THE IMMUNE RESPONSE TO LIVE VACCINES AGAINST BOVINE ANAPLASMOSIS

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ABSTRACT

Anaplasma marginale causes bovine anaplasmosis, a hemolytic disease which is a serious problem for the cattle industry in tropical and sub-tropical regions worldwide. *A. centrale* is a less pathogenic species which is closely related to *A. marginale* and used as a live vaccine against anaplasmosis. Unfortunately the *A. centrale* vaccine can only be produced from the blood of infected cattle, risking contamination with other pathogens. This project compared the *A. centrale* live vaccine with *A. marginale* UFMG1, a low pathogenicity Brazilian strain which has been proposed as a potentially safer live vaccine derived from cell culture. Calves were infected with UFMG1 or *A. centrale*, and then challenged with the pathogenic Israeli *A. marginale* Gonen strain.

Previous infection with UFMG1 did not significantly reduce the severity of disease caused by challenge with the Gonen strain, whereas *A. centrale* infection did provide cross-protection against Gonen. In comparison to the antibody response to UFMG1 infection, the response to *A. centrale* infection had higher overall levels of IgG and showed higher cross-reactivity to Gonen strain antigen. The antibody response to *A. centrale* also had higher levels of IgG2, and showed more opsonophagocytic activity. All of these characteristics correlated significantly with protection from disease upon challenge. Understanding how *A. centrale* infection stimulates this effective immune response would be a valuable direction for future vaccine research.

Keywords: *Anaplasma marginale*, bovine anaplasmosis, immune response, opsonophagocytosis, live vaccines.

RÉSUMÉ

Le rickettsies *Anaplasma marginale* provoque l'anaplasmose bovine, une maladie hémolytique qui est un grave problème pour l'industrie bovine dans les régions tropicales et subtropicales du monde entier. Lô*A. centrale* est une espèce moins pathogène qui est étroitement liée à lô*A. marginale* et elle est utilisée comme vaccin vivant contre l'anaplasmose. Malheureusement, le vaccin *A. centrale* ne peut être produit quà partir du sang de bovins infectés, qui risquent dêtre contaminés par d'autres agents pathogènes. Ce projet a comparé le vaccin vivant *A. centrale* avec *A. marginale* UFMG1, une souche brésilienne faiblement pathogène qui a été proposée comme un vaccin vivant issu de culture cellulaire et potentiellement plus sûr. Les veaux ont été infectés par UFMG1 ou *A. centrale*, puis inoculés avec la souche israélienne pathogène *A. marginale* Gonen.

Lôinfection précédente avec UFMG1 n'a pas significativement réduit la sévérité de la maladie causée par la souche inoculée avec le Gonen, alors que l'infection par *A. centrale* a fournit une protection croisée contre le Gonen. Par rapport à la réaction des anticorps à lôinfection UFMG1, la réaction à l'infection par *A. centrale* avait des niveaux globaux plus élevés d'IgG et a montré une réactivité croisée ultérieure à l'antigène de la souche Gonen. La réaction des anticorps à *A. centrale* avait également des niveaux plus élevés d'IgG2, et a montré une activité plus opsonophagocytaire. Toutes ces caractéristiques sont en corrélation significative avec la protection contre la maladie lors de l'épreuve. Comprendre comment l'infection par *A. centrale* stimule cette réponse immunitaire efficace serait une piste de recherche vaccinale prometteuse.

Mots-clés : *Anaplasma marginale*, anaplasmose bovine, réponse immunitaire, opsonophagocytose, vaccins vivants.

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CHAPTER 1: GENERAL INTRODUCTION

1.1. WHAT IS *A. MARGINALE*?

1.1.1. Classification

Anaplasma marginale Theiler 1910 is a gram negative rickettsia and the causative agent of bovine anaplasmosis - a tick-transmitted hemolytic disease which causes serious economic problems to the cattle industry in tropical and sub-tropical areas worldwide (Kocan *et al.*, 2010). At present, there is no safe and globally effective vaccine against bovine anaplasmosis, and vaccine development remains an area of active research.

A. marginale belongs to the order Rickettsiales and the family Anaplasmataceae. This family consists of small, obligate intracellular bacteria, which are found within membrane-bound vacuoles in the cytoplasm of host cells (Dumler *et al.*, 2001). Within the Anaplasmataceae family, the genus *Anaplasma* includes several species of particular economic interest: *A. marginale,* the type species for the genus; *A. centrale,* sometimes described as a sub-species of *A. marginale* (Shkap *et al.*, 2008); and *A. phagocytophilum,* which was formerly known by several different names - *Ehrlichia phagocytophila, E. equi,* or the causative agent of Human Granulocytic Ehrlichiosis (Dumler *et al.,* 2001).

A. marginale was first isolated from cattle by Theiler (1910), who inaugurated the genus *Anaplasma.* He named the type species *A. marginale* after the 'marginal points' that the rickettsia formed in the infected erythrocytes. *A. marginale* infection has since been found in a wide range of wild and domestic ruminants, including cattle, bison, buffalo, wildebeest, deer and elk. However, reports of severe disease have exclusively been confined to cattle - experimental infection of other ruminant species produced only mild symptoms of anaplasmosis (Kuttler, 1984).

Within the ruminant host, *A. marginale* primarily infects erythrocytes. Endothelial cell infection has also been detected *in vivo* (Carreño *et al.*, 2007), but this does not appear to play an important role in the *A. marginale* life cycle (Wamsley *et al.*, 2011).

1.1.2. Transmission

A. marginale can be transmitted biologically by ticks, mechanically by biting flies or blood-contaminated fomites, and transplacentally (Kocan *et al.*, 2010). Around 20 different species of ticks have been implicated as vectors for biological transmission, with the most important genera being *Dermacentor* and *Rhipicephalus.* Across the majority of the tropical and sub-tropical range of *A. marginale, Rhipicephalus (Boophilus)* spp. are the most critical tick vectors, in particular *R. (B). microplus* and *R. (B). annulatus.* Since these species were eradicated from the United States in the first half of the 20th century, *Dermacentor* spp. particularly *D. andersonii* and *D. variabilis,* and *D. albipictus,* have taken over as the predominant biological vectors for *A. marginale* in the US. In Europe, multiple tick species have been proposed to play a role in anaplasmosis transmission (De la Fuente *et al.*, 2005). Of the species suggested, *D. reticulatus* has the strongest evidence of being an important *A. marginale* vector in Europe: it is capable of experimental transmission of *A.marginale* (Zivkovic et al., 2007), *A. marginale* DNA has been isolated from *D.reticulatus* ticks during an anaplasmosis outbreak in Hungary (Hornok et al., 2012), and its range runs from France, through Eastern Europe to Central Asia, covering many of the areas in which *A. marginale* has been found (Karbowiak, 2014).

Tick transmission can be transstadial or intrastadial; transovarial transmission has not been reported (Stich *et al.*, 1989). When the tick feeds on infected cattle, the rickettsia are taken up as part of the blood meal, infect the gut cells of the tick, and then spread to other tissues. These tissues include the salivary glands, from where *A. marginale* can then be transmitted to new hosts during tick feeding (Ge *et al.*, 1996).

On a global scale, biological transmission by ticks is considered the most common route of *A. marginale* infection. But not all strains of *A. marginale* are tick-transmissable, and in some regions no tick vector species occur. Therefore, although it has lower transmission efficacy, mechanical transmission can also play an important role in the spread of *A. marginale* (Scoles *et al.*, 2005). Mechanical transmission can be through various genera of biting flies (*Tabanus, Stomoxys*) or mosquitoes (*Culex* and *Aedes*), or through blood-contaminated fomites such as de-horning saws, ear-tagging devices, or castration instruments.

Transplacental transmission has been measured at around 15 % by Potgieter and Van Rensburg (1987), and as such is also likely to contribute to *A. marginale* epidemiology.

1.1.3. Disease

In the vertebrate host, *A. marginale* primarily infects erythrocytes. As part of the host response, these infected cells - along with considerable numbers of uninfected erythrocytes - are then destroyed by the reticuloendothelial system. Baker *et al.* (1961) measured up to a ten-fold increase in the rate of erythrocyte phagocytosis during acute *A. marginale* infection. This considerable loss of erythrocytes leads to anaemia and icterus, without hemoglobinemia or hemoglobinuria (Richey, 1981). Other symptoms of bovine anaplasmosis include fever, lethargy, weight loss, lowered milk production, and abortion. It is often fatal in older cattle if they are not treated early (Kocan *et al.*, 2010). The severity of bovine anaplasmosis increases with age: calves under six months of age rarely become ill; between 6-12 months they usually develop mild disease, which is more acute at 1-2 years old; finally, adults over two years old suffer acute and often fatal disease (Roby *et al.*, 1961; Aubry and Geale, 2011). The reason behind this increasing severity with age is as yet unknown.

If cattle recover from acute disease, they usually remain persistently infected, often at microscopically undetectable levels ($<10^7$ rickettsia/ml). Throughout persistent infection, cattle show cyclical rises in rickettsemia (Eriks *et al.*, 1993). These peaks in rickettsemia represent the generation of antigenic variants, which escape immune control and multiply rapidly, before they stimulate a variant-specific immune response that brings the infection under control again (French *et al.*, 1999). Due to this consistent stimulation, persistently infected cattle maintain a strong immune response against *A. marginale,* and are protected from disease when subsequently challenged with the homologous *A. marginale* strain. Unfortunately they also act as reservoirs of infection for naïve cattle (Palmer *et al.*, 1999).

1.1.4. Economic Impact in Endemic Areas

Bovine anaplasmosis has a serious economic impact on the cattle industry of endemic areas. Its effect is most serious in areas of endemic instability, where the transmission rate is too low to ensure that all cattle become infected as calves Aubrey and Geale, 2011). When cattle are first exposed to *A. marginale* as adults, they are much more likely to develop serious disease (Roby *et al.*, 1961).

Anaplasmosis causes direct losses through mortality, abortion, lowered weight of beef cattle and lowered milk production from dairy cattle. In addition, anaplasmosis frequently limits breed improvement (Lombardo, 1976; Ocampo Espinoza *et al.*, 2006). In Mexico, a highly endemic area,

anaplasmosis is estimated to cause up to a quarter of total deaths in national cattle improvement programmes (Rodriguez-Camarillo *et al.*, 1999). When pedigree *Bos taurus* cattle are imported from temperate areas to endemic tropical or sub-tropical areas, the new cattle are likely to be extremely susceptible to *A. marginale* infection. This is due to both a lack of previous exposure, and to the greater susceptibility of *B. taurus* breeds (e.g. Holstein, Hereford) to tick-borne diseases in comparison to *B. indicus* cattle (Kocan *et al.*, 2003).

There have been relatively few studies quantifying the economic impact of bovine anaplasmosis. In the 1970s, McCallon (1973) estimated that the disease caused annual losses of over 300 million US dollars (USD) to the American cattle industry. More recently, Tanzania was estimated to lose 47.3 million USD solely due to the direct costs of bovine anaplasmosis (Kivaria, 2006).

A. marginale is often considered in conjunction with the protozoan parasites *Babesia bovis and B. bigemina,* as all three are tick-borne pathogens which cause serious disease to cattle in tropical and sub-tropical regions of the world, and may often co-exist in the same animal. For example in Brazil, the three pathogens are referred to as one complex of disease, 'Tristeza Parasitária Bovina' (Gonçalves, 2000). This complex was estimated to cause a loss of 875 million USD to the cattle industry in South American countries (Brown, 1997).

Global climate change is likely to increase the range of tick vector species, and so increase the regions affected by the pathogens they can transmit (Howden *et al.*, 2010). As such, an ever-increasing number of countries are working to develop a deeper understanding of tick-borne diseases such as anaplasmosis.

1.2. IMMUNOLOGY

Multiple parts of the immune system appear to play a role in the immune response to *A. marginale.* The serological response has been identified as an important part of protection after vaccination (reviewed by Palmer *et al.*, 1999), and yet immune serum alone is not protective (Gale *et al.*, 1992). Cell-mediated responses have also been shown to play a role in protection (Brown *et al.*, 1998), but the T-cell response is inhibited by the pathogen to facilitate persistent infection (Han *et al.*, 2010). Due to this complexity it remains an ongoing task to clarify which parts of the immune system can or should be targeted by *A. marginale* vaccines.

1.2.1. Serological Response

High antibody levels, particularly of the subclass IgG2 (Brown *et al.*, 1998; Barigye *et al.*, 2004; Vega *et al.*, 2007) or against major surface proteins (MSPs), (Tebele *et al.*, 1991) have frequently been shown to correlate with protection. Antibodies can act through several routes: neutralization, opsonization, and complement-mediated bactericidal activity (Siegrist, 2012).

Palmer and McGuire (1984) demonstrated that polyclonal immune sera could neutralize the infectivity of *A. marginale* for calves, and Palmer *et al.* (1987) showed the same neutralization effect with anti-MSP1a monoclonal antibody.

Opsonophagocytosis, i.e. antibody-enhanced phagocytosis, has been proposed as an important mechanism to clear *A. marginale* infection (Palmer *et al.*, 1999). An opsonin, which literally means 'a flavoring', is any substance which makes whatever it binds to more likely to be 'eaten' by phagocytic cells (Murphy *et al.*, 2012). The antibody classes IgM and IgG are the most effective opsonins. Within IgG, different IgG subclasses have different levels of efficacy in opsonization; in cattle, the IgG2 subclass is the best opsonin (McGuire and Musoke, 1981).

In the presence of immune serum, antigen-specific antibodies coat the surface of bacteria, allowing the C1 complement protein to bind to the antibody-antigen complex. Once bound, C1 is activated and triggers the complement cascade, binding and activating a sequence of complement proteins including the opsonins C3 and C4. Phagocytic cells will then bind greatly enhanced numbers of these opsonized or 'flavored' bacteria, through their receptors for C3 and C4 complement proteins, and for the Fc portion of antibody (Murphy *et al.*, 2012). In the absence of any antibody, bacteria can still be recognized by phagocytic cells via pathogen recognition receptors (PRRs) which bind to common pathogen components such as lipopolysaccharide (LPS). The complement protein C3 also binds at low levels directly to the bacterial surface, leading to its activation and recognition by the C3 receptor on the phagocyte (Murphy et al., 2012). An overview of the opsonophagocytosis of bacteria is shown in Figure 1.

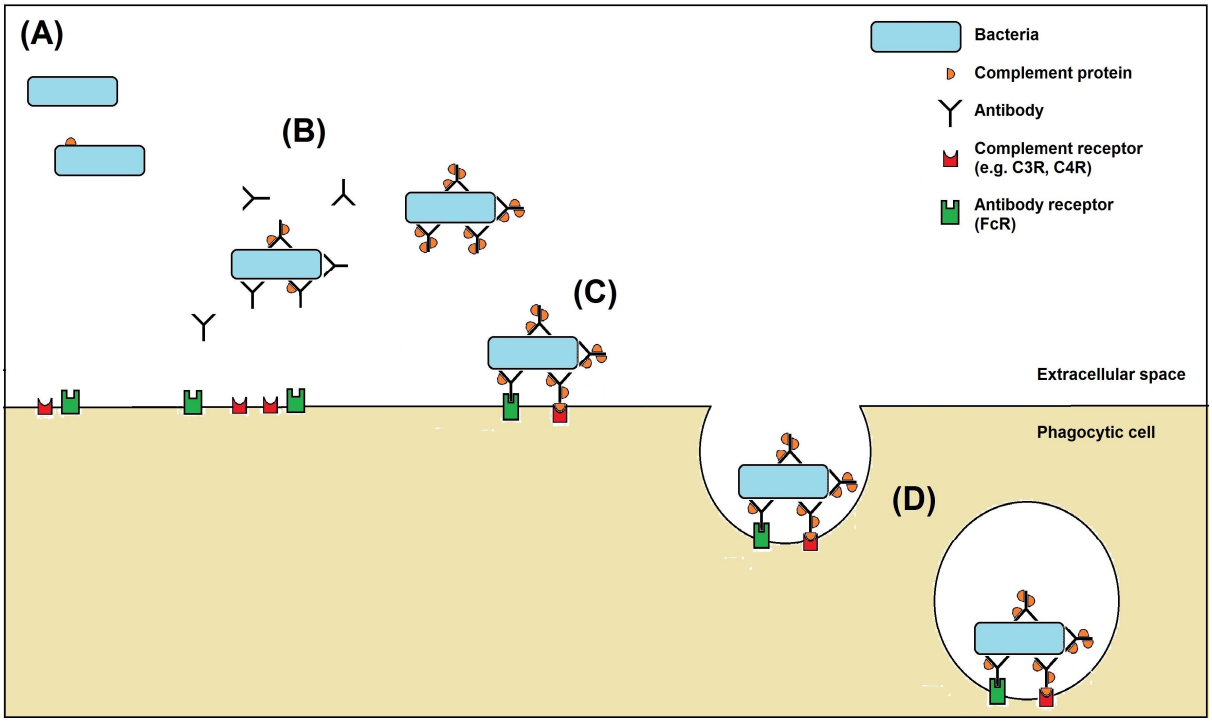

Figure 1: An overview of opsonophagocytosis of bacteria by immune sera.

(A) Without immune sera, only a small amount of complement protein will bind to bacteria.

(B) When immune serum is added, antibodies bind to epitopes on the surface of the bacteria. High amounts of complement proteins then bind to the antibody-antigen complex that has formed on the bacteria.

(C) Phagocytic cells recognize opsonized bacteria through the cell's receptors for antibody (FcR) and for complement proteins (C3R, C4R).

(D) The binding of opsonized bacteria to these receptors triggers the cell to internalize the bacteria in a membrane vesicle (the phagosome). The phagosome then fuses with other vesicles called lysosomes, which contain a range of antimicrobial components, and the bacteria are broken down by toxic reactive oxygen species and nitrogen oxides, acidification, proteolytic enzymes, and antimicrobial peptides.

Cantor *et al.* (1993) demonstrated that sera from MSP1-immunized cattle could significantly enhance *in vitro* opsonization of *A. marginale*. Melendez (2005) showed that immune sera from naturally infected cattle enhanced phagocytosis of both infected and uninfected erythrocytes, suggesting that opsonophagocytosis could be a double-edged sword: both limiting the spread of *A. marginale,* and contributing to the severe anemia that characterizes the disease.

Serum bactericidal activity against *A. marginale* has not yet been assessed.

Despite the demonstrated activity of antibodies against *A. marginale*, immune serum alone is not sufficient to prevent disease. In a classic passive protection experiment, Gale *et al.* (1992) showed that transfer of serum from hyper-immune to naïve calves was insufficient to protect the latter from disease. In addition, immune suppression by splenectomy or drugs leads persistently infected asymptomatic cattle to suffer a relapse in symptoms within 1-2 weeks ï this relapse occurs before circulating IgG levels show a significant fall (Jones *et al.*, 1968; Kuttler and Adams, 1977).

1.2.2. Cell-mediated Immune Response

There is ample evidence that cell-mediated immunity also plays a role in the host response to *A. marginale*. In early experiments, protection against bovine anaplasmosis correlated with inhibition of leukocyte migration (Buening, 1976) and development of cutaneous hypersensitivity (Carson *et al.*, 1976), indicating an association with T-cell responsiveness and macrophage activation, respectively. Brown *et al.* (1998) demonstrated that after cattle were vaccinated with outer membrane proteins, their protection from challenge correlated with their level of CD4+ T-cell proliferation, and with functions associated with CD4+ T-cells, namely the production of IFNɔ and IgG2.

These findings led to the current paradigm for a protective immune response to vaccination, proposed by Palmer *et al.* (1999). This model centers around antigen-specific CD4+ T-cells, and their production of IFNɔ, which activates macrophages and stimulates B-cells to produce more IgG2. Antigen-specific IgG2 opsonises the rickettsia, increasing their uptake by the activated macrophages and leading to control of the infection.

This model has been challenged by more recent research, which suggests that CD4+ T-cells do not appear to be essential to protection from anaplasmosis. When calves were thymectomized (removing their ability to develop new T-cells), and then had their existing CD4+ T-cells depleted, they were still able to control *A. marginale* infection. They showed no significant difference in disease severity compared to untreated calves (Valdez *et al.*, 2002). There is a caveat to this study in that CD4+ T-cell depletion was not absolute (as this is very difficult to achieve).

Interestingly, a natural depletion of T-cells during infection appears to be a persistence strategy of *A. marginale* (Han et al, 2010). Vaccination with *A. marginale* surface proteins MSP2 (Abbott *et al.*,

2005) or MSP1a (Han *et al.*, 2008) induced high levels of antigen-specific CD4+ T-cells; these high levels rapidly fell after the cattle were infected with *A. marginale.* Later in infection only low and sporadic T-cell responses were seen. This phenomenon was not the general immunosuppression seen after *A. phagocytophilum* infection. The T-cell reduction was confined to those cells which responded to *A. marginale* antigen, with the level of CD4+ T-cells specific for a control antigen remaining high after *A. marginale* infection (Han *et al.*, 2010).

The inhibition of a T-cell response to *A. marginale* is very likely to contribute to its long-term persistence in infected cattle. However, the inhibition may also benefit the host. Continuous CD4+ T-cell activity in response to the cyclically high bacterial loads seen in persistent *A. marginale* infection would likely lead to systemic inflammation, damaging the host (Han *et al.*, 2010). In fact, down-regulation of T-cell responses is frequently seen in long-term persistent infections where antigen loads remain high (Jenson *et al.*, 2002; Xu *et al.*, 2002).

The majority of studies on the T-cell response to *A. marginale* have focused on alpha-beta T-cells. These are the most abundant T-cell type, encompassing CD4+ and CD8+ T-cells, and a range of regulatory T-cells. Another type of T-cell which may also play a role in the response to *A. marginale* infection is the gamma-delta T-cell. Gamma-delta T-cells are particularly abundant in ruminants, and are at their highest levels in younger animals ï the age at which anaplasmosis causes the mildest symptoms (Hein and MacKay, 1991; Roby *et al.*, 1961). Gamma-delta T-cell clones specific for *A. marginale* MSP2 have been shown to respond to antigen by proliferating, producing IFNɔ, and expressing a range of chemokines which recruit inflammatory cells (Lahmers *et al.*, 2005; 2006). However, the dynamics of gamma-delta T-cells during infection with *A. marginale* have not yet been reported.

Much of the nature of the T-cell response to *A. marginale* remains to be clarified - in particular, whether the effects of T-cell deletion during *A. marginale* infection prevent the T-cell response from having any protective effect against bovine anaplasmosis.

1.3. CONTROL MEASURES AGAINST *A. MARGINALE*

Control measures vary in different areas, according to their expense, practicality, and the preferences of the region. Controlling arthropods with acaricide treatment is useful for limiting the spread of various vector-borne diseases, including anaplasmosis. But as *A. marginale* can also be transmitted mechanically and transplacentally, acaracides cannot totally eliminate the spread of this rickettsia. In addition, regular use of acaricides is expensive and can hasten the development of resistance (Kocan *et al.*, 2010).

1.3.1. Chemotherapy

Tetracycline antibiotics are by far the predominant treatment for bovine anaplasmosis. Trials of imidocarb (Roby *et al.*, 1972) and enrofloxacin (Facury-Filho *et al.*, 2012) have also shown good results, but they are not commonly used (Coetzee *et al.*, 2006). Repeated doses of tetracycline can eliminate persistent *A. marginale* infections, although complete elimination is not always achieved (Swift and Thomas, 1983).

Tetracycline is most effective in the earlier stages of the disease (Kuttler *et al.*, 1980), so it can be difficult to catch the disease early enough in range cattle (Kocan *et al.*, 2010). Therefore it is sometimes used prophylactically, particularly in the US (Kocan *et al.*, 2010). Such frequent use of antibiotics has the potential to cause selection of resistant strains, but to date this has not been reported as a problem. The withholding period in antibiotic-treated cattle before they can be used for meat or milk can be an issue for farmers, particularly with long-lasting oxytetracycline preparations (Lew-Taylor, 2012).

In some regions, tetracyclines are used for the 'infection-treatment' control method: cattle are inoculated with *A. marginale*-infected erythrocytes, and then treated early in the patent phase of disease by low doses of tetracycline drugs. The aim is the development of persistent infection without acute disease. As the animals have the opportunity to develop an immune response, they will subsequently be immune to challenge with homologous strains. However, the infection-treatment method requires careful monitoring to ensure acute disease does not develop, and so can be unsuitable for large herds of cattle (Kocan *et al.* 2003).

1.3.2. Vaccination

Despite the effectiveness of antibiotic treatment, demand for a vaccine is still high (Spath *et al.*, 1990; Kocan *et al.*, 2010). Existing vaccines can be effective control methods for bovine

anaplasmosis, but developing improved vaccines which are safer and globally effective has long been an objective of the cattle industry (Palmer *et al.*, 1989).

No vaccines tested to date reliably induce sterile immunity. Instead, when vaccinated cattle are challenged, they develop persistent infection without acute disease, and so can still act as reservoirs of infection for naïve cattle (Bock and deVos, 2001). Various types of vaccine against bovine anaplasmosis have been studied. Killed and live vaccines have been frequently used in the field; recombinant protein, outer membrane, and DNA vaccines have so far been confined to small research trials (Kocan *et al.,* 2010).

1.3.2.1 Killed vaccines

The commercial vaccine '*Anaplaz'* consisted of killed *A. marginale* derived from the blood of infected cattle (Fort Dodge Laboratories, Fort Dodge, Iowa). *'Anaplaz'* was marketed for several decades in the US before being withdrawn due to company restructuring (Kocan *et al.*, 2003). Killed vaccines have several advantages over live vaccines: less chance of contamination with other pathogens, greater ease of storage, and low post-inoculation reactions. However, they offer limited cross-protection against strains from different regions (Kuttler *et al.*, 1984), and require yearly boosters since they only induce short-term immunity (Brock *et al.*, 1965). Moreover, expensive purification procedures are needed to remove erythrocyte debris (McCorkle-Shirley *et al.*, 1985). If the rickettsia are insufficiently purified from erythrocyte stroma, repeated vaccination may stimulate isoantibodies against erythrocyte proteins. Calves ingesting colostrum from cows with high isoantibody titres may then develop hemolytic anemia (Dennis *et al.*, 1970). The occurrence of this problem was reduced by improved purification protocols (Hart *et al.*, 1990), and the introduction of guidelines not to vaccinate cows in the later stages of pregnancy (Luther *et al.*, 1989)**.**

1.3.2.2. Outer Membranes, Recombinant Proteins, and DNA Vaccines

Outer membrane proteins (OMPs) are common vaccine targets as they will be exposed to the host's immune system during infection (Grandi, 2010). The *A. marginale* proteins that have been considered as vaccine candidates are therefore OMPs, with the majority of research being focused on the most highly expressed OMPs. These are the most abundant and immunogenic proteins on the surface of *A. marginale,* and are known collectively as the Major Surface Proteins (MSPs), MSP1a, MSP1b, MSP2, MSP3, MSP4, and MSP5 (Agnes *et al.*, 2011).

MSP1a and MSP1b form the MSP1 complex, which is involved in cell entry. MSP1a is necessary and sufficient for attachment to both erythrocytes and tick cells (de la Fuente *et al.*, 2003), while MSP1b only plays a role in attachment to erythrocytes (de la Fuente *et al.*, 2001). MSP2 and MSP3 are both transcribed from large multi-gene families. They vary considerably between isolates (Alleman *et al.*, 1997; Palmer *et al.*, 1994), and even express variant sequences over the course of persistent infection (Brayton *et al.*, 2003; Barbet *et al.*, 2001; French *et al.*, 1999). MSP4 and MSP5 are both encoded by single-copy genes, and are highly conserved. As such they are useful for phylogenetic analysis (de la Fuente *et al.*, 2002b) and diagnostics, with MSP5 used for a commercial diagnostic ELISA (de Eschaide *et al.*, 1998).

Outer membrane protein vaccines

Outer membrane protein (OMP) vaccines are a complex mix of proteins of varying immunogenicity. At least 25 different antigenic OMPs have been identified (Lopez *et al.*, 2005; Noh *et al.*, 2008). Several studies have demonstrated that OMP-based vaccines can induce protection against homologous challenge (Tebele *et al.*, 1991; Brown *et al.*, 1998; Noh *et al.*, 2008), but Palmer *et al.* (1994b) found they did not significantly reduce disease symptoms after heterologous challenge. In addition, OMP vaccines are expensive to prepare and difficult to standardize (Noh *et al.*, 2013).

Recombinant protein vaccines

Recombinant protein vaccines are often less protective than OMP vaccines. This is perhaps unsurprising, as they usually consist of a single protein rather than the complex mix of immunogens seen in OMP vaccines (Palmer and McElwain, 1995).

The majority of recombinant protein vaccines have used MSPs. Despite good results with native protein (Palmer *et al.*, 1989), recombinant MSP1 (in the form of the MSP1a/MSP1b complex) has had mixed results, with some studies finding protection against homologous strains (Camacho-Nuez *et al.*, 2000), and others not (Palmer and McElwain, 1995). Although MSP2 and MSP3 are highly immunogenic (Palmer *et al.*, 1999), their high variability makes them unsuitable targets for a widely protective vaccine (Albarrak *et al.*, 2012). There has been very limited study of recombinant MSP4 and MSP5; the former induced protection against homologous challenge, the latter not (A.F. Barbet, unpublished data, cited in Palmer and McElwain, 1995).

The high variability of the most abundant OMPs (the immunodominant MSP2 and MSP3) has led

some groups to focus on sub-dominant OMP antigens. Candidate sub-dominant antigens are those which are highly conserved between strains and over the course of infection, and are still immunogenic despite being less abundant. Multiple antigens have been identified which fit these requirements (Lopez *et al.*, 2005; Noh *et al.*, 2008; Sutten *et al.*, 2010; Agnes *et al.*, 2011), but so far only one, AM779, has been tested as a recombinant protein vaccine. It was immunogenic but not protective, raising the possibility that multiple sub-dominant antigens may have to be combined create an effective recombinant protein vaccine (Albarrak *et al.*, 2012).

DNA vaccines

DNA vaccines have been tested in mice (Kano *et al.*, 2008) and cattle (Arulkanthan *et al.*, 1999; Mwangi *et al.*, 2007). Interestingly, Arulkanthan *et al.* (1999) found that the bovine antibody response to a MSP1a DNA vaccine was largely restricted to IgG1, which is not associated with protection (Brown *et al.*, 1998). However, an MSP1b-based DNA vaccine has been shown to induce partial protection from disease (de Andrade *et al.*, 2006).

1.3.2.3. Live vaccines

Live vaccines have been used against bovine anaplasmosis for over a century, beginning shortly after *A. marginale* was first identified (Theiler, 1912). Today live vaccines are perhaps still the most widely used type (Kocan *et al.*, 2003). As live vaccines lead to a persistent infection in vaccinated cattle, they provide them with lifelong protection against severe disease. They produce a stronger immune response than killed vaccines, with the generation of antigenic variants during persistent infection leading to a broader immune response (Palmer *et al.* 1999).

The practise of deliberately establishing a persistent infection with a less pathogenic strain in order to create immunity against a more severe infection is known as premunition or premunisation (Kuttler *et al.*, 1984b). Vaccines for premunisation are customarily produced from the blood of splenectomized calves, which develop high levels of rickettsemia after infection (Kocan *et al.*, 2003). The infected blood must then be kept chilled (for use within a week) or cryopreserved (for longer term storage, and quality control) (McElwain, 2008). Natural variation can be a problem with live vaccines: it can lead to variants which either cause unexpectedly serious disease, or do not maintain sufficient infectivity for successful premunisation (Bock *et al.*, 2003).

Attenuated *A. marginale*

Several approaches have been used to attenuate pathogenic *A. marginale* to make them suitable for

use as a live vaccine: passage through sheep (Ristic *et al.*, 1968), passage through deer (Kuttler and Zaugg, 1988), or ^{60}Co irradiation (Sharma and Bansal, 1986). The most successful attenuated vaccine was produced in sheep by Ristic *et al.* (1968). It induced protection with limited side effects in several trials (Welter and Ristic, 1969; Osorno *et al.*, 1975; Ristic and Carson, 1977). However, other investigators found severe post-vaccination reactions (Anziani *et al.*, 1981; Henry *et al.*, 1983).

Naturally low pathogenic *A. marginale* strains

Low pathogenic strains of *A. marginale* cause mild or no disease without needing deliberate attenuation. Several low pathogenic strains have been tested as live vaccines: the Dawn strain from Australia (Bock *et al.* 2003; Carter *et al.*, 2006); the Yucatan strain from Mexico (Rodriguez-Camarillo *et al.*, 2008); and the UFMG1 strain from Brazil (Bastos *et al.*, 2010). A low pathogenicity field isolate from Colombia has also been tested as part of a combination babesiosis-anaplasmosis live vaccine (Benavides *et al.*, 2000).

All these strains, with the exception of the combination vaccine, induced good protection against homologous strains, and against heterologous *A. marginale* strains from the same geographic area. However, none have yet successfully progressed into wider use outside their country of origin.

