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EVALUATION OF IgG 2 REACTIVITY IN CATTLE INOCULATED WITH RECOMBINANT OUTER MEMBRANE PROTEIN 7, 8 AND 9 OF *ANAPLASMA MARGINALE*

BY

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THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTER OF PHILOSOPHY DEGREE IN ANIMAL SCIENCE

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DECLARATION

I hereby declare that this thesis which is submitted to the University of Ghana, for the award of Master of Philosophy in Animal Science degree, is the result of my own original work and that no part of it has been presented for another degree in this university or elsewhere. All assistance towards the production of this work and all the references contained herein have been duly credited.

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DEDICATION

I dedicate this work to the Almighty, most gracious and merciful God in whom I live and move and have my being.

To my beloved husband; Ing. Collins Adu - Poku and my children, Nana Kofi, Kwame Yeboah, Yaa Kessewaa, Yaw and the last and most accommodating Kwame Nyarko Adu -Poku

To my parents, siblings and all loved ones whom without their support, prayers and encouragement I would not have been this far.

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TABLE OF CONTENT

Contents

DECLARATION ii
DEDICATION iii
ACKNOWLEDGEMENT iv
LIST OF FIGURES vii
ABSTRACT viii
LIST OF ACRONYMES AND ABBREVIATIONS x
CHAPTER ONE 1
1.0 INTRODUCTION 1
1.1 Background 1
1.2 Hypothesis 2
1.3 Specific Aim: 3
1.4 Main objective 3
1.5 Sub-objective 3
1.5.1 Specific objectives 3
1.6 Justification 3
CHAPTER TWO 5
1 LITERATURE REVIEW 5
2.1 Tick borne pathogens and their related diseases 5
2.2 Bovine Anaplasmosis 5
2.3 Geographical distribution of bovine anaplasmosis 6
2.4 Anaplasma infection of cattle 7
2.5 Theileriosis 7
2.6 Babeiosis 8
2.7 Control strategies of anaplasmosis 9
2.8 Problems associated with disease control in the absence of vaccines 9
2.9 Anaplasma target vaccines that have proved less successful 10
2.10 Current vaccine development strategies 11
2.11 Outer membrane protein protection of cattle against anaplasmosis 13
2.12 Interleukins 14
2.13 IgG 2 reactivity 15
CHAPTER THREE 17
MATERIALS AND METHODS 17
3.1 Experimental Design: 17

University of Ghana http://ugspace.ug.edu.gh

3.1.1 Specific Aim: 17
1.6.1 Strategy 17
3.1 Experimental Procedures 18
3.1.1 Location of study 18
3.1.2 Source of Plasmid DNA and confirmation of the presence of Omp7-9 Inserts 18
3.2 Expression of Omp 7 – 9 Proteins 18
3.3 Protein Purification Under Native Conditions 20
3.5 *Omp7-9* Immunisation of Cattle for increased IgG 2 Secretion 20
3.5.1 Source of Calves and DRB3* Haplotypes 20
3.5.2 Verification of the Immune Status of Calves 21
3.6 Blood Sampling, Serum and Genomic Extraction 21
3.7 Multiplex PCR and Infection Prevalence 22
3.8 Immunisation of Calves with Omp 7 – 9 Proteins 22
3.9 Measurement of the Induced Immune Response: 23
3.10 Ethical Statement 25
CHAPTER FOUR 26
4.0 RESULTS 26
4.1 Eco RI digest of omp 7-9 in plasmid preps 26
4.2 Successful expression of rOmp 7-9 in bacterial cells 27
4.3 Molecular concentration of *rOmp 7 – 9* 28
4.4 Infection status of calves 29
4.5 Prevalence of *Anaplasma marginale* Infection in calves 30
4.6 Immune status of calves 31
4.7 Cytokine secretion - IL 6 32
4.8 Purified Omp 7-9 triggers TNF – α secretion in cattle 33
4.9 Purified *rOmp 7-9* triggers IgG 2 Production in cattle 34
4.10 Comparison Between Artificially Stimulated and Naturally Infected 35
CHAPTER FIVE 37
5.0 DISCUSSION 37
5.1 rOmp 7-9 stimulates innate immunity in calves 38
5.2 rOmp 7-9 Increases IgG 2 secretions in calves 39
CHAPTER SIX 41
6.0 CONCLUSION AND RECOMMENDATION 41
6.1 CONCLUSION 41
6.2 RECOMMENDATION 42
REFERENCES 43

