a TomTec plate harvester, and counted on a Perkin-Elmer scintillation counter. After stimulation indices (cpm in antigen stimulated wells/cpm in unstimulated wells) in vaccinates exceed an average of 10, cells should be analyzed using flow cytometric techniques. To determine proliferation and intracellular cytokine production using flow cytometry, PMBCs are stained with 1:10 CellTrace Violet (eBioscience, Thermo Fisher Scientific) solution, according to manufacturer's recommendations,

and resuspended in complete RPMI media. PMBCs are plated at 10^6 per well onto 96-flat bottom plates, and left unstimulated, stimulated with killed *B. melitensis* antigen, or stimulated with Concanavalin A (ConA; 0.5 ug/well; Sigma). Plates should be incubated for 7 days at 37° C with 5% CO_2. Approximately 16 hours prior to harvest, all wells are treated with a 1x Golgistop solution (eBioscience, Thermo Fisher Scientific) or a 1x Cell Stimulation Cocktail plus Golgistop solution (eBioscience, Thermo Fisher Scientific), according to manufacturer's recommendation. After incubation, cells are centrifuged at 300x g at RT, washed in 1x Dulbecco's phosphate buffered saline (DPBS) (Thermo Fisher Scientific) twice and once in FACS buffer (0.5% fetal bovine serum (FBS) in PBS). After resuspension by gentle vortexing, cells should be incubated with anti-goat γδ and αβ (CD4 and CD8) primary antibodies for 15 minutes at RT and then with fluorescently labeled secondary antibodies for an additional 15 minutest at RT. After two washes in FACS buffer, cells are fixed and permeabilized for intracellular staining using a permeabilization kit according to the manufacturer's recommendations (BD Biosciences, San Jose, CA). Intracellular cytokine staining should be performed using anti-goat IFN-γ antibody, washed twice in wash buffer, once in FACS, and then resuspended in FACS buffer until analysis. Data should be collected using the BD Symphony flow cytometer (BD Biosciences) using the DIVA software, and analyzed using FlowJo® (FlowJo, Ashland, OR) [17,18].

4.6. OBSERVATION AND RECORDING OF CLINICAL SIGNS

All groups should be followed rigorously and equally throughout the study. Attrition due to drop-out or non-compliance is anticipated; the sample size has been designed to accommodate such losses. Incentives may be provided to encourage continued participation.

The study is extended over 12 months. Animals should be monitored for clinical signs of brucellosis every four weeks post-vaccination. Owners' observations of clinical signs should also be recorded to establish the date of infection. Data are collected as shown in Table 2.

4.7. OUTCOME MEASUREMENT AND ANALYSIS

The primary outcome is that the *B. melitensis* recombinant strain generates effective humoral and cellular responses against *B. melitensis* in goats following vaccination. The secondary outcome is that *B. melitensis* recombinant decreases the incidence of clinical signs associated with *B. melitensis,* including abortion, retained placenta, stillbirths, weak offspring, and mastitis, in goats

For the primary outcomes, serological and cellular immune responses will first be assessed for normality. Continuous outcomes that meet the assumptions of normality will be analyzed using repeated measures ANOVA. When significant overall differences are detected, the Tukey post-hoc test will be applied to identify specific group differences. For non-parametric continuous outcomes, such as certain cellular response measures, we will use Friedman's test, followed by Dunn's post-hoc test to determine significant group differences.

For the secondary outcomes, the attributable risk should be calculated to quantify the contribution of vaccination to preventing clinical signs associated with *B. melitensis*. For example, the attributable risk (AR) is calculated to compare the incidence of retained placenta in goats vaccinated with the *B. melitensis* recombinant strain against those vaccinated with the Rev 1 vaccine under field conditions. The AR can be determined using 2×2 contingency table: AR = (a)/(a + b) - (c)/(c + d). In this formula, (a) and (b) represent the number of goats with and without retained placenta in the recombinant vaccine group, while (c) and (d) represent those in the Rev

1 group. This measure estimates the difference in the risk of developing retained placenta between the two vaccine groups. For instance, if 100 goats were vaccinated with the recombinant vaccine and the Rev 1 vaccine, with 4 and 12 of them exhibiting retained placenta, respectively, the AR would be calculated as follows: AR = (4)/ (100) - (12)/(100) = -0.08. This indicates that the recombinant vaccine reduces the risk of retained placenta by 8% compared with the Rev 1 vaccine under field conditions.

Statistical comparisons of proportions are performed using the chi-square test, and 95% confidence intervals are calculated for estimates of vaccine effectiveness. To account for potential confounding factors, such as age and sexually mature and non-mature females, multivariable regression analyses should be conducted, specifically using modified logistic regression to estimate relative risk.

4.8. ETHICAL CONSIDERATIONS

- Ethical approval is obtained from the relevant ethics committee or institutional review board, as well as compliance with animal welfare standards.
- Biosafety regulations for handling *B. melitensis* will strictly be followed.
- The quality of the randomized controlled field trial will be assessed using a checklist (Table 3) specifically designed to evaluate the risk of bias in randomized controlled trials (19).
- Consent will be obtained from the farm owner before the trial begins.