Anaplasma centrale

Anaplasma centrale is a low pathogenic species closely related to *A. marginale,* which was first identified in South Africa by Sir Arnold Theiler, shortly after he isolated *A. marginale* (Theiler, 1912). As only a few, quite variable, *A. centrale* isolates have been sequenced, it is difficult to determine whether *A.centrale* should be identified as a variant of *A.marginale,* a sub-species, or a separate species. 16S rRNA analysis based on a Japanese *A. centrale* isolate classified it as a distinct species (Inokuma *et al.*, 2001), but analysis of two South African *A.centrale* (including the vaccine strain, *A. centrale Theiler, 1911)* by 16S rRNA (Dumler *et al.,* 2001) and GroEL sequences (Lew *et al.,* 2003) classified *A. centrale* as an *A. marginale* sub-species.

As the name suggests, *A. centrale* inclusions are generally seen in the centre of erythrocytes, as opposed to the 'marginal points' of *A. marginale* (Theiler, 1912). Theiler determined that animals infected with *A. centrale* only showed mild disease or were asymptomatic, and were subsequently protected from challenge with pathogenic *A. marginale*. In the decades after this discovery, *A. centrale* was exported to various countries to be used as a live vaccine against bovine anaplasmosis

ï not only to other countries in Africa, but further afield to Israel, Australia, and parts of South America (Shkap *et al.*, 2008; Bock and de Vos, 2001; De Wall, 2000; Melendez *et al.*, 2003).

In all these regions, *A.centrale* is generally very effective at protecting cattle against bovine anaplasmosis (Bock and de Vos, 2001). Nevertheless there have been some reports of vaccine failure, particularly against highly pathogenic *A. marginale* strains (Wilson *et al*., 1980; Payne *et al.*, 1990; Turton *et al.*, 1998; Brizuela *et al.*, 1998; Bock and de Vos, 2001), and *A. centrale* itself has been reported to cause disease if administered to adult cattle (Pipano, 1976; Potgieter 1979; Bigalke 1980; Pipano *et al.*, 1985).

As with all the live vaccines that have been used in the field so far, *A. centrale* is produced from the blood of infected splenectomized calves (Pipano, 1995). Blood-derived vaccines risk accidental transmission of other blood-borne pathogens. For example when one calf used for vaccine production became accidentally infected with bovine leucosis virus, over 10,000 *A. centrale* vaccines doses were contaminated (Rogers *et al.*, 1988).

1.3.2.4. Tick-cell culture as a source of vaccines

Tick cell culture has been proposed as a safer and more easily standardized source of live vaccines. *A. centrale* has never successfully been cultivated *in vitro,* but multiple *A. marginale* strains have been propagated in tick cell lines (Passos, 2012). *In vitro*-derived *A. marginale* remain infective for cattle and ticks (Munderloh *et al.*, 1996), and keep the same antigenic composition through repeated passages in tick cell culture (Barbet *et al.*, 1999). Tick cell-derived *A. marginale* have been shown to be immunogenic when used as a killed (Kocan *et al.*, 2001; de la Fuente *et al.*, 2002) or live inoculum (Bastos *et al.*, 2010; Hammac *et al.*, 2013). When compared with blood-derived *A. marginale* of the same strain, they induced similar levels of protection (de la Fuente *et al.*, 2002; Bastos *et al.*, 2010).

Tick cell culture-derived *A. marginale* would be more easily standardized and safer than blood-derived vaccines, with a lower risk of contamination by other pathogens, and no potential auto-immune complications from contaminating erythrocyte proteins. Therefore tick cell culture-derived *A. marginale* are a focus of current research into new live vaccines.

1.4. THESIS STRUCTURE

The aim of this project was to investigate the immune response to live vaccines against bovine anaplasmosis. *A. centrale,* the current 'gold-standard' live vaccine, was compared with a naturally low pathogenic Brazilian *A. marginale* strain (UFMG1), which has been successfully established in cell culture and so has the potential of being a cell culture-derived live vaccine.

Chapter 2 describes a trial comparing the effectiveness of *A. centrale* and UFMG1 in protecting calves against challenge with a pathogenic Israeli strain, *A. marginale* Gonen.

Chapter 3 investigates the serological response induced by *A. centrale* and *A. marginale* UFMG1.

Chapter 4 investigates the cell-mediated response to *A. centrale* and *A. marginale* UFMG1 ï namely PBMC proliferation and IFNɔ production.

Chapter 5 describes the development of an *in vitro* assay for serum opsonophagocytosis activity (OPA) against *A. marginale,* based on flow cytometric measurement of the oxidative burst response to phagocytosis. OPA has been suggested as a correlate of protection for bovine anaplasmosis, but this has never been experimentally confirmed.

Chapters 2 and 3 are partially based on:

R.Kenneil, V. Shkap, B.Leibovich, E. Zweygarth, K. Pfister, M.F. Ribeiro, L.M.Passos (2013). Cross-protection between geographically distinct *Anaplasma marginale* isolates appears to be constrained by limited antibody responses. *Journal of Transboundary and Emerging Diseases* 60 Suppl.2: 97-104.

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CHAPTER 2: CLINICAL RESULTS OF LIVE VACCINE TRIAL OF *A. MARGINALE* UFMG1 AND *A. CENTRALE*

2.1. INTRODUCTION

2.1.1. *A. centrale* as a live vaccine against *Anaplasma marginale*

Live vaccination with *Anaplasma centrale* is the most widely used method to prevent bovine anaplasmosis (Kocan *et al.*, 2003). It has been used as a live vaccine from 1912, when it was first identified by Sir Arnold Theiler (Theiler, 1912). It is currently used throughout Israel, Australia, and parts of Africa and South America (Shkap *et al.*, 2008; Bock and de Vos, 2001; De Wall, 2000; Melendez *et al.*, 2003). It is not licensed for use in the EU, the USA and Mexico (Kocan *et al.*, 2003). Cattle infected with *A. centrale* develop a persistent infection with mild or no disease. When vaccinated cattle are subsequently exposed to pathogenic *A. marginale,* they still become infected but are protected from serious illness (Shkap *et al.*, 2008).

Although vaccination with *A. centrale* is generally very effective against bovine anaplasmosis, there have been reports of severe side effects in older cattle (Pipano *et al.*, 1985), and of vaccine failure, especially against high pathogenic *A. marginale* strains (Turton *et al.*, 1998, Brizuela *et al.*, 1998, Guglielmone and Vanzini, 1999; Payne *et al.*, 1990; Bock & de Vos, 2001). An additional problem is that the vaccine is produced from the blood of infected splenectomized calves (Pipano, 1995), which risks accidental transmission of other blood-borne pathogens. Furthermore, using calves for vaccine production is laborious, expensive, and should be replaced wherever practical with more ethical alternatives.

2.1.2. Cell culture-derived *A. marginale* UFMG1 as a potential vaccine

Unfortunately, despite many attempts *A. centrale* has never been cultivated *in vitro*. In contrast, several *A. marginale* strains have been successfully established in tick cell culture (as reviewed by Bell-Sakyi *et al.*, 2007; Passos, 2012). Vaccines derived from cell culture would have improved safety and reproducibility over those produced from blood. Initial studies with inactivated cell culture-derived *A. marginale* have shown that they remain immunogenic and can induce protection against homologous strain challenge (Kocan *et al.*, 2001; de la Fuente *et al.*, 2002b). Cell culture-derived *A. marginale* also remain infective for cattle and ticks (Munderloh *et al.*, 1996), and so can

be used as live vaccines. Bastos *et al.* (2010) demonstrated that infection with UFMG1, a naturally low pathogenic Brazilian *A. marginale* strain, protected cattle from disease on subsequent challenge with UFMG2, a heterologous high pathogenic Brazilian strain. Blood- or cell culture-derived UFMG1 induced statistically similar levels of protection. Bastos *et al.* (2010) therefore proposed that UFMG1 could be a potential cell culture-derived live vaccine against bovine anaplasmosis.

UFMG1 is a strain with an inclusion appendage which was first isolated from an infected calf in the Minas Gerais province of Brazil by Ribeiro *et al.* (1997). It is not transmissible by *Rhipicephalus (Boophilus) microplus*, the main tick vector of *A. marginale* in Brazil (Gonçalves-Ruiz *et al.*, 2005). However, it has been successfully cultivated *in vitro* in BME26, an *R. (B). microplus*-derived cell line (Esteves *et al.*, 2009), and in IDE8, an *Ixodes scapularis*-derived cell line (Bastos *et al.*, 2009), despite *I. scapularis* not being a natural *A. marginale* vector. When UFMG1 was tested as an inactivated vaccine by Lasmar *et al.* (2012), it did induce seroconversion but did not achieve the protection against the high pathogenic strain UFMG2 that was seen by Bastos *et al.* (2010). This echoes previous results that live vaccines are more efficacious than killed (reviewed by Kocan *et al.*, 2003).

2.1.3. The problem of cross-protection between strains

Bastos *et al.* (2010) demonstrated that UFMG1 infection could protect cattle against disease caused by UFMG2, a heterologous strain originating from the same country. However, one of the main problems with controlling *A. marginale* is the high variability of strains from different regions. Over two hundred different strains have been reported across the global range of *A. marginale* (Cabezas-Cruz *et al.*, 2013), and even within one herd there may be multiple strains circulating (Alamzan *et al.*, 2008; Pohl *et al*., 2013). Since there is often limited cross protection between heterologous strains, this high level of diversity is a major barrier to developing an effective global vaccine against bovine anaplasmosis. A good vaccine in one region may only offer limited protection in another (Palmer *et al.*, 1994; Ocampo Espinoza *et al.*, 2006).

2.1.4. Protection with or without infection-exclusion

ôInfection-exclusionô is a theory that persistent infection with one *A. marginale* strain excludes secondary infection with a challenge strain. This theory was proposed by de la Fuente *et al.* (2002a), as a possible explanation for the action of live anaplasmosis vaccines. Infection-exclusion was demonstrated between two strains *in vivo* in cattle and ticks, and *in vitro* in bovine erythrocytes and tick cell culture (de la Fuente *et al.*, 2002a; 2003). However, infection-exclusion does not occur with the *A. centrale* vaccine (Shkap *et al.*, 2008), or with all combinations of *A. marginale* strains (Palmer *et al.*, 2004; Bastos *et al.*, 2010). Rodriguez *et al.* (2005) determined that the deciding factor was the *msp2* pseudogene repertoire of the strains tested. When the two strains had overlapping *msp2* repertoires, infection-exclusion was seen; in contrast, strains with distinct *msp2* repertoires developed co-infections. Live anaplasmosis vaccines can sometimes cause infection exclusion, but since *msp2* is highly variable (Futse *et al.*, 2008) this means of protection will only work against a limited number of strains. The most important aspect of any vaccine proposed for widespread use is its ability to induce protection without infection exclusion.

UFMG1 protected cattle against UFMG2 without infection-exclusion occuring (Bastos *et al.*, 2010). Interestingly, when co-infection with UFMG1 and UFMG2 developed after challenge, a new UFMG2 *msp1a* tandem repeat variant was found in several cattle. Co-infection has previously been implicated as a driver for *msp2* diversity (Futse *et al.*, 2008). The *msp1a* gene is also under positive selection pressure (Cabezas-Cruz *et al.*, 2013). Therefore it is possible that co-infections, whether natural or deliberate, may also be a selective pressure driving the diversity of *msp1a* sequences.

This could present an issue for research into the epidemiology of *A. marginale,* as at present the most common way of differentiating between strains is through their *msp1a* sequence ï namely by sequencing the N-terminal tandem repeat region of *msp1a*, and then determining how closely the amino acid sequence of the repeats match to previously published strains*. This is used because the MSP1a tandem repeat region been sequenced for the majority of published strains, and sufficient variation is seen between different *A. marginale* isolates that it can serve as a useful way of estimating the *A. marginale* variability in a population of infected cattle, and can be used to track strains for epidemiological studies**. Within one isolate, several studies** have shown that MSP1a tandem repeats remains unchanged through *A. marginale* infection in cattle or ticks, and through transmission between hosts. If co-infection with two or more *A. marginale* strains provides selective

pressure which leads to changes in MSP1a repeat sequences, this would make consistent identification of different strains much more unreliable.

2.1.5. Aims of the vaccine trial

Bastos *et al.* (2010) has already shown that UFMG1 can protect cattle against a heterologous Brazilian strain. The next step in characterizing the potential of UFMG1 as a vaccine is to investigate whether it can also induce protection against non-Brazilian *A. marginale* strains. A broadly cross-reactive response would imply it could potentially be useful in affected areas across the world. Therefore in this study UFMG1 was tested for its ability to protect cattle against a pathogenic Israeli *A. marginale* strain. The effectiveness of UFMG1 was compared to the current standard live vaccine, *A. centrale*. After challenge, cattle were tracked for establishment of co-infections (i.e. protection without infection exclusion), and for changes to the *msp1a* sequence.

2.2. MATERIALS AND METHODS

2.2.1. *A. marginale* and *A. centrale* strains

A. marginale UFMG1 (*msp1α* sequence GenBank EU676176), was originally isolated from a naturally infected calf in Minas Gerais State, Brazil, as described by Ribeiro *et al.* (1997). At the time of the trial UFMG1 had been continuously maintained in IDE8 tick cells for approximately 5 years at the Universidade Federal de Minas Gerais, Brazil, and then the Ludwig-Maximilians-Universität, Munich, Germany, using standard culture methods described by Bastos *et al.* (2010).

The *A. centrale* vaccine strain (full genome sequence GenBank CP001759) used in the trial is routinely used as a live vaccine in Israel (Shkap *et al.*, 2008). It has been maintained through inoculation of splenectomized calves at the Kimron Veterinary Institute, Bet Dagan, Israel.

A. marginale Gonen (hereafter referred to as Gonen) originated from a field case at the Gonen farm in the north of Israel. It was determined to have one of the most common *msp1a* genotypes in Israel at the time the strain was isolated (Molad *et al.*, 2009). Blood collected from the original infected cow (*msp1α* sequence GenBank EU678755) was subcutaneously inoculated into a splenectomized calf, resulting in minimum hematocrit of 9 % and maximum rickettsemia of 55 % before the calf was treated with oxytetracycline to prevent death (V. Shkap, written communication). Blood taken from the calf before antibiotic treatment was used to infect another splenectomized calf, which showed similar symptoms. Blood cryostabilate prepared from the latter calf was used in this trial.

To obtain sufficient infective material for cattle inoculation, *A. centrale* and *A. marginale* UFMG1 and Gonen were first individually subcutaneously inoculated into 3 splenectomized calves. The UFMG1 inoculate was rickettsia partially purified from infected IDE8 tick cells by repeated syringing ten times through a 26G needle to fragment cells, followed by centrifugation at 200 x g for 10 minutes to pellet cell debris and leave rickettsia in the supernatant. *A. centrale* and Gonen inoculates were blood cryostabilates frozen in 15 % DMSO. Once splenectomized calves reached over 10 % rickettsemia, infected blood was collected and used to inoculate trial cattle. This also standardized all strains as fully viable and blood-derived to allow for a more direct comparison between UFMG1 and *A. centrale.*

2.2.2. Trial design

The trial design is outlined in Figure 1. Twelve Israeli Friesian calves (*Bos taurus)* were used, aged 3-5 months at the start of the trial ï close to the average age at which calves are vaccinated with *A. centrale* (Bock and de Vos, 2001). They were kept under tick- and fly-free conditions, and confirmed to be negative for pre-existing *A. marginale* and *A. centrale* infections by PCR and MSP5 competitive ELISA (cELISA) (VMRD, Pullman, WA). The Kimron Veterinary Institute Animals Welfare Committee and the Israeli Ministry of Health approved all experiments in cattle (licence number 020_b1731).

Calves were randomly divided into 3 groups of 4 animals each, and subcutaneously inoculated with $1x10^6$ *A. marginale* UFMG1, $1x10^6$ *A. centrale*, or 2 ml PBS alone (control). The infection was allowed to resolve without treatment. Sixty days after initial infection (2 weeks after the last point rickettsemia was Ó0.1 %), the calves were challenged with $1x10^7$ Gonen strain rickettsia in 2 ml PBS.

Progress of infection was monitored at least thrice-weekly, and daily during acute infection, by rickettsemia, hematocrit, and rectal temperature. Rickettsemia was measured in Giemsa-stained blood smears, using blood taken by ear vein puncture. Percentage rickettsemia was calculated by the number of infected erythrocytes divided by total number of erythrocytes, counted over a minimum of 20 fields in 100x oil immersion. Hematocrit was measured by microhematocrit technique (Schalm et al, 1975), using capillary blood from the ear vein.

The threshold of disease severity at which cattle would be treated with antibiotics was set before the trial as a hematocrit remaining below 20 % for 3 days.

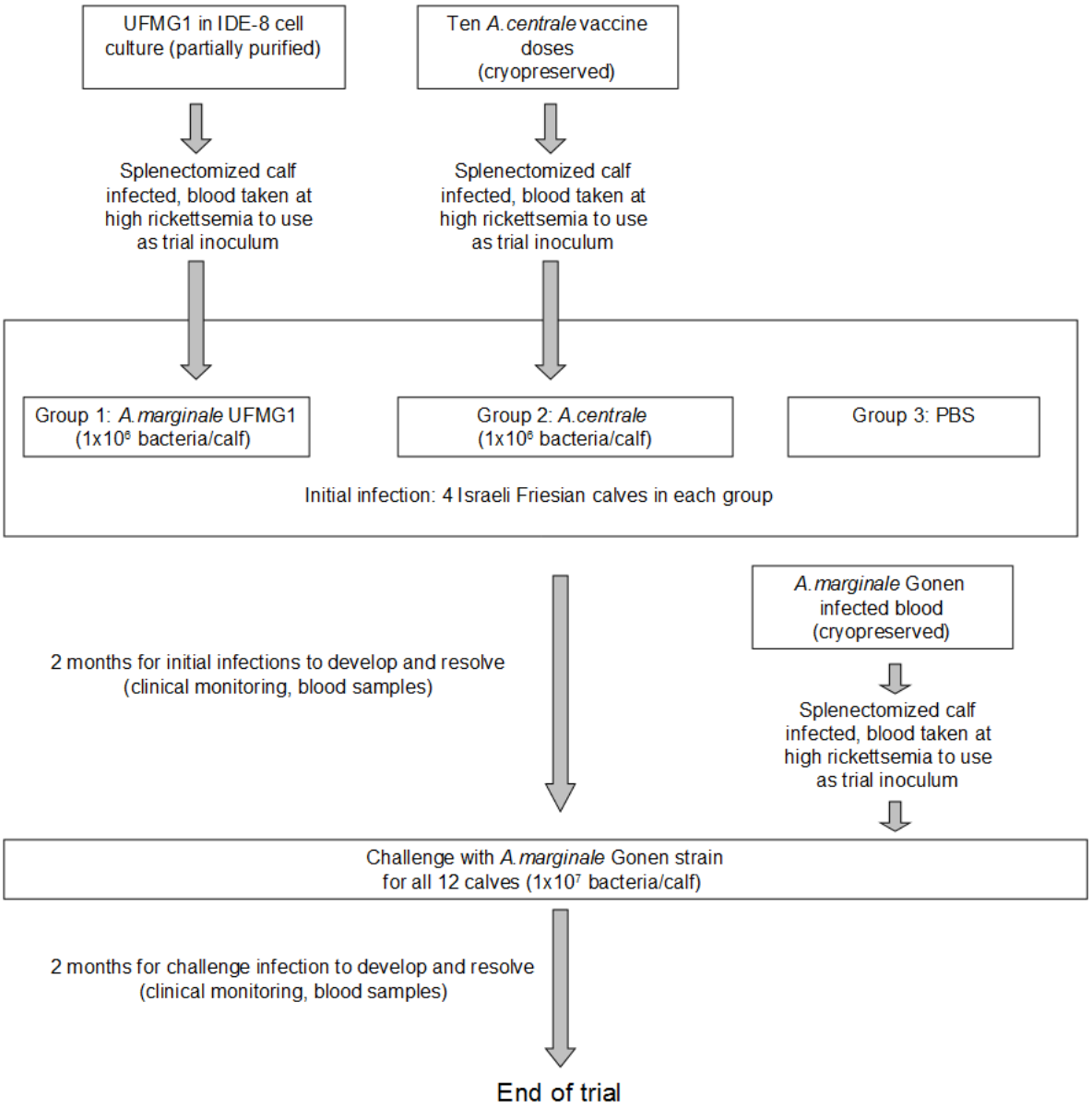

Figure 1: Design of live vaccine trial.

2.2.3. Statistical Analysis

The statistical significance of differences in clinical parameters between different groups was calculated by ANOVA and Tukey HSD post-analysis (Yandell, 1997), using the Minitab software package (Minitab Inc., State College, Pennsylvania, USA).

2.2.4. Confirmation of infection and genotype analysis

For DNA analysis, blood samples were collected into EDTA-Vacutainers (Becton Dickinson, Franklin Lakes, New Jersey), diluted with an equal volume of cold PBS, and then washed three times in cold PBS by centrifugation at 2600 g for 20 minutes at 4 °C. The buffy coat was removed after each centrifugation. Packed erythrocytes were resuspended in an equal volume of PBS and frozen at -70 °C to lyze the cells. Samples were then thawed to room temperature, and DNA extracted by the QiaAmp DNA Mini Kit (Qiagen, Hilden, Germany) according to manufacturer's instructions. The DNA concentration post-extraction was measured by NanoDrop spectrophotometer (NanoDrop ND-1000, PeqLab Erlangen, Germany).

All PCR primers and reaction conditions are described in detail in Table 1. Initial confirmation of *A. marginale* infection was through a real-time *msp1b* PCR, as described by Carelli *et al.* (2007). *A. centrale* does not have an MSP1 protein, so a conventional *msp4* PCR was used to confirm *A. centrale* infection, after Shkap *et al.* (2008).

A. marginale strains are typically characterized by the amino acid sequence of their MSP1a tandem repeat region (reviewed by Cabezas-Cruz *et al.*, 2013). The *msp1a* sequence of *A. marginale* in infected animals was amplified by a hemi-nested PCR as described by Lew *et al.* (2002). For animals with a dual infection of *A. marginale* Gonen and UFMG1 strains, the shorter UFMG1 *msp1a* tandem repeat sequence was preferentially amplified. Therefore in dual infections with Gonen and UFMG1 strains, Gonen *msp1a* was amplified using the original reverse primers, and self-designed forward primer F2, which was specific for the Gonen strain *msp1a* (based on sequences successfully amplified from singly-infected cattle).

All conventional PCRs used the same reaction mixture, namely a final volume of 50 εl containing: 5 εl purified DNA as template, 1× PCR buffer, 1 εM of each primer, 0.2 mM of each deoxynucleotide triphosphate, and 1.25 U of HotStar Taq (all from Qiagen, Hilden, Germany). The reaction was performed in a Eppendorf Mastercycler (Eppendorf, Hamburg, Germany), and PCR products were visualized on a 2 % agarose gel dyed with 1x Gel RedÊ Nucleic Acid stain (Biotium Hayward, USA). The real time *msp1b* PCR used a final volume of 25 εl, containing: 5 εl purified DNA as template, 1 x TaqMan Gene Expresssion Mastermix (Applied Biosystems, USA), 0.9 εM of the forward and reverse primers, and 0.2 εM of the probe. Reactions were performed in a 7500-

fast-Real-Time PCR System (Applied Biosystems, Darmstadt, Germany).

PCR target (purpose)	**Primers**	**Reaction conditions**	**Reference**
A. marginale msp1b (Pre-trial screen and initial confirmation of infection)	AM-For TTGGCAAGGCAGCAGCTT, AM-Rev TTCCGCGAGCATGTGCAT, probe AM-Pb6FAM-TCGGTCTAACATCTCCAGGCTTTCAT-BHQ.	95 °C for 10 min; 45 cycles of 95 °C for 45 s, and 60 °C for 1 min.	Carelli *et al.* (2007)
A. centrale msp4 (confirmation of *A. centrale* infection)	F: CATGGGGCATGAATCTGTG, R: AATTGGTTGCAGTGAGCGC	95 °C for 3 min; 35 cycles of 98 °C for 10 s, 55 °C for 30 s, and 72 °C for 30 s; with final extension at 72 °C for 5 min.	Shkap *et al.* (2008)
A. marginale msp1a (strain identity, sequence changes)	Hemi-nested PCR: initial reaction used 1733Fð 5ÑTGGTGCTTATGGCAGACATTTCC3ôand 3134Rð 5ÑTCACGGTCAAAACCTTTGCTTACC3Ñj Second reaction used 1733F and 2957Rð 5ÑjAACCTTGTAGCCCCAACTTATCC3Ñj	95 °C for 15 minutes; 40 cycles of 94 °C for 30 s, 55 °C for 60 s, and 72 °C for 120 s; with final extension at 72 °C for 7 min. The second reaction (using 5ε l of the first reaction as a template) was as above but used an annealing temperature of 60 °C instead of 55 °C.	Lew *et al.* (2002)
A. marginale msp1a, specific for the Gonen strain.	Hemi-nested PCR: Initial reaction used F2 - 5ôCGTATGTTACAATCAGGCACGCTG3ô and 3143R (see above). Second reaction used F2 and 2957R (see above).	Reaction conditions as with general *A. marginale msp1a* PCR above.	None

Table 1: PCR conditions for all assays used to test trial samples.

Positive *msp1a* samples were purified using QIAquick PCR Purification Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol, and sequenced (in forward and reverse directions) by Eurofins MWG Operon (Ebersberg, Germany), using 1733F and 2957R primers for UFMG1, and F2 and 2957R primers for Gonen. Results were analyzed by Chromas Lite®, reverse sequences were reversed and complemented with Reverse Complement (http://www.bioinformatics.org/sms/rev_comp.html), and alignment performed with ClustalW2

(http://www.ebi.ac.uk/Tools/msa/clustalw2/). The amino acid sequence of the tandem repeat region was determined by ExPASy translate (http://web.expasy.org/translate/).

2.3. RESULTS

2.3.1. Clinical response to initial infection with *A. marginale* UFMG1 and *A. centrale*

The rickettsemia, hematocrit, and temperature of calves in response to UFMG1 or *A. centrale* infection are summarized in Table 2. On average, calves infected with UFMG1 developed a clearly detectable rickettsemia (Ó0.1 %) 24 days post infection, while *A. centrale* rickettsemia was detectable after 35 days. Once infection was detected, the rickettsemia remained measurable for around an average 32 days for UFMG1 and 13 days for *A. centrale*, peaking at an average of 10% for the UFMG1 group, and 5 % for the *A. centrale* group.

UFMG1 infection caused a more pronounced drop in hematocrit than *A. centrale.* UFMG1-infected calves had minimum hematocrits of 18-21 %, compared to minimums of 21-33 % for *A. centrale*-infected calves, and 28-31 % for control group calves. This represents an average reduction of 13 % hematocrit points (39% of total) for UFMG1-infected calves, 7.5% points (20.3% of total), for *A. centrale*-infected calves, and 5 % (13.2 % of total) for the control group. The rate of decrease in hematocrit was twice as fast for UFMG1 as for *A. centrale.*

In all groups the animals had similar maximum body temperatures, with no significant differences between them.

For both peak rickettsemia and minimum hematocrit, UFMG1 group values were significantly different from the control group ($p<0.01$), whereas *A. centrale* group values were not ($p>0.05$). All infections spontaneously resolved without antibiotic treatment (pre-determined treatment threshold described in Methods).

2.3.2. Clinical response after challenge

The Gonen strain caused relatively mild infection in all groups, and no calf showed disease symptoms severe enough to require treatment. Post-challenge rickettsemia, hematocrit, and temperature are summarised in Table 2. The average clinical response to initial infection and challenge for each group are shown in Figure 2.

Period	Group	Peak rickettsemia (%)	Days to peak rickettsemia	Days of measurable rickettsemia (≥0.09%)	Minimum hematocrit (%)	Percentage hematocrit reduction (% of total)	Maximum temperature (°C)
Initial infection	UFMG1	10 ± 3.1	29±3	32 ± 4	19.8 ± 1.5	39.4 ± 12.2	39.8±0.25
	A. centrale	5 ± 3.6	39±4	13 ± 1	26 ± 5.3	20.3± 25.6	39.6±0.51
	Control	0 ± 0	n/a	0 ± 0	29± 1.2	13.2 ± 14.0	39.5±0.52
Gonen challenge	UFMG1	2.6 ± 3.2	28±2	15 ± 11	21.5 ± 2.4	34.3± 12.7	40.4± 0.77
	A. centrale	1.0 ± 1.4	33±2	4 ± 4	28.3 ± 4.3	15.3 ± 13.1	40.1±0.95
	Control	3.05 ± 3.1	29±6	20 ± 17	22 ± 2.8	37.2 ± 3.5	39.8±0.36

Table 2: Summary of average clinical parameters for all groups, during initial infection with UFMG1 or A. centrale, and during challenge with the Gonen strain.

Rickettsemia after challenge was low in all groups ï no individual calf exceeded 7 %, and the group averages were 2.6 % for UFMG1, 1.0 % for *A. centrale*, and 3.0 % for the control group. There was high variability within each group, as shown in Figure 2, and no statistically significant difference was seen between any of the groups.

The UFMG1 and control groups showed a similar level of red blood cell loss after challenge. Their minimum hematocrit values ranged between 19-24 % (average reduction of 12 % points) and 20-26 % (average reduction of 13 % points) respectively. Calves in the *A. centrale* group were less affected, with minimum hematocrit values between 22-32 % (average reduction of 5 % points), and only one calf showing a similar level of red blood cell loss to the UFMG1 and control groups (see Figure 3). There was a significant difference between minimum hematocrit values of UFMG1 and *A. centrale* infected calves after challenge ($p<0.05$), but neither UFMG1 nor *A. centrale* groups were significantly different from the control group. There were no significant differences in body temperature between the groups post-challenge.

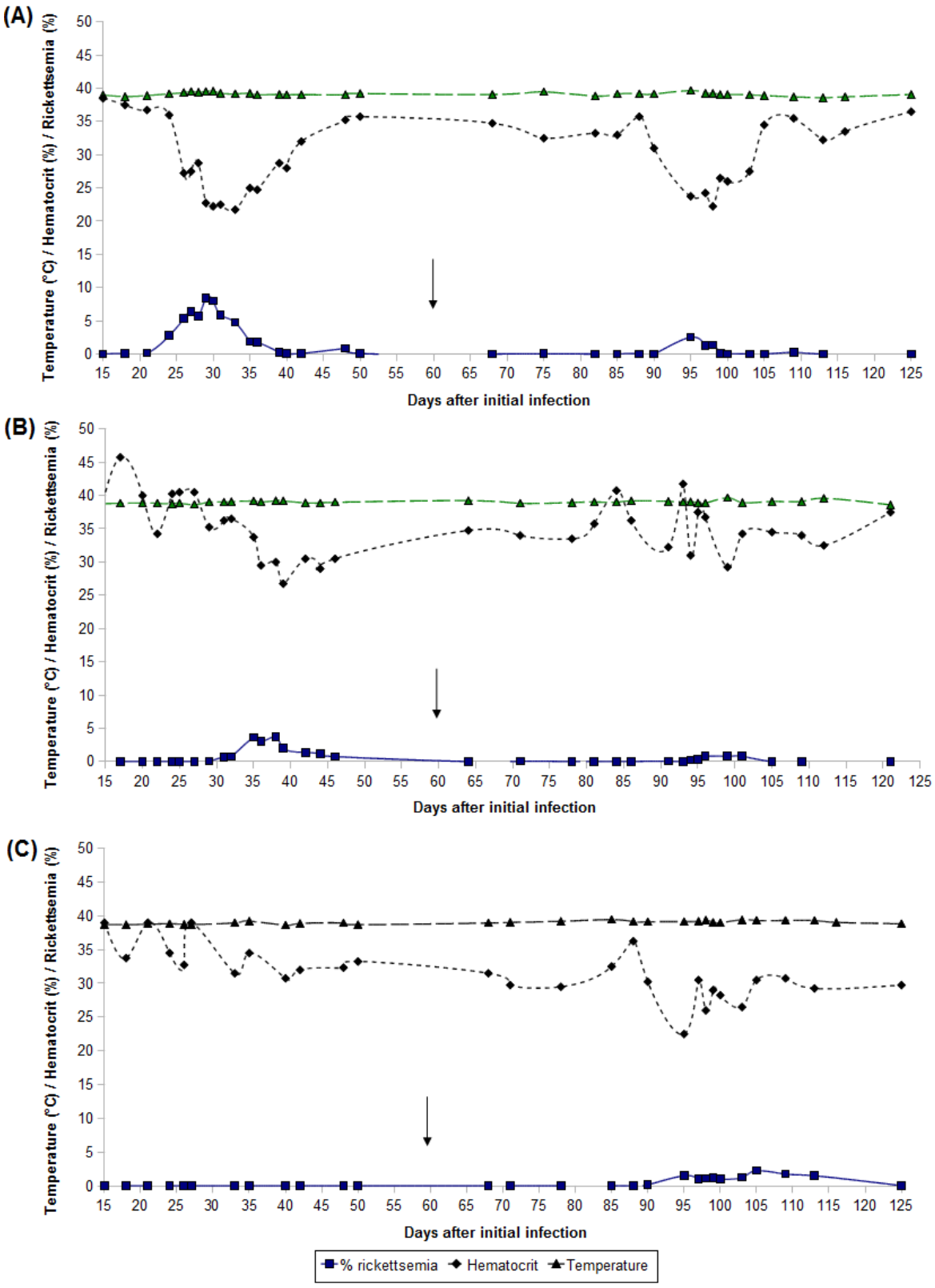

Figure 2: The course of rickettsemia, hematocrit, and temperature over initial infection and challenge (median values/group) for (A) UFMG1 group, (B) A. centrale group, and (C) control group. Arrow indicates point of challenge.