University of Ghana http://ugspace.ug.edu.gh

LIST OF FIGURES

Figure 1. Bovine Anaplasmosis Distribution in the world in 2018 (OIE,2019) 6

Figure 2: General scheme of Anaplasma marginale vaccine development 11

Figure 3. The digest of stored plasmids with DNA inserts of omp 7 – 9 of Anaplasma marginale. 26

Figure 4: Coomassie Stained SDS Gel of Expressed rOmp 7,8 and 9 of Anaplasma marginale. 27

Figure 5: The display of protein concentration of rOmp 7 – 9 of Anaplasma marginale using A280 Protein parameter of NanoDrop 2000c Spectrophotometer 28

Figure 6: PCR Screening of calve herd for Anaplasma marginale infection status.. 29

Figure 7: Anaplasma marginale Infection prevalence in Calves using PCR 30

Figure 8: Immune status of Calf Herd.. 31

Figure 9: Quantity of IL-6 in pg/ml 32

Figure 10. Mean quantity of TNF–α in pg/ml 33

Figure 11: Effect of rOmp 7-9 on IgG 2 production 34

Figure 12: Comparison between Naturally Infected and Artificially stimulated...... 35

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ABSTRACT

Presently, there is a surge in vaccine development strategies with focus on the subunit vaccines using outer membrane proteins (OMP) which are sub-dominant surface antigens. The specific antigens that induce protective immunity against *A. marginale* are not known. Immunization with individual OMPs has been unsuccessful in providing protection against homologous strain challenge in the natural bovine host as compared to that achieved by immunization with OM. The current study examines if Omps serve as better immunogens for the development of cross-protective vaccine.

Omp7-9 have encoded CD4+ T cell epitope that are universally conserved among geographic isolates of A. marginale. This finding has positioned OMPs 7 – 9 of *A. marginale* as prospective vaccine candidate. The goal of this study was to determine whether immunization of cattle with recombinant Omp (rOmp) 7 - 9 proteins will trigger IgG 2 secretions as a surrogate for adaptive immunity.

The current study has demonstrated, for the first time in Ghana, > 500mg/ml of Omp 7 – 9 of *A. marginale* can be expressed and purified as a 45kDa recombinant protein (rOmp 7 – 9). Also, for the first time in Ghana, when administered to calves subcutaneously, rOmp 7-9 stimulated innate immune responses resulting in the development of IgG production, consistent with the induction of adaptive immune response. Firstly, experimental calves have expressed significant levels of the essential biomarkers for innate immunity. This included increased expression of IL – 6 ($p < 0.05$) from 100pg/ml to 1000pg/ml and of TNFα within 48 hours. Innate immune responses progressed to adaptive immunity by the significant rise in IgG 2 secretion ($p = 0.02$) as compared to control cohorts.

This high amount of IgG 2 readout in inoculated calves compared with non - inoculated calves ($p = 0.014$) signifies the potential Omp 7 – 9 in inducing protective immunity and therefore a candidate for vaccine development against worldwide prevalent *Anaplasma*

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marginale. Most importantly, the IgG 2 secretions in experimental calves injected with rOmp 7 – 9 comparable to IgG 2 secretions prevailing in the population of cattle naturally infected with *A. marginale* ($p = 0.10$).

Together, these results provide empirical evidence that vaccination with recombinant Omp7 - 9 would trigger the development of significant amounts of IgG Friesian x Sanga F1 crossbred calves.

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LIST OF ACRONYMES AND ABBREVIATIONS

CD	-	Cluster of differentiation
cELISA		Competitive Enzyme – linked immunosorbent assay
ELISA	-	Enzyme–linked immunosorbent assay
GM–CSF	-	Granulocyte macrophage colony stimulating factor
IgA	-	Immunoglobulin A
IgG	-	Immunoglobulin G
IgM	-	Immunoglobulin M
IL	-	Interleukin
INF α	-	Interferon alpha
INF γ	-	Interferon gamma
LIF	-	Leukaemia inhibitor factor
M–CSF	-	Macrophage colony stimulating factor
MHC		Major Histocompatibility Complex
mPCR		Multiplex Polymerase Chain Reaction
MSP		Major Surface Protein
NK–cells	-	Natural killer cells
OM		Outer Membrane
OMP		Outer Membrane Protein
PCR		Polymerase Chain Reaction