Table 3 Quality Assessment of a Randomized Controlled Field Trial

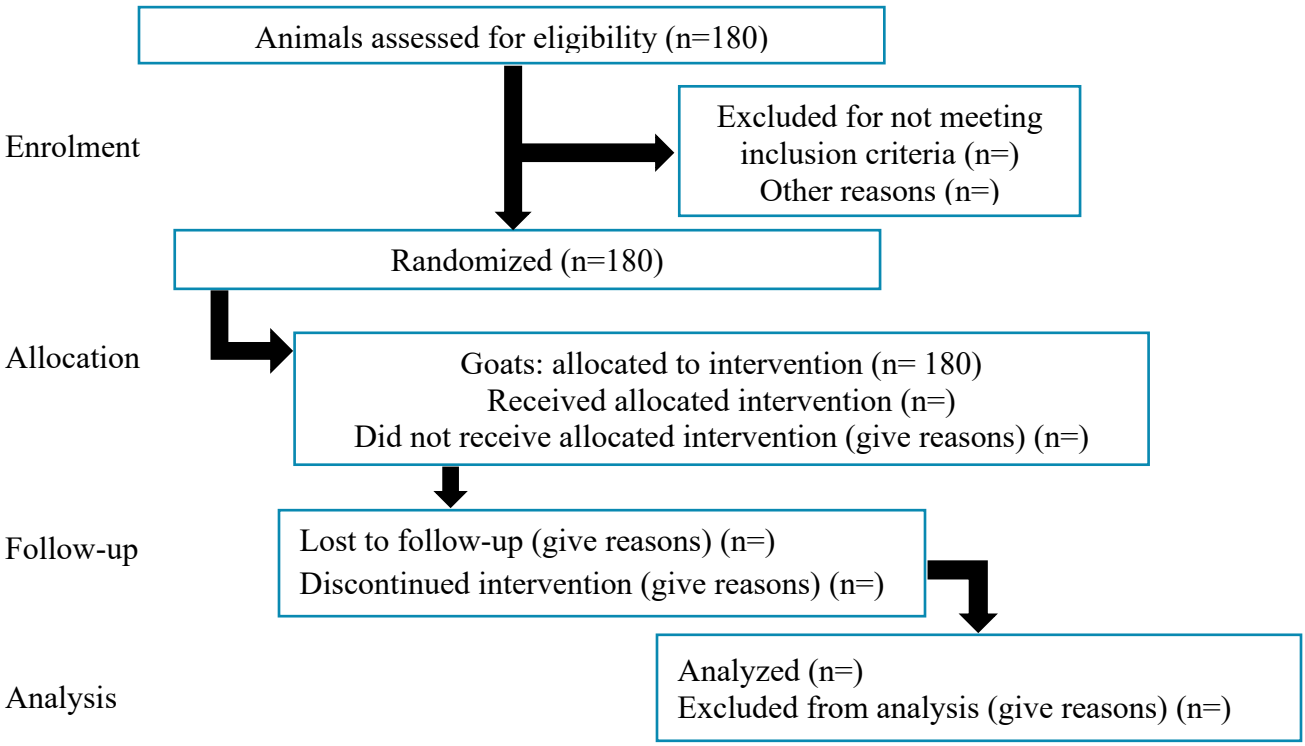
Joanna Briggs Institute (JBI) Critical Appraisal Checklist for Randomized Controlled Trials	YES	NO	Unclear	NA
Was true randomization used for the assignment of participants to treatment groups?				
Was allocation to treatment groups concealed?				

Were treatment groups similar at the baseline?				
Were participants blind to treatment assignment?				
Were those delivering treatment blind to treatment assignment?				
Were outcomes assessors blind to treatment assignment?				
Were treatment groups treated identically other than the intervention of interest?				
Was follow up complete and if not, were differences between groups in terms of their follow up adequately described and analyzed?				
Were participants analyzed in the groups to which they were randomized?				
Were outcomes measured in the same way for treatment groups?				
Were outcomes measured in a reliable way?				
Was appropriate statistical analysis used?				
Was the trial design appropriate, and any deviations from the standard RCT design (individual randomization, parallel groups) accounted for in the conduct and analysis of the trial?				
Overall appraisal (Include -Exclude - Seek further information)				
Comments (Including reason for exclusion)				

4.9. RESULTS

The data will be presented as follows.

4.9.1 Participant Flow Diagram



4.9.2. Baseline demographics for each group

Participant characteristics	Farm 1			Farm 2			Farm3		
Group	1	2	3	1	2	3	1	2	3
Age (years)									
Goat breed									

4.9.3. Occurrence of Clinical Signs of Brucella Infection Under Natural Exposure

Vaccine Type	Farm	No. of Animals	Abortion	Retained placenta	stillbirths	weak offspring	mastitis
Recombinant strain	1						
	2						
	3						
Rev1 strain	1						
	2						
	3						
placebo	1						
	2						
	3						
Attributable Risk (AR)	-						
P-value	-						

4.10. LIMITATION

This field study is subject to several environmental variables, including farm management practices, which may influence the outcomes and cannot be entirely controlled. The findings may have limited generalizability regarding the use of the *B. melitensis* recombinant strain in other geographical regions or under different environmental conditions, as the study is conducted in a single endemic country. The vaccine's efficacy against other *Brucella* species and livestock remains to be determined. Unknown parameters or variables may be present that will influence vaccine responses/efficacy. However, by randomizing animals at each farm for assignment to all 3 treatments, the influence of these variables will be minimized. Although measures are

implemented to minimize confounding factors, unforeseen influences, such as concurrent infections or unreported health conditions in the animals, may affect the results. Additionally, reliance on farm owners for certain data collection introduces the potential for reporting errors or omissions due to misinterpretation or observation inaccuracies.

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CHAPTER 5 – DISEASE MANAGEMENT APPROACHES FOR *BRUCELLA MELITENSIS* IN ENDEMIC REGIONS: AN EVALUATION OF SCIENTIFIC AND PRACTICAL OPTIONS[4]

ABSTRACT

Brucellosis, particularly infections caused by *Brucella abortus* and *Brucella melitensis*, remains a significant threat to human health worldwide. While most developed countries have eradicated brucellosis, it remains widespread in the Mediterranean, Middle East, Central Asia, and Latin America, making it a persistent global concern. Approximately 2.1 million new human brucellosis cases are reported annually, with animals and animal products identified as the primary sources of infection. *B. melitensis* is the main agent of small ruminant brucellosis and is the most common species linked to human cases. *B. melitensis* has also been found in cattle and camels in Gulf countries, including Saudi Arabia and Kuwait. A comprehensive surveillance and control system has not been established to address human and animal brucellosis in these countries. Controlling *B. melitensis* has proven more challenging than managing bovine brucellosis caused by *B. abortus*. Vaccination in conjunction with testing and removal of presumed infected animals is widely regarded as one of the most cost-effective control and eradication measures. However, the currently available vaccines alone are insufficient for eliminating *B. melitensis* in small ruminants, cattle, and camels. Therefore, three key strategies for effectively controlling and eradicating *B. melitensis* in sheep, goats, and cattle include: enforcing strict biosecurity and management practices at the farm level, as well as at border sites for imported animals, developing a comprehensive system to manage animal movement that incorporates efficient disease