Multiple calves, even in the control group, showed low levels of rickettsemia after challenge. Nonetheless, Gonen still caused similar levels of hematocrit reduction as the initial infection with UFMG1 despite having lower rickettsemia (Figure 2).

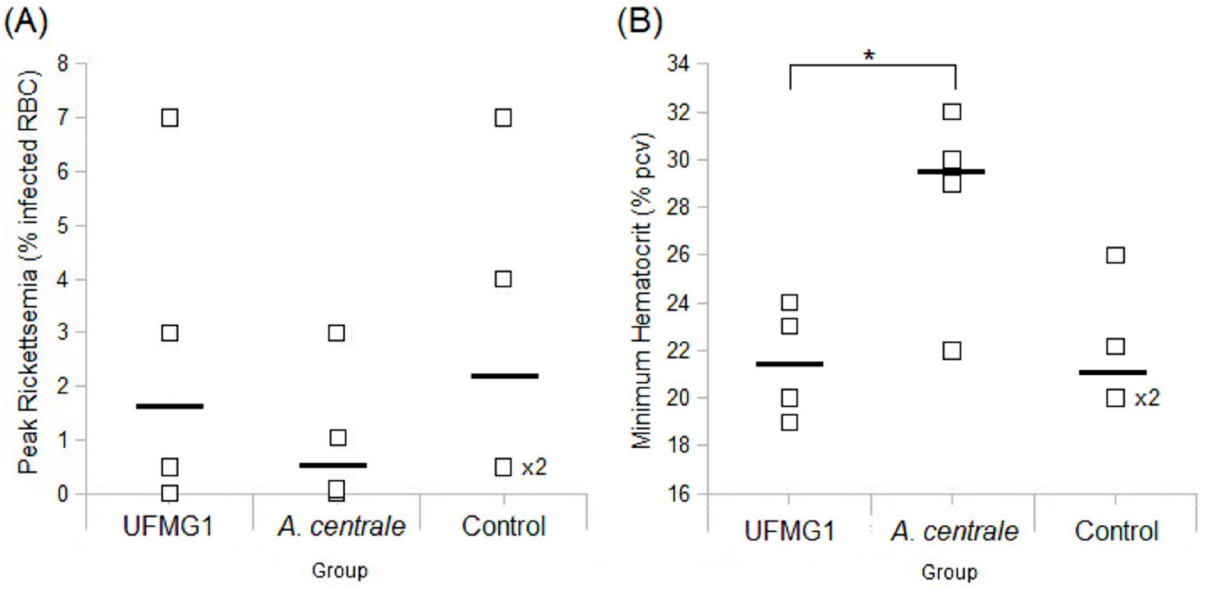

*Figure 3: Peak rickettsemia (A) and minimum hematocrit (B) post-challenge. Horizontal bars represent median group value. * = p<0.05 significance of difference between groups.*

Within all groups, the individual response to initial infection and challenge was highly variable, as can be seen in Figure 3. This was particularly noticeable for the levels of rickettsemia. There was no significant correlation between the severity of symptoms during initial infection, and their severity after challenge (data not shown).

2.3.3. Establishment and persistence of infection

All calves inoculated with *A. marginale* UFMG1 or *A. centrale* remained infected throughout the whole trial (four months in total for initial infection and challenge). After challenge, all calves but one became PCR-positive for the Gonen strain, and the infection remained detectable until the trial ended, two months after challenge. One animal in the UFMG1 group (calf #1) did not become PCR-positive for the Gonen strain at any point after challenge ï this calf also showed no disease symptoms after challenge.

MSP1a genotype over the course of initial infection and challenge

UFMG1 and Gonen MSP1a tandem repeat sequences remained stable in almost all calves over the course of infection, whether the host was infected with one strain or two. There was one exception: post-challenge, one animal in the *A. centrale* group showed a single nucleotide base change in the Gonen strain *msp1a*, giving rise to a single amino acid change in the MSP1a amino acid tandem repeat sequence. The MSP1a tandem repeat sequences of *A. marginale* UFMG1 and Gonen strains are illustrated in Table 3; *A. centrale* does not have an *msp1a* gene.

(A)

Strain	Tandem repeats	Tandem repeats in altered genotype
UFMG1	13 / 42 / 13 / 18	No changes
Gonen	77 / 25 / 3 / 25 / 3 / 3	77 / 25 / 3 / 25 / 162 / 3

(B)

Strain	Tandem repeat no.	Tandem repeat amino acid sequence
UFMG1	13	TDSSSASGQQQESSVLSQSDQASTSSQLG
	42	TDSSSASGQQQESSVLPQSGQASTSSQSG
	18	TDSSSASGQQQESSVLSQSDQASTSSQSG
Gonen	77	ADSSSVSGQQQESSVLSQSGQASTSSQLG
	25	ADSSSASGQQQESSVLSQSSQASTSSQLG
	3	ADSSSASGQQQESSVLSQSGQASTSSQLG
	162	ADSSSASCQQQESSVLSQSGQASTSSQLG

Table 3: MSP1a tandem repeat structure in UFMG1 and Gonen strains.

(A): Original and altered A. marginale UFMG1 and Gonen MSP1a tandem repeats.

(B): amino acid sequences of tandem repeats. Nomenclature according to that proposed by de la Fuente et al. (2007) and tandem repeat numbers updated by Cabezas-Cruz et al. (2013) – tandem repeat sequence 162 has not previously been described.

2.4. DISCUSSION

2.4.1. Response to initial infection with UFMG1 vs. *A. centrale*

UFMG1 infection gave rise to symptoms which were not severe enough to require intervention. But the symptoms were greater than those from *A. centrale,* and came close to the predetermined threshold for antibiotic treatment. This level of disease was similar to that seen in the calf from which UFMG1 was originally isolated (Ribeiro *et al.*, 1997), and less severe than that seen in the calves infected in the initial UFMG1 protection trial by Bastos *et al.* (2010).

Unfortunately, inactivation of UFMG1 appears to abrogate any protective effect it causes. A study by Lasmar *et al.* (2012) using cell culture-derived UFMG1 as a beta-propiolactone-inactivated vaccine demonstrated no protection against the pathogenic Brazilian strain UFMG2. In contrast, live cell culture-derived UFMG1 induced significant protection against UFMG2 under otherwise similar conditions (Bastos *et al.*, 2010).

While UFMG1 appears unlikely to cause fatal disease in calves at the target age for vaccination, any pronounced pathogenicity raises concerns about its safety as a live vaccine. The calves used in this trial were under one year old; this is the stage at which live vaccination is recommended as any symptoms tend to be mild (Bock and de Vos, 2001). If, however, a vaccine strain is transmitted from vaccinated calves to naïve adult cattle by tick or mechanical transmission, the disease is very likely to be fatal in the adult ï as seen in experimental infection of adult cattle with *A. centrale* (Pipano *et al.*, 1985).

The risk of accidental infection of naïve adults occurring after *A. centrale* vaccination is limited by its extremely restricted biological transmission - only reported to date with the African tick *Rhipicephalus simus,* from which it was initially isolated (Theiler, 1912; Potgieter and Van Rensburg, 1987), and at an extremely low transmission efficiency by *Dermacentor andersoni* (Ueti *et al.,*2009). Several other tick species that act as *A. marginale* vectors cannot transmit *A. centrale,* namely *Hyalomma excavatum*, *R. sanguineus,* and *R.* (*Boophilus*) *annulatus* (Shkap *et al.*, 2009).

Gonçalves-Ruiz *et al.* (2005) showed that *A. marginale* UFMG1 was not transmitted by *Rhipicephalus (Boophilus) microplus* ticks, which are the major biological vector of *A. marginale* in

Brazil. However, *A. marginale* is known to be transmissible by at least 20 tick species (Kocan *et al.*, 2010), and mechanical transmission is always a possibility; therefore any further studies on UFMG1 as a live vaccine should investigate both its transmissibility by other tick vector species, and the effect of UFMG1 infection on adult cattle.

2.4.2. Protection from challenge

Previous UFMG1 infection had a negligible effect on disease caused by the Gonen strain. The lack of protection from UFMG1 against the heterologous Israeli Gonen strain tested here contrasts with the complete protection that UFMG1 induced against the high pathogenic Brazilian strain UFMG2 (Bastos *et al.*, 2010). UFMG1 infection caused significantly less reduction in disease symptoms after challenge than *A. centrale,* the current live vaccine of choice, and so does not appear to be a candidate for replacing it.

The low pathogenicity of the Gonen strain in this trial made assessing protection from fatal infection impossible. The Gonen strain was selected due to its high prevalence in Israel (Molad *et al.*, 2009), and the serious symptoms seen after deliberate infection of splenectomized cattle with this strain (V. Shkap, unpublished results described in Methods). However, splenectomized cattle are much more susceptible to anaplasmosis (Kocan *et al.*, 2003), and from the symptoms seen here the Gonen strain cannot be considered highly pathogenic in intact calves. The young age of the calves used here is also likely to explain the lower symptoms ï although some *A. marginale* strains such as UFMG2 can induce serious disease in young calves (Bastos et al., 2010), in general symptoms of anaplasmosis will be milder in younger animals. If time and money had permitted, Gonen infection should have been tested before the trial with an intact calf of the same age as those used in the trial, to confirm the pathogenicity of the strain in conditions more similar to the trial. The optimum scenario would have been to vaccinate the calves and then wait until they were at least one year old before challenging them with the Gonen strain. However, keeping the calves in tick and fly-free conditions for this length of time would be prohibitively expensive.

Nonetheless, although the Gonen strain could not be used to assess protection from fatal levels of disease, in this trial it caused a similar level of symptoms as the challenge strains used in several other vaccine studies (Kocan *et al.*, 2001; de la Fuente *et al.*, 2002). The inclusion of *A. centrale* provided a benchmark to compare how UFMG1 reduced the symptoms of Gonen infection. Given

that UFMG1 was less effective than *A. centrale,* and *A. centrale* is itself reported to have problems protecting cattle against some highly virulent *A. marginale* strains (Carter *et al.*, 2006), it seems unlikely that UFMG1 would have induced significant protection against a more pathogenic strain.

2.4.3. Variation in response to infection

There was considerable variation within each group for the level of disease caused by initial infection with UFMG1 or *A. centrale,* and then for the level of protection against the Gonen strain. For example, while the majority of the *A. centrale* group had mild or no disease after challenge, one calf appeared unprotected, with a similar level of symptoms as the unvaccinated calves of the control group. Such a varied response to the *A. centrale* vaccine has frequently been reported in the field (Bock and de Vos, 2001). One controlled study showed considerable variation even when identical inocula were used: when Wilson *et al.* (1980) infected 18 cattle with *A. centrale,* 10 were protected, the other 8 were not. This high variability in response should not be unexpected in cattle, as the animals are not specifically inbred for laboratory studies. But it does highlight the difficulties in finding a vaccine capable of inducing complete coverage across a diverse population, where there may be genetic predisposition to vulnerability or resistance.

Some other naturally low pathogenic *A. marginale* strains have been previously proposed as live vaccines: the Dawn strain from Australia (Bock *et al.* 2003; Carter *et al.*, 2006), and the Yucatan strain from Mexico (Rodriguez *et al.* 2008). However, despite promising early results neither has yet progressed into wider use. In the case of the Dawn strain, it showed greater protection than *A. centrale* against a heterologous Australian strain (Bock *et al.*, 2003), but was not protective against a high pathogenic African strain (Carter *et al.*, 2006). The Mexican Yucatan strain has not been tested against strains from outside Mexico. It increasingly appears that the prospect of a globally effective anaplasmosis vaccine still remains distant.

There are many potential live *A. marginale* vaccines, including UFMG1, that are effective against heterologous strains from the same geographic area, and are likely to have a longer lasting effect than killed vaccines. These could be useful vaccines when targeted to their area of efficacy. But such 'local' vaccines are a poor business investment, and with cattle movement spreading *A. marginale* strains around the world, they are far from guaranteed to stay fully effective.

2.4.4. MSP1a tracking

UFMG1 and Gonen strains established a co-infection in three out of four calves in the UFMG1 group. Infection exclusion doesn't appear to happen between these two strains, suggesting that they do not have overlapping *msp2* pseudogene repertoires (Futse *et al.*, 2008). This ensures that the protection (or lack thereof) measured here is likely to be due to aspects of the immune response other than MSP2 reactivity.

The single calf in the UFMG1 group which did not establish a Gonen infection after challenge is an interesting case. It is unlikely to be due to infection exclusion, as this was not seen in any of the other calves in the group. It is possible that it had an unusually effective immune response to UFMG1 infection (see Chapter 3). This calf should have been re-challenged after the main trial was over, to determine if it was a chance occurrence or a true example of sterile immunity.

The MSP1a tandem repeat sequence is used as a way to track strain identity because it shows sufficient global variation to be a good marker of different strains, but remains constant within both the cattle and tick hosts, and over transmission between them (as reviewed by Cabezas-Cruz *et al.*, 2013). The minor Gonen *msp1a* genotype change seen here in one calf co-infected with *A. centrale* may be only a single nucleotide change, but since the sequencing was done from total PCR product rather than cloned sequences, it was apparently the new predominant sequence in that calf.

The small tandem repeat change upon co-infection is similar to that seen by Bastos *et al.* (2010) in cattle co-infected with UFMG1 and UFMG2. A variant UFMG2 *msp1a* genotype had two tandem repeats with a one nucleotide change leading to one amino acid change. De la Fuente *et al.* (2001) proposed slip-strand mispairing as a mechanism for generating new *msp1a* tandem repeats, with duplications and deletions of entire or partial tandem repeat sequences. Although small, the single base changes seen in this study and in Bastos *et al.* (2010) could also represent an example of the generation of new sequences through small nucleotide changes, which accumulate to contribute to the many tandem repeat sequences characterized to date (Cabezas-Cruz *et al.* 2013). It is interesting to speculate whether co-infection is a particular driver in this variation, as no changes were seen in singly-infected animals. But with only one co-infected calf showing a variant sequence, no firm conclusion can be drawn on this.

2.4.5. Conclusions

UFMG1 infection produced greater side effects than *A. centrale,* casting doubt on its safety as a live vaccine. Moreover, it had a limited effect on symptoms of disease caused by the geographically distant Israeli Gonen strain, suggesting it may not effectively protect cattle against distantly related *A. marginale* strains. The relatively low pathogenicity of the Israeli Gonen strain in this trial makes it impossible to determine if UFMG1 could have reduced fatal disease, but overall it does not appear to be a strong candidate to replace *A. centrale* as a live vaccine against anaplasmosis in Israel.

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CHAPTER 3: SEROLOGICAL RESPONSE TO INFECTION WITH *A. MARGINALE* OR *A. CENTRALE*

3.1. INTRODUCTION

3.1.1. The importance of the antibody response in protection against bovine anaplasmosis

The antibody response plays an important role in protecting cattle from anaplasmosis. Although it must work in conjunction with other parts of the immune system to be protective (Gale *et al.,* 1992), a high antibody response ï and in particular a strong IgG2 response - has previously been identified as a correlate of protection against bovine anaplasmosis after vaccination (reviewed by Palmer *et al.*, 1999).

3.1.2. Characteristics of a protective antibody response

A correlation between the strength of the IgG response to vaccination and subsequent protection from disease has been seen in several studies (Wilson *et al.*, 1980; Tebele *et al.*, 1991; Brown *et al.*, 1998). However, the challenge strains used in these studies were either homologous (Tebele *et al.*, 1991; Brown *et al.*, 1998) or antigenically very similar (Wilson *et al.*, 1980) to the vaccine strains. Many candidate *A. marginale* vaccines are first tested against homologous challenge strains, because heterologous challenge is frequently unsuccessful (Kuttler *et al.*, 1984; Palmer *et al.*, 1994; Carter *et al.*, 2006; Ocampo-Espinoza *et al.*, 2006). Therefore the cross-reactivity of the antibody response to infection with live vaccine strains has hardly been investigated. In this trial, the relative cross-reactivity of the antibody response to UFMG1 and *A. centrale* is likely to be a critical factor in their effectiveness against the heterologous Israeli Gonen challenge strain.

Cattle have two IgG subclasses: IgG1 and IgG2. Several previous studies have shown that an IgG2-biased antibody response is associated with protection from anaplasmosis (Brown *et al.*, 1998; Barigye *et al.*, 2004; Vega *et al.*, 2007). Palmer *et al.* (1999) proposed that the importance of IgG2 was its effectiveness in opsonising bacteria, leading to more rapid clearance of *A. marginale*. Opsonophagocytosis (antibody-enhanced phagocytosis) has been demonstrated *in vitro* with *A. marginale* and immune sera (Cantor *et al.*, 1993; Melendez 2005). In cattle, both IgG1 and IgG2 can increase phagocytosis; however, IgG2 is considerably more rapid and effective at enhancing phagocytosis by neutrophils and peripheral blood monocytes (McGuire and Musoke, 1981).

The main model of protective immunity largely drew on the results of immunization with killed or outer membrane protein (OMP) vaccines (Palmer *et al.*, 1999). Despite the success of *A. centrale* (Bock *et al.*, 2003), relatively little research on correlates of protection against anaplasmosis has been derived from live vaccines. Research on the immune response to *A. centrale* has largely focused identifying conserved antigens between *A. centrale* and *A. marginale* (Agnes *et al.*, 2011), to inform the development of future recombinant protein vaccines. But more basic immunology research on why *A. centrale* is less pathogenic and more protective than *A. marginale* could clarify which properties are most important for an effective vaccine to induce.

3.1.3. Aims

From the clinical results of the live vaccine trial (Chapter 2), *A. centrale* infection caused less serious symptoms than UFMG1 infection, and had more of a protective effect against disease after challenge with the Israeli Gonen strain. The antibody response to infection with UFMG1 and *A. centrale* will be investigated to examine the underlying causes of these differences. In particular, the strength, subclass, and cross-reactivity of the antibody response will be investigated to determine how these factors correlate with protection or pathology. This will provide information on the underlying factors behind the clinical results described in Chapter 2, and increase knowledge of the antibody response to live vaccines against anaplasmosis.

3.2. MATERIALS AND METHODS

3.2.1. Sample collection

For serological tests, blood samples were collected weekly in Serum Separation Vacutainer tubes (Becton Dickinson, Franklin Lakes, New Jersey, USA), and processed according to manufacturerôs instructions. Sera samples were stored at -70 ºC until tested.

3.2.2. Source of *A. marginale* and *A. centrale* antigen material

The *Anaplasma* antigenic material used for ELISA assays was derived from infected blood or tick cell culture. Infected blood came from the splenectomised calves used as sources of inocula in the trial (as described in Chapter 2). Tick cell cultures of *A. marginale* UFMG1 and UFMG2 have been continuously maintained in IDE8 tick cells for approximately 5 years at the Universidade Federal de Minas Gerais, Brazil, and Ludwig-Maximilians-Universität München, using standard culture

methods described by Bastos *et al.* (2010). Tick cell cultures of *A. marginale* Gonen were initiated from a blood sample taken from the splenectomised calf used to provide inoculum for the trial (K.Lis, manuscript in preparation).

3.2.3. Measurement of seroconversion

Seroconversion of infected calves was measured using an anti-MSP5 competitive ELISA (VMRD, Pullman, WA). Samples were tested according to manufacturerôs instructions.

3.2.4. IgG ELISAs

Antigen preparation

IgG levels against *A. marginale* or *A. centrale* were measured by an indirect ELISA using purified rickettsia as the coating antigen, adapted from the protocol described by Shkap *et al.* (1990). The rickettsia were purified from infected blood or IDE-8 tick cell cultures (sources described in 3.2.2.). Purification from blood used a protocol similar to that of Palmer and McGuire (1984). Briefly, blood was collected in EDTA Vacutainers (Becton Dickinson, Franklin Lakes, New Jersey, USA), diluted into an equal volume of cold PBS, and then washed three times in cold PBS by centrifugation at 2600 g for 20 minutes at 4 °C. The buffy coat was removed after each centrifugation. Packed erythrocytes were resuspended in an equal volume of PBS and frozen at -70 °C to lyse the cells. The samples were then thawed at room temperature, and washed in PBS eight times by centrifugation at 23000 g for 15 minutes at 4 °C (Sorvall SS-34 rotor, Thermo), until no visible pink haemoglobin remained in the pellet. The final pellet was resuspended in 20 ml PBS, sonicated at 100 W for 2 minutes, and spun at 500 g for 5 minutes at 4 °C. The supernatant was retained, and pelleted at 5000 g for 10 minutes at 4 °C. This pellet containing purified rickettsia was resuspended in 1ml PBS, and protein concentration measured by Bradford protein assay (Sigma, Munich, Germany).

A. marginale was purified from infected IDE-8 tick cell cultures using a Percoll (GE Healthcare, Uppsala, Sweden) density gradient (K.Lis, manuscript submitted). Briefly, cell cultures at >70 % infection were centrifuged at 3000 g for 20 minutes to pellet infected cells and rickettsia. The pellet was resuspended in Tris-Sucrose buffer (33 mM Tris-HCl, 0.25 M sucrose, pH 7.4), and homogenized for 2 minutes in a Dounce homogenizer to disrupt cells and release the rickettsia. The homogenate was then spun at 200 g for 10 minutes to pellet the larger cell debris, and the supernatant containing rickettsia and smaller cell debris retained. The supernatant was layered over

30 % Percoll diluted in 0.25 M sucrose, and centrifuged at 25000 g for 60 minutes. The upper layer containing cell debris was removed, and the cloudy base layer containing rickettsia retained. The rickettsia were washed twice with PBS (15000 g, 15 minutes), resuspended in a small volume of PBS, and protein concentration measured by Bradford protein assay.

Total IgG ELISA

High protein binding microtiter plates (Nunc, Rochester, New York, USA) were coated with 100 εl of 5 εg/ml *A. marginale* or *A. centrale* antigen diluted in carbonate/bicarbonate buffer pH 9.5, and incubated overnight at 4 °C. After washing 4 times with PBS with 0.05 % Tween 20 (PBS-T), plates were blocked with 200 εl diluent (5 % heat-inactivated horse serum in PBS-T) for 2 hours at room temperature (RT), shaking. 100 εl of each serum sample was then added at a final 1:80 dilution. After incubation and wash steps as before, 100 εl rabbit anti-bovine IgG conjugated to alkaline phosphatase (Thermo Scientific, Waltham, Massachusetts, USA) was added at 1:5000 dilution, and incubated as before. After washing, 100 εl p-nitrophenyl phosphate (pNPP) substrate in diethanolamine buffer (Thermo Scientific, Waltham, Massachusetts, USA) was incubated for 30 minutes, and the reaction stopped by the addition of 50 εl 2N NaOH. Absorbance values were read at 405 nm using a LEDETECT 96 plate reader (Labexim Products, Lengau, Austria).

Subclass IgG ELISA

The protocol for the IgG subclass ELISA was identical to the total IgG ELISA for antigen coating, blocking, and sera addition steps. A dilution series of an IgG reference sample (Bethyl Laboratories, Montgomery, Texas, USA) with known concentrations of IgG1 and IgG2 was also added, to allow the concentration of IgG1 and IgG2 in the test samples to be calculated from the standard curve.

Then 100 εl of sheep anti-bovine IgG1 or anti-bovine IgG2 (Bethyl Laboratories, Montgomery, Texas, USA) diluted to 1:15000 was incubated for 90 minutes at RT before plates were washed 4 times. 100 εl donkey anti-sheep IgG conjugated to horseradish peroxidase was added at 1:100000 and plates incubated and washed as in the previous step. 100 εl of 3,3′,5,5′-tetramethylbenzidine (TMB) substrate (Sigma, Munich, Germany) was added and the reaction allowed to develop for 30 minutes, before being stopped by addition of 100 εl 1N HCl. Absorbance values were read at 450 nm (reference wavelength 690 nm) by a LEDETECT 96 plate reader.

3.2.5. Data analysis

Antibody levels were expressed as either raw absorbance values; fold change, calculated by: absorbance of immune sample divided by absorbance of pre-immune sample; or antibody

concentration (εg/ml), calculated from the standard curve of the IgG reference. The Minitab software package (Minitab Inc., State College, Pennsylvania, USA) was used for statistical analysis. ANOVA and Tukey HSD post-analysis were used to calculate the significance of parameter differences between groups (Yandell, 1997). Linear correlations between parameters were calculated by the Pearson product-moment correlation coefficient.

3.3. RESULTS

3.3.1. Seroconversion

Serum samples from the trial were tested with an anti-MSP5 competition ELISA as a confirmation of infection and an initial assessment of immune response. All calves were seronegative before the trial started. After infection with UFMG1 or *A. centrale*, they rapidly seroconverted, and antibody levels remained high until point of challenge and beyond. When undiluted serum samples were tested (as by manufacturerôs instructions), infection with UFMG1, *A. centrale,* and Gonen induced similar antibody levels, all reaching a maximum inhibition level of around 90 %. To determine whether these samples had reached the maximum inhibition level of the cELISA, samples were diluted 1:64. Under these conditions (shown in Figure 1), UFMG1 and Gonen both induced higher anti-MSP5 total antibody levels than *A. centrale*, although after challenge the anti-MSP5 antibody levels of *A. centrale*-infected calves rose to reach a similar level to UFMG1-infected calves.

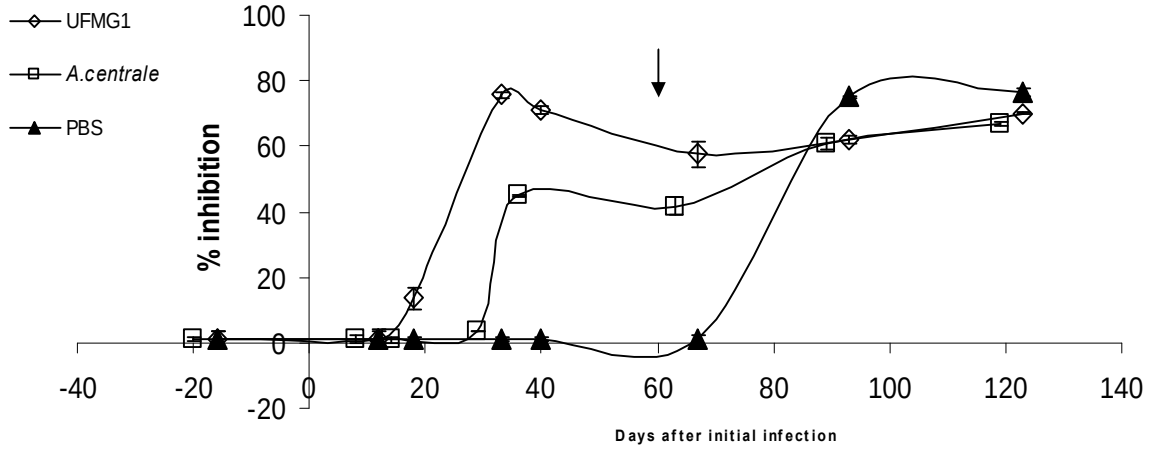

Figure 1: Seroconversion after infection, measured by MSP5 cELISA with samples diluted 1:64. Each data point represents the average value for each group of four calves. Arrow indicates point of challenge with Gonen strain.

3.3.2. Total IgG ELISA development

The ELISA for total levels of IgG against *A. marginale* or *A. centrale* was optimized for several factors: antigen concentration, coating buffer, blocking buffer (horse serum, foetal bovine serum, bovine serum albumin) and serum concentration. An antigen concentration equivalent to 5 εg/ml extracted protein was found to be the best condition for high absorbance values without using excessive quantities of antigen (data not shown). A carbonate/bicarbonate coating buffer (pH 9.5) was slightly more effective than PBS (data not shown). A blocking buffer of 5 % inactivated horse serum was the most effective (data not shown), and produced a sigmoidal serum dilution curve, as shown in Figure 2. To conserve limited serum volumes, a single dilution of 1:80 was selected from the linear portion of the dilution curve, and used for all samples in future assays.

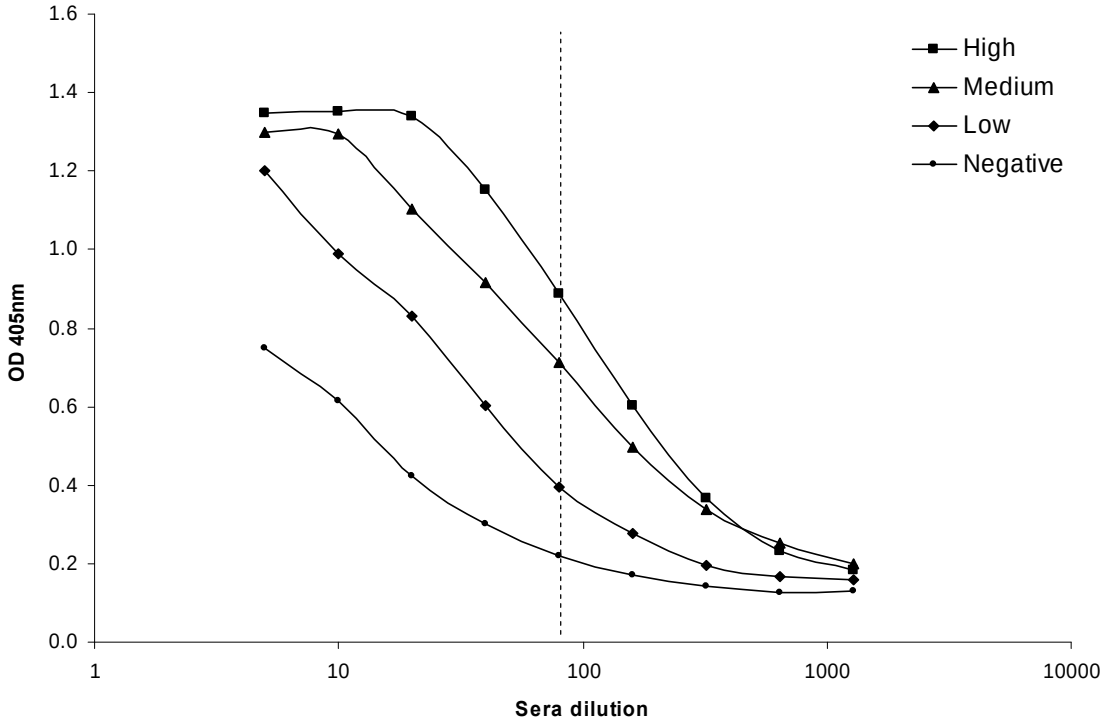

Figure 2: Dilution curve of representative serum samples, using 5 % heat-inactivated horse serum as diluent. Final concentration used for future assays shown by dashed line.

Antigen purified from red blood cells or tick cell cultures gave statistically similar results, as seen in Figure 3. However, *A. centrale* cannot be cultivated *in vitro* and must be derived from red blood cells. Therefore to allow a better comparison between strains, red blood cell-derived rickettsia were used for all assays unless otherwise stated.

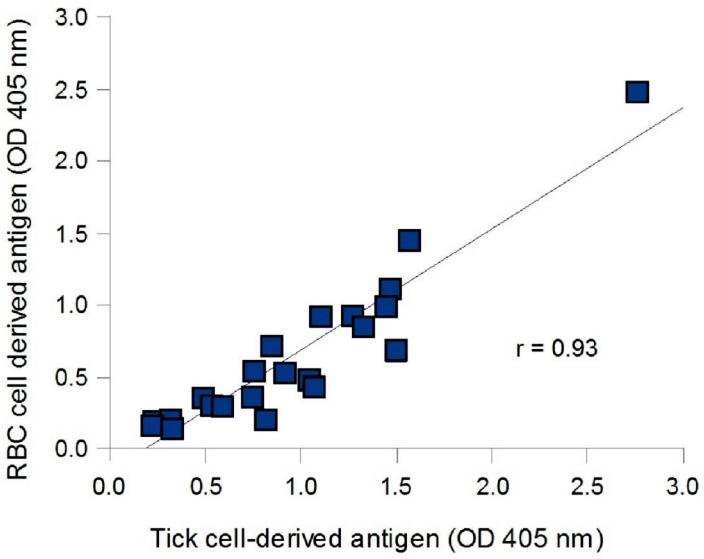

Figure 3: Comparison of tick-cell derived and blood-derived rickettsia (Gonen strain) as IgG ELISA antigen. Correlation was statistically significant (Pearson correlation co-efficient $r=0.93$, $r^2=0.87$, $p<0.01$).