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PFAM		Protein Family Database
rOMP		Recombinant Outer Membrane Protein
SDS		Sodium dodecyl sulphate–polyacrylamide gel electrophoresis
TBDs		Tick Borne Diseases
Th 1 cells	-	Type 1 helper T cells
TNF – α	-	Tumour necrosis factor alpha

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CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

One of the major problems associated with tick-borne disease control is lack of vaccines. In the absence of vaccines farmers resort to uncontrolled use of acaricides that has led to the emergence of resistant pathogen and vector strains. This practice subjects the pathogen to the host immune selection pressure that culminates in antigenic variation of vaccine-relevant molecules displayed by the pathogen. Over time, it becomes very difficult to find stable antigens to develop effective vaccines.

Presently, there is a surge in vaccine development strategies with focus on the subunit vaccines using outer membrane proteins (OMP) which are sub-dominant surface antigens. Purified *Anaplasma marginale* outer membrane and cross-linked outer membrane protein (Omp) complexes induced protection against experimental tick-borne challenge (Noh *et.al*., 2008). The composition has been defined for strains circulating in the Americas, allowing progression to define vaccine testing. Whether these Omps are structurally and immunologically conserved with endemic strains circulating in Ghana and other countries in West Africa is unknown. Addressing this knowledge gap is critically important for implementation of new vaccines in Africa. Answering the current question will therefore direct whether region-specific vaccine approaches are required. Specifically, Omp immunisation partially protected cattle against Anaplasmosis (Noh *et.al*., 2008). Using protein complex - *Omp1,* Major surface proteins (*Msp1a, Msp2, Msp3, Msp4*) to immunize cattle indicated that the surface protein subset of the outer membrane cross-linked were capable of inducing protective immunity (Lopez *et.al*., 2007; Marcelino *et.al.,* 2012).

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Whether this ability to protect can be extended to lesser-known surface molecules including *omp7-9* has not been investigated.

Analysis of the primary structure indicated that OMPs are highly conserved among tick-borne pathogen strains circulating in Ghana and revealed their relative conservation in strains isolated in the Americas (Futse *et.al*., 2019). Results from sequence and immunologic analyses of the AM854 and AM779 represent this conservation where there are no significant antigenic differences in the OMPs of the endemic Ghanaian strains (including the most diverse structurally) as compared to the OMPs of the North American reference strain (St. Maries). This would imply that a broadly cross protective vaccine is achievable and that full-length recombinant proteins may be optimal. In contrast, the conservation of a defined CD4+ T cell epitope in *Omps 7-9* among all strains suggests that vaccination should use an "epitope-defined" approach. In combination, these outcomes suggest a hybrid approach where full-length OMPs can be combined with an epitope-specific immunogen. This approach could be realized using recombinant proteins either expressed individually and linked to the T cell epitope or a unique chimera. These characteristics have positioned *Omps 7 to 9* of *A. marginale* as prospective vaccine candidate as they can be recognised by immune serum IgG 2 and T cells in cattle immunised with *A. centrale* vaccine strain (Deringer *et.al.*, 2017).

1.2 Hypothesis

Inoculation of cattle with recombinant *Omp 7 – 9* protein will amplify the magnitude of IgG 2 activity against *Anaplasma marginale* challenge on the field.

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1.3 Specific Aim:

Determine if vaccination with conserved Omp7-9 induces IgG and relevant immune components in immunologically naive calves

1.4 Main objective

To determine whether immunization of cattle with recombinant *Omp 7-9* proteins will trigger IgG 2 secretions.

1.5 Sub-objective

1. Determine if omp7 – 9 isolates circulating in cattle from Ghana could be expressed in bacterial cell and purified
2. Determine if *Omp7-9* immunisation of cattle will result in increased IgG secretion.
3. To determine whether increased IgG 2 expression correlates with decrease disease severity.

1.5.1 Specific objectives

1. To express and purify recombinant *omp 7-9* proteins of *Anaplasma marginale*.
2. To inoculate cattle with expressed *rOmp 7-9* and determine their IgG 2 responses.

1.6 Justification

Although there have been several vaccinations attempts against anaplasmosis involving the use of dominant surface coat proteins to trigger natural immune response, they have all proved less successful. These dominant surface coat proteins were highly variable, and thus, were not stable vaccine targets. and appeared to have evolved under positive selection

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pressure (Palmer *et.al.*, 1999). Some vaccines have efficiently prevented clinical anaplasmosis in cattle but have failed to block *A. marginale* infection (Kocan *et.al.,* 2003).