[4] Disclaimer: This chapter is formatted for potential publication in a scientific journal.

traceability mechanisms, establishing a coordinated surveillance program to detect, monitor, and assess the presence of brucellosis in livestock populations, along with implementing control and eradication measures such as test-and-slaughter programs and vaccinations animal populations. In managing brucellosis in camels, vaccinating young camels and promoting the pasteurization of camel milk to minimize the risk of human infection should be used. By adopting these strategies, we could significantly reduce the global prevalence of brucellosis and its associated zoonotic infections.

Keywords: Brucellosis; DIVA vaccine; Small ruminant, cattle, and camels; Control and eradicate

5.1. INTRODUCTION

Animal brucellosis, particularly infections caused by *B. abortus* and *B. melitensis*, remains a significant challenge for animal health officials and producers worldwide. This is due to the wide variety of host species that can become infected, the limitations of current diagnostic and preventive tools, and the complex nature of the disease's epidemiology. Effectively controlling and ultimately eradicating animal brucellosis is critical for addressing the issue of human brucellosis. This review focuses on using scientifically based approaches with practical consideration of options for control strategies, including the possibility of application of DIVA vaccines. It is essential to understand the complex and evolving epidemiological landscape of animal brucellosis when assessing specific situations, as some Brucella species can occasionally be found in host species outside their typical associations [1,2]. A noteworthy example of this complexity is the increasing presence of *B. melitensis* in cattle and camels in several Mediterranean countries, including Saudi Arabia, Egypt, and Kuwait [3,4].

The control of animal brucellosis has been a significant concern since the disease was first linked to animals in 1905 [5]. The methods used for controlling and eradicating brucellosis vary

widely between countries due to different national circumstances. While some countries have made considerable progress in controlling animal brucellosis, achieving complete eradication is challenging in many developing countries. To our knowledge, no comprehensive surveillance and control system addresses human and animal brucellosis in regions endemic with *B. melitensis* in animal populations. Therefore, it is essential to implement additional brucellosis control initiatives tailored explicitly to low-resource countries [6-7].

B. melitensis is the leading bacterial zoonotic disease globally, accounting for approximately 2.1 million new cases annually [8,9]. The pathogen causes human brucellosis, which is derived from small ruminants. If not treated, the disease can lead to severe complications, including arthritis, miscarriages, and death (10,11). Brucellosis also results in considerable economic losses in livestock due to abortions and infertility. Transmission to humans occurs through contact with contaminated dairy products or infected animals. Controlling the disease in its animal hosts is essential to prevent human brucellosis, as humans are dead-end hosts (12). Vaccination of animals is the most cost-effective method of control, complemented by the early detection and removal of infected individuals [13]. The *B. melitensis* strain Rev1 has been used to prevent brucellosis in small ruminants since the early 1950s [11,14, and 15]. While this vaccine is efficacious, it has limitations, including the potential to cause abortions in pregnant animals, the long-term serological responses in vaccinated animals that cannot be differentiated from infections with field strains, the shedding in the milk of lactating animals (which can lead to human infections), and its high virulence in humans.

Despite significant efforts to control or eliminate animal brucellosis, outcomes have not consistently met expectations, particularly in the case of *B. melitensis* in small ruminants, cattle, and camels. Managing this form of brucellosis has proven to be more challenging than controlling

bovine brucellosis caused by *B. abortus*. Not using a DIVA vaccine and other factors, such as the environmental resilience of *B. melitensis*, complicates the situation (16). To our knowledge, in Gulf countries, significant obstacles to brucellosis control and eradication include the inability to identify circulating *Brucella* strains, co-infection, and improper veterinary quarantine measures applied to imported sheep, goats, and camels at border crossing sites. Host-related factors, including traditional farming practices such as communal pastures and transhumance, which are typical for small ruminants, and further complicate disease control (17).

This chapter presents the conclusion of a doctoral dissertation focused on exploring management strategies for controlling and eradicating brucellosis caused by *B. melitensis,* including the use of alternatives to Rev1 vaccines for application in small ruminants, cattle, and camels. It proposes guidelines for a brucellosis management strategy tailored to Gulf countries.

5.2. Disease Management Components for Controlling or Eradicating Brucellosis in Small Ruminants, Cattle, and Camels

An animal disease strategy consists of three key components, which are most effective when implemented together: enforcing strict management and biosecurity measures at the farm level and on imported animals, developing an animal movement control, disease traceability system and quarantines, establishing a coordinated surveillance program to detect and monitor the presence of the disease (e.g. brucellosis) in animal populations, and control and eradication strategies that include implementing test and slaughter programs and vaccinating susceptible populations.

Details of each component, specifically for *B. melitensis* in livestock in the Gulf region, as a model for an endemic area, are elaborated below. The findings and conclusions from the other chapters of the dissertation have been incorporated into these details.

5.2.1. Enforcing strict management and biosecurity measures at the farm level and country border sites of imported animals

Robust biosecurity measures significantly reduce disease incidence, improving animal health, welfare, and productivity. The most common pathways for *B. melitensis* to enter a disease-free farm include: (I) the purchase of infected animals that can shed the bacteria into the environment, thereby putting susceptible individuals at risk; and (II) contact with contaminated materials or pastures [18]. Consequently, implementing effective biosecurity measures is essential to prevent the introduction of the disease into a susceptible epidemiological unit. Recommended measures include purchasing from reliable sources with known and trusted herd health programs, quarantining new animals before their introduction and testing them within 10 days of arrival, isolating animals of unknown or uncertain health status, and isolating pregnant females—particularly first-time dams—before they give birth [19, 20]. The online 'Farm Biosecurity Action Planner' developed by Animal Health Australia [21] can be used, which enables farmers to perform structured self-audits and identify areas for improvement. Modifications, however, should be made to the biosecurity form to address biosecurity factors related to Gulf countries and *B. melitensis*.