3.3.3. IgG response to the homologous strain

Figure 4 shows the maximum IgG response of each calf after infection with UFMG1, *A. centrale* or Gonen (for the latter, samples were taken from control group post-challenge), when tested against homologous strain antigen. UFMG1 infection had the lowest IgG response (average 3-fold increase above pre-immune levels). The response to Gonen infection was higher (5.1-fold increase), and the *A. centrale* response the highest (7-fold). There was considerably more variation in the latter two groups, each having both high- and low-responding calves.

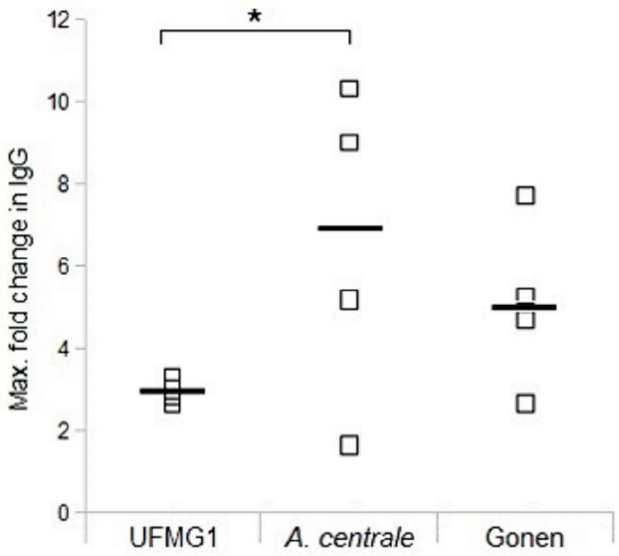

Figure 4: Maximum IgG response against homologous strain antigen after infection with UFMG1, A. centrale, or Gonen. Response to UFMG1 and A. centrale infection were significantly different (= $p<0.05$ for group difference). Bar represents median value for the group.*

3.3.4. IgG response to the challenge strain

Serum samples from all groups were tested for IgG reactivity to the Gonen challenge strain. Maximum anti-Gonen IgG levels during infection with UFMG1, *A. centrale* or Gonen are shown in Figure 5. The antibody response to UFMG1 infection had minimal cross reactivity with the Gonen strain, while the antibody response to *A. centrale* had a significantly higher level of binding to Gonen antigen ($p<0.05$). The cross-reactivity of the IgG response correlated significantly with protection from disease symptoms. The higher the level of cross-reactive IgG against the Gonen strain induced during initial infection with UFMG1 or *A. centrale*, the lower the subsequent drop in hematocrit after challenge with the Gonen strain ($p<0.05$; see Figure 6).

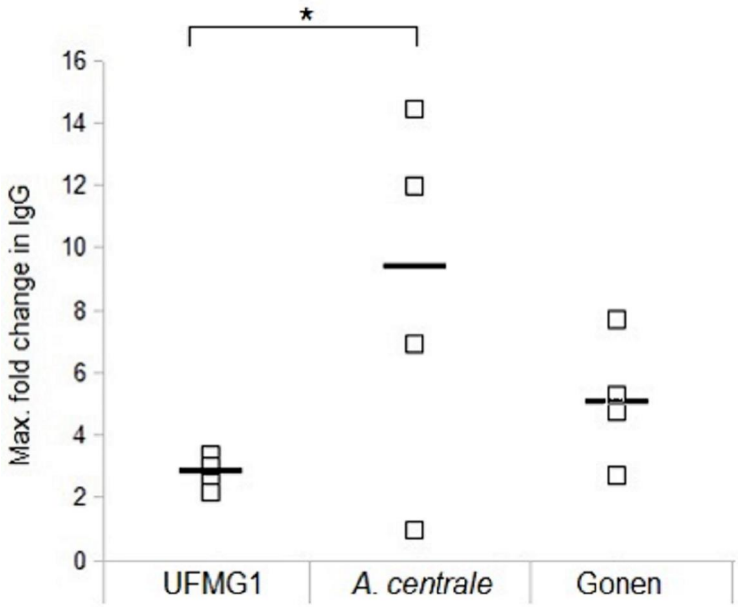

Figure 5: Maximum IgG response against Gonen strain antigen. Cross-reactive response to UFMG1 and A. centrale infection were significantly different (= $p<0.05$ for group difference). Bar represents median value for the group.*

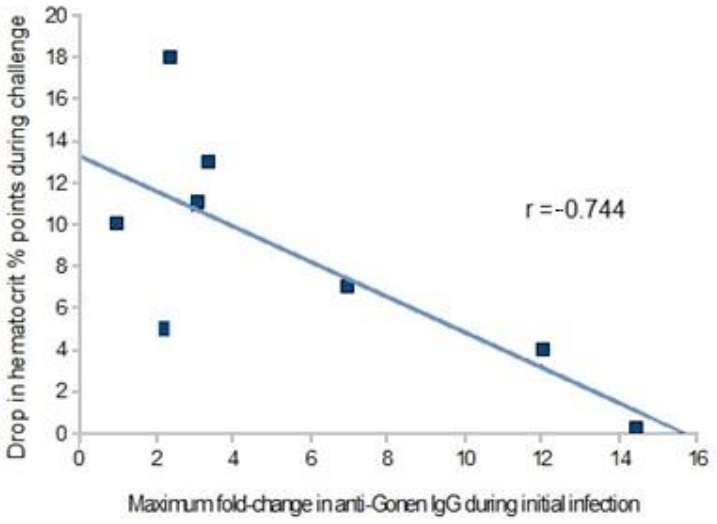

Figure 6: Correlation between the cross-reactivity of the IgG response to UFMG1 or A. centrale (measured by maximum fold change in anti-Gonen IgG during initial infection) and subsequent drop in hematocrit values after challenge with Gonen. Pearson correlation co-efficient $r=-0.744$, $r^2=0.554$, $p<0.02$.

3.3.5. Cross-reactivity of the UFMG1 IgG response against Brazilian vs. Israeli strains

In a previous study of UFMG1 as a live vaccine (Bastos et al, 2010), UFMG1 infection had a protective effect against challenge with the high pathogenic Brazilian strain UFMG2. Therefore the antibody response to UFMG1 in this trial was tested for reactivity against both UFMG2 and Gonen strain antigen. The homologous response to UFMG1 antigen was used as a positive control.

The IgG response after UFMG1 infection had similar reactivity to UFMG1 and UFMG2 antigen. The IgG reactivity to UFMG2 was slightly, but non-significantly, lower than reactivity to the homologous strain UFMG1. In contrast, as seen in Figure 7, IgG cross-reactivity to the Israeli Gonen strain was significantly lower than IgG reactivity to both the Brazilian strains UFMG1 and UFMG2.

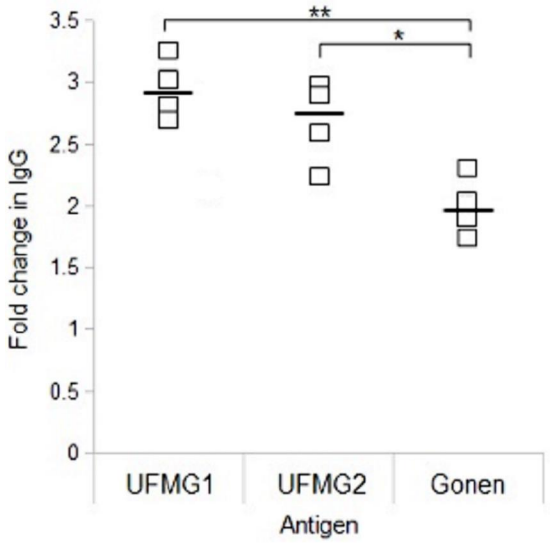

*Figure 7: The IgG response to UFMG1, UFMG2, and Gonen antigen (all derived from IDE8 cell culture). Horizontal bar represents median value. Significance of difference between conditions: *= $p<0.05$; **=$p<0.01$.*

3.3.6. IgG subclass of the antibody response

The time points with the highest total IgG levels after infection with UFMG1, *A. centrale*, or Gonen alone were tested for relative levels of IgG1 and IgG2. As shown in Figure 8, levels of IgG1 and IgG2 were similar for the majority of samples. The *A. centrale* response had more calves with greater IgG2 than IgG1, while the response to the UFMG1 and Gonen strains had more IgG1. However, the differences in IgG1:IgG2 between groups were non-significant. A greater IgG2 concentration and greater IgG2 bias in the response to initial infection were significantly correlated with a reduced loss of red blood cells after challenge ($p<0.05$; see Figure 9), but there was no significant association with temperature or rickettsemia. There was no significant correlation between IgG1:IgG2 or IgG2 concentration in post-challenge serum samples and the clinical response post-challenge (data not shown).

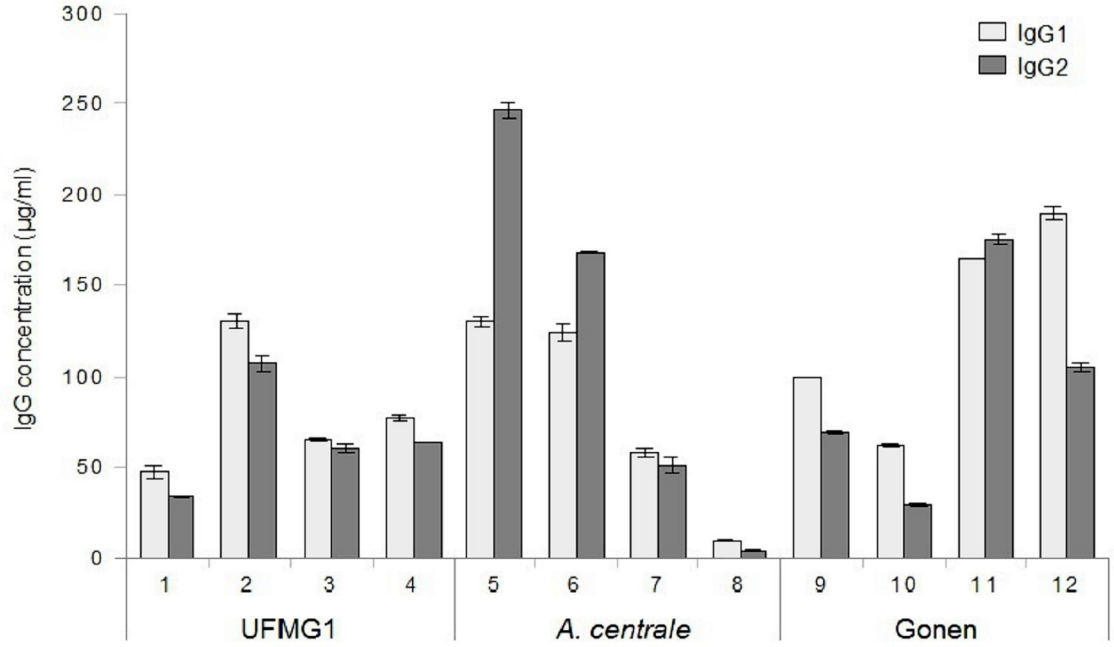

Figure 8: IgG1 and IgG2 antibody responses to infection with UFMG1 (calves 1-4, samples taken from the UFMG1 group before challenge with Gonen), A. centrale (calves 5-8, samples taken from the A. centrale group before challenge with Gonen), or Gonen alone (calves 9-12, samples taken from the control group post-challenge).

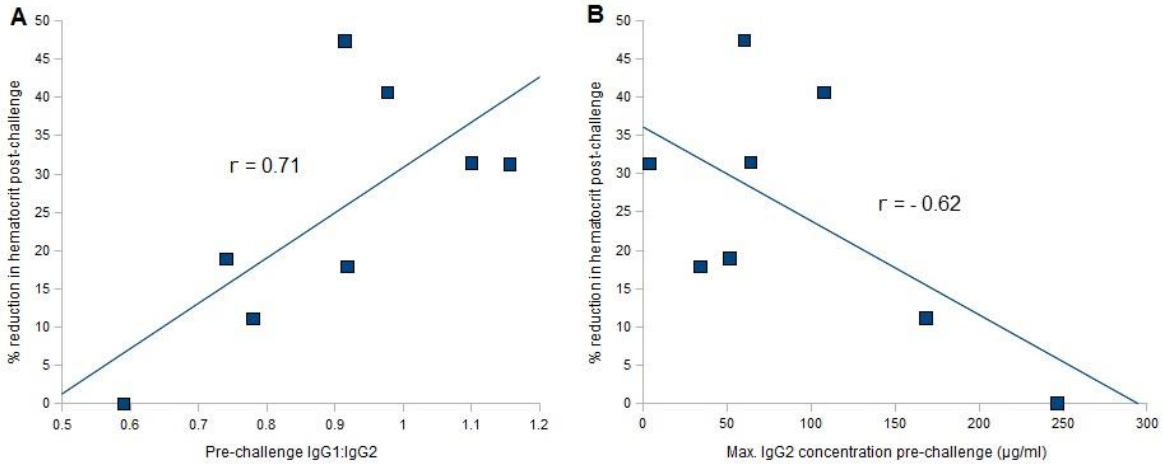

Figure 9: Correlations between the level of red blood cell loss post-challenge and (A) the pre-challenge IgG2 bias (the lower the IgG1:IgG2, the greater the IgG2 bias)($r=0.71$, $p<0.05$) and (B) the pre-challenge IgG2 concentration ($r=-0.62$, $p<0.1$).

3.3.7. Dynamics of the IgG response to infection

The dynamics of the IgG response were measured over the course of initial infection with UFMG1 or *A. centrale,* and subsequent challenge with the Gonen strain. Figure 10 shows the results when serum samples from all groups were tested against Gonen strain antigen.

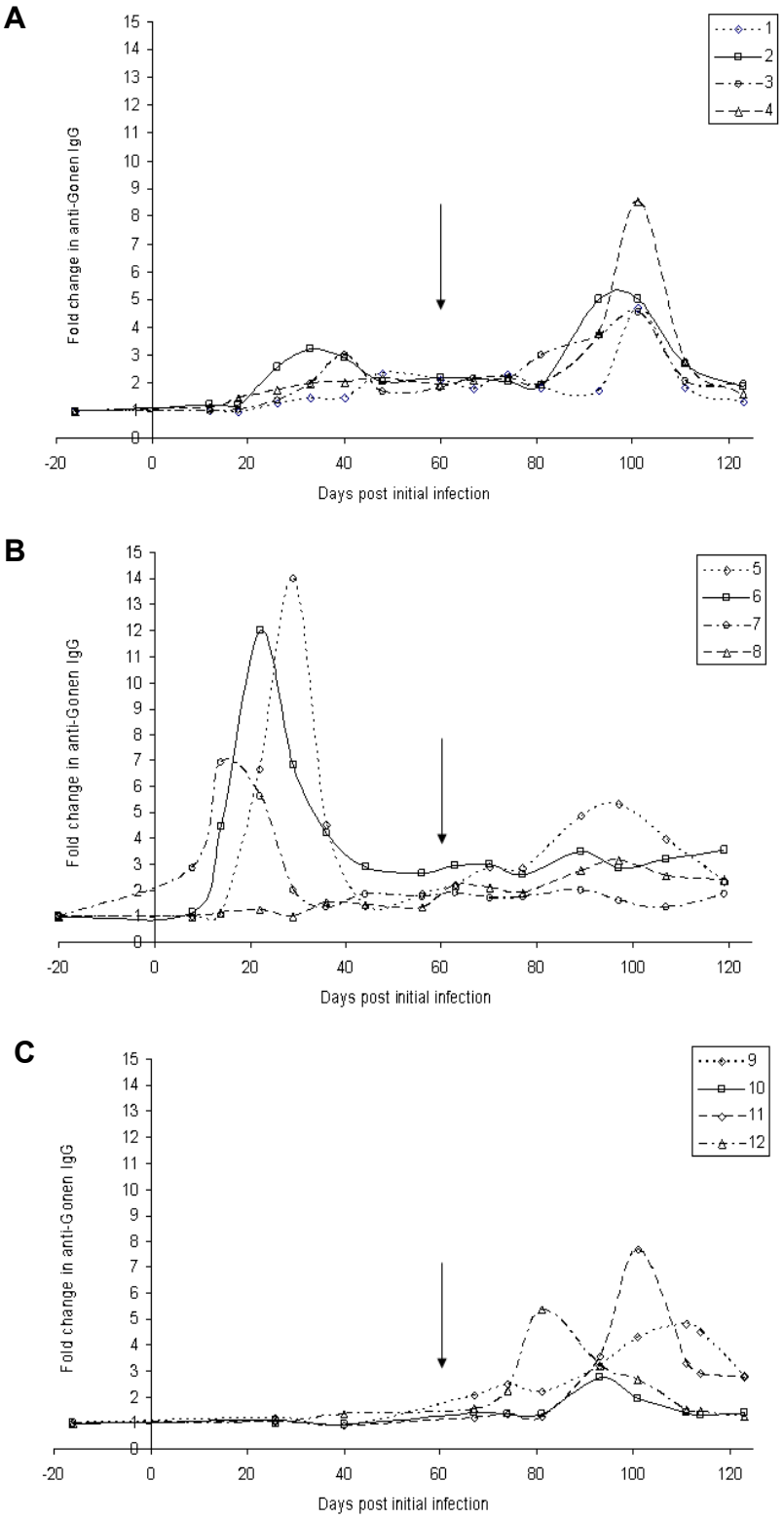

Figure 10: Dynamics of the anti-Gonen IgG response to infection in the (A) UFMG1, (B) A. centrale, or (C) control groups. Arrow indicates the point of challenge with the Gonen strain. Similar IgG response dynamics were seen when UFMG1 and A. centrale group samples were tested against antigen from their respective homologous strains (data not shown).

As shown in Figure 10.A., the cross-reactive IgG response to UFMG1 peaked on average 40 days after infection at an average 2.7-fold increase, declined, and then peaked again at 40 days post-challenge with a 5.7-fold increase. All calves in this group had relatively low cross-reactive anti-Gonen IgG responses after UFMG1 infection, which then increased after challenge with the Gonen strain.

Calves infected with *A. centrale* showed a more variable IgG response, as seen in Figure 10.B. The average fold-change in anti-Gonen IgG in response to *A. centrale* infection was 9.5. One calf had a very rapid response, peaking at 15 days post-infection; two calves peaked at around 25-30 days, and one (with a very low response) at around 35 days. IgG levels increased to a lower level following challenge with the Gonen strain, with an average 3.4-fold rise. Only one calf in this group (calf #8) had higher anti-Gonen IgG levels post-challenge than during initial *A. centrale* infection. This calf also had the most serious symptoms of the *A. centrale* group in response to challenge with Gonen.

The strength of the IgG response to initial infection with UFMG1 or *A. centrale* appeared to influence how the calves responsed to challenge - both the severity of the symptoms of Gonen infection, and the strength of the post-challenge IgG response. Calves which had high IgG responses to initial infection then showed milder symptoms during Gonen infection and lower post-challenge rises in IgG. Calves with low IgG during initial infection had more serious symptoms during Gonen infection and and higher rises in IgG post-challenge. Only one calf (#4 in the UFMG1 group) was an exception to this. It had a very high IgG response post-challenge (four-fold increase) and relatively mild symptoms. However, overall a higher IgG response to initial infection with UFMG1 or *A. centrale,* and then a lower IgG response to subsequent challenge with Gonen was significantly associated with reduced symptoms during Gonen infection (Figure 11; $p<0.1$).

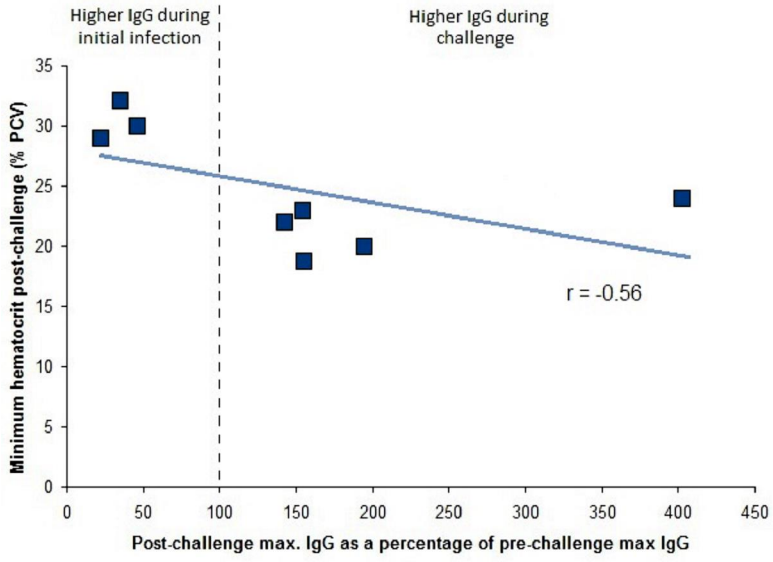

*Figure 11: Correlation between relative pre/post-challenge IgG levels [(post-challenge max.IgG / pre-challenge max. IgG) *100] and severity of challenge response (minimum hematocrit after challenge). r = - 0.56; p<0.1.*

3.4. DISCUSSION

A. centrale gave rise to a highly cross-reactive IgG response which appeared to protect calves without a pronounced secondary rise in IgG levels post challenge. Despite good seroconversion after UFMG1 infection, the level of cross-reactive IgG induced in response to UFMG1 was low. This correlated with a lower level of protection from challenge with the heterologous Israeli Gonen strain.

3.4.1. Seroconversion

The MSP5 cELISA was used to give an early confirmation of seroconversion during the trial. Interestingly, results from the MSP5 cELISA contrasted with those from the total IgG ELISA. The MSP5 cELISA showed higher antibody levels induced by UFMG1 infection compared to *A. centrale* infection, while the IgG ELISA gave the opposite result.

Measuring the IgG response against all major surface proteins, either by ELISA as used here, or by Western blot as in Brown *et al.* (1998), appears to give more valuable information on the likely degree of cross-protection induced by vaccination. MSP5 is a highly conserved protein, demonstrated by the fact that this cELISA can be used to detect antibodies after *A. marginale, A. centrale,* and *A.phagocytophilum* infections (Dreher *et al.*, 2005; Molloy *et al.*, 1999). However, *A. marginale* immunity is highly strain-specific, and antibodies against the conserved MSP5 protein do not appear to correlate well with protection (Agnes et al, 2011).

The cELISA measures all antibody classes. Therefore the higher antibody response it showed could be largely due to IgM. Although IgM is mainly produced in early infection, it can persist for up to 80 days after *A. marginale* infection (Klaus and Jones, 1968; Murphy *et al.* 1966). Therefore IgM is likely to increase cELISA results over the course of the whole trial. However, IgG is the main class of antibody that is produced after secondary exposure to an antigen. Any remaining IgM at the point of challenge is likely to contribute to clearance of *A. marginale.* But when assessing the likely long-term effectiveness of a vaccine, IgG levels are of more interest than IgM.

3.4.2. Cross-reactivity between *A. marginale* strains and *A. centrale*

While the antibody response has been shown to be insufficient for protection on its own (Gale *et al.* 1992), it still appears to be a very important component of a protective immune response. Many previous studies have shown a high antibody response to be associated with protection (Wilson *et*

al., 1980; Tebele *et al.*, 1991; Brown *et al.*, 1998). That association was also seen in this study, with high IgG titers correlating significantly with less severe anemia after challenge.

UFMG1 infection induced only a low level of cross-reactive IgG, and had very little effect on disease caused by the subsequent challenge with the Gonen strain. In contrast, the *A. centrale* vaccine strain induced considerably higher levels of cross-reactive IgG and was significantly more protective (Chapter 2). It is hard to determine whether the higher level of anti-Gonen IgG induced by *A. centrale* infection is principally due to a fundamentally higher IgG response, or to greater cross-reactivity within that response. Both of these factors are likely to contribute to its success as a vaccine.

The importance of a cross-reactive antibody response can be seen in the higher level of IgG cross-reactivity between the Brazilian strains UFMG1 and UFMG2, and significantly lower cross-reactivity between UFMG1 and Gonen. This corresponds to the cross-protection between the two Brazilian strains (Bastos *et al.*, 2010) and the lack of cross-protection between the Brazilian UFMG1 and Israeli Gonen strains seen here.

From the results of this study, broad antibody cross-reactivity appears to be critical for cross-protection between heterologous strains. This suggests that *in vitro* measurement of antibody cross-reactivity between different strains would be a good way of estimating cross-protection before clinical trials.

Past tests of heterologous protection between *A. marginale* strains have often shown very mixed results, ranging from no protection (Ocampo-Espinoza *et al.*, 2006), partial protection (Palmer *et al.*, 1994; Carter *et al.*, 2006) or reasonable reduction of disease (Tebele and Palmer 1991). The better results were generally seen with live vaccines. Palmer *et al.* (1999) proposed that live vaccines stimulated a broader immune response due to exposure to antigenic variants of the immunodominant proteins MSP2 and MSP3 which are generated during infection.

It is curious that *A. centrale* infection induces a more cross-reactive IgG response to the Gonen strain than UFMG1 infection did, as *A. centrale* and Gonen are more distantly related than UFMG1 and Gonen. The high, cross-reactive antibody response is likely to be a principal factor behind why *A. centrale* is a more widely protective live vaccine than low pathogenic or attenuated *A. marginale* strains (Kocan *et al.*, 2003). However, the reasons behind the differing level of antibody response to

A. centrale and *A. marginale* remain unknown.

3.4.3. Subclass of the IgG response

In this study, a higher IgG2 response to initial infection correlated significantly with reduced symptoms after challenge. There were a slightly higher number of calves with an IgG2 biased antibody response after *A. centrale* infection as opposed to UFMG1 or Gonen infection. However, for most calves the differences between levels of the two IgG subclasses were relatively small.

The association of IgG2 and protection agrees with previous studies (Brown *et al.*, 1998; Barigye *et al.*, 2004; Vega *et al.*, 2007). These studies measured the antibody response to immunization with killed or OMP vaccines, rather than after live infection, as was used in this trial. When looking at previous studies on the subclass response after challenge with live *A. marginale,* the picture becomes less clear-cut, as there have been very few studies focusing the subclass of the IgG response to natural infection, and most have had very small sample sizes. Han *et al.* (2010) used two animals - both produced very similar levels of IgG1 and IgG2 in response to infection with South Idaho strain *A. marginale*. Murphy *et al.* (1966) studying natural infection in cattle of varying ages and severity of infection surprisingly found considerably higher levels of 'electrophoretically fast ɔG globulin' (IgG1) compared to 'slow ɔG globulin' (IgG2) during the acute phase of disease, and did not associate IgG subclass with severity of infection.

It appears while an IgG2-biased response is still associated with protection after live vaccines, the IgG2 bias is much less pronounced than that seen with killed or OMP vaccines. This could be due to the T-cell deletion during infection (Han *et al.*, 2010). This would reduce the number of CD4+ T-cells producing IFNɔ, which increases IgG2 production by B-cells (Estes et al, 1994; Estes and Brown, 2002).

A possible alternative explanation is suggested by the results of Vega *et al.* (2007). After vaccination with inactivated rickettsia, the majority of calves produced high IgG2 and low IgG1. However, after challenge, IgG2 titers fell from high pre-challenge levels until IgG1 titers were higher than IgG2. They speculated that this may be due to IgG2 concentrating in the lymph nodes and spleen, where the elimination of *A. marginale* occurs. The proposal that IgG2 could be concentrated in immune tissues during active infection is an interesting one, and may explain the reason for the less pronounced IgG2 bias after live vaccines. Immunohistochemistry on spleen and lymph nodes for IgG subclasses, to see which are actually present and active during the elimination

of *A. marginale,* would be a way to confirm this theory.

3.4.4. Overall antibody levels and possible link to T-cell function and regulation

The considerably higher level of IgG production in response to *A. centrale* infection compared to UFMG1 and Gonen is likely to contribute to its success as a vaccine. As previously mentioned, a model of immunity against *A. marginale* suggests a central role for antigen-specific CD4+ T-cells and their IFNɔ production to stimulate antibody class switching of B-cells, causing high levels of IgG production, and in particular increased IgG2 (Palmer *et al.*, 1999). Differences in CD4+ T-cell function and their level of IFNɔ production, would therefore be a likely candidate to correlate with differences in IgG levels between and within groups; investigating this possibility is the subject of Chapter 4. *A. marginale* infection can down-regulate high pre-challenge, recombinant vaccine-induced CD4+ T-cell responses (Abbott *et al.*, 2005; Han *et al.*, 2008). Han *et al.* (2008) proposed this to be due to deletion of antigen-specific T-cells after overstimulation during infection. This phenomenon has not been investigated in *A. centrale* infection ï if it does not occur, this could be a central factor behind the higher antibody response seen here in the *A.centrale* group.

There was a smaller difference in IgG levels between *A. marginale* strains, with UFMG1 infection stimulating lower IgG levels than the Gonen strain. This may be due to UFMG1 being maintained in tick cell culture for a number of years before inoculation into the splenectomized calf. Bastos *et al.* (2010) found no significant difference in the level of protection provided by tick cell- or blood-derived UFMG1, but in that trial tick cell cultures were used less than one year after initialization from blood stabilate, as opposed to more than 5 years in this trial. It is possible that prolonged tick cell culture may have had an enduring effect on UFGM1 gene expression. For example, MSP1a is an important antigen for both cellular and humoral responses (Brown *et al.*, 2001; Palmer and McGuire, 1984). *A. marginale* expresses *msp1*Ŭ at a higher level in blood compared to tick cell culture (Garcia-Garcia *et al.*, 2004), and blood-derived *A. marginale* stimulates higher levels of anti-MSPa IgG than tick cell-derived *A. marginale* (de la Fuente *et al.,* 2002). Therefore if prolonged tick cell culture did have a lasting effect on UFMG1 gene expression, the differentially expressed proteins in tick cell-derived rickettsia could influence their immunogenicity and effectiveness as vaccines.

3.4.5. Variation in response to infection

The level of disease caused by the *A. marginale* strains and *A. centrale* varied considerably within each group, both during initial infections and after challenge. The severity of symptoms after challenge correlated to very variable levels of *Anaplasma*-specific IgG production during initial infection with UFMG1 or *A. centrale*. A variable response to vaccination against anaplasmosis is frequently seen in research studies (Brown *et al.*, 1998; Morse *et al.*, 2012) and in the field (Bock and de Vos, 2001), and was suggested by Brown *et al.* (2003) to reflect genetic differences between cattle. This can influence the response to multiple diseases, as can be seen on a large scale by the differing susceptibility of *Bos indicus* and *Bos taurus* to tick-borne disease (Morris, 2007).

3.4.6. Dynamics of the IgG response to initial infection and challenge

The association of protection with a reduced secondary IgG response after challenge is interesting, as in the classical model of immunity the secondary immune response should be higher and more rapid than the primary response (Siegrist, 2012). Live vaccines tend to lead to more prolonged antibody responses due to continual exposure to the antigen. But for most calves in this trial there was a clear decline in IgG levels after the initial infection, followed by a distinct secondary peak in IgG after challenge.

This pattern of higher antibody responses post-challenge being associated with lack of protection was also described by Wilson *et al.* (1980). They noted strong antibody responses to initial infection with an Australian *A. marginale* strain or *A. centrale.* However, after subsequent challenge with a heterologous Australian strain, those calves which were protected from disease showed relatively weak secondary rises in antibody level. Barigye *et al.* (2004) also noted this as occurring after successful vaccination with inactivated *A. marginale*: high antibody titers were induced, which dropped after the last inoculation and did not increase again after challenge with live *A. marginale*.

I would speculate that in protected cattle, the infection is rapidly controlled before it reaches a level that would stimulate a strong secondary immune response. In contrast, a lack of protection would lead to uncontrolled infection after challenge, which then causes the host immune system to respond with a pronounced rise in antibody levels. This model was described for malignant catarrhal fever by Russell *et al.* (2012), and complements the clinical results of this study (see Chapter 2). After challenge with the Gonen strain, calves protected from disease had only minimal rises in rickettsemia and low to no increase in IgG levels. Presumably the circulating antibodies after initial infection were of a sufficient level and specificity to control the infection with the challenge strain

before it reached a sufficient level to provoke a strong secondary immune response. In contrast, in unprotected animals the circulating antibody was insufficient to contain the infection, and rickettsemia increased, triggering a secondary immune response and leading to the high IgG levels seen post-challenge. A caveat to this potential explanation is that it does not fit for one calf: after challenge this animal (calf #1; UFMG1 group) was PCR-negative for Gonen infection and had no symptoms of disease (see Chapter 2) but still had a post-challenge IgG response of a similar level to other calves in the group. This case of apparently sterile immunity does not fit the pattern of controlled infection leading to lower antibody responses. Unfortunately since sterile immunity against anaplasmosis is extremely rare (Kocan *et al.*, 2003), there is no information on the typical immune responses in such cases.

3.4.7. Conclusions

In conclusion, a high, cross-reactive, and IgG2-biased antibody response to initial infection was associated with protection after challenge, without a strong secondary IgG response upon challenge being necessary. Prior UFMG1 infection had only a limited protective effect on disease caused by the heterologous Israeli Gonen strain. The low pathogenicity of the Israeli Gonen strain in this trial makes it impossible to determine if UFMG1 could have reduced fatal disease. However, the limited cross-reactivity of the IgG response to UFMG1 suggests that it would also be less protective than *A. centrale* against a more pathogenic challenge strain.