Recently, there have been new vaccine development strategies which include the Subunit Vaccines such as Outer membrane proteins (OMP). These molecules are sub-dominant surface antigens. Evidence showed that Omps immunization partially protected cattle against *Anaplasma marginale* infection (Noh *et.al*., 2010).

Protein complexes *Omp 1, Omp 7 – 9, Msp1a, Msp2a, Msp3*, and Msp4 used to immunize cattle indicated that a surface protein subset of the outer membrane is cross-linked and were capable of inducing protective immunity and serves to direct vaccine development (Lopez *et.al*.,2007; Marcelino *et.al.,* 2012).

Globally-conserved T cell epitope in *Omp 7 – 9* of *Anaplasma marginale*, has been successfully identified and found to share sequence identity only with the *Omp7-9* in the vaccine strain *A. marginale* subsp. *centrale*. *Omps 7-9* of *A. marginale* has the potency of being a vaccine candidate because they are surface exposed, present in the outer membrane immunogen and protective crossed – linked outer membrane protein. These OMPs can be recognised by immune serum IgG 2 and T cells of cattle immunized with outer membrane (OM) and also recognised by immune serum IgG 2 from cattle immunised with the *A. centrale* strain (Deringer *et al.,*2017). OMP's are also relatively stable in the face of immune pressure, they can be expressed in bacterial cells and co-expression of 2-3 proteins is feasible (Ducken *et al*., 2015).

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CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Tick borne pathogens and their related diseases

Ticks and tick-borne diseases (TBDs) are of global economic importance as they affect livestock, human and other animals. The annual global losses associated with ticks and TBDs in cattle was estimated to be between US$ 13.9 billion and US$ 18.7 billion (Marcelino *et.al.*, 2012). Ticks are responsible for extreme economic losses in the tropics and in most African countries where the majority of cattle owners are poorly resourced (Regitano and Prayaga 2010; Sungirai *et. al.*, 2016).

In Ghana, *A. marginale* is the most prevalent of the three targeted tick-borne pathogens of three vegetational zones (Guinea savannah, Coastal Savannah and Semi-deciduous forest); *A. marginale* being 45–75 % followed by *Theileria spp*. (13–34 %) and *B. bigemina* (3 %) with the least prevalence (Beckley *et al*., 2016).

2.2 Bovine Anaplasmosis

One of the world's most economically significant tick-borne diseases of ruminants is bovine Anaplasmosis, triggered by *Anaplasma marginale*, a gram-negative, obligate intra-erythrocytic pathogen of the Rickettsiales order and the Anaplasmataceae family (Aubry and Geale, 2011; Hove et.al., 2018). Also, the most common vector-borne cattle pathogen on all six inhabited continents of the globe is *Anaplasma marginale* (Ducken *et.al.*,2015; Tabor *et.al.*,2019). The disease is spread biologically by ixodide ticks and can also be mechanically transmitted by biting flies or blood-contaminated fomites, unlike the other protozoan pathogens (Kocan *et.al.*, 2000; Bilgic *et.al*, 2013).

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2.3 Geographical distribution of bovine anaplasmosis