In contaminated environments, in addition to the above biosecurity practices, stringent hygienic measures are vital for limiting and controlling the bacterial load in the environment to diminish the possibility of contact with viable *Brucella* spp. These measures should be consistently applied and may include the removal and safe handling of reproductive tissues following abortions,

thorough cleaning and disinfection of facilities, elimination of infected manure, incineration of contaminated materials, and use of personal protective equipment (22, 23).

Small ruminants, particularly sheep and goats, are essential to Gulf countries' food supply and cultural traditions. In Saudi Arabia, around five million ruminants are imported each year, with at least two million arriving before the pilgrimage season, which elevates the risk of introducing Brucellosis [24]. Similarly, the United Arab Emirates (UAE) relies on live animal imports for approximately 90% of its food requirements as of 2021, a situation further complicated by climate-related challenges that limit domestic production [25]. This heavy reliance on imported animals significantly increases the risk of introducing Brucellosis unless stringent biosecurity measures are implemented. To mitigate these risks, it is crucial to establish robust importation protocols, including sourcing from reputable suppliers and enforcing a clearly defined quarantine period before animals are imported. Upon their arrival, imported animals should be housed in secure, separate pens to prevent contact with native livestock. Adhering to strict cleaning and disinfection protocols is essential. During the Hajj season, additional precautions are necessary to maintain a clear separation between imported and native animals. Designated slaughter facilities must follow rigorous disinfection procedures and handle waste as a potential health hazard [26].

5.2.2. Animal Movement Control, Disease Traceability System, and Quarantines

Animal traceability systems assist in preventing, controlling, and eradicating infectious livestock diseases like Brucellosis. While disease prevention remains the primary objective, it is equally essential to establish an effective and rapid response mechanism in the event of an outbreak. The movement of livestock significantly contributes to the transmission of brucellosis, making the ability to swiftly trace infected and exposed animals essential [27]. Rapid traceability enables animal health officials to identify, isolate, and contain sources of infection, thus

interrupting the spread of the disease and restoring normal animal movement patterns [28]. A typical outbreak investigation involves backward tracing to locate the source of the infection and forward tracing to identify animals or premises at risk. Several countries have implemented national animal identification systems that serve as foundational tools in veterinary public health [29]. For these systems to succeed, they must find an optimal balance among cost-effectiveness, technological reliability, physical durability, field feasibility, data transfer speed, and user confidentiality. A comprehensive livestock traceability system comprises three integrated components: premises identification, individual animal identification, and tracking of animal movements.

Premises identification associates each herd or flock with a specific registered location, enabling veterinary authorities to efficiently trace and manage the source of infection. Individual animal identification ensures that the health status and vaccination history of each animal are accurately recorded, enabling targeted interventions such as testing, culling, or imposing movement restrictions. Additionally, tracking animal movements strengthens control by documenting transfers between farms, markets, and regions, which is vital for tracing infection pathways and preventing the wider spread of disease.

These measures contribute to a comprehensive strategy for managing animal movements related to brucellosis in small ruminants, cattle, and camels. They provide the foundation for imposing movement restrictions on specific units when warranted, even if the formal case definition is not fully satisfied. Furthermore, they guarantee that any allowed movements from restricted herds occur only to approved destinations and reinforce limits on sales for slaughter to designated facilities, such as abattoirs and markets. Furthermore, identification and movement records support interregional brucellosis testing and the establishment of movement control zones

by enabling authorities to verify and enforce which animals and herds fall within these zones. Collectively, these tools form a robust operational backbone for effective surveillance, control, and eradication strategies against brucellosis.

All infected animals must be quarantined until the herd is confirmed free of brucellosis or sold for slaughter. Three consecutive negative herd blood tests are required to be released from quarantine. The first test must be conducted 30 to 45 days after all infected animals, referred to as reactors, have been removed and slaughtered. The second test should occur 90 to 120 days following the removal and slaughter of all reactors. The third test, known as the releasing test, must be conducted 180 to 210 days or more after all reactors have been removed and slaughtered. Additionally, all young sheep and goats within an affected herd are subject to these quarantine restrictions. These kids and lambs may only be moved if they comply with the specified movement requirements [30].

5.2.3. SURVEILLANCE SYSTEM

5.2.3.1. Components of Brucellosis Surveillance System in the Gulf Countries

A Brucellosis Surveillance System (BSS) includes systematic collection, collation, and timely dissemination of information [31]. Effective surveillance is crucial to any program aimed at controlling and eradicating brucellosis. Components of a brucellosis surveillance system in the Gulf Countries include data collection, analysis, action, and evaluation (Figure 1). The four main surveillance objectives determine the type of data required to be collected. If a pathogen or agent is known to be present in the country, the first objective of surveillance can be to identify the circulating *Brucella* species, particularly *B. abortus* and *B. melitensis*. The second objective can be related to estimating the national prevalence of these organisms. The two initial objectives of

this surveillance in Gulf countries can be achieved through conducting a scientifically based seroprevalence survey. The results of these investigations are vital for improving the planning and implementing brucellosis surveillance systems concerning both pathogens across various livestock species. More information on designing and implementing the seroprevalence survey can be found in Annex 1. The third objective of surveillance can be to evaluate the geographical and demographic distribution of brucellosis among cattle, sheep, goats, and camels. The final objective is to provide decision-makers with the necessary data to develop and implement an effective disease control action plan. For more detailed discussions on designing animal health surveillance systems, readers are encouraged to refer to the existing literature [32,33].