The high levels of cross-reactive IgG induced by *A. centrale* infection are likely to be a critical factor in its success as a vaccine in a wide range of countries. However, further research is needed to characterize the factors causing the differing antibody responses, and to determine if these factors can be harnessed for other vaccines.

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CHAPTER 4: CELL-MEDIATED IMMUNE RESPONSE TO INFECTION WITH *A. MARGINALE* OR *A. CENTRALE*

4.1. INTRODUCTION

4.1.1. Cell-mediated immunity also plays a role in protection from bovine anaplasmosis

Antibody is an important part of host defence against bovine anaplasmosis (Palmer *et al.*, 1999). But several experiments have demonstrated that on its own, antibody is not sufficient to prevent disease. Firstly, when persistently infected calves are immunosuppressed by splenectomy (Jones *et al.*, 1968) or drugs (Kuttler and Adams, 1977), there is a rapid relapse in symptoms, which begins even before circulating antibody levels fall significantly. Secondly, although calves borne to immune dams have reduced symptoms compared to those borne to non-immune dams, they are not completely protected by colostral transfer of *Anaplasma*-specific antibodies (Zaugg and Kuttler, 1984). Finally, serum from a hyper-immune protected steer did not convey passive protection to susceptible calves (Gale *et al.*, 1992). Therefore protection against bovine anaplasmosis is not solely mediated through humoral immunity.

Multiple studies have shown that cell-mediated immunity also plays a role in the response to anaplasmosis. In early experiments, development of protection correlated with inhibition of leukocyte migration (Buening, 1976), and with cutaneous hypersensitivity (Carson *et al.*, 1976). These results are consistent with a role for T-cells and macrophage activation respectively (Palmer *et al.*, 1995).

More specifically, Brown *et al.* (1998a) demonstrated that after vaccination with *A. marginale* OMPs, protected calves had CD4+ T-cells with high levels of *in vitro* antigen-specific proliferation and IFNɔ production. Palmer *et al.* (1999) proposed that IFNɔ produced by CD4+ T-cells might play a central role in the immune response to anaplasmosis: by stimulating B-cells to produce high levels of IgG2 (Estes et al, 1994; Estes and Brown, 2002), and by activating macrophages to increase their phagocytosis and killing of rickettsia (Adler *et al.*, 1994; Stich *et al.*, 1998). Multiple T-cell epitopes have been identified in *A. marginale* MSPs (Brown *et al.*, 1998; Shkap *et al.*, 2002; Lopez *et al.*, 2008). However, the majority of *A. marginale* T-cell research has focused on the response to killed, OMP, or recombinant protein vaccines. Studies of the T-cell response to natural *A. marginale* infection suggest that CD4+ T-cells and IFNɔ may not play such a central role in the

immune response to live vaccines.

4.1.2. Cell-mediated immune responses to live vaccines

High levels of *A. marginale*-specific T-cells induced by OMP or recombinant protein vaccination fall sharply after infection with live *A. marginale* (Abbott *et al.*, 2005; Bautista-Garfias *et al.*, 2003; Han *et al.* 2008 and 2010). This reduction in CD4+ T-cell numbers has been proposed to be an immune evasion mechanism by *A. marginale* to facilitate the development of persistent infection (Han *et al.*, 2010). Curiously, CD4+ T-cell depletion of thymectomized calves did not prevent them from controlling *A. marginale* infection, and they showed no significant difference in disease severity compared to untreated calves (Valdez *et al.*, 2002). There is a caveat to this study, in that CD4+ T-cell depletion was not absolute (as this is very difficult to achieve).

Gale *et al.* (1997) treated four calves with a monoclonal antibody that reduced IFNɔ in peripheral blood to undetectable levels; there was no significant effect on the severity of their symptoms after *A. marginale* infection. This suggests that IFNɔ is not as critical to protection as suggested by Palmer *et al.* (1999), although it is possible that IFNɔ could have persisted undetected in treated animals, for example in the lymphoid tissues.

Taken together, these experiments suggest that the T-cell response may not have such a significant role in protection with live vaccines as it does with killed, OMP, or recombinant vaccines (Brown *et al.*, 1998; Palmer *et al.*, 1999). To date, the T-cell response to the most common live vaccine, *A. centrale,* has been largely unstudied. Gale *et al.* (1996) found that peripheral blood mononuclear cells (PBMCs) from an *A. centrale*-infected calf proliferated in response to both *A. centrale* and *A. marginale* antigen preparations. However, this was only one time point from one splenectomised calf, taken after antibiotic treatment had reduced the infectious burden. Therefore it is uncertain if a more typical *A. centrale* infection would lead to the reduction in antigen-specific T-cells that is seen with *A. marginale* infection. If the T-cell response to *A. centrale* is not inhibited, the resulting increased 'help' to B-cells (Brown *et al.*, 1998; Grant *et al.*, 2012) could contribute to the higher antibody response to *A. centrale* that was reported in the previous chapter.

4.1.3. Aims

This chapter describes the measurement of cell-mediated responses, specifically PBMC proliferation and IFNɔ production, during infection with *A. marginale* or *A. centrale.* Any relationship to protection from disease would provide an insight into the role of cell-mediated immunity in the response to live vaccines against bovine anaplasmosis.

4.2. MATERIALS AND METHODS

4.2.1. Sample collection and processing

IFNɔ levels were measured in plasma: the plasma fraction was taken from blood collected into EDTA Vacutainers (Becton Dickinson, Franklin Lakes, New Jersey, USA), then stored at -70 ºC.

For PBMC purification, blood was collected in a heparin Vacutainer (Becton Dickinson, Franklin Lakes, New Jersey, USA). Blood was diluted 1:1 with PBS, layered carefully over 1.084 g/ml Ficoll Premium Plus (GE Healthcare, Uppsala, Sweden) in a 3:1 ratio, and spun at 900 g for 45 minutes at 18 °C. The resulting cloudy PBMC layer was carefully collected and washed in PBS to remove any remaining Ficoll. Contaminating erythrocytes were lysed by osmotic shock, induced by resuspending the cell pellet in 5 ml 10 % PBS for 20s. 45 ml PBS was then added to restore osmolarity and the cells washed and resuspended in RPMI 1640 media (Biochrom AG, Berlin, Germany). Cells were counted and either used directly in a proliferation assay, or prepared for freezing by resuspension in a freezing media of FBS with 5 % DMSO to a final cell concentration of approximately $1x10^7$ cells/ml. Cryovials were slowly cooled to -70 ºC in a Mr Frosty freezing container (Thermo Scientific, Waltham, Massachusetts, USA) for at least 24 hours, before being transferred to liquid nitrogen for long-term storage.

4.2.2. PBMC proliferation assays

PBMCs were either tested directly after isolation from blood (for two time points, one pre- and one post-challenge with the Gonen strain) or after cryopreservation (8 time points spread throughout the trial). Cryopreserved samples were thawed rapidly in a 37 °C water bath, diluted in RPMI 1640 media and centrifuged at 500 g for 5 min to remove the DMSO cryopreservative. The pellet was resuspended in RPMI 1640, cell concentration and viability measured, and cells resuspended at the required final concentration (see Results) for the assay.

Proliferation assays were set up with 2.5 or $5x10^5$ PBMCs per well in a 200 µl total volume, in a 96-well U-bottom plate (TPP, Trasadingen, Switzerland). Cells and antigen were diluted in RPMI 1640 supplemented with 10 % FBS, 2 mM L-glutamine, 100 U penicillin / 100 µg/nl streptomycin, and 50 µM beta-mercaptoethanol. *A. marginale* and *A. centrale* antigen was prepared from infected red blood cells or IDE-8 tick cell culture as described in Chapter 3. Cells were incubated with antigen at varying concentrations (see Results). The mitogen concanavalin A (ConA; Sigma, Munich, Germany) was used as a positive control at 1 µg/ml (fresh PBMCs) or 5 µg/ml (cryopreserved PBMCs). Following 6 days incubation at 37 °C and 5 % CO_2, bromodeoxyuridine (BrdU) (Roche, Mannheim, Germany) at a final dilution of 1:1000 was incubated with cells for a

further 8 hours. The cells were transferred to a flat-bottomed 96 well plate (TPP, Trasadingen, Switzerland), and centrifuged at 300 g for 10 minutes. Supernatants were carefully removed and frozen at -70 °C for later testing for IFNɔ production. Cells were fixed onto the 96-well plate by heating at 60 °C for one hour. A BrdU cell proliferation ELISA (Roche, Mannheim, Germany) was used to detect BrdU incorporation into proliferating cells, according to manufacturerôs instructions. Final levels of BrdU incorporation were indicated by OD_{450} absorbance, read by a LEDETECT 96 plate reader (Labexim Products, Lengau, Austria).

The stimulation index (SI) of different conditions was calculated by:
OD_{450} (stimulated cells) ÷ OD_{450} (media control cells).

4.2.3. IFNγ ELISA

IFNɔ levels were measured using a bovine IFNɔ ELISA kit (MABTECH, Nacka Strand, Sweden), according to manufacturerôs instructions. Briefly, a high protein binding ELISA plate (Nunc, Rochester, NY, USA) was coated with an anti-bovine IFNɔ mAb diluted to 2 µg/ml in PBS, and plates incubated overnight at 4 °C. They were then washed twice with PBS, and blocked for 1 hour at room temperature with diluent (0.05 % Tween20 and 0.1 % bovine serum albumin in PBS). After blocking, plates were washed 5 times with wash buffer (0.05 % Tween20 in PBS). Undiluted plasma samples or the IFNɔ standard (dilution range of 50-500 pg/ml in diluent) were added to wells, and incubated for 2 hours. Plates were washed as before, 100 µl of 0.25 µg/ml of a different anti-bovine IFNɔ mAb in diluent added, and incubated for 1 hour. Plates were washed again, 100 µl streptavidin-HRP added (at 1:1000 dilution) and incubated for 1 hour, and washed again. Finally, 100 µl 3,3N,5,5N-tetramethylbenzidine (TMB) substrate (Sigma, Munich, Germany) was added, and incubated for 30 minutes, before being stopped by 100 µl 1N HCl. Plates were read at 450 nm with a reference wavelength of 690 nm by a LEDETECT 96 plate reader (Labexim Products, Lengau, Austria). The results of the IFNɔ standard was used to draw a standard curve, from which the IFNɔ concentration in plasma samples was calculated.

4.2.4. Statistical analysis

The Minitab software package (Minitab Inc., State College, Pennsylvania, USA) was used for statistical analysis. ANOVA and Tukey HSD post-analysis were used to calculate the significance of parameter differences between groups (Yandell, 1997). Linear correlations between parameters were calculated by the Pearson product-moment correlation coefficient.

4.3. RESULTS

4.3.1. Optimization of freezing procedure and PBMC proliferation assay

To determine the optimum conditions for PBMC cryopreservation, various cell concentrations ($1x10^6$, $1x10^7$, $1x10^8$ cells/ml) and freezing media (FBS + 5 % DMSO; FBS + 10 % DMSO; horse serum + 5 % DMSO) were tested. Freezing cells at $1x10^7$ cells/ml in FBS + 5% DMSO consistently gave the best viability and proliferation in response to ConA after thawing (data not shown). Therefore this protocol was used for trial samples.

Conditions of the PBMC proliferation assay were optimized, namely: incubation time (4, 5, or 6 days), cell number ($2.5x10^5$ or $5x10^5$ cells/well), assay volume (100 or 200 µl/well), and the effect of a resting period between thawing and antigen stimulation. Figure 1 shows the effect of assay incubation period on the proliferation of cryopreserved cells in response to 5 µg/ml ConA. Although the difference was not significant, 6 days incubation consistently gave slightly higher values.

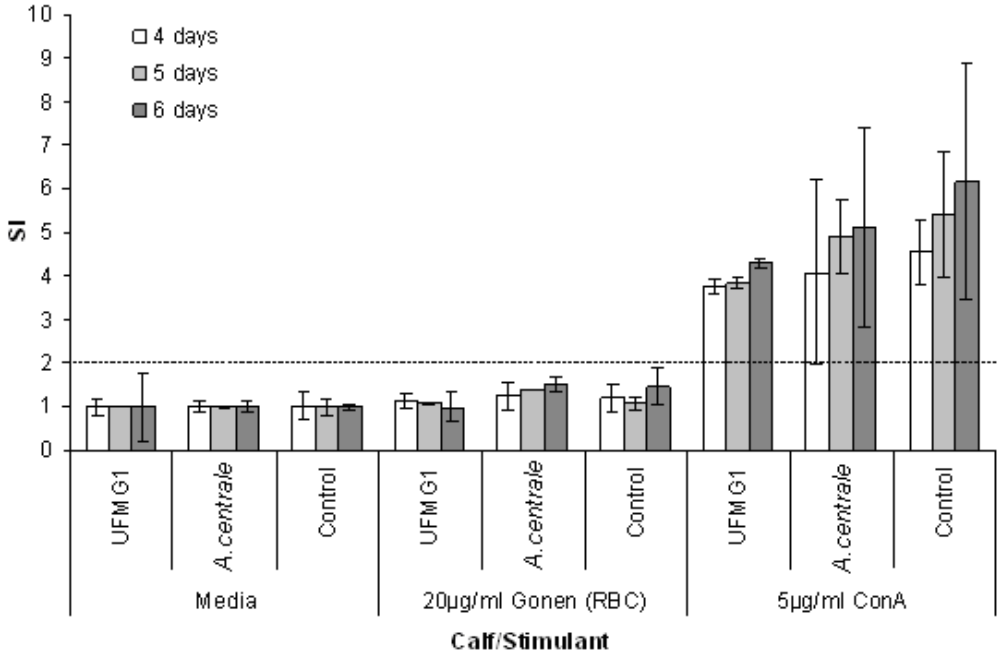

Figure 1: Optimization of incubation period for PBMC proliferation assay with cryopreserved cells. A PBMC sample from one calf of each group was used, taken 56 days post-challenge. Similar results were seen from cryopreserved naïve cells. Dashed line shows SI=2, the lowest cut-off for a positive result.

There was around a 20 % increase in proliferation in response to 5 µg/ml ConA when $5x10^5$

cells/well were used rather than $2.5x10^5$ cells/well (data not shown). An assay volume of 200 µl/well gave consistently higher results than 100 µl (data not shown). A rest period between thawing and stimulation of cells did not consistently increase proliferation (data not shown), and so was not used. Final conditions for subsequent proliferation assays were therefore set at: no rest period after thawing of cryopreserved PBMCs, which were then incubated with antigen or ConA for 6 days, at $5x10^5$ cells/well in a total volume of 200 µl.

4.3.2. Proliferation of fresh PBMCs

Fresh PBMCs were tested for antigen-specific proliferation at two time points during the trial: one during initial infection shortly before challenge with the Gonen strain, and one 3 weeks post-challenge.

As the initial infection assay was the first time that immune samples were available to test the antigen-specific response to *A. marginale,* multiple antigen concentrations were used: 1, 5, and 10 µg/ml. A stimulation index (SI) value of 2 or greater (i.e. at least twice the level of proliferation as seen in the media control) was considered a positive result (Brown *et al.*, 1998).

The initial infection assay (8 weeks post-infection) showed all samples were viable and proliferated in response to ConA, although often at a relatively low level (data not shown). No proliferation was seen in response to *A. marginale* UFMG1 or *A. centrale* antigen at 1, 5, or 10 µg/ml (data not shown). *A. marginale* Gonen antigen was not available for testing due to a shortage of infected blood pre-challenge (this strain was not established in cell culture until after the trial was completed).

Post-challenge PBMCs were tested against UFMG1, *A. centrale,* and Gonen antigen at higher concentrations (10 and 20 µg/ml). Positive proliferation responses were seen in 2 calves of the UFMG1 group, 3 calves of the *A. centrale* group, and all calves of the control group (Figure 2). Overall values were still low (SIs of 2-4), but the highest proliferation values were seen in response to the Gonen antigen. Of this, the highest SIs were seen in the control group (calves 9-12): at the point of testing (pre-patent period of challenge), the control group were the only calves to have reached a mean rickettsemia over 0.1 %. However, SI differences between groups were not statistically significant.

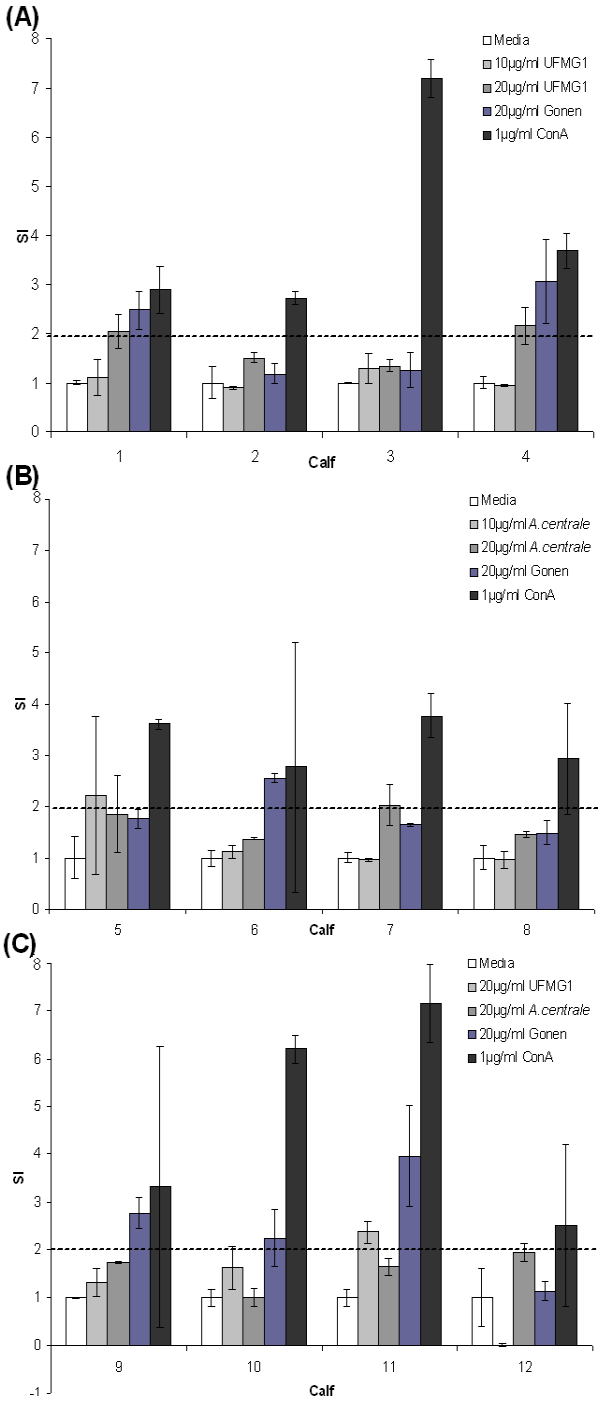

Figure 2: Proliferation response of freshly-isolated PBMCs in response to A. marginale and A. centrale antigen, with samples taken 3 weeks post-challenge with the Gonen strain. (A) UFMG1 group; (B) A. centrale group; (C) control group. PBMCs from UFMG1 and A. centrale groups were tested against 10 and 20 µg/ml of their respective homologous strain antigen, and 20 µg/ml Gonen strain antigen. PBMCs from the control group were tested against 20 µg/ml UFMG1, A. centrale, and Gonen strain antigens. Dashed line represents SI=2, the lowest cut-off for a positive result.

4.3.3. Proliferation of cryopreserved PBMCs

Viability of cryopreserved PBMC samples

PBMC samples were cryopreserved at fortnightly intervals over the course of the trial, and stored in liquid nitrogen until transported on dry ice in two batches from Israel to Munich. Samples were then stored long-term in liquid nitrogen. On resuscitation of the PBMC samples, cells appeared viable by

trypan blue exclusion, but the majority did not proliferate in response to the positive control mitogen ConA. For all time points available, a sample from each of the three experimental groups was tested. As shown in Figure 3, only two time points showed consistent proliferation in response to ConA.

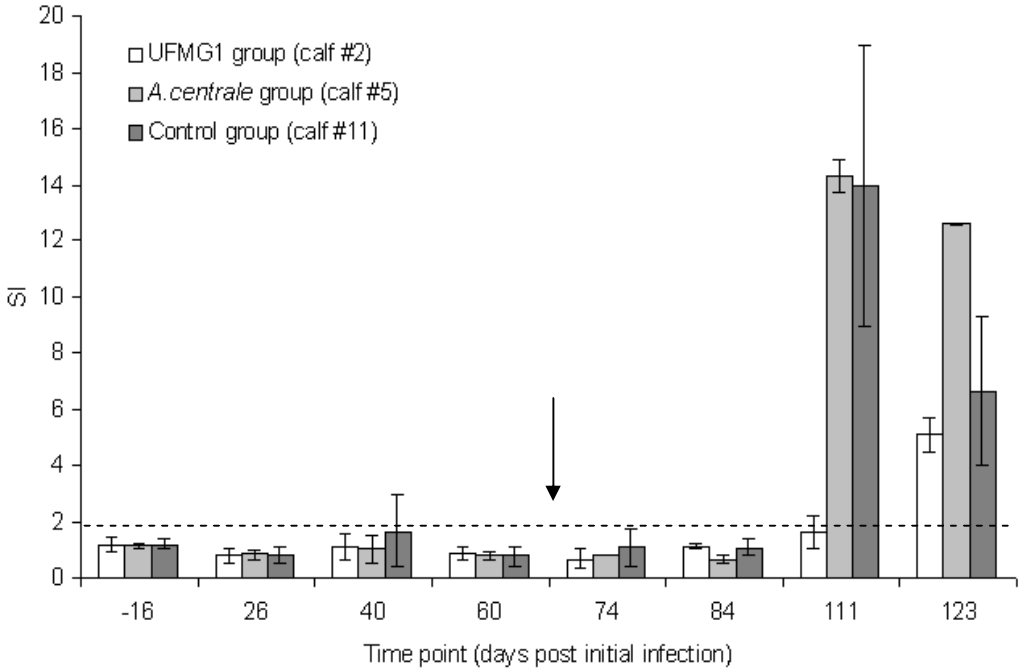

Figure 3: Proliferation of cryopreserved PBMCs from different time points throughout the trial, in response to the mitogen ConA (5 µg/ml). Cryopreserved PBMCs from one calf per group were tested for all time points. Dashed line indicates SI=2, the lowest cut-off for a positive result; the arrow indicates point of challenge with Gonen strain, 60 days after initial infection with UFMG1 or A. centrale.

Analysis of PBMC samples capable of proliferation

PBMCs from the two time points which showed proliferation in response to ConA were tested further for antigen-specific proliferation and IFNɔ production. From the proliferation of fresh cells, 20 µg/ml antigen gave the best results. This was the highest concentration previously tested with fresh PBMCs ï therefore a range of higher concentrations of Gonen antigen from 20-100 µg/ml were tested against cryopreserved samples from 56 days post-challenge (Figure 4). None of the concentrations gave a positive proliferative response.

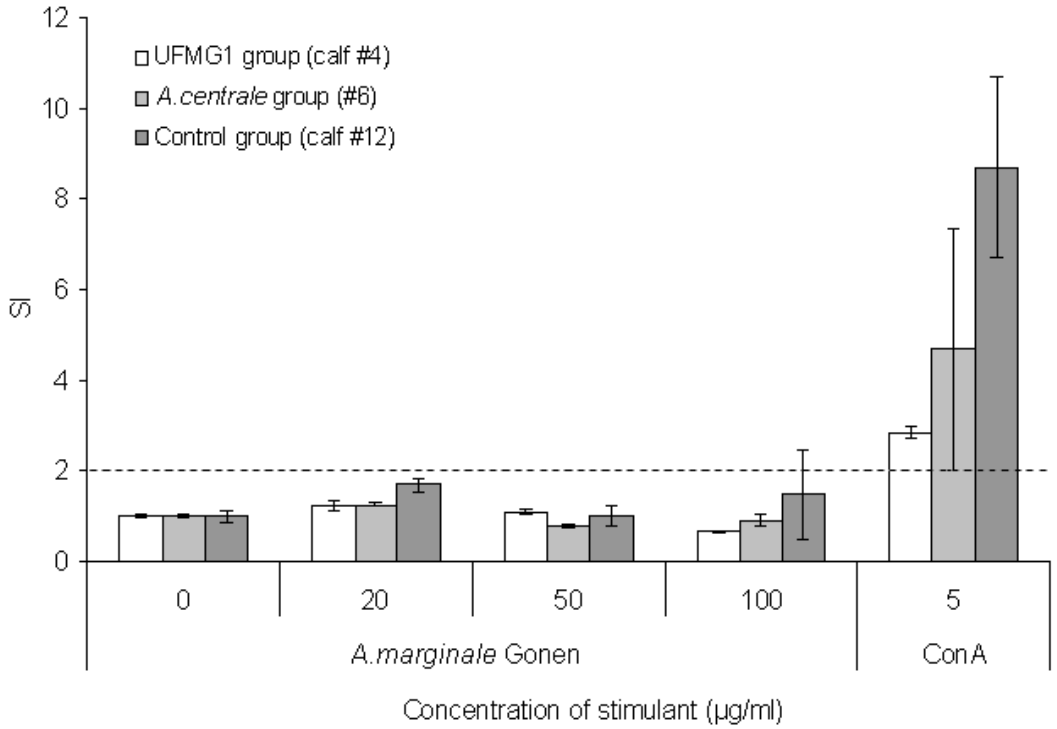

Figure 4: Effect of A. marginale Gonen antigen concentration on proliferation of cryopreserved PBMCs. PBMCs from one calf per group were tested. Dashed line indicates SI=2, the lowest cut-off for a positive result.

Therefore, as it was successful with fresh PBMCs, 20 µg/ml antigen was used for testing all samples. Results are shown in Figure 5. The first sample date, 44 days post-challenge (Figure 5.A.), showed a low level of antigen-specific proliferation for 2 calves of the UFMG1 group. The second sample date, 56 days post-challenge (Figure 5.B.), had no calves with detectable antigen-specific PBMC proliferation.

Proliferation in response to ConA varied considerably between different calves, and between the two time points. Several calves had only very low levels of proliferation after ConA stimulation, and one calf (from the 44 day post-challenge time point) even gave a negative result (i.e. SI < 2).

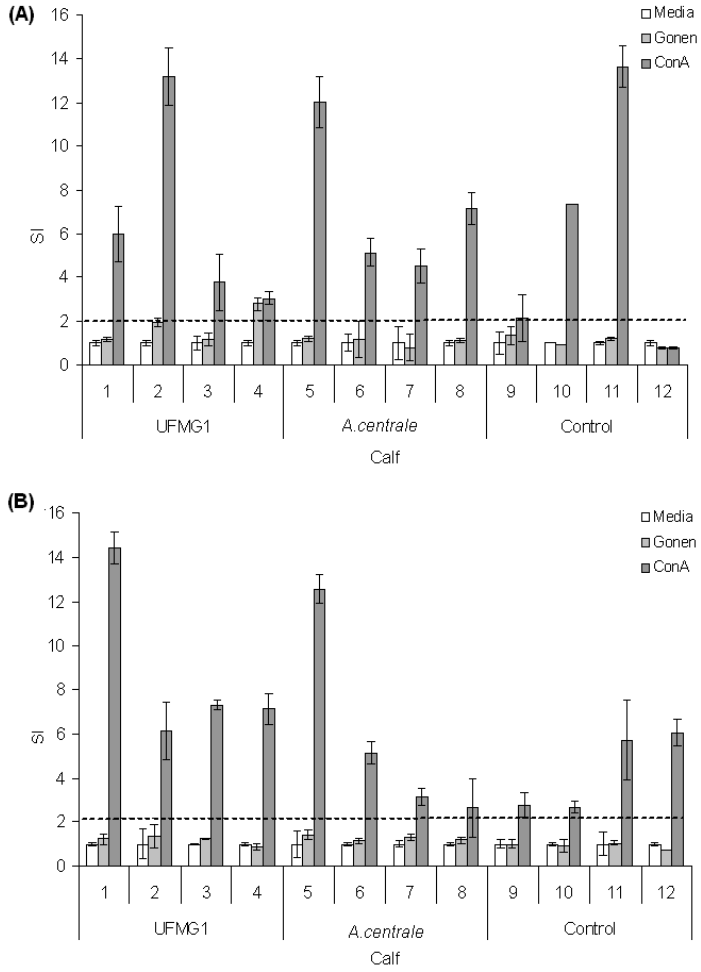

Figure 5: Proliferation of cryopreserved PBMCs post-challenge in response to A. marginale Gonen antigen. (A) 44 days post-challenge. (B) 56 days post-challenge. Dashed line indicates SI=2, the lowest cut-off for a positive result.

4.3.4. IFNγ production *in vitro* and *in vivo*

4.3.4.1. *In vitro* IFNγ production in antigen stimulation assays

IFNγ production by fresh PBMCs

IFNɔ production by fresh PBMCs (Figure 6) in response to antigen or ConA was high - however, PBMCs incubated with media alone also produced high levels of IFNɔ. The IFNɔ levels in media controls were higher than in the *A. marginale* Gonen samples for almost all calves, and also higher than the ConA positive control for two calves. There was no significant correlation between IFNɔ production and PBMC proliferation.

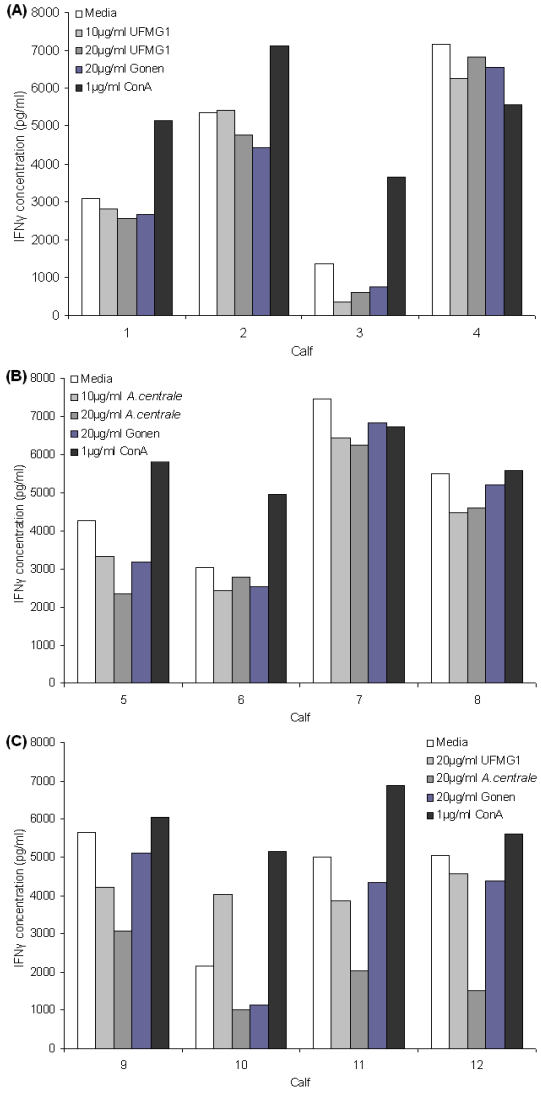

Figure 6: IFNγ production by fresh PBMCs 3 weeks post-challenge. (A) UFMG1 group, (B) A. centrale group and (C) Control group calves. Similar results were seen with the antigen stimulation assay using PBMCs from the initial infection (data not shown).

IFNγ production by cryopreserved PBMCs

The two sample dates which had PBMCs capable of responding to the mitogen ConA were tested for IFNɔ production. There was very variable IFNɔ production between calves, with no significant correlation to proliferation, and on average, cryopreserved PBMCs produced lower levels of IFNɔ than fresh PBMCs. There was no significant difference in IFNɔ production between UFMG1, *A. centrale* and control groups for either of the two post-challenge sample dates (Figure 7).