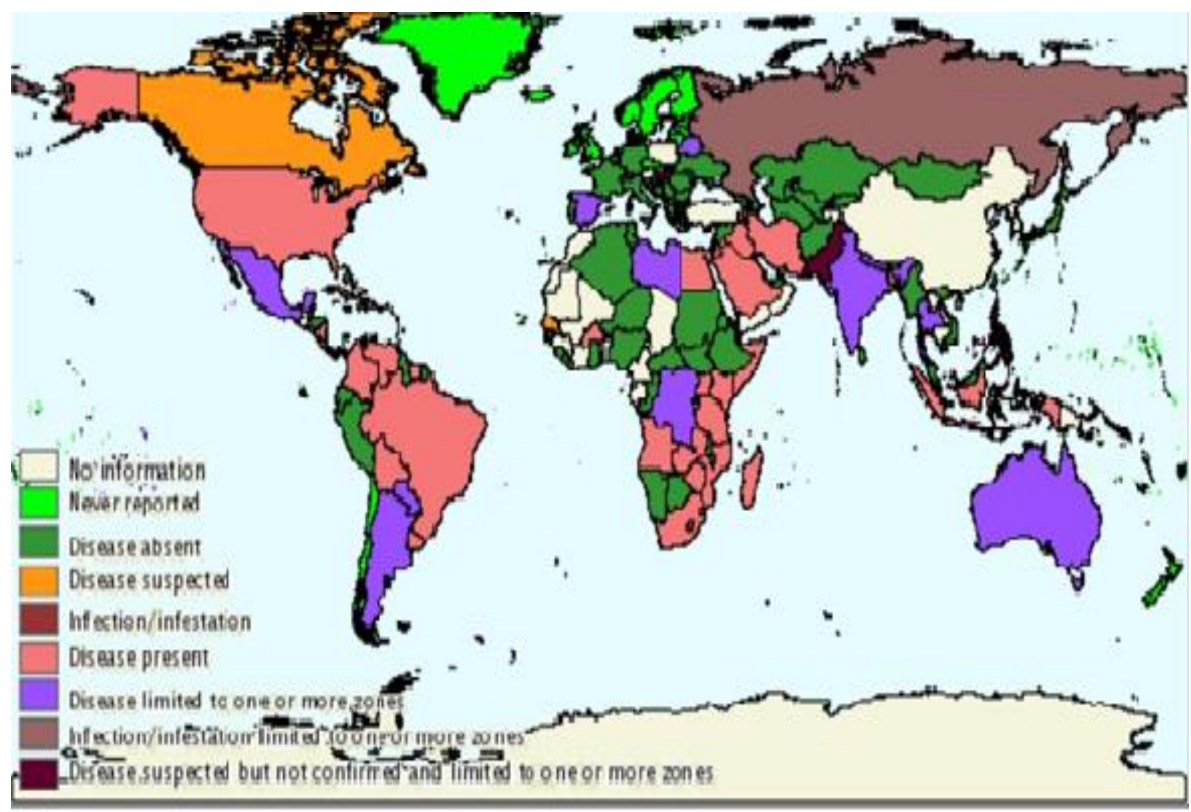

***Figure 1*. Bovine Anaplasmosis distribution in the world in 2018 (OIE,2019)**

Anaplasmosis exists globally in tropical and subtropical regions (Figure 1) and is the principal constraint in many nations for cattle production (McCallon, 1973; Kocan *et.al.*, 2003). Nevertheless, it has been recorded in nearly every U.S. state and this may be due to enhanced cattle transport creating an enabling environment for transmission from persistently infected asymptomatic cattle. Bovine anaplasmosis is also prevalent but enzootic in most Latin American countries in Mexico, Central and the Caribbean Islands with the exception of desert regions or mountain ranges (Guglielmone Andes, 1995; Kocan *et.al.*, 2003). For the year 2019, bovine anaplasmosis was amongst the list of 117 animal diseases, infection and infestation and also amongst 13 cattle diseases and infections (OIE, 2019).

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2.4 Anaplasma infection of cattle

Cattle anaplasmosis is characterized clinically by two distinct stages. The acute infection and the persistent infection stages. Acute symptoms are fever, anaemia, weight loss and jaundice. Other symptoms are abortion, lowered milk production and finally death (Morrision, 2015). Persistent infection in cattle is asymptomatic and bacteraemia cannot be detected by microscopy except by specialized serological assays and carrier herds remain infective to mates (Kocan *et.al.*,2003). Detection is hence a necessity, as carrier livestock they play a critical role in TBD epidemiology serving as infection reservoirs for naïve ticks and the disease gets introduced into new locations. (Burridge *et.al.*, 1974; Kiltz *et. al.*, 1986; Molad *et.al.*, 2000; Bilgic *et.al.*, 2013).

In addition, serological tests, such as the enzyme-linked immunosorbent assay (ELISA), may lack adequate sensitivity to detect proof of infection in low-parasite cattle, and this may have immediate consequences for disease control as outbreaks may happen as a result of poorly diagnosed cattle being transported from one location to another. Polymerase Chain Reaction assays were designed to detect single species and multiplex polymerase chain reaction (mPCR) is a modified PCR developed for simultaneous detection of *T annulate, B. bovis* and *A. marginale* in cattle-derived blood (Bilgic *et.al.*, 2013).