5.2.3.1.1. Data Streams

Disease reporting systems are based on the reporting of animal health-related events to the veterinary authority. In the context of the BSS, data streams can be divided into on-farm and off-farm sources. Both categories of data provide critical epidemiological insights that contribute to the effectiveness of disease reporting systems. On-farm data sources might include serological surveys, whereas off-farm data can encompass abortion rates and the submission of tissue samples to veterinary diagnostic laboratories for culture analysis, diagnostic laboratory databases regarding milk and blood testing, as well as bacteriological and serological examinations of tissues and blood from breeding-age cattle or small ruminants at markets or slaughterhouses.

5.2.3.1.2. Analysis

The analysis component of the BSS involves the systematic assessment of disease occurrence by estimating incidence and prevalence within livestock populations. This process integrates serological data, bacteriological confirmations, and epidemiological information to define the numerator, identifying clinical cases of infection accurately. The denominator, which reflects the

population at risk, is derived from livestock census data, herd registration records, or field sampling frameworks that provide estimates of the total number of animals by species and geographic location. More details on the case definition for surveillance can be found in Annex 1. By stratifying results according to host species and geographic distribution, the analysis provides valuable insights into transmission dynamics. This stratification illustrates how *Brucella* species circulate among and within animal populations, emphasizing species-specific reservoirs, cross-species transmission pathways, and regional clustering of infections. These findings are essential for informed decision-making, facilitating the enhancement of control programs and the strategic allocation of resources for brucellosis eradication in Gulf regions.

5.2.3.1.2.1. Expected Outcomes

Expected outcomes include producing annual comprehensive reports that detail the brucellosis status in the livestock sector of endemic countries. These reports will focus on identifying trends and areas of concern, reducing disease prevalence across the host species, evaluating the effectiveness of intervention measures, assessing the level of disease awareness among stakeholders, and measuring efforts to reduce the annual incidence of brucellosis in specific host species, including humans.

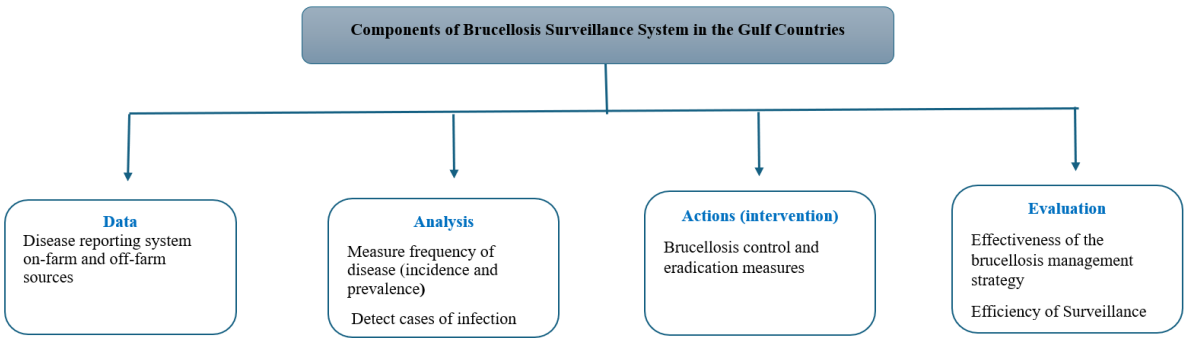

Figure 1. Components of Animal Brucellosis Surveillance System in Gulf Countries.

5.2.3.1.3. Actions (Intervention)

5.2.3.1.3.1. Control and Eradication Requirements

As part of these strategies, Rev 1 vaccine or the availability of DIVA vaccine against *B. melitensis* in small ruminants, cattle, and camels is needed to control or eradicate the infection. The *B. abortus* strain RB51 or S19 vaccine is necessary to control or eradicate infections caused by *B. abortus* in cattle. DIVA vaccines facilitate the differentiation between infected animals and those that are vaccinated and are crucial for eradicating the infection in endemic countries. The Rev 1 vaccine and the available DIVA vaccine have not been fully evaluated regarding their immunogenicity and protective efficacy against *B. melitensis* in cattle and camels. Similarly, there is limited data on the assessment of the RB51 and S19 vaccines for their effectiveness in protecting small ruminants and camels against *B. abortus*. Therefore, these vaccines should be evaluated before use in surveillance interventions. More information on the characteristics of these vaccines can be found in Annex 1 (Table S1).

The control and eradication of brucellosis in small ruminants, cattle, and camels depend on several critical factors. It is crucial to identify flocks and herds and to ensure that veterinary services are able to swiftly implement interventions across the targeted population. Adequate funding must also be secured to cover the costs of these interventions. At the same time, engaging breeders through awareness campaigns and educational initiatives, along with the involvement of relevant stakeholders (Government veterinary authorities, public health authorities, livestock breeders and farmers' associations), is essential for building support and cooperation. Equally important is a thorough understanding of the disease status, which requires conducting cross-border assessments, monitoring the occurrence of brucellosis in humans, and collecting accurate

information about the circulating *Brucella* species in livestock. If eradication is set as the ultimate goal, all of these requirements are critical. Special emphasis should be placed on the individual identification of animals and the control of their movements, at least within the intervention area and ideally throughout the country. Farmers should also be compensated for culled animals, either through reimbursement at market value or by offering incentives for attaining a "brucellosis-free" status. Furthermore, eradication demands informed political decisions and a sustained commitment from all relevant authorities and stakeholders [34].