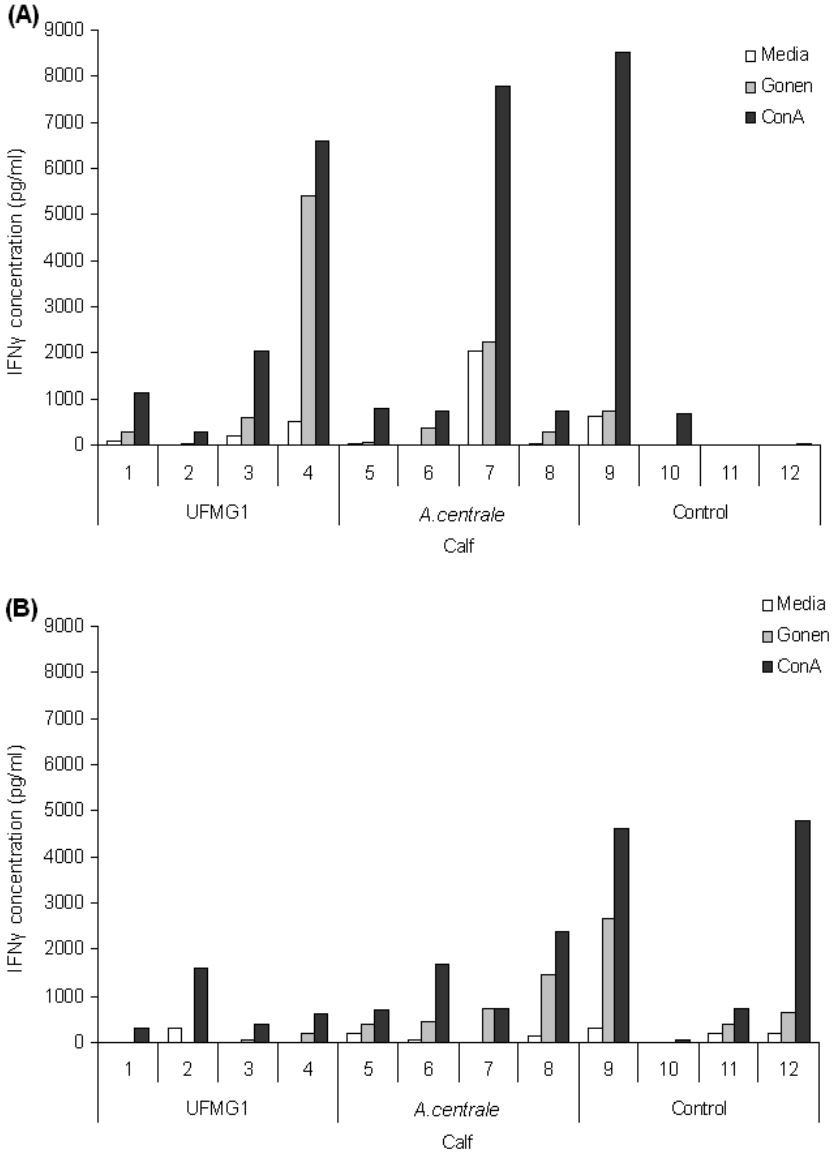

Figure 7: IFNγ production by cryopreserved PBMCs post-challenge in response to 20 µg/ml A. marginale Gonen antigen. (A) 44 days post-challenge. (B) 56 days post-challenge.

4.3.4.2. IFNγ levels *in vivo* during initial infection and challenge

IFNɔ levels were measured in plasma samples taken over the course of initial infection with *A. marginale* UFMG1 or *A. centrale,* and after subsequent challenge with *A. marginale* Gonen (Figure 8).

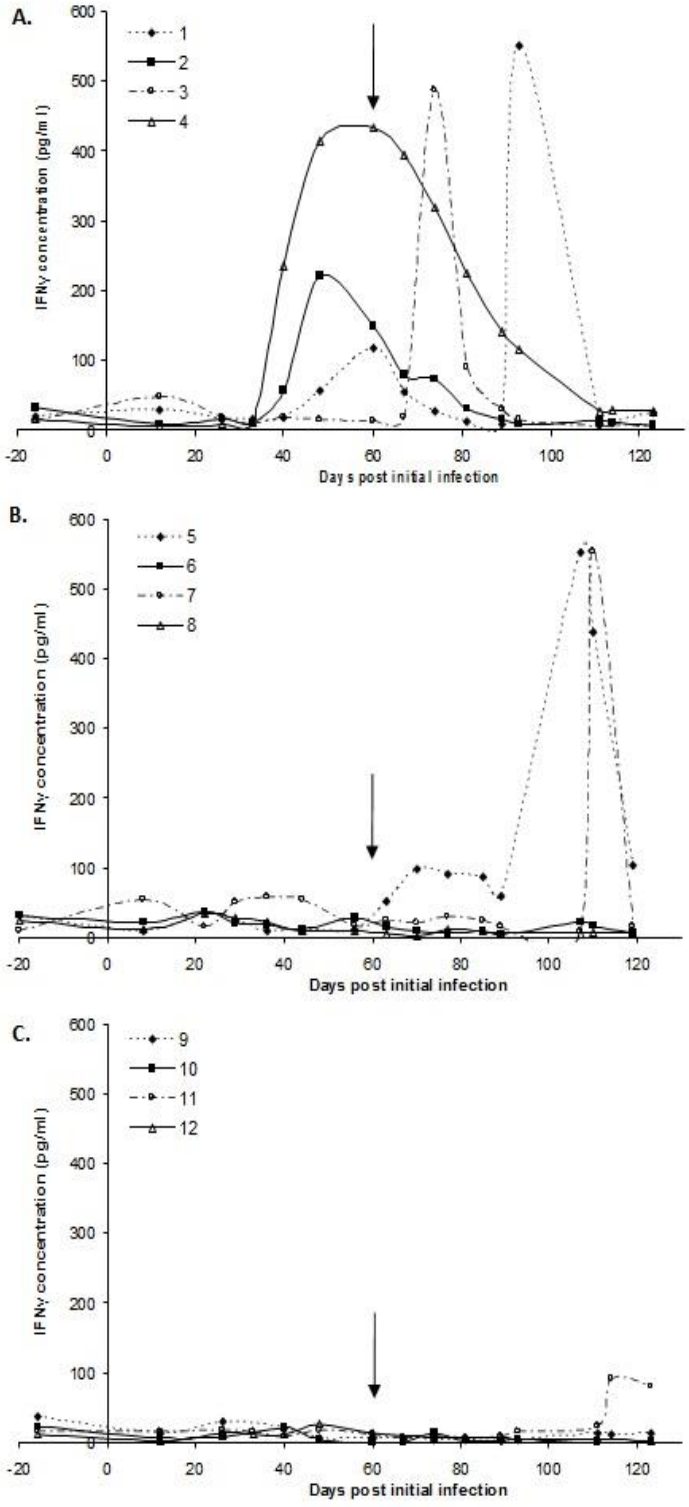

Figure 8: IFNγ levels over the course of initial infection and challenge.
(A) UFMG1 group,
(B) A. centrale group,
(C) control group.
Arrow indicates point of challenge with the Gonen strain, at 2 months after initial infection with UFMG1 or A. centrale.

UFMG1 and *A. centrale* infection induced different patterns of IFNɔ production, with peak IFNɔ levels in UFMG1-infected calves being significantly higher ($p<0.05$). Calves in both groups showed low levels of IFNɔ during the first month, only slightly elevated above the background level of uninfected calves. However, in the second month of infection, IFNɔ levels remained low in *A. centrale*-infected calves (around 50 pg/ml), but rose steeply in 3 out of 4 UFMG1-infected calves, to peaks of 100, 200, and 400 pg/ml respectively. Calf #1 of the UFMG1 group, which was PCR-negative for the Gonen strain after challenge, was one of these calves, peaking at 100pg/ml IFNɔ.

After all calves were challenged with the Gonen strain, calves from both the UFMG1 and *A. centrale* groups had a similar response, with two out of four calves from each group showing steep and very high rises in IFNɔ. The two UFMG1 group calves which had the highest response to initial infection with UFMG1 (#2 and #4) did not show a second peak in IFNɔ after challenge, although their IFNɔ responses to the initial UFMG1 infection persisted at levels considerably above background. The calf which had a lower IFNɔ peak of 100 pg/ml during UFMG1 infection (#1) had returned to background IFNɔ levels shortly after challenge, and subsequently had a higher secondary peak in IFNɔ (550 pg/ml) at around 40 days post-challenge. There was no statistically significant difference in peak IFNɔ levels post-challenge in UFMG1 and *A. centrale* group calves; both were significantly higher than peak IFNɔ levels in the control group ($p<0.05$).

Calves in the control group, which were solely infected with *A. marginale* Gonen, showed very little IFNɔ response post-challenge. In the 2 months of measurement post-challenge, only one calf showed a rise above background levels, peaking at around 100 pg/ml. The post-challenge IFNɔ response was therefore considerably higher in previously infected calves than in calves solely infected with the Gonen strain.

Plasma samples for IFNɔ testing were taken approximately every 7 days. The majority of high IFNɔ responses were relatively transient; four out of the six calves which had levels of 100 pg/ml only registered these high responses at one or two time points tested. Two calves - both in the UFMG1 group - were an exception to this, with one having IFNɔ levels that remained at over 100 pg/ml for more than a month.

Association of IFNγ levels and clinical parameters

Higher levels of IFNɔ (>100 pg/ml) were generally seen late in infection ï consequently they were usually after the most severe symptoms (the period of maximum rickettsemia, maximum fever, or

minimum hematocrit) had passed. Therefore when IFNɔ concentrations in plasma samples were compared to clinical parameters from the same time points, there were no significant correlations between IFNɔ concentration and rickettsemia, hematocrit, or temperature (data not shown).

For each calf, the maximum IFNɔ level measured over the course of infection was compared with the overall severity of symptoms they showed (maximum rickettsemia, maximum temperature, minimum hematocrit). There was a significant correlation ($p<0.05$, data not shown) between the magnitude of the IFNɔ response during initial infection with UFMG1 or *A. centrale,* and the corresponding maximum rickettsemia and minimum hematocrit. This was largely due to UFMG1 infection, which had both more severe symptoms and higher IFNɔ levels during infection. However, post-challenge with the Gonen strain, there was no statistically significant relationship between IFNɔ and any of the clinical parameters.

Association of IFNγ levels and other immunological parameters

IFNɔ enhances the production of IgG2 by bovine B-cells (Estes and Brown, 2002). Therefore IFNɔ levels in plasma were compared to the ratio of IgG1: IgG2. There was no significant correlation between IFNɔ levels and IgG1:IgG2 during initial infection, but a strongly significant correlation between the two parameters during challenge with *A. marginale* Gonen ($p<0.02$). As shown in Figure 9, the higher the IFNɔ levels in plasma during challenge, the greater the bias towards an IgG2 response.

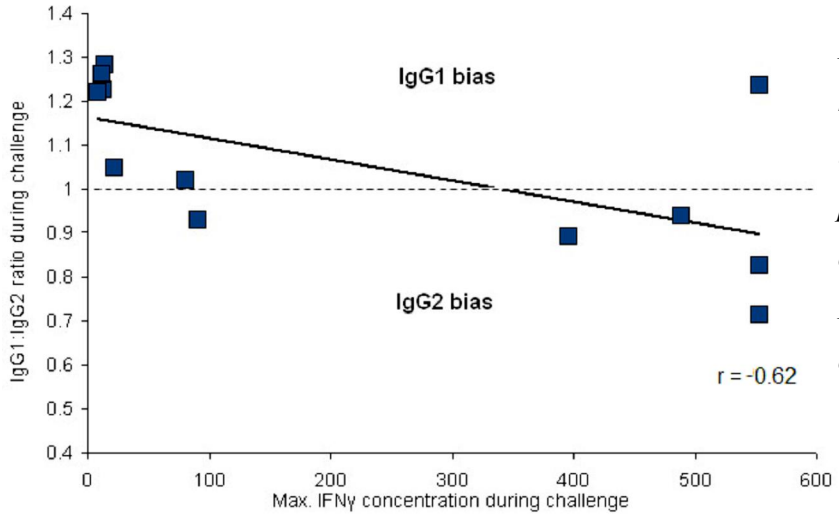

Figure 9: Correlation between maximum post-challenge IFNγ levels and IgG1:IgG2(r=-0.62; p<0.02). An IgG1:IgG2 ratio of one or greater implies an IgG1 bias; a ratio of less than one implies an IgG2 bias.

4.4. DISCUSSION

4.4.1. PBMC proliferation

A. marginale infection has been reported to lead to a fall in levels of antigen-specific T-cells (Abbott *et al.*, 2005; Han *et al.*, 2008 and 2010). Therefore it was not unexpected that this trial found no or low levels of PBMC proliferation in response to UFMG1, *A. centrale* or Gonen antigen.

Some antigen-specific proliferation was seen post-challenge from fresh and cryopreserved PBMCs. However, there was no significant difference in levels of PBMC proliferation between the UFMG1, *A. centrale,* or control groups. This suggests that the greater protection seen after the *A. centrale* live vaccine was not due to a stronger T-cell response. However, this hypothesis cannot be firmly rejected on the basis of this trial, as there were multiple issues with the scope and reliability of the PBMC data.

Firstly, there was extremely limited data on PBMC proliferation from calves infected with only *A. marginale* UFMG1 or *A. centrale* – i.e. using samples taken before challenge. The PBMC response to this initial infection would be likely to play a very important role in subsequent protection from later challenge: through T-cell amplification of the IgG response (Grant *et al.*, 2012), and through the development of pre-activated T-cells which can respond more rapidly upon challenge (Zinkernagel and Hengartner, 2006).

Unfortunately, cryopreserved PBMCs from pre-challenge time points were non-viable. For fresh PBMCs, only one assay was performed prior to challenge, at 8 weeks post-infection, since purified antigen was not available any earlier. Unfortunately, by 8 weeks post-*A.marginale* infection, any T-cell response is likely to be waning (Bautista-Garfias *et al.*, 2003). In addition, this assay used a very low antigen concentration. 10 µg/ml was the highest concentration tested; the subsequent post-challenge assay tested 10 and 20 µg/ml, and found that generally only 20 µg/ml induced PBMC proliferation. Therefore it is likely that the antigen concentration used in the pre-challenge PBMC assay was too low to stimulate any antigen-specific T-cells present.

The second, and main problem, with the *in vitro* PBMC data is that the PBMC assay protocol was insufficiently optimized. As there was no prior access to *A. marginale*-infected cattle, only the response to ConA and cryopreservation conditions could be tested before the trial began. During the trial, a limited number of assays were done with freshly-isolated PBMCs. The bulk of optimization

and testing was intended to be done with cryopreserved cells, prepared from time points throughout initial infection and challenge. This would allow simultaneous testing of multiple time points against antigens from all strains, for a more valid comparison of results. As described in the next section, this was unfortunately not possible.

More assay optimization should have been done with fresh cells early in the infection, and these optimized conditions used to test fresh PBMCs from more time points. Although this would have meant fewer samples could have been cryopreserved, it would have allowed a much better assessment of the response from fresh PBMCs. Since freshly isolated cells are more sensitive to antigenic stimulation (Weinberg *et al.*, 1998), any negative proliferation results could be more confidently assumed to genuinely reflect the *in vivo* response, rather than being a possible result of experimental shortcomings.

Cryopreserved PBMCs

Cryopreserved PBMCs were transported by air from Israel to Munich, on dry ice in two separate flights. All samples were checked on arrival in Munich, appeared to still be frozen, and were stored in liquid nitrogen for later analysis. Only PBMC samples from the second flight were capable of proliferating in response to ConA, a very strong inducer of T-cell proliferation. As samples from the two batches were otherwise treated identically, there may have been an issue with the transportation of the first batch of PBMCs. However, as all samples appeared to still be frozen after transport, there is no clear explanation for why the PBMCs from the first batch were not capable of proliferating.

An unexpectedly low response to ConA was seen in multiple cryopreserved PBMC samples, and in fresh PBMCs from both infected and uninfected calves. As this low proliferation was seen in PBMC samples from uninfected calves, it is unlikely to be general immunosuppression as a result of the infection, as seen with *A.phagocytophilum* (Whist *et al.*, 2003). It may instead be due to shortcomings in the PBMC purification protocol or in the assay itself. Low proliferation in response to ConA, an extremely strong mitogen, makes it less likely that any antigen-specific proliferation would be high enough to measure.

To conclude, the PBMC data is of very limited value due to shortcomings in the experimental design of assays with fresh cells (assays were too few, too late, and insufficiently optimized), and in the sample transport of cryopreserved PBMCs (which prevented many time points from being

tested). Therefore it is impossible to draw any firm conclusions from this data about the relative strength of the *A. marginale* and *A. centrale* T-cell proliferation response, and its role in protection from anaplasmosis.

4.4.2. IFNγ production by PBMCs

The caveats on interpretation of PBMC proliferation data previously discussed also affect the data on *in vitro* IFNɔ production by PBMCs. There was an additional aspect which was curious: freshly isolated PBMCs produced high levels of IFNɔ in media control samples. This could have been due to cross-contamination from other samples during the IFNɔ ELISA, but this would be unlikely to cause such consistently high levels of IFNɔ in so many samples. It could be genuine reaction by PBMCs in response to a component of the media used. However, the high reaction to media was not seen in cryopreserved samples, which used an identical media recipe. It is possible that the different batches of FCS used for fresh and cryopreserved PBMCs could be responsible for different background levels of IFNɔ.

This adds more data to the overall conclusion that the results from isolated PBMCs, whether fresh or frozen, cannot be used to draw any firm conclusions on the cell-mediated immune response to *A. marginale* and *A. centrale*.

4.4.3. *In vivo* IFNγ levels during *A. marginale* and *A. centrale* infection

In his model of vaccine-induced immunity to *A. marginale*, Palmer *et al.* (1999) proposed a central role for the cytokine IFNɔ. This pro-inflammatory cytokine has a wide range of effects, as reviewed in Schroder *et al.* (2004). Most critically for the response to anaplasmosis, IFNɔ plays a role in the humoral immune response by stimulating B-cells to produce IgG2 (Estes et al, 1994; Estes and Brown, 2002). It also activates macrophages, leading to increased levels of antigen presentation and phagocytosis, and a greater ability to kill phagocytosed microbes (Stich *et al.*, 1998; Adler *et al.*, 1994).

Several studies have detected *in vitro* IFNɔ production by PBMCs in response to *A. marginale* antigen (Brown *et al.*, 1998; Bautista-Garfias *et al.*, 2003; Barigye *et al.*, 2004). However, only two studies have measured *in vivo* IFNɔ levels during *A. marginale* infection (Bautista-Garfias *et al.*, 2003; Nazifi *et al.*, 2012). Bautista-Garfias *et al.* (2003) experimentally inoculated 1-year-old cattle and measured IFNɔ over the course of acute infection. But as they did not quantify IFNɔ levels beyond arbitrary ELISA units, IFNɔ concentrations cannot be closely compared with this trial.

Nazifi *et al.* (2012) did quantify IFNɔ levels, and found considerably lower IFNɔ concentrations than seen here. In that study, infected cattle had average IFNɔ levels of 0.5 pg/ml. In this trial, the average peak IFNɔ concentration in infected cattle was 180 pg/ml, over 300 times higher. However, Nazifi *et al.* (2012) measured adult cattle with a naturally-acquired persistent infection. In this trial, calves were sampled during acute infection when IFNɔ levels are likely to be at their highest, and the number of infectious units used here to inoculate the calves is likely to be considerably higher than the low levels passed on during natural infection (Pacheco *et al.*, 2004).

In this trial, many peaks of IFNɔ were very briefly seen - for most calves higher IFNɔ levels were measured in one or two time points only. Prolonged high levels of IFNɔ without immune control would lead to prolonged inflammatory responses, which would be likely to damage the host (Martin *et al.*, 2001) However, the brevity of cytokine responses combined with relatively infrequent sampling means that it is possible that IFNɔ peaks could have been missed in some calves if high IFNɔ levels did not correspond to a sampling point.

With that caveat, there was a higher IFNɔ response to UFMG1 infection than to *A. centrale* infection. However, the IFNɔ peaks were seen late in UFMG1 infection, and so are unlikely to have contributed to the more severe symptoms caused by the UFMG1 strain. High IFNɔ responses seen only late in infection (from 51 days post-infection) was also reported by Bautista-Garfias *et al.* (2003). The IFNɔ response to UFMG1 was also significantly higher than the naïve response to Gonen infection (i.e. IFNɔ in the control group post-challenge). As UFMG1 and Gonen infections produced similar levels of anemia, it confirms that IFNɔ production does not directly relate to pathology (Gale *et al.*, 1996).

After challenge with Gonen, calves previously infected with UFMG1 or *A. centrale* showed a higher IFNɔ response than the naïve control group. In the case of two calves of the *A. centrale* group, this high post-challenge response was despite not having a high IFNɔ response to initial *A. centrale* infection. This suggests that T-cell responses primed during initial infection produced a considerably amplified secondary response on re-exposure. A higher IFNɔ response on re-infection compared to initial *A. marginale* infection was also seen by Bautista-Garfias *et al.* (2003).

During challenge with the Gonen strain, higher IFNɔ levels were significantly associated with an IgG2-biased antibody response. This was not unexpected, as it has been previously demonstrated *in vitro* that IFNɔ stimulates bovine B-cells to produce high levels of IgG2 (Estes *et al.*, 1994).

Curiously, during initial infection with UFMG1 or *A. centrale,* there was no such clear association: surprisingly *A. centrale* infection stimulated the highest IgG2 bias despite a low IFNɔ response. The regulation of IgG subclass expression is complex (Estes and Brown, 2002), and it is likely that other factors beyond IFNɔ are responsible for the higher IgG2 production in *A. centrale* infection.

Higher IgG2 is associated with protection from anaplasmosis (Brown *et al.*, 1998; Barigye *et al.*, 2004; Vega *et al.*, 2007; see Chapter 3). Therefore it would be interesting to investigate some of the other factors potentially responsible for the higher IgG2 expression after *A. centrale* infection ï for example, interferon alpha or TGF-beta, which *in vitro* can also stimulate IgG2 production from B-cells (Estes *et al.,* 1998).

Because IFNɔ has been proposed as central to protection from anaplasmosis (Palmer *et al.*, 1999), previous vaccine studies have tried to stimulate high IFNɔ production in vaccinees. Tuo *et al.* (2000) used an IL-12 adjuvant with a recombinant MSP2 vaccine, and successfully stimulated high levels of IFNɔ in three calves. However, only one of these calves produced detectable antigen-specific IgG2 ï and this was the calf with the lowest level of IFNɔ.

Clearly the regulation of the antibody response to *A. marginale* infection is too complex to simply aim for a vaccine which stimulates high levels of IFNɔ. In this trial the immune response to *A. centrale* was significantly more protective than the response to UFMG1, despite stimulating lower IFNɔ production. The successful development of future vaccines could be helped considerably by investigating which other factors lie behind *A. centrale* infection stimulating the higher IgG level and greater IgG2 bias seen in this study.

4.4.4. Conclusion

No solid conclusions could be drawn from the *in vitro* PBMC proliferation and IFNɔ production data. The *in vivo* IFNɔ data showed that previous infection with UFMG1 or *A. centrale* leads to amplified IFNɔ production on subsequent challenge with the Gonen strain. This amplified post-challenge IFNɔ response correlates with higher levels of IgG2. But other factors beyond IFNɔ appear to also have a role in enhancing IgG2 production, as seen in *A. centrale* infection, and should be further investigated.

It has been proposed that the role of IgG2 in protecting cattle from anaplasmosis is the effectiveness of IgG2 in enhancing phagocytosis (Palmer *et al.*, 1999). Studies have previously demonstrated that

immune sera against *A. marginale* have opsonophagocytic activity (Cantor *et al.*, 1993; Melendez *et al.*, 2005). However, neither of these studies investigated if the opsonophagocytic activity was actually related to IgG2 levels, or more importantly, correlated with protection from disease. Developing a functional assay to determine this information will be the subject of the next chapter.

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CHAPTER 5: OPSONOPHAGOCYTIC ACTIVITY OF IMMUNE SERA AGAINST *A. MARGINALE*

5.1. INTRODUCTION

5.1.1. The value of measuring antibody function as a correlate of protection

All antibodies are not created equal. Even if an antibody binds to a pathogen, it will not necessarily have a protective effect against disease (Plotkin 2008). Antibodies have three main functions against pathogens: neutralization, opsonization, and bactericidal activity. Different antibodies vary in their effectiveness at these functions. Therefore while ELISAs are a simple and practical tool to measure the immunogenicity of a vaccine, an assay for antibody function may correlate better with protection (Johnson *et al.*, 1999).

Functional antibody assays are used to assess vaccines against many diseases. For example, it was found that for mice infected with *Streptococcus pneumoniae*, the opsonophagocytic activity of their serum samples was a better indicator of protection than IgG titers (Johnson *et al.*, 1999). Standardized *in vitro* opsonophagocytosis assays are now often used to assess the performance of pneumococcal vaccines (Romero-Steiner *et al.*, 2006). Serum bactericidal activity is used to assess vaccines against *Neisseria meningitidis* (Plotkin *et al.*, 2008), and growth inhibition assays are used in malaria vaccine research (Bergmann-Leitner *et al.*, 2006).

Therefore the functional activity of antibodies can be an excellent and useful correlate of protection to measure. Correlates of protection can identify which immune mechanisms are most important against different diseases, which guides further vaccine development. They also provide a useful alternative to challenge trials, especially if these are dangerous or logistically difficult (Plotkin *et al.*, 2008). While challenge trials for *Anaplasma marginale* are relatively straightforward in endemic areas, establishing good correlates of protection would be very useful in estimating the effect of vaccines against a wider range of strains than it would be practical to challenge cattle with.

5.1.2. Opsonophagocytosis as a correlate of protection for bovine anaplasmosis

IgG2 levels induced by inactivated or recombinant protein vaccines correlated with protection from anaplasmosis in several studies (Brown *et al.*, 1998; Barigye *et al.*, 2004; Vega *et al.*, 2007). Palmer *et al.* (1999) proposed that the importance of IgG2 in reducing the symptoms of anaplasmosis was

due to its effectiveness in opsonising bacteria, leading to better clearance of *A. marginale* by phagocytic cells.

In cattle, both IgG1 and IgG2 can fix complement and mediate phagocytosis; however, IgG2 is considerably more rapid and effective at enhancing phagocytosis by neutrophils and peripheral blood monocytes (McGuire *et al.*, 1979; McGuire and Musoke, 1981).

Opsonophagocytosis has been demonstrated *in vitro* with immune sera and *A. marginale* by Cantor *et al.* (1993) and Melendez (2005). However, neither of these studies investigated if the opsonophagocytic activity (OPA) was actually related to IgG2 levels, or more importantly, whether OPA correlated with protection from disease. These theories could be tested by using an opsonophagocytosis assay to test serum samples from the live vaccine trial described in Chapter 2, and comparing results to severity of disease (Chapter 2) and levels of IgG2 (Chapter 3).

5.1.3. Measuring opsonophagocytosis

Many *in vitro* assays have been developed to measure opsonophagocytosis. These assays either detect the uptake of micro-organisms or other targets, the killing of micro-organisms, or the production of reactive oxygen species (ROS) (Lehmann *et al.*, 2000). Phagocytic cells use ROS, which include nitric oxide (NO), superoxide anion (O_2^-), and hydrogen peroxide (H_2O_2), to kill the micro-organisms they have engulfed. The production of these ROS is known as the respiratory burst or oxidative burst, and is stimulated by phagocytosis. The ingestion of micro-organisms causes the phagocytic cell to assemble the multi-subunit enzyme NADPH oxidase. Once assembled, NADPH oxidase converts molecular oxygen to O_2^-, which is further converted by other enzymes to produce a range of toxic ROS (reviewed by Bylund *et al.*, 2010).

The simplest but most subjective method for measuring phagocytosis is by microscopy. A conventional microscope can be used to see phagocytosed objects inside Giemsa-stained cells (Melendez 2005), or to see the nitroblue tetrazolium precipitation products which form on contact with ROS (Park *et al.*, 1968). A fluorescent microscope can be used to more reliably visualize intracellular bacteria when they are either stained directly with a fluorescent dye or tagged with a fluorochrome-conjugated antibody (Cantor *et al.*, 1993). While microscopy is very useful for small-scale studies, it has disadvantages: it is highly labor-intensive and time-consuming, and can be subjective ï results are likely to vary from operator to operator (Chaka *et al.*, 1995).

Phagocytosis can also be tested through measuring the killing of target micro-organisms. After incubating the target with phagocytic cells, the level of killing is assessed either by plating out samples after the assay (Leijh *et al.*, 1979), level of [3H]-thymidine uptake (White and Walker, 1981), or viability indicator dyes (Goldner *et al.*, 1983). Batch-to-batch variation in the initial viability and growth rate of the bacterial stocks used in these assays can easily affect the results (Hampton *et al.*, 1999).

There is a range of more high-throughput and reliably quantifiable methods. Chemiluminescence assays rely on the fact that the oxidative burst results in electronically excited oxidation products ï when these relax back to their ground state it leads to photon emission. Adding luminol or lucigenin boosts the photon emission to the point that the chemiluminescence can be detected and quantified by a luminometer (Allen 1977). Chemiluminescence is a highly sensitive method to quantify phagocytosis, but reagents vary in effectiveness between different cell types, and the signal can be easily affected by any contaminating red blood cells (Kopprasch *et al.*, 2003; Easmon *et al.*, 1980).

Currently, flow cytometry is possibly the most high-throughput, objective, and flexible method for measuring phagocytosis. It is fast, so large numbers of samples can be measured. Within each individual sample, information from tens of thousands of cells can be collected, so it is considerably more objective than microscopy, in which limited numbers of cells can be counted (Lehmann *et al.*, 2000). It can be used to measure bacterial uptake, level of killing, or ROS production.

The uptake of bacteria can be measured by staining them with a fluorophore. This can be done by using an antigen-specific fluorescent-tagged antibody, but binding of the tagged antibodies may then inhibit immune sera from binding to relevant epitopes (Lehmann *et al.*, 2000). A more efficient alternative is to use fluorescent dyes which integrate into the nucleic acid or the cell membrane of bacteria.

The oxidative burst resulting from phagocytosis can be measured by indicator dyes. These dyes are incubated with and taken up by phagocytic cells; if these cells then phagocytose a micro-organism and trigger the oxidative burst reaction, the indicator dye will fluoresce when it reacts with the oxidative burst ROS products. Examples of these indicator dyes are hydroethidine, xylenol orange, dichloro-dihydro-fluorescein diacetate (DCFH-DA), and the widely used and extremely sensitive dihydrorhodamine 123 (DHR123) (Vowells *et al.*, 1995; Lehmann *et al.*, 1997). DHR123 is non-fluorescent, but converts to fluorescent rhodamine 123 in the presence of ROS (Rothe *et al.*, 1988).

Over the past few decades, opsonophagocytosis assays have become increasingly more objective, easier to standardize, and more high-throughput. Therefore they have become an increasingly practical tool for assessing samples from vaccine trials.

5.1.4. Aims

Antibodies are important in protection against bovine anaplasmosis, and due to the association of higher IgG2 levels with protection, opsonophagocytosis has been proposed as an important mechanism of antibody action against *A. marginale.* There is evidence that immune sera against *A. marginale* have opsonophagocytic activity (OPA), but no evidence to date that OPA correlates with IgG2 levels, or with protection against anaplasmosis.

Both studies on opsonophagocytosis and *A. marginale* to date have used microscopy. While this is a very effective method, it is time-consuming and subjective, and therefore of limited use when testing large numbers of samples from vaccine trials.

The aim of this chapter is therefore the development of a high-throughput, practical and objective opsonophagocytosis assay for *A. marginale,* which could in future serve as a more efficient tool for vaccine assessment. This assay will be used to measure the sera from the live vaccine trial of *A. marginale* UFMG1 and *A. centrale* reported in previous chapters. The OPA of serum from calves in the trial will be compared with the calves' corresponding clinical symptoms, IgG production, and IFNɔ levels, to help build up a picture of the role of opsonphagocytosis in protection against bovine anaplasmosis.

5.2. MATERIALS AND METHODS

5.2.1. Sera

Sera were taken from calves infected with *A. marginale* or *A. centrale* as described in Chapter 3, aliquoted and stored at -80ºC until used.

5.2.2. Bacteria

Purification of bacteria

A. marginale Gonen strain bacteria were purified from IDE-8 tick cell culture as described in Chapter 3. The quantity of bacteria was estimated by RT-PCR, after the method of Carelli *et al.*

(2007). Briefly, DNA was extracted from bacteria with the QIAamp DNA Mini Kit (Qiagen, Germany), and tested in an *msp1b* RT-PCR (protocol described in Chapter 2) alongside a quantified *msp1b* plasmid standard. The standard was produced as described by Lis *et al.* (manuscript submitted): an *A. marginale msp1b* gene fragment was cloned into the pGEM®-T easy vector (Promega, Medison, USA), propagated into competent *Escherichia coli* cells, and plasmids purified with QIAprep Spin Miniprep Kit (Qiagen, Germany). The *msp1b* copy number was then calculated from the OD of the purified DNA.

Staining of bacteria

For some experiments, bacteria were labeled with nucleic acid stains. The nucleic acid stain SYTO9 (Invitrogen, Darmstadt, Germany) has an optimum excitation wavelength of 485 nm and emission wavelength of 498 nm. It is membrane-permeable, and so is taken up by viable and non-viable cells. SYTO9 was tested at concentrations from 50 nM ï 20 µM, diluted in Dulbecco's PBS (DPBS). 100 µl bacteria were added to an equal volume of SYTO9 (at double the final concentration of dye), and incubated, shaking, for 30 minutes at room temperature. After incubation, any free dye was removed by washing samples six times (spinning at 16000 g for 5 minutes), and the pellet of bacteria re-suspended in 100 µl DPBS.

The nucleic acid stain propidium iodide (PI) (Invitrogen, Darmstadt, Germany) has an optimum excitation wavelength of 535 nm and emission wavelength of 617 nm. It is membrane impermeable and therefore generally only taken up by non-viable cells with compromised membranes. Therefore before staining, bacteria were killed by incubation with 1 % formaldehyde, 70 % ethanol, or at 56 °C heat (all for 1 hour, followed by washing once at 16000 g for 5 minutes). Bacteria were then stained with 50-200 µg/ml PI, using the same procedure as described for SYTO9, but with an incubation period of 1 hour.