2.5 Theileriosis

Theileriosis is a disease triggered by tick-borne by *Theileria spp. T parva* and *T annulata*, which cause extensive deaths of cattle in tropical and subtropical regions, and other cattle-related species. *Theileria spp.* uses WBCs and RBCs successively in mammalian hosts to complete their life cycle. *Theileria's* pathogenic species replicates primarily within the

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host WBCs, whereas less pathogenic species primarily multiply in RBCs. The existence of the disease depends on the particular tick vectors being distributed geographically. Indigenous cattle have an inherent amount of resistance in certain endemic regions, which means that mortality is comparatively small, but cattle introduced are rather susceptible and there is no evidence of increased resistance in calves less than six months old. Depending on the level of challenge, clinical signs vary from unseemly or mild to severe and deadly. Typically, lymph node swelling (pronounced and generalized) happens 7–10 days after parasites are transmitted by feeding ticks. The remaining signs are anorexia, fast condition loss, nostril tearing and discharge. Terminally, dyspnea is prevalent and death generally happens 18-24 days after infection. However, cattle recovering from comparable strains become immune to subsequent infection, they may be susceptible to some heterologous strains and the majority of those recovering or being immunized stay carriers of infection. (Morrison, 2015)

2.6 Babeiosis

Bovine babesiosis is primarily caused by tick-borne apicomplexan parasites such as *B. bovis*, *B. Bigemina,, B. divergens*. Acute disease-related clinical symptoms include fever, hemolytic anemia, anorexia, lethargy, hemoglobinuria, tachycardia, and icterus. *B. Bovis* may also cause severe disease that usually leads to cerebral babesiosis, with symptoms such as seizures, hyperesthesia, and paralysis associated with brain capillary parasites. Shock and respiratory distress are common causes of death in acutely infected cattle (Brown and Palmer, 1999 ; Suarez and Noh, 2011).

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2.7 Control strategies of anaplasmosis

The main tick control strategy has been the use of acaricide but the unintended immergence of acaricide-resistant tick strains coupled with the presence of acaricide residues in milk and meat is major public health concern. This can eventually disrupt the enzootic stability making cattle susceptible to tick borne diseases (Regitano and Prayaga, 2010; Quiroz-Castañeda *et.al.,* 2016). Chemoprophylaxis is quite efficient but not enduring, so mixed control techniques that are more efficient and sustainable such as vaccines to control TBDs should be developed (Minjauw and Mcleod, 2003; Marcelino *et.al*., 2012) as the host immunity possess the facility to complement other control strategies and reduce the need for chemical control. The use of acaricides to control tick vectors and long-acting, rickettsicidal tetracyclines, such as the most frequently used oxytetracycline, are mainly involved in chemical control and therapy measures. The impacts of tick-borne diseases on livestock throughout the globe are often synergistic, with livestock infected with more than one pathogen at a moment (DeWaal *et.al.*, 2000: Hove *et.al.,* 2018).

2.8 Problems associated with disease control in the absence of vaccines

One of the major problems associated with tick-borne disease control in the absence of vaccines is the excessive and uncontrolled use of acaricides that has led to the emergence of resistant pathogen and vector strains because the pathogen is subjected to the host's increased immune selection pressure resulting in antigenic variation making it difficult to find a stable vaccine target. Other factors include the high cost of available acaricides and drugs which leads to higher budgetary constraints of farmers (Regitano and Prayaga, 2010; Marcelino *et.al*., 2012).

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2.9 Anaplasma target vaccines that have proved less successful

Although, there has been some interventions of vaccines against Anaplasmosis, these has however, proved less successful. These involved the use of dominant surface coat proteins to trigger natural immune response. Major surface proteins (MSPs) and adhesion proteins play a critical role in *A. marginale* host cell's interaction. MSPs from multigene families undergoing antigenic shift and selection in cattle help maintain persistent infections. These dominant surface coat proteins were highly variable, thus, were not stable vaccine targets. Some phylogenetic studies about isolates of *A. marginale* that use *Msp1* and *Msp4* genotypes in vaccines revealed that these MSPs seem to evolve under positive selection pressure. Therefore, new procedures are needed that may be capable of preventing clinical disease and at the same time preventing infection in cattle and ticks, thereby eliminating these hosts as infection reservoirs. Although some vaccines in cattle have efficiently prevented clinical anaplasmosis, they have not blocked *A. marginale* infection. (Kocan *et.al.,* 2003).

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2.10 Current vaccine development strategies