5.2.3.1.3.2. Control and Eradication Programs against B. melitensis or B. abortus in small ruminants, and cattle

If *B. melitensis* is identified as a circulating species in small ruminants, and cattle in the Gulf countries, as determined by the survey findings, the following control and eradication strategies are recommended for implementation. Rev 1 or the available DIVA vaccine is recommended for use in control and eradication programs. When the prevalence of a disease is high (>10%), a mass vaccination strategy is the most effective way to control it. Vaccination of most flocks/herds of young and adult female sheep, goats, and cattle should be conducted every year or every two years (Table 1). It is estimated that, based on the current replacement rates in endemic countries, approximately 30-40 percent of the cattle population and 20-30 percent of small ruminants will be susceptible to disease every two years [36]. The ideal timeframe for vaccination is when the animals are neither pregnant, lactating, nor nearing the end of their calving season or lambing period.

An eradication program based on testing and slaughtering in the absence of vaccination can be recommended only when the prevalence found in the flocks/herds is low (4-1%) (Table 1). However, under low to moderate prevalence conditions (10-5%), a test-and-slaughter strategy

combined with the vaccination of young replacements is the eradication approach of choice to achieve “brucellosis-free” status. This approach has been applied successfully in several countries [37-38].

Vaccination of young replacements with the DIVA vaccine, if it is available, is necessary during eradication programs (Table 1). The vaccine is indicated for use in female sheep and goats 3 to 6 months of age and female calves 4 to 12 months of age that are not pregnant. Vaccinated animals must be identified using the standard official vaccination ear tag, with a vaccination tattoo in the right ear.

If *B. abortus* is detected among the circulating *Brucella* species in small ruminants, and cattle in the Gulf countries, as indicated by survey results, it is advisable to implement the control and eradication strategies outlined in Table 1 using a different type of vaccine. In cases where the prevalence is high (>10%), the *B. abortus* strain RB51 or S19 vaccine is an effective option for managing *B. abortus* in the affected populations. Conversely, if the prevalence is below 10%, the *B. abortus* strain RB51 vaccine is a suitable option for controlling and eradicating *B. abortus* in these populations.

Herds are classified as "suspect” if at least one animal tests positive by the Rose Bengal test (RBT) and the ELISA. Herds are designated as "infected” when Brucella is isolated or detected in PCR. Animals that test positive for RBT and ELISA and are part of infected herds are considered "infected animals" and must be slaughtered within 15 days of receiving the test results. Furthermore, female animals born to positive sheep, goats, or cattle that are 6 to 18 months of age are regarded as at risk and are subject to slaughter. Herds with positive animals are monitored with follow-up serological tests until they are released from quarantine or declared free from Brucellosis [36].

Table 1 Field-Based Approaches to the Control and Eradication of *B. melitensis* in Small Ruminants and Cattle

Phase 1: Control Program -high prevalence (>10%)

Year	Activity	Details
1	Implementation of the surveillance system	Estimate initial flock/herd prevalence and identify the circulating *Brucella* species.
	Vaccination	Vaccinate the flock/herd of young and adult female sheep, goats, and cattle using a Rev1 or DIVA vaccine, if it is available. Apply identification to premises and animals
2	Implementation of the surveillance system	Estimate disease prevalence.
	Vaccination	Repeat the vaccination of the flock/herd of young and adult female sheep, goats, and cattle. No testing of vaccinated flocks/herd – rely on natural replacement. Apply identification to premises and animals
3	Implementation of the surveillance system	Estimate disease prevalence.
	Vaccination	Repeat the vaccination of the flock/herd of young and adult female sheep, goats, and cattle. No testing of vaccinated flocks/herd – rely on natural replacement. Apply identification to premises and animals
4	Decision point	If prevalence is ≤ 10%, transition to the Eradication Program.

Phase 2: Eradication Program - low to moderate prevalence (5–10%)

Year	Activity	Details
5	Begin Eradication	Administer the available DIVA vaccine to lambs and kids aged 3–6 months and calves aged 4–12 months. Apply individual animal identification.
6	Implementation of the surveillance system	- Continue vaccination of young animals. - Conduct a serological survey to detect infected animals at the flock/herd level. - Slaughter all confirmed positive adult animals. - Implement quarantine and movement control procedures. - Apply identification to premises and animals
7	Implementation of the surveillance system	- Continue vaccination of young animals. - Conduct a serological survey to detect infected animals at the flock/herd level. - Slaughter all confirmed positive adult animals. - Implement quarantine and movement control procedures. - Apply identification to premises and animals
8	Implementation of the surveillance system	- Continue vaccination of young animals. - Conduct a serological survey to detect infected animals at the flock/herd level. - Slaughter all confirmed positive adult animals.

		- Implement quarantine and movement control procedures. - Apply identification to premises and animals
9	Implementation of the surveillance system	- Continue vaccination of young animals. - Conduct a serological survey to detect infected animals at the flock/herd level. - Slaughter all confirmed positive adult animals. - Implement quarantine and movement control procedures. - Apply identification to premises and animals
10	Implementation of the surveillance system	- Estimate disease prevalence. If the infection is not detected in sheep, goats, and cattle for one year, discontinue use of the vaccine and transition to phase 3.

Phase 3: Eradication Program - low prevalence (4-1%)

Year	Activity	Details
11	Implementation of the surveillance system	- Conduct a serological survey to detect infected animals at the flock/herd level. - Slaughter all confirmed positive animals. - Implement quarantine and movement control procedures. - Apply identification to premises and animals

12	Implementation of the surveillance system	- Conduct a serological survey to detect infected animals at the flock/herd level. - Slaughter all confirmed positive animals. - Implement quarantine and movement control procedures. - Apply identification to premises and animals
13	Surveillance Phase	Continue surveillance activities and monitor the animals using the Milk Ring Test (MRT) or RBT and the ELISA. When a milk sample is positive on the MRT, the adult animals of the contributing herds will be blood-tested. The test and slaughter strategy will be applied. Implement quarantine and movement control procedures.