5.2.3. Cell preparation

Separation of leukocytes

Blood was collected into heparin Vacutainers (Becton Dickinson, Franklin Lakes, New Jersey) from the jugular vein of adult Fleckvieh cattle (*Bos taurus*), spun at 1000 g for 15 minutes, and the plasma fraction removed. The remaining cell fraction was treated to lyse erythrocytes: 1 volume of cells was mixed with 2 volumes of 0.02 M NaCl for 30 seconds, and osmolarity restored by addition of 0.5 volumes 0.164 M NaCl. The leukocytes remaining were spun at 400 g for 5 minutes, and washed twice in 5 mM glucose in DPBS (DPBS-G) to remove the debris of lysed erythrocytes. Cells were then resuspended in 10 ml DPBS-G, counted, and finally resuspended at $2x10^6$ cells/ml

in DPBS-GCM assay buffer (5 mM glucose, 1.2 mM $CaCl_2$, 1 mM $MgSO_4$ in DPBS). 100 µl of cells were added to 96-well microtiter plates (TPP, Trasadingen, Switzerland), and incubated at 38 °C, 5 % CO_2 for 2 hours before use in the assay.

Staining of leukocytes

For some experiments, the leukocytes were stained with dihydrorhodamine 123 (DHR123) (Invitrogen, Darmstadt, Germany). 5 µl of 400 µg/ml DHR123 was added to 100 µl cells in the microtiter plate, for a final DHR123 concentration of 20 µg/ml. The plate was then incubated shaking for 5 minutes to allow cells to take up the dye before being used in the phagocytosis assay.

5.2.4. Assay set-up

Bacteria were diluted to required concentration (see Results) in DPBS-GCM assay buffer. 10 µl diluted bacteria and 10 µl serum were added to a 96-well microtiter plate. Serum samples were tested in duplicate. Sufficient assay buffer was added to each well to bring the total volume to 50 µl, and samples mixed by pipetting. The plate was then sealed with an air-permeable film (Sigma, Munich, Germany) and incubated at 38 °C, 5 % CO_2 for one hour to allow antibodies to bind to bacteria. After one hour, 50 µl of cells were added to the wells (cells unstained or after 5 minutes staining with DHR123), and the plate re-incubated at 38 °C for 15-45 minutes (see Results). The assay was stopped by addition of ice-cold DPBS + 0.02 % EDTA.

5.2.5. Flow cytometry

Samples were read using a MACSQuant VYB flow cytometer (Miltenyi Biotec, Bergisch Gladbach, Germany). For each sample, 100 µl was read, containing approximately 25000 cells. DHR123 and SYTO9 were both excited by a 488 nm laser, with fluorescence collected by a 525/50 nm bandpass filter. A 561 nm laser was used to excite PI, with resulting fluorescence collected by a 615/20 nm bandpass filter.

Flow cytometry data were analyzed using Weasel® software (Walter and Eliza Hall Institute of Medical Research, Victoria, Australia). Doublets and clumps were excluded from analysis by gating on FSC-A vs. FSC-H. Granulocytes were then selected by their size and density (FSC-A vs. SSC-A). Fluorescence in this population was then taken as the level of phagocytosis.

To ensure that the threshold for positive events (i.e. cells which had phagocytosed bacteria) was set consistently between assays, the threshold gate was set in every assay to include 10 % of cells in the

fluorescence histogram of the cell only control. This gate was then applied to all test samples, and level of phagocytosis quantified as the fluorescence index (FI): % cells gated x mean intensity of gated cells (Li *et al.*, 2006).

5.2.6. Statistical analysis.

The Minitab software package (Minitab Inc., State College, Pennsylvania, USA) was used for statistical analysis. ANOVA and Tukey HSD post-analysis were used to calculate the significance of parameter differences between groups (Yandell, 1997). Linear correlations between parameters were calculated by the Pearson product-moment correlation coefficient.

5.3. RESULTS

5.3.1. Using bacterial staining to measure opsonophagocytosis

Testing nucleic acid dyes

Two nucleic acid dyes, SYTO9 and propidium iodide (PI), were tested for their efficacy in staining *A. marginale*.

SYTO9 successfully stained bacteria. However, when these stained bacteria were incubated with leukocytes, sufficient dye leached from the bacteria that the nucleus of the leukocytes also became stained (assessed by fluorescence microscopy, data not shown). This was despite six wash steps ï any further washing reduced the numbers of bacteria too much to be practical.

PI also successfully stained bacteria, and when these PI-stained bacteria were incubated with leukocytes, no dye transferred to the cell nuclei (data not shown). Therefore the PI staining protocol was optimized further for use in the opsonophagocytosis assay.

PI is only taken up by non-viable cells with compromised membranes. When *A. marginale* bacteria were stained directly after being purified from tick cell culture, many did not pick up the dye (assessed by fluorescence microscopy, data not shown). This agrees with results from Lis *et al.* (manuscript submitted) that Percoll gradient-purified rickettsia retain a high level of viability. Several killing methods were investigated to improve the uptake of PI, namely: heat killing, ethanol, and formaldehyde (see Materials and Methods). Heat killing and ethanol caused the bacteria to clump, which made it more difficult to add a standardized number of bacteria to each

well. Formaldehyde did not have this problem, and increased the percentage of bacteria which were successfully stained (data not shown).

Formaldehyde-killed bacteria stained with PI were tested in an *in vitro* opsonophagocytosis assay. Leukocytes were incubated for 30 minutes with stained bacteria, which were either untreated or had been pre-incubated with immune sera. As shown in Figure 1, leukocytes alone had a very low level of background fluorescence. When stained, untreated bacteria were added, only low numbers of bacteria were taken up or attached to the cells ï as shown by a small increase in numbers of fluorescent cells. The addition of immune sera opsonized the bacteria, leading to considerably increased phagocytosis or attachment ï shown by increased numbers of fluorescent cells and higher intensity of fluorescence (the latter indicating greater numbers of bacteria taken up by or attached to each leukocyte).

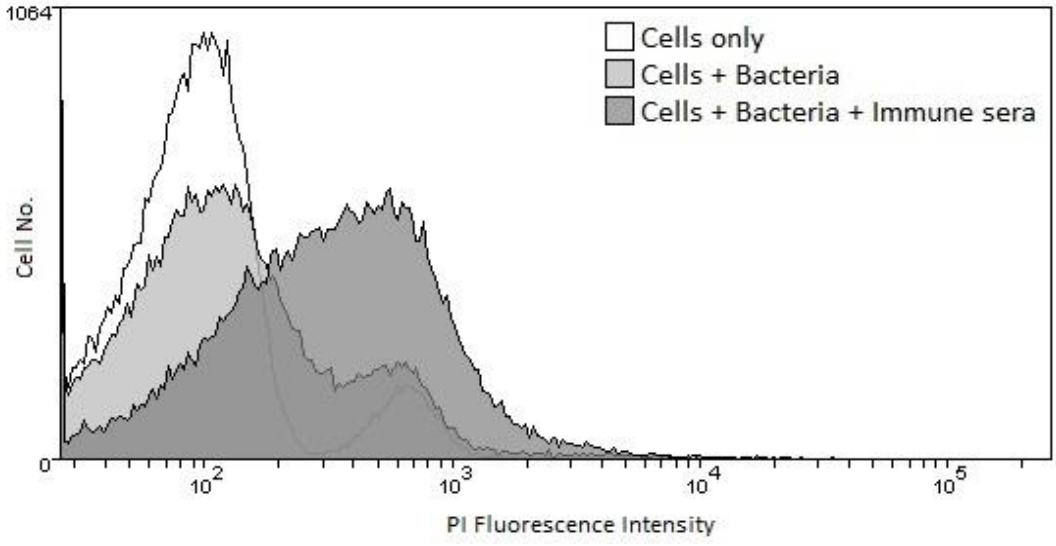

Figure 1: Overlay histograms of the increase of leukocyte fluorescence after incubation with PI-stained bacteria (untreated or pre-incubated with immune sera).

Determining Attachment vs. Uptake

When phagocytic cells bind bacteria, the bacteria may be phagocytosed, or may remain attached to the surface of the cell rather than being fully internalized and killed. Therefore it is important to ensure that phagocytosis assays only measure fully internalized bacteria (Hed, 1986).

The simplest way to compare levels of internalized vs. attached bacteria is to run the same phagocytosis assay in parallel at the physiological temperature of the cells, and at a lower temperature. At physiological temperature, cells will be active and able to internalize bacteria. At the lower temperature, any internalization should be negligible, and so any fluorescence from the cells can be attributed to bacteria which are only attached to the outside of the cell (Hed, 1986; Giunta *et al.*, 2008).

PI-stained bacteria were tested in the OPA assay at 38 ºC (bovine physiological temperature) and 4 ºC, with results shown in Figure 2. The fluorescence at 38 ºC was only around 30 % higher than the fluorescence seen at 4 ºC ï indicating that the majority of the signal in this assay at 38 ºC is likely to be from attached rather than internalized bacteria.

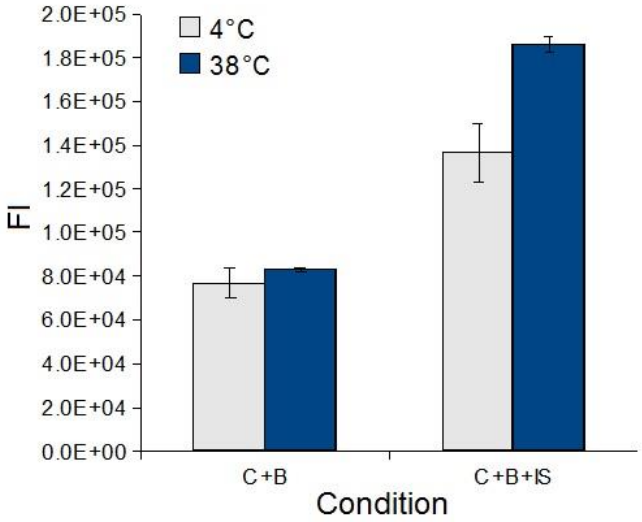

Figure 2: Results of the OPA assay using PI-stained bacteria at 4 ºC and 38 ºC. C+B = Cells + Bacteria. C+B+IS = Cells+Bacteria+Immune sera.

5.3.2. Using the oxidative burst to measure opsonophagocytosis

DHR123 is a non-fluorescent substrate which converts to fluorescent rhodamine-123 in the presence of ROS. Therefore when cells are stained with DHR123, their level of fluorescence becomes an indicator of the level of the oxidative burst response. As the oxidative burst is triggered by phagocytosis (Bassoe *et al.*, 2000), using DHR123 in an opsonophagocytosis assay removes the need to distinguish attachment from internalization.

DHR123-stained leukocytes were incubated for 30 minutes with bacteria alone, or with bacteria pre-incubated with immune sera. As shown in Figure 3, there was some background fluorescence for cells alone. When untreated bacteria were added, there was a small increase in fluorescence. The addition of bacteria pre-incubated with immune sera led to considerably increased fluorescence ï indicating a much higher uptake of opsonized bacteria and subsequently higher level of oxidative burst by the leukocytes. Therefore an OPA assay using DHR123-stained leukocytes was developed further.

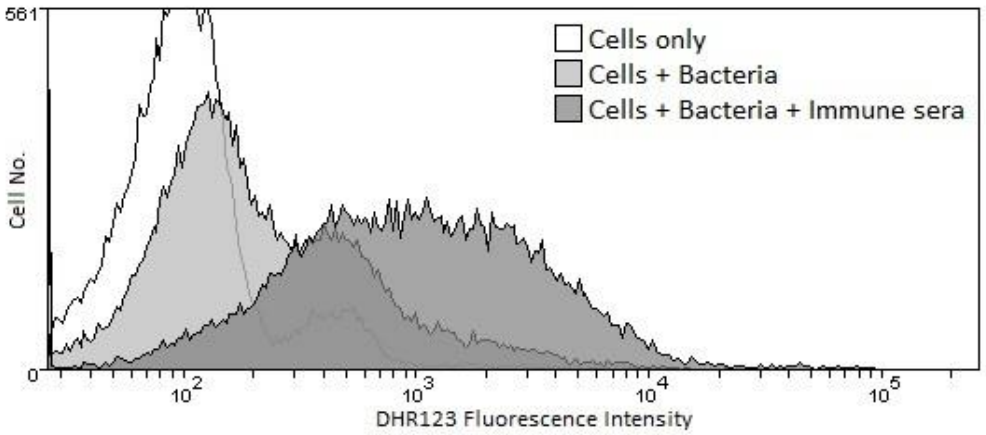

Figure 3: Overlay histograms of the increase in fluorescence of DHR123-stained leukocytes after incubation with bacteria (untreated or pre-incubated with immune sera).

5.3.3. Defining and analyzing the *in vitro* phagocytosis assay

The two most abundant groups of phagocytic cells are mononuclear phagocytes (monocytes and macrophages) and polymorphonuclear granulocytes (Silva, 2010). Lymphocytes may also show a very low level of phagocytosis from B-cells (Sunyer, 2012).

The leukocytes used in the opsonophagocytosis assay contained granulocytes, monocytes, and lymphocytes. The gating strategy to analyse these cells is shown in Figure 4. First, in Figure 4.A., single cells were selected, as any doublets would give a disproportionately high fluorescent signal. Then different cell populations were identified on the basis of their differing size and granularity. Figure 4.B. shows the phagocytic activity of the different cell types. In this assay, lymphocytes showed no detectable phagocytic activity and monocytes only a very low level. Only the granulocyte population showed clearly measurable phagocytic activity. Therefore further work on the opsonophagocytosis assay was based only on the fluorescence signal from the granulocyte population.

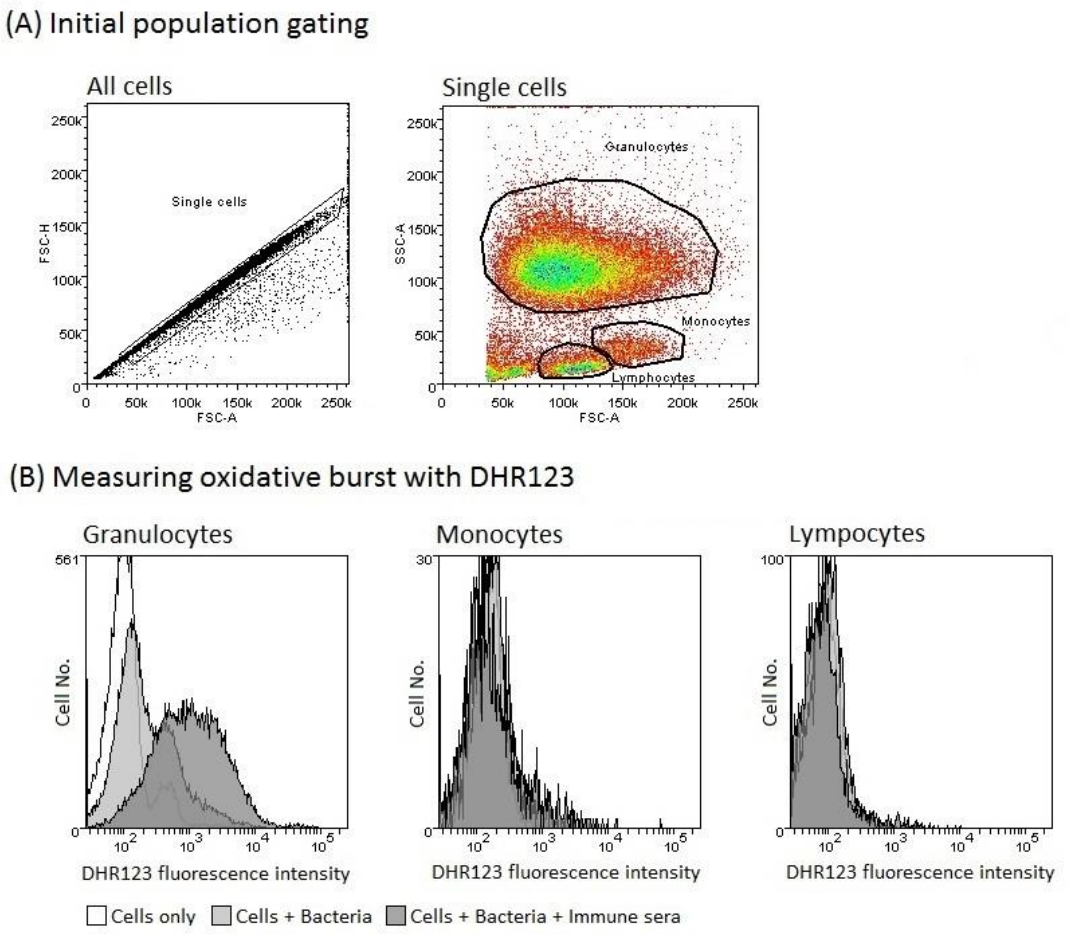

Figure 4: Gating strategy to determine which cell populations showed increased oxidative burst activity when incubated with bacteria and immune sera.
(A) Initial gating strategy of selecting single cells based on FSC-A vs. FSC-H, then identifying distinct cell populations based on size (FSC-A) vs. granularity (SSC-A).
(B) Level of oxidative burst in different cell populations. Only granulocytes showed a clear increase in fluorescence with the addition of opsonized bacteria.

When DHR123-stained cells phagocytose bacteria, there are an increased number of fluorescent cells, and an increase in the intensity of their fluorescence. In order to capture both of these properties, the results of the opsonophagocytosis assay are expressed as the Fluorescence Index (FI). The FI is calculated from percentage of fluorescent cells multiplied by the mean intensity of their fluorescence. Therefore it incorporates both the percentage of cells which phagocytose the target, and the level of phagocytosis within those cells. The calculation of FI is shown in Figure 5.

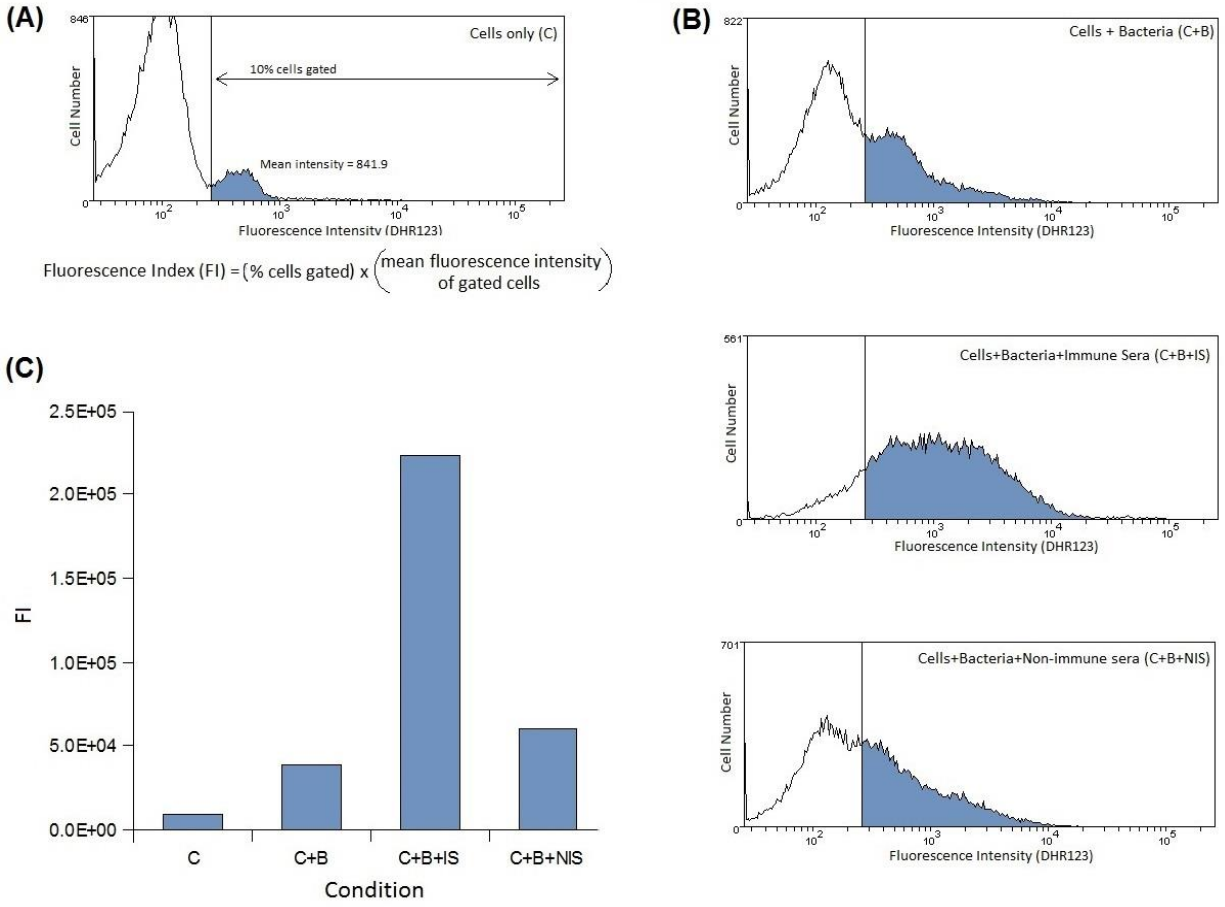

Figure 5: Calculating the fluorescence index (FI). (A) cell only control, showing 10% gate (shaded population), and formula for calculation of fluorescence index (FI). (B) Test samples, showing the increased % of cells gated and the increased intensity of fluorescence with the addition of bacteria and sera. (C) Histogram of FI values for samples shown in (A) and (B).

Figure 5.A. shows the cell only control. The cut-off for background fluorescence needed to be standardized between assays to compensate for inter-assay differences in flow cytometer settings and cell activity. Therefore the fluorescence gate on the cell only control is set to include 10% of the cells. Any cells showing fluorescence levels beyond this gate are counted as positive events, and included in the calculation of FI (see Figure 5.A.). Figure 5.B. shows histograms from test samples showing increased fluorescence with the addition of bacteria, which are either untreated or pre-incubated with immune or non-immune sera. The FI values of the samples in Figure 5.A. and 5.B are shown in Figure 5.C.

5.3.4. Optimization of the opsonophagocytosis assay

The conditions of the OPA assay were optimized to maximize the difference between the FI of immune sera (IS) and the background signal.

First, DHR123 concentrations from 5-20 εg/ml were tested. As shown in Figure 6, 20 εg/ml DHR123 gave the greatest separation between the 'bacteria only' and the 'bacteria and immune sera' samples. Therefore this concentration was used for future assays.

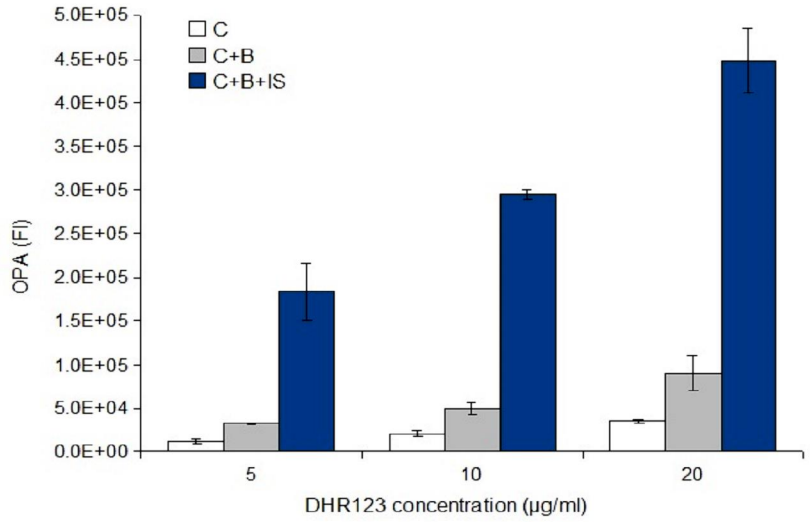

Figure 6: effect of DHR123 concentration on level of OPA measured. C=cells only, C+B=cells+bacteria, C+B+IS=cells+bacteria+immune sera.

The primary incubation period - when bacteria and sera were combined - was kept constant at one hour. The secondary incubation period - after leukocytes were added - was varied, and 15, 30, and 45 minute incubation periods were tested for several bacteria:cell ratios (Figure 7).

FI increased with the length of secondary incubation - with a sharp increase from 15 to 30 minutes, and then a lower increase from 30 minutes to 45. For all bacterial concentrations tested, a secondary incubation time of 45 minutes gave the greatest difference between the FI of bacteria with immune sera and control condtions (bacteria alone or with non-immune sera). The highest bacteria:cell ratio tested, 6200:1, gave the best results.

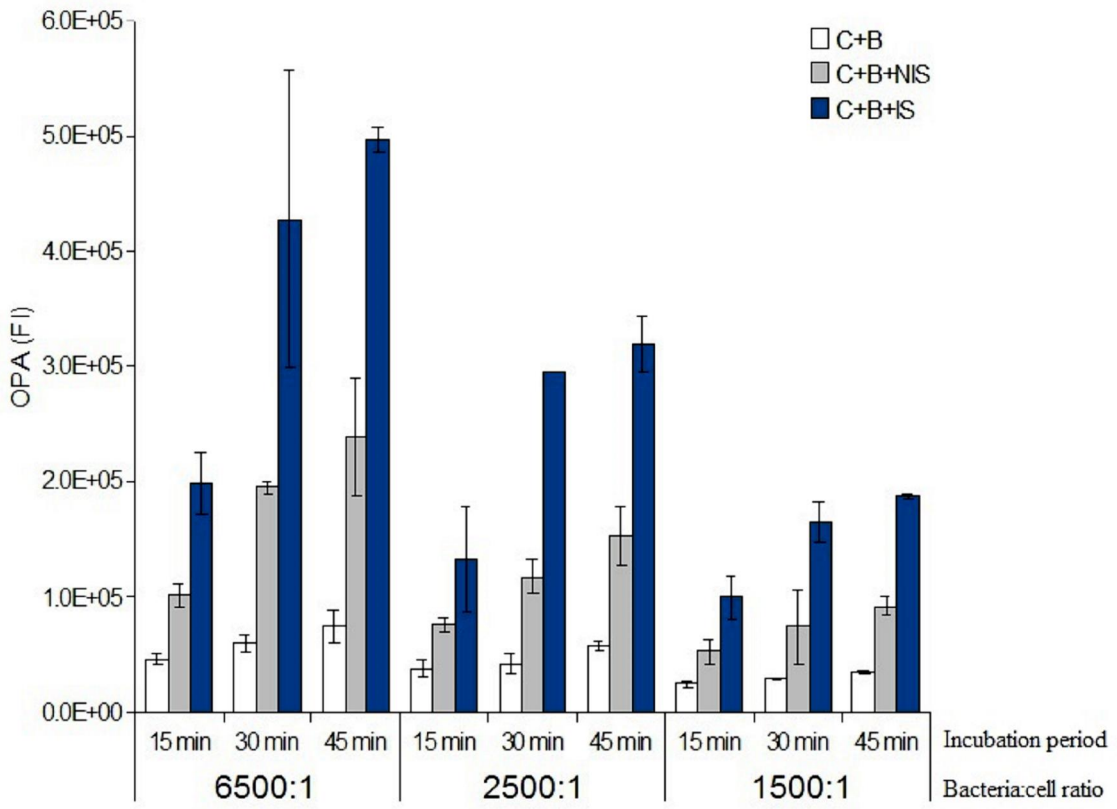

Figure 7: Effect of the length of OPA assay secondary incubation period and bacterial concentration on FI. C+B=cells+bacteria, C+B+IS=cells+bacteria+ non-immune sera, C+B+IS = cells+ bacteria+immune sera. 6500, 2500, or 1500 bacteria per cell were tested.

The ratio of bacteria per cell is critically important for the level of oxidative burst (Anding *et al.*, 2003), and so was tested in more detail. Accurately quantifying the bacteria purified from tick cell culture was problematic. As the *A. marginale* rickettsia are very small, reliably distinguishing them from co-purified tick cell mitochondria by an optical microscope was difficult, and an electron microscope was not available. Therefore the concentration of bacteria was estimated by a quantitative PCR targeting *A. marginale msp1b* (see Methods).

The oxidative burst response at different bacteria:cell ratios is shown in Figure 8. The response to immune sera increased rapidly with bacteria:cell ratio from 200 bacteria per cell up to 800 bacteria per cell, and then showed small and inconsistent increases as bacterial numbers increased further. The ratio with the greatest separation between the FIs of immune and non-immune sera was 3250 bacteria per cell.

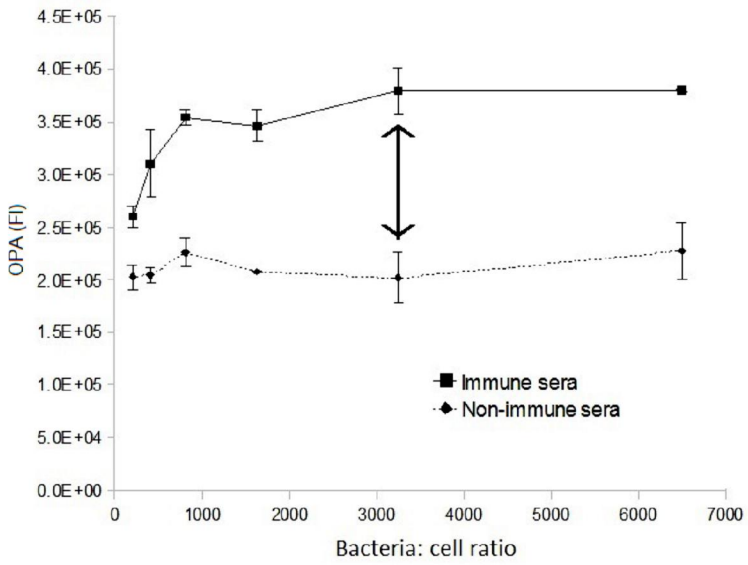

Figure 8: Effect of different bacteria:cell ratios on the differentiation between OPA of immune sera and non-immune sera. The double-headed arrow indicates ratio with greatest differentiation between immune serum and non-immune serum samples (3250 bacteria per cell).

One complete 96-well microtiter plate took approximately two hours for all samples to be analyzed by flow cytometry. To determine whether the time of reading would make a significant difference in the fluorescent signal, replicates of an IS and NIS sample was tested at approximately hourly intervals over a three-hour period (Figure 9). The non-immune sample showed no change over the three hours; the immune sample showed a gradual but non-significant increase in FI.

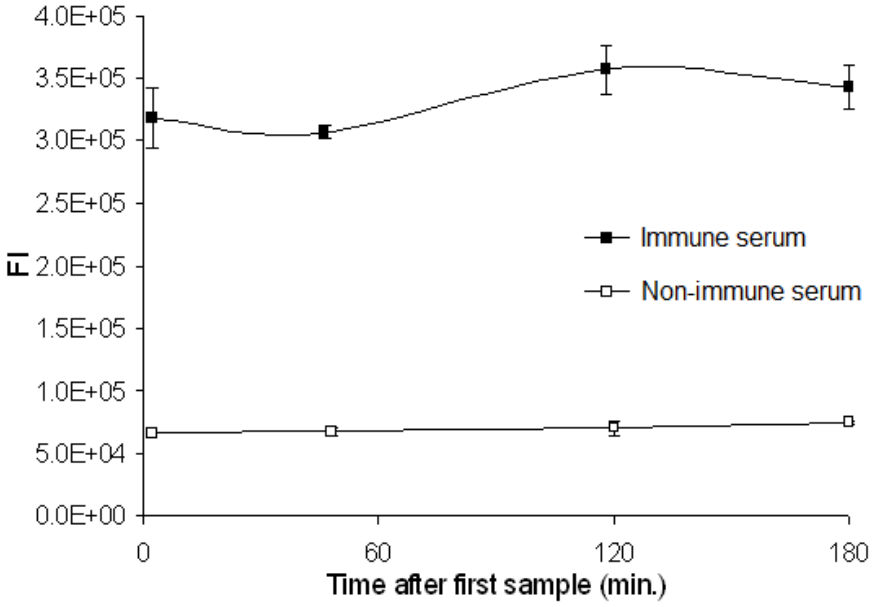

Figure 9: The effect of read-time on the fluorescence of immune (IS) and non-immune (NIS) samples.

Therefore the final optimum conditions of the assay were 20 εg/ml DHR123 used to stain cells, which were then incubated with bacteria for 45 minutes at a bacteria:cell ratio of 3250:1. No more than one full 96-well microtiter plate was used in any assay to prevent the slight increase in signal over time from influencing the results.

5.3.5. Testing serum samples from the live vaccine trial against *A. marginale* Gonen

The optimized assay conditions were used to measure the OPA of serum samples from the live vaccine trial described in Chapter 3. The challenge strain in the trial, *A. marginale* Gonen, was used as the target of the OPA assay.

A comparison of the OPA against the Gonen strain that was induced by infection with UFMG1, *A. centrale* or Gonen strain is shown in Figure 10. The average OPA response to *A. centrale* was higher than to UFMG1 or Gonen infection, but the difference was not significant.

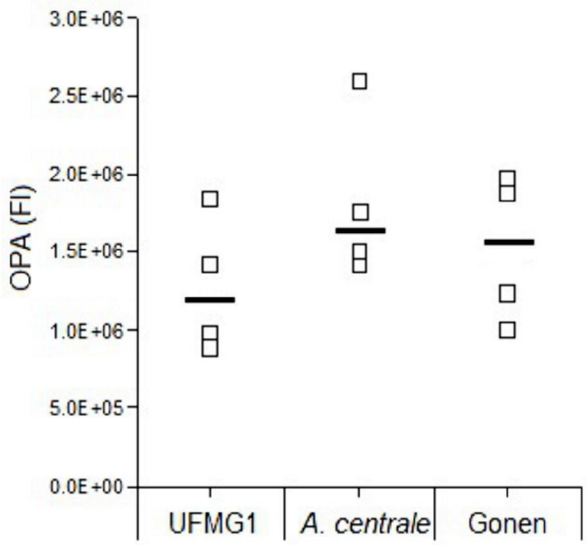

Figure 10: Opsonophagocytic activity induced by infection with UFMG1, A. centrale or Gonen. Results for Gonen infection were taken from the control group post-challenge. Horizontal bars indicate the median for each group.

The maximum OPA results for each calf during initial infection, and after challenge with the Gonen strain, are shown in Figure 11. Three out of four calves infected with UFMG1 showed very similar OPA levels during initial infection with UFMG1 and challenge with Gonen. This contrasts with IgG levels in the UFMG1 group (see Chapter 3), which rose sharply after challenge.

OPA levels for calves infected with *A. centrale* showed similar dynamics for overall IgG levels and OPA levels. Three out of four calves showed a higher OPA response to initial infection with *A.*

centrale than to challenge with Gonen.

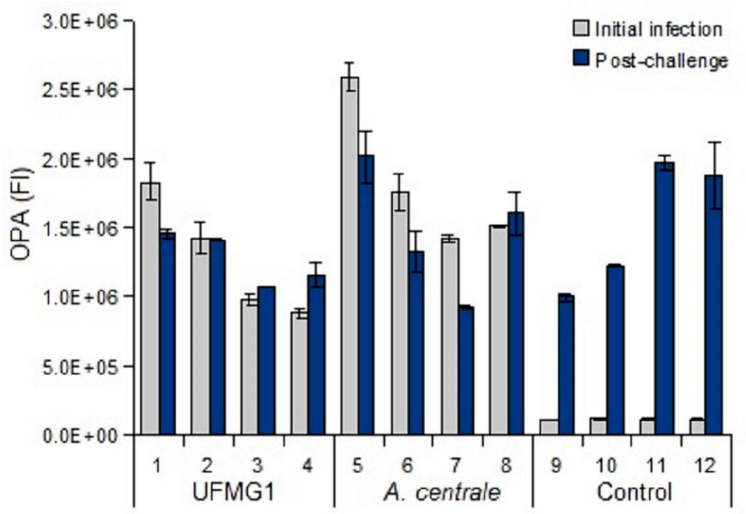

Figure 11: OPA of sera during initial infection and challenge, for the UFMG1 group (calves 1-4), A. centrale group (calves 5-8), and control group (calves 9-12).

5.3.6. Comparing OPA levels to clinical symptoms, IgG2, and IFNγ

Correlations between OPA and clinical symptoms

During initial infection with UFMG1 or *A. centrale*, higher OPA correlated significantly with milder disease symptoms ï specifically with a lower temperature ($p<0.02$), less reduction in hematocrit ($p<0.01$), and a lower rickettsemia ($p<0.01$), as shown in Figure 12.

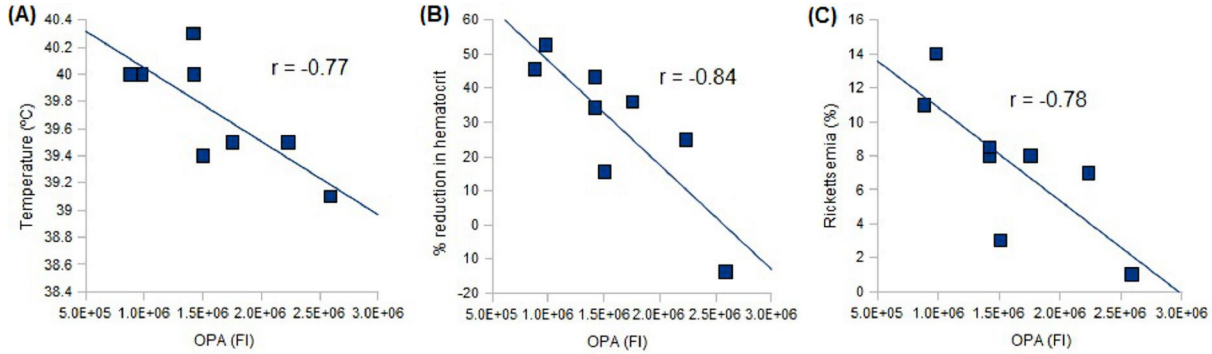

Figure 12: Correlations between OPA and clinical symptoms during initial infection with UFMG1 or A. centrale. From left to right, graphs show maximum OPA compared to (A) maximum temperature, (B) maximum reduction in hematocrit, and (C) maximum rickettsemia per calf, over the course of initial infection.

When the maximum level of OPA found in sera during initial infection was compared to the clinical response to challenge, high pre-challenge OPA correlated significantly ($p<0.01$) with less severe post-challenge anemia (see Figure 13). There was no such correlation with temperature or rickettsemia.

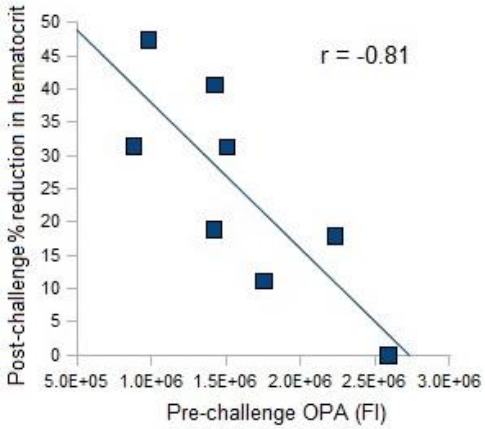

Figure 13: Correlation between pre-challenge OPA and post-challenge reduction in haematocrit ($r = -0.81$, $p<0.01$).

Correlations between OPA and other immunological parameters

Levels of OPA were compared to levels of total IgG, IgG2, and IFNɔ during initial infection with UFMG1 or *A. centrale* and challenge with the Gonen strain. When OPA was compared with the IFNɔ concentration, there was no significant correlation during initial infection or challenge. During initial infection with UFMG1 or *A. centrale,* OPA did show significant correlations with IgG1: IgG2 ratio ($p<0.06$), total IgG2 concentration ($p<0.1$), and increase in IgG ($p<0.05$) as shown in Figure 14. Considering the period after challenge with the Gonen strain in isolation, there was no significant correlation between OPA levels and IgG parameters.

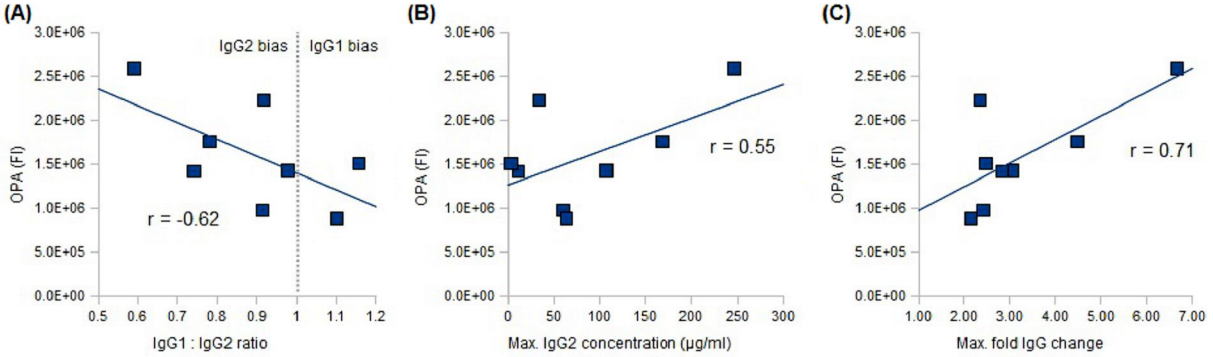

Figure 14: Correlations between OPA during initial infection with UFMG1 or A. centrale and (A) IgG1:IgG2, (B) IgG2 concentration, and (C) fold change in IgG.

5.4. DISCUSSION

5.4.1. Measuring phagocytosis through the uptake of fluorochrome-stained bacteria

Initially, the OPA assay was intended to detect phagocytosis by measuring the uptake of fluorochrome-stained bacteria. This was found to be impractical as an unsuitably high proportion of the fluorescent signal came from extracellular bacteria attached to the outside of phagocytic cells.

Extracellular fluorescence can be reduced by a quenching agent, which absorbs the fluorescence of any bacteria remaining outside the cell. Trypan blue is the most common quenching agent, as it will not enter living cells and so will not quench the fluorescence of any internalized bacteria (Bjerknes and Bassoe, 1984). Unfortunately the absorption spectrum of trypan blue does not fully cover the emission spectra of PI, and initial experiments found it to be ineffective in quenching PI fluorescence. SYTO9, the other nucleic acid dye which was tested, can be quenched by trypan blue, but had problems with the dye leaching from stained bacteria. The leaching of SYTO9 might be reduced by formaldehyde fixation of the stained bacteria, but unfortunately fixation decreases fluorescence emission for SYTO dyes (Lebaron *et al.*, 1998).

5.4.2. Measuring phagocytosis through the oxidative burst

The final OPA assay measured phagocytosis by detecting the level of oxidative burst. Within the mixed leukocyte population that was used, only the data from granulocytes was analyzed. This was because they were the only population to show a clear increase in oxidative burst when incubated with opsonized bacteria. The relatively low oxidative burst response from the monocyte population is likely to be due to the long incubation time in the *in vitro* assay. Bassoe *et al.* (2000) found that after more than 5 minutes incubation with the target, ROS production by monocytes began to decrease. In contrast, ROS production from granulocytes increased with longer incubation periods.

When comparing the ROS production of monocytes and granulocytes, van Pelt *et al.* (1996) and Bassoe *et al.* (2000) found that monocytes produced some ROS even without phagocytosis. In contrast, granulocytes required phagocytosis to trigger ROS production. Therefore analyzing data from granulocytes provides a reassurance that analysing the oxidative burst response will not overestimate the level of phagocytosis.

The optimum bacteria:cell ratio found here - 3250 bacteria per cell - was extremely high in comparison to other *in vitro* OPA assays. Cantor *et al.* (1993) used 50 *A. marginale* per macrophage.

The method used in that study to quantify bacteria was very approximate, based on percentage of infected erythrocytes before rickettsia were purified. However assays with other targets also used similar numbers: Vogel *et al.* (1994) used 10 *Haemophilus influenzae* for every cell, and Bassoe *et al.* (2000) 40-60 opsonin-coated beads.

It is likely that the 3250 bacteria per cell calculated here considerably over-estimated the actual number of intact bacteria per cell. The rtPCR quantification method used here provides a way to compare different batches of bacteria, but it is very likely to overestimate the number of bacteria that will be effective targets in the OPA assay. Epitopes on the surface of the bacteria must be intact for them to be opsonized by immune serum; with qPCR, DNA from broken up or damaged bacteria will also be counted.

It would be valuable to improve quantification methods for *A. marginale* to be used in *in vitro* assays. At present, numbers of purified bacteria are often simply estimated from the percentage of infected cells in the original blood sample or tick cell culture. Unfortunately as rickettsia are so small, it is very difficult to count them accurately once they are purified. Quantifying them by flow cytometry, using a nucleic acid stain such as SYTO9, or an *A. marginale*-specific antibody and then a fluorescent-tagged secondary antibody, could be considerably more accurate, but their propensity for clumping would still add a degree of uncertainty to the final count.

Vaccine trials often produce large numbers of samples which should if possible be tested simultaneously to allow for a more valid comparison of results. Therefore in this OPA assay, the slight increase in fluorescence when samples are read at a later time point could be problematic when larger numbers of samples have to be analyzed. This increase in fluorescence may be able to be reduced by formaldehyde fixing of samples, but this can often alter the results of oxidative burst assays (C. Brookes, personal communication). More accurate results with a smaller number of samples were therefore preferable.

The OPA assay could be further improved, for example: finding an effective bacterial stain would allow the simultaneous measurement of phagocytosis and oxidative burst. Access to a heated plate mixer would be likely to reduce both the number of bacteria and the incubation time required for the assay (Hampton *et al.*, 1999), saving time and resources. Nevertheless, overall the OPA assay developed here is effective, and allows the analysis of up to 42 duplicated samples per assay. It is considerably faster and more objective than the equivalent OPA assays using microscopy, and

therefore more useful in assessing the functional antibody response to *A. marginale* vaccines.

5.4.3. OPA response to *A. marginale* and *A. centrale* infection

During infection with UFMG1 or *A. centrale,* a higher level of OPA correlated with milder clinical symptoms. A stronger OPA response to these initial infections also correlated with a reduced anemia upon subsequent challenge with the Gonen strain. There was a lack of correlation between OPA levels after challenge and the severity of symptoms ï possibly, because as proposed in Chapter 3, a strong antibody response to initial infection may control the challenge infection before it can reach a level that provokes a strong new immune response.

Overall, these results corroborate the theory of Palmer *et al.* (1999) that OPA plays an important role in clearing *A. marginale* and protecting cattle from anaplasmosis. The model of Palmer *et al.* (1999) was based on studies showing that IgG2, the IgG subclass that is most effective at opsonization, has repeatedly been linked to protection (Chapter 3; Brown *et al.*, 1998; Barigye *et al.*, 2004; Vega *et al.*, 2007), and that immune sera can opsonize *A. marginale* (Cantor *et al.*, 1993; Melendez 2005).

The two previous studies of opsonophagocytosis with *A. marginale* confirmed that it occurs but could not determine whether it correlated with protection. Cantor *et al.* (1993) demonstrated that sera from calves immunized with purified MSP-1 significantly increased the phagocytosis of *A. marginale* by bovine macrophages. They tested sera samples from five calves, and found statistically significant differences in OPA activity of their sera. However, as all the calves used were completely protected from challenge after immunization (Palmer *et al.*, 1989), it was not possible to determine if the differences in OPA correlated with level of protection from challenge.

Melendez (2005) demonstrated that immune serum could opsonize *A. marginale*-infected red blood cells, but as the sera used came from only one acutely infected and one immunized calf it was impossible to infer the importance of OPA in reducing disease symptoms.

Therefore this is the first study that explicitly demonstrated an association of OPA with reduced disease symptoms, confirming and expanding on considerable supporting evidence from previous studies (summarized in Palmer *et al.*, 1999).

5.4.4. The relationship of OPA to other immune parameters

A significant correlation was seen between IgG2 concentration and OPA levels during infection with UFMG1 or *A. centrale.* This was expected, as several studies have shown that IgG2 is considerably more rapid and effective than IgG1 at enhancing phagocytosis by neutrophils and peripheral blood monocytes (McGuire *et al.*, 1979; McGuire and Musoke, 1981).

In the model of opsonophagocytosis by Palmer *et al.* (1999), IFNɔ played an important role in activating macrophages to increase their level of phagocytosis. However in this trial, serum IFNɔ concentration did not significantly correlate with OPA levels. This is perhaps not surprising as the assay here measured OPA in granulocytes, which are largely neutrophils (Murphy *et al.*, 2012). Although neutrophils can be activated by IFNɔ (Marchi *et al.*, 2014), as 'first-responder' cells, they are more involved in producing IFNɔ than in responding to it (Kumar and Sharma, 2010).

5.4.5. Conclusions and Future Research

Overall, OPA appears to be a useful correlate of a protective immune response to *A. marginale*. The relatively high-throughput *in vitro* OPA assay developed here could be very useful in estimating the effect of candidate vaccines against a wider range of strains than it would be practical to challenge cattle with.

More specialized OPA assays could also be used as a tool to investigate the immune response to *A. centrale* in more detail. Identifying antigens recognized by IgG2 from *A. centrale*-immunized cattle has already been used to find novel target proteins for vaccine research (Agnes *et al.*, 2011). The reasoning behind this study was that IgG2 is associated with protection due to its central role in OPA (Palmer *et al.*, 1999). But the opsonophagocytic activity of serum is not solely determined by IgG2 titer ï other factors such as antibody avidity also play a role (Johnson *et al.*, 1999). An OPA assay could be used to more directly identify which proteins are targeted by functional antibodies. Coating microsphere beads with recombinant protein antigens allows individual proteins to be evaluated for their importance as a target of the functional antibody response (Lehmann *et al.*, 1997). This technique has been used for assessing meningococcal (Lehmann *et al.*, 1999; Plested *et al.*, 2001) and streptococcal (Fabrizio *et al.*, 2010) antigens. Applied to *A. centrale,* this would be a valuable screening tool for identifying more effective vaccine candidates.

At the more basic level used here, the OPA assay provided an effective and relatively straightforward technique for evaluating serum samples from the live vaccine trial. *A. centrale*

infection induced higher levels of OPA in calves than UFMG1 infection. The higher OPA corresponded to a greater level of protection from disease on challenge with the heterologous Gonen strain. This further supports the evidence from the previous chapters that *A. centrale* induces a more effective immune response than UFMG1, enabling it to be a significantly more effective live vaccine.

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6. GENERAL CONCLUSIONS

6.1. Rationale for the Project

The aim of this project was to investigate the immune response to live vaccines against bovine anaplasmosis. *A. centrale,* the current 'gold-standard' live vaccine, was compared with UFMG1, a naturally low pathogenic Brazilian *A. marginale* strain, which has been proposed as a potentially safer cell culture-derived live vaccine. Calves were premunised with *A. centrale* or UFMG1, before being challenged with the heterologous Israeli *A. marginale* Gonen strain. The immune response of the calves was then compared with their level of protection from disease. This study therefore evaluated UFMG1 as a live vaccine, and elucidated correlates of protection from bovine anaplasmosis to help guide future vaccine development.

6.2. Main Findings of the Project

Chapter 2 described the trial comparing the effectiveness of *A. centrale* and *A. marginale* UFMG1 in protecting calves against the Israeli *A. marginale* Gonen challenge strain. *A. centrale* was significantly more effective than UFMG1 at reducing disease symptoms after challenge. UFMG1 was proposed as a potential live vaccine against *A. marginale* by Bastos *et al.* (2010), because it successfully protected calves from a heterologous and high pathogenic Brazilian *A. marginale* strain. Unfortunately, UFMG1 was not as effective when tested against a Israeli *A. marginale* strain. After challenge with the Israeli Gonen strain, anaplasmosis symptoms in calves previously infected with UFMG1 were similar to those in the naïve calves of the control group. In addition, UFMG1 infection itself caused a concerning level of disease symptoms, approaching close to the threshold for antibiotic treatment. The young calves used in the trial were the age group least affected by *A. marginale* infection. This suggests that the use of UFMG1 as a live vaccine is highly likely to cause problems in the field, where mechanical and biological transmission from vaccinated calves to more vulnerable adult cattle is always a possibility.

Chapter 3 investigated the serological response induced by *A. centrale* and *A. marginale* UFMG1. *A. centrale* infection induced a higher level of IgG, with a greater bias to IgG2, and also showed more cross-reactivity to antigen from the Gonen challenge strain. The IgG2 concentration, and level of IgG cross-reactivity to the challenge strain correlated significantly with subsequent protection from challenge.

Chapter 4 investigated the cell-mediated response to *A. centrale* and *A. marginale* UFMG1 ï namely levels of PBMC proliferation and IFNɔ production. No consistent antigen-specific PBMC proliferation was seen in samples from either group. However, there were numerous problems with the PBMC proliferation assays, which made it impossible to draw any firm conclusions from these experiments. When *in vivo* IFNɔ concentrations were measured, previous infection with UFMG1 or *A. centrale* resulted in amplified levels of IFNɔ production after challenge with Gonen. This amplified post-challenge IFNɔ response correlated with higher levels of IgG2, associated with protection from challenge. However, other factors beyond IFNɔ appear to also have a role in enhancing IgG2 production, as during initial *A. centrale* infection there was a low IFNɔ response but high IgG2 concentrations.

Chapter 5 described the development of an *in vitro* assay for serum opsonophagocytosis activity (OPA) against *A. marginale.* The OPA assay was based on flow cytometric measurement of the oxidative burst response to phagocytosis. OPA has previously been suggested as a correlate of protection for bovine anaplasmosis, but this had not been experimentally confirmed. In this study, calves which had higher OPA in their antibody response to initial infection were better protected from subsequent challenge with the heterologous Gonen strain. As expected, serum samples with higher IgG2 concentrations showed higher levels of OPA. Taken in combination with previous studies, these results suggest that OPA is an effective correlate of protection against bovine anaplasmosis.

Overall, this study showed that *A. centrale* was considerably more effective as a live vaccine than UFMG1, as only premunisation with *A. centrale* effectively protected calves against disease. Several characteristics of the serological response to premunisation significantly correlated with reduced disease upon subsequent challenge with Gonen: a high and cross-reactive IgG response, an IgG2 bias, and high OPA levels. *A. centrale* was more effective at consistently inducing this type of protective immune response than UFMG1.

The current model for a protective immune response against bovine anaplasmosis is largely based on studies from recombinant protein vaccination (Palmer *et al.,* 1999). However, these vaccines are so far confined to research trials, with live vaccination being the most effective approach in the field. From the results of this project, it appears that are likely to be differences in the mechanisms by which protein vs. live vaccination lead to protection from anaplasmosis.

The model of protection after protein vaccination proposed by Palmer *et al.* (1999) centers around antigen-specific CD4+ T-cells and their production of IFNɔ, which activates macrophages and stimulates B-cells to produce more IgG2. Antigen-specific IgG2 opsonises *A. marginale,* leading to increased phagocytosis by the activated macrophages and control of the infection.

However, during infection with *A. marginale,* there is large-scale selective deletion of *Anaplasma* antigen-specific CD4+ T-cells (Han *et al.,* 2010). Despite this removal of the CD4+ T-cells, calves which survive infection with *A. marginale* are generally well protected against homologous strains (Bock *et al.,* 2001). Therefore it seems unlikely that CD4+ T-cells can play such a central role in immunity induced by live vaccination.

The data on the T-cell response to live vaccination from this project was inconclusive, but other aspects of Palmerôs model appear to still apply here, namely, the correlation of IgG2 and opsonophagocytic activity with protection. However, the vast majority of phagocytic activity was seen from granulocytes, rather than macrophages as proposed by Palmer.

It is possible that granulocytes may be even more important in protection against anaplasmosis, as neutrophils can fulfil several of the functions that were assigned to CD4+ T-cells in Palmerôs model. These functions are centralized in the spleen, which has been shown to be critical for controlling *A. marginale* infection (Jones *et al.,* 1968). Once neutrophils have phagocytosed circulating bacteria, they transport them to the marginal zone of the spleen (Balázs *et al.*, 2002). Splenic neutrophils then serve as helpers for marginal zone B-cells: these B-cells specialize in rapidly producing T-cell independent antibody responses to blood-borne micro-organisms (Puga *et al.* 2011). The splenic neutrophils stimulate B-cell survival, antibody production, class switching, and somatic hypermutation. As neutrophils produce IFNγ (Etuin *et al.,* 2004), they can bias the antibody response towards production of IgG2, with its greater opsonic activity. Therefore, there is the intriguing possibility that the CD4+ T-cells central to Palmerôs model - but deleted during *A. marginale* infection - could have their role in protection at least partially filled by neutrophils.

6.3. Future Research

Many trials of *A. marginale* vaccines use homologous challenge strains, which is an excellent initial step to test candidate vaccines. But one of the main problems with existing *A. marginale* vaccines is the lack of cross-protection between the highly diverse strains. This was seen here again with

UFMG1, which was protective against a heterologous Brazilian strain (Bastos *et al.*, 2010), but not against the more distantly related Israeli strain used in this study.

Testing candidate vaccines against a wide range of heterologous strains is an important part of evaluating their potential. But performing challenge trials against multiple strains can be prohibitively expensive, taking considerable time and resources. Reliable correlates of protection that can be measured *in vitro* would be a valuable method for gauging the potential cross-protection induced by candidate vaccines.

The results here confirm previous studies that IgG2 appears to be a good correlate of protection, and show for the first time that the OPA of the immune response to live vaccines also correlates significantly with later protection from challenge. The *in vitro* OPA assay developed here has the potential to be used to test immune serum samples against a range of *A. marginale* strains, and to identify specific antigens targeted by the functional antibody response.

Previous infection with UFMG1 did not significantly reduce the severity of disease after challenge with the heterologous Gonen strain, whereas *A. centrale* did provide cross-protection. As seen here, *A. centrale* infection led to high production of IgG, high cross-reactivity within that IgG response, a strong IgG2 bias, and high opsonophagocytic activity. All of these characteristics are likely to be important factors behind the widespread success of the *A. centrale* vaccine.

Understanding how *A. centrale* infection stimulates this effective immune response would be a valuable direction for future vaccine research. This could either be through identifying protective antigens, to suggest a more rational design of a subunit vaccine; or by determining critical cytokines induced by *A. centrale* infection, which could suggest suitable adjuvants to add to vaccine formulations.

A. centrale has some problems as a vaccine: safety concerns due to it being derived from blood, and reports of failure against high pathogenic strains. However, *A. centrale* is still used extensively and successfully in many countries. Possibly the most practical and cost-effective approach to developing a better anaplasmosis vaccine would be to focus research on making the *A. centrale* vaccine safer and more consistently able to induce the type of protective immune response identified here.

6.4. REFERENCES

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7. APPENDIX: SUMMARY OF CLINICAL AND IMMUNOLOGICAL DATA

Initial infection

Group	Calf	Peak rickettsemia (% iRBCs)	Peak temperature (°C)	Minimum PCV (% pcv)	Reduction in PCV (% pcv)	% Reduction in PCV
A. marginale						
UFMG1	**1**	7.00	39.50	21.00	7.00	25.00
	2	8.00	40.00	21.00	11.00	34.38
	3	14.00	40.00	18.00	20.00	52.63
	4	11.00	40.00	19.00	16.00	45.71
	Median	**9.5**	**40.0**	**20.0**	**13.5**	**40.0**
A. centrale	**5**	1.00	39.10	33.00	-4.00	-13.79
	6	8.00	39.50	23.00	13.00	36.11
	7	8.00	40.30	21.00	16.00	43.24
	8	3.00	39.40	27.00	5.00	15.63
	Median	**5.5**	**39.5**	**25.0**	**9.0**	**25.9**
Control	**9**	0.00	39.70	29.00	7.00	19.44
	10	0.00	39.00	31.00	0.00	0.00
	11	0.00	39.10	30.00	4.00	11.76
	12	0.00	40.10	28.00	10.90	28.02
	Median	**0.0**	**39.4**	**29.5**	**5.5**	**15.6**

Post-challenge with *A. marginale Gonen*

Group	Calf	Peak rickettsemia (% iRBCs)	Peak temperature (°C)	Minimum PCV (% pcv)	Reduction in PCV (% pcv)	% Reduction in PCV
A. marginale						
UFMG1	**1**	0.01	41.20	23.00	5.00	17.86
	2	7.00	39.70	19.00	13.00	40.63
	3	3.00	40.80	20.00	18.00	47.37
	4	0.50	39.70	24.00	11.00	31.43
	Median	**1.8**	**40.3**	**21.5**	**12.0**	**36.0**
A. centrale	**5**	1.09	40.50	29.00	0.00	0.00
	6	0.02	41.30	32.00	4.00	11.11
	7	0.09	39.30	30.00	7.00	18.92
	8	3.00	39.40	22.00	10.00	31.25
	Median	**0.6**	**40.0**	**29.5**	**5.5**	**15.0**
Control	**9**	4.00	40.10	22.00	14.00	38.89
	10	0.70	39.40	20.00	11.00	35.48
	11	7.00	40.10	20.00	14.00	41.18
	12	0.50	39.60	26.00	12.90	33.16
	Median	**2.4**	**39.9**	**21.0**	**13.5**	**37.2**

Table 1: Summary of clinical data (see chapter 2 for further information).

Initial infection

Group	Calf	Max. IgG (fold change)	Max. IFNγ (pg/ml)	IgG1:IgG2	Max. IgG2 (µg/ml)	OBA activity
***A. marginale* UFMG1**	**1**	2.34	117.47	1.39	33.93	1.83E+06
	2	3.05	221.18	1.21	107.47	1.42E+06
	3	2.41	47.65	1.08	60.27	9.80E+05
	4	2.15	434.36	1.21	64.00	8.81E+05
	Average	2.49	205.16	1.22	66.42	1.28E+06
	Std Dev	0.39	168.61	0.13	30.47	4.38E+05
A. centrale	**5**	6.66	35.17	0.53	246.55	2.59E+06
	6	4.47	82.29	0.74	168.16	1.75E+06
	7	2.84	58.07	1.59	11.29	1.42E+06
	8	2.47	34.37	2.55	3.61	1.51E+06
	Average	4.11	52.47	1.35	107.40	1.82E+06
	Std Dev	1.91	22.71	0.92	119.81	5.34E+05
Control	**9**	1.20	37.33	1.81	3.53	1.22E+05
	10	1.11	21.97	1.74	3.53	1.26E+05
	11	1.04	15.89	1.75	3.64	1.19E+05
	12	1.89	26.83	1.75	3.32	1.21E+05
	Average	1.31	25.51	1.76	3.51	1.22E+05
	Std Dev	0.39	9.07	0.03	0.13	2.92E+03

Post-challenge with *A. marginale Gonen*

Group	Calf	Max. IgG (fold change)	Max. IFNγ (pg/ml)	IgG1:IgG2	Max. IgG2 (µg/ml)	OBA activity
***A. marginale* UFMG1**	**1**	4.69	552.78	0.55	240.02	1.46E+06
	2	5.03	79.96	1.06	563.07	1.41E+06
	3	4.55	487.65	0.95	185.11	1.08E+06
	4	8.54	395.66	0.82	600.39	1.16E+06
	Average	5.70	379.01	0.85	397.15	1.28E+06
	Std Dev	1.90	209.53	0.22	214.85	1.87E+05
A. centrale	**5**	5.30	552.78	1.07	250.84	2.02E+06
	6	3.57	21.76	2.55	30.50	1.33E+06
	7	2.01	552.92	2.01	58.07	9.35E+05
	8	3.18	11.73	2.47	62.36	1.60E+06
	Average	3.51	284.80	2.03	100.44	1.47E+06
	Std Dev	1.36	309.55	0.68	101.25	4.56E+05
Control	**9**	4.83	12.45	1.28	109.32	1.00E+06
	10	2.76	13.09	2.11	29.37	1.23E+06
	11	4.10	90.32	0.94	175.57	1.97E+06
	12	5.36	7.66	1.80	105.42	1.88E+06
	Average	4.26	30.88	1.53	104.92	1.52E+06
	Std Dev	1.13	39.70	0.52	59.77	4.77E+05

Table 2: Summary of immunological data (see Chapters 3,4, and 5 for further details).

8. ABBREVIATIONS

B	Bacteria
BrdU	Bromodeoxyuridine
C	Cells
C3R	C3 complement protein receptor
C4R	C4 complement protein receptor
ConA	Concanavalin A
DCFH-DA	Dichloro-dihydro-fluorescein diacetate
DHR123	Dihydrorhodamine 123
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's Phosphate Buffered Saline
DPBS-G	DPBS + 5 mM glucose
DPBS-GCM	DPBS + 5 mM glucose + 1.2 mM $CaCl_2$ + 1mM $MgSO_4$
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FcR	Antibody Fc Receptor
FI	Fluorescence Index
FSC-A	Forward scatter ï area of signal
FSC-H	Forward scatter ï height of signal
IFNɔ	Interferon gamma
IgG	Immunoglobulin G
IgM	Immunoglobulin M
IS	Immune sera
LPS	Lipopolysaccharide
MSP	Major Surface Protein
NIS	Non-immune sera
OD	Optical Density
OMP	Outer Membrane Protein
OPA	Opsonophagocytic Activity
PBMC	Peripheral Blood Mononuclear Cell
PBS	Phosphate Buffered Saline
PBS-T	PBS with 0.05 % Tween 20
PCV	Packed Cell Volume
PI	Propidium Iodide
pNPP	p-nitrophenyl phosphate
PRR	Pathogen Recognition Receptor
RBC	Red blood cell
RT	Room Temperature
ROS	Reactive Oxygen Species
SSC-A	Side scatter ï height of signal
SI	Stimulation Index
TGF-beta	Transforming Growth Factor beta
TMB	Tetramethylbenzine
UFMG1	Universidade Federal de Minas Gerais strain 1
UFMG2	Universidade Federal de Minas Gerais strain 2
USD	United States Dollars

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7: Appendix: Summary of Clinical and Immunological Data

10. CURRICULUM VITAE

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