5.2.3.1.4. Evaluation of disease management strategy and its surveillance

Animal health authorities should establish and monitor annual Key Performance Indicators (KPIs) to evaluate the effectiveness of the brucellosis management strategy. Control and eradication programs, as well as surveillance activities, will be adjusted in response to the results of these KPIs. Examples of such indicators include the incidence rate, which refers to the number of new brucellosis cases reported in animals at the herd level each year, and the prevalence rate, which represents the total number of existing cases within a population at a given point in time. In addition, the annual herd vaccination rate serves as a key measure, reflecting the percentage of herds that receive vaccination at least once per year. Complementing this, the annual animal vaccination rate accounts for the number of animals present in the herd during the respective

year—taking into consideration an average replacement rate of 20%—and vaccinated from the beginning of the program or within the eligible animal population. Another important KPI is the annual proportion of outbreak investigations by host species, which includes measuring the average time required to identify and respond to brucellosis outbreaks. Furthermore, the incidence and prevalence rates of human brucellosis must be tracked, with specific attention to differentiating infections caused by *B. abortus* and *B. melitensis*.

5.3. CONTROL OF CAMEL BRUCELLOSIS

Camel brucellosis is becoming more common and warrants immediate intervention by all parties, including camel owners, to prevent further expansion. The incidence of human brucellosis is significantly high in camel-rearing countries, mainly due to the consumption of raw camel milk. It has been reported that camelid brucellosis due to *B. melitensis* or *B. abortus* is endemic in most camel-rearing countries, with occurrence levels closely linked to husbandry and breeding practices [39].

In Gulf countries where camels are reared, they are highly valued by their owners, both economically and culturally. Dromedaries' value can be particularly high in camel-racing nations. Most camels infected with brucellosis appear clinically healthy, and owners may not allow such serologically positive animals to be culled. Thus, it has been suggested that a suitable strategy to stop the spread of *B. melitensis* would be to castrate serologically positive bulls and avoid breeding positive females [40]. However, castrating positive bulls and not breeding positive females is not the correct strategy. Venereal transmission has not been reported as a mode of transmission for *B. abortus* or *B. melitensis* in animals. Moreover, infected camels have shown no notable clinical signs [40]. Further research is needed to investigate these areas. Given the difficulties inherent in the management of camel brucellosis, we propose that the best tools currently available to control

and mitigate the spread of infection among camel populations and human cases of camel brucellosis are: vaccination of young camels (three months to one year) using the DIVA or Rev1 vaccine, enforcing strict management and biosecurity measures at the farm level and country border sites of imported animals, develop a coordinated surveillance program to detect and monitor brucellosis in animal populations, and pasteurize camel milk.

5.4. CONCLUSION

Brucellosis remains a significant threat to public and animal health in the Gulf region, primarily due to the high prevalence of *B. melitensis* among small ruminants, cattle, and camels, and its zoonotic potential. This chapter explores management strategies for controlling and eradicating brucellosis caused by *B. melitensis*. Key challenges to effectively eradicating brucellosis include the limited use of the DIVA vaccine, difficulties in identifying circulating *Brucella* strains, risks of co-infection in small ruminants, cattle, and camels, and inadequate veterinary quarantine measures for imported animals at border sites. We propose several strategies to control and eradicate *B. melitensis* in sheep, goats, cattle, and camels. These strategies are organized into three interrelated components: enforce stringent biosecurity and management practices at the farm level and border sites for imported animals, develop a robust system to control animal movement that integrates effective disease traceability mechanisms, establish a coordinated surveillance program to detect, monitor, and assess the presence of brucellosis within livestock populations and implement targeted control and eradication measures, such as test-and-slaughter programs and vaccinating susceptible animal populations. For camels, where clinical signs of disease are often absent, and transmission typically occurs through contact with infected small ruminants, it is crucial to prevent interspecies contact, ensure vaccination of young camels, and promote the pasteurization of camel milk to reduce the risk of human infection. By implementing

these strategies, we could significantly decrease the global prevalence of brucellosis and the associated zoonotic infections.

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ANNEX 1

1.1. Surveillance system

1.1.1. Steps to design and implement the seroprevalence survey

Step 1. Identify survey objective(s)

The first step of the surveillance system for animal brucellosis in Gulf countries is to conduct a scientifically based seroprevalence survey. The objective of conducting a seroprevalence survey is to estimate the national prevalence of Brucella species, particularly *B. abortus* and *B. melitensis*, and detect circulating *Brucella* organisms in Gulf countries.

Step 2. Define the population of interest

Animal populations targeted for brucellosis surveillance in Gulf countries can be classified into two distinct categories: the target population and the study population. The target population for sampling may include all adult cattle, small ruminants, and camels in a particular herd/flock, or region. Definitions of "adult" vary by species: for cattle, it refers to animals that are 18 months or older or possess central incisor teeth; for sheep and goats, adults are those aged 6 to 8 months or older; and for camels, adults are classified as 18 months or older. The study population includes sexually intact cattle, sheep, goats, and camels. Sampling should prioritize herds with a history of brucellosis, especially in regions where the disease is endemic. Databases from veterinary diagnostic laboratories and field investigations would be utilized to identify herds with evidence of exposure to brucellosis.

Step 3. Choose the survey design

A stratified sampling method should be used for this survey. Stratification means dividing the population into separate, exclusive groups (strata) and then randomly sampling units from each

stratum. This approach can be used to ensure that each group in the population is represented in the survey. For the seroprevalence survey, stratification may be conducted by species-specific independent livestock herds, such as dairy cattle, sheep, goats, and camels, as well as Brucella species (*B. melitensis* and *B. abortus*), and by geographic area.

Step 4. Determine the sample size

There are online sample size calculators available (Epitools) that can greatly assist with determining the sample size. Several parameters are required, as shown in the table below.

Ingredient	Description
Expected prevalence	Level of disease or another characteristic expected to be present
Margin of error	The precision associated with the estimate (usually the width of a confidence interval)
Confidence level	Confidence that the true value lies in the confidence interval (typically 95% or 90%)
Population size	Number of sampling units in the population
Test sensitivity	The probability that a test will identify a true positive as a test positive
Test specificity	The probability that a test will identify a true negative as a test negative

Step 5. Samples Collection

Serum samples should be collected from dairy cattle, sheep, goats, and camel flocks or herds. For vaccinated cattle, samples should be taken from animals aged 18 to 20 months that have received the RB51 or S19 vaccine. For vaccinated sheep and goats, samples should be collected from all animals over six months of age, regardless of whether they are unvaccinated or vaccinated with the Rev1 vaccine. Samples should be drawn from camels that are either vaccinated with the

Rev1 vaccine or from non-vaccinated camels aged nine to twelve months or older. 18 months or older

Step6. Laboratory tests

Blood samples should be collected to estimate the seroprevalence and circulation of *B. abortus* and *B. melitensis* in the Gulf countries. There are two options for testing. The first option is a test series that includes the Buffered Acidified Plate Antigen (BAPA) assay, commonly known as the Rose Bengal Plate or Brucella card, along with the Enzyme-Linked Immunosorbent Assay (ELISA) and Polymerase Chain Reaction (PCR) or culture. The second option involves using the Fluorescent Polarization Assay (FPA) and PCR or culture. Preferred samples for culture should include milk, vaginal discharge, and tissues from aborted fetuses, such as the liver, spleen, lung, blood, bronchial lymph nodes, gastric contents, and rectal swabs.

Step 7: Analyze and report the results of the laboratory

The key result from the survey will be an estimate of the prevalence of flocks and herds with Brucellosis-positive animals, which indicates prior infection with the disease, as well as detecting the circulating *Brucella* species in Gulf countries. However, caution must be exercised when interpreting the seroprevalence survey results, as most animals might have been vaccinated with the Rev1 vaccine before or after initiating the Brucellosis surveillance system. This vaccine can elicit long-term serological responses that are indistinguishable from those caused by infections with field strains. Therefore, it is crucial to gather records confirming whether the animals have been vaccinated. Additionally, positive results from serological tests should be corroborated with culture or PCR examinations to ensure a more accurate assessment.

1.1.2. Case Definition

I. Laboratory tests

During the implementation of the surveillance system, the following assays are recommended. For the isolation and identification of agents, Brucella species can be presumptively identified through modified acid-fast staining of organisms and by examining the morphology of *Brucella* species in aborted materials or vaginal discharges. This identification is especially robust when corroborated by serological tests. *Brucella* species should be isolated using plain or selective media inoculated with samples from uterine discharges, aborted fetuses (including stomach contents, spleen, and/or lung), udder secretions, or selected tissues such as lymph nodes or reproductive organs. Species identification should be performed using cultural techniques, PCR, and biochemical assays. In terms of serology, these tests are generally effective for screening both herds and individual animals [41]. A series of two tests is recommended: the BAPA assay (also known as the Rose Bengal Plate or Brucella card) and either ELISA or the FPA. Conducting these assays in series enhances both sensitivity and specificity. Furthermore, the milk ring test, applied to milk samples, serves as an effective method for screening and monitoring animals.

II. Case Definition

- Suspect case: A susceptible species that has:
    - Clinical signs consistent with infection with *Brucella* (the presence of abortions in tandem with any positive test results).
    - Epidemiological information showing exposure to *B. abortus* or *B. melitensis* (Known association with epidemiologically linked herds proven and suspected to be infected or infected humans).
    - Serum or milk ring test results suggest infection but are inconclusive.
- Presumptive positive case: A suspect case in which:

- Serum or milk test results indicate that the sheep, goat, cattle, and camels have been exposed to and infected with *B. abortus* or *B. melitensis*.
- *Brucella* organisms are identified microscopically.
- Paired samples demonstrate a significant rise in serological titers.

- Confirmed positive case: A presumptive positive case:
  - *B. abortus* or *B. melitensis* is isolated.
  - A positive confirmatory antigen test, such as Polymerase chain reaction (PCR), is performed.

Table S1: General characteristics of Animal brucellosis vaccines

Vaccines	Target animal species	Advantages	Disadvantages	Comments
B. melitensis Rev 1	Sheep, goats, camels, and cattle.	-High efficacy in control programs. -Effective against *B. melitensis* (13,14,15). -A single dose provides lifelong protection (6)	-Cause abortions in pregnant animals. -Long-term serological responses in vaccinated animals that cannot be differentiated from infections with field strains (13,14,15). -Shedding in the milk of lactating animals (which can lead to human	-There is limited data on the immunogenicity and protection against experimental challenges with *B. melitensis* in cattle and camels under both experimental and field conditions.

			infections) (13,14,15). - high virulence in humans (13,14,15).	
B. abortus S19	Cattle	-High efficacy in control programs. -Effective against B. abortus (43). -A single dose provides lifelong protection (43). -Slightly more efficacious than RB51 under experimental conditions (43).	-Cause abortions in pregnant cattle (42). -Long-term serological responses in vaccinated animals that cannot be differentiated from infections with field strains (42).	-There is limited data on immunogenicity and protection against experimental challenges with B. abortus and *B. melitensis* in sheep, goats, and camels under both experimental and field conditions. -Data on the immunogenicity and protection against *B. melitensis* challenges in cattle are limited under both experimental and field conditions.
B. abortus RB51	Cattle	-High efficacy in control/eradication programs (43). -Effective against B. abortus (43) -Classified as DIVA vaccine (43).	-Cause abortions in pregnant cattle. -Slightly less efficacious than S19 under experimental conditions (43).	-There is limited data on immunogenicity and protection against experimental challenges with *B. abortus* and *B. melitensis* in sheep, goats, and camels under both experimental and field conditions.

				-Data on the immunogenicity and protection against *B. melitensis* challenges in cattle are limited under both experimental and field conditions.
Availability of DIVA against *B. melitensis*	Sheep, goats, cattle, and camels	-	-	-Immunogenicity and protective against experimental challenge with *B. melitensis* in natural hosts are lacking. -A randomized controlled field trial also still needs to be performed on natural hosts to assess the effectiveness of the vaccine in terms of reducing clinical infection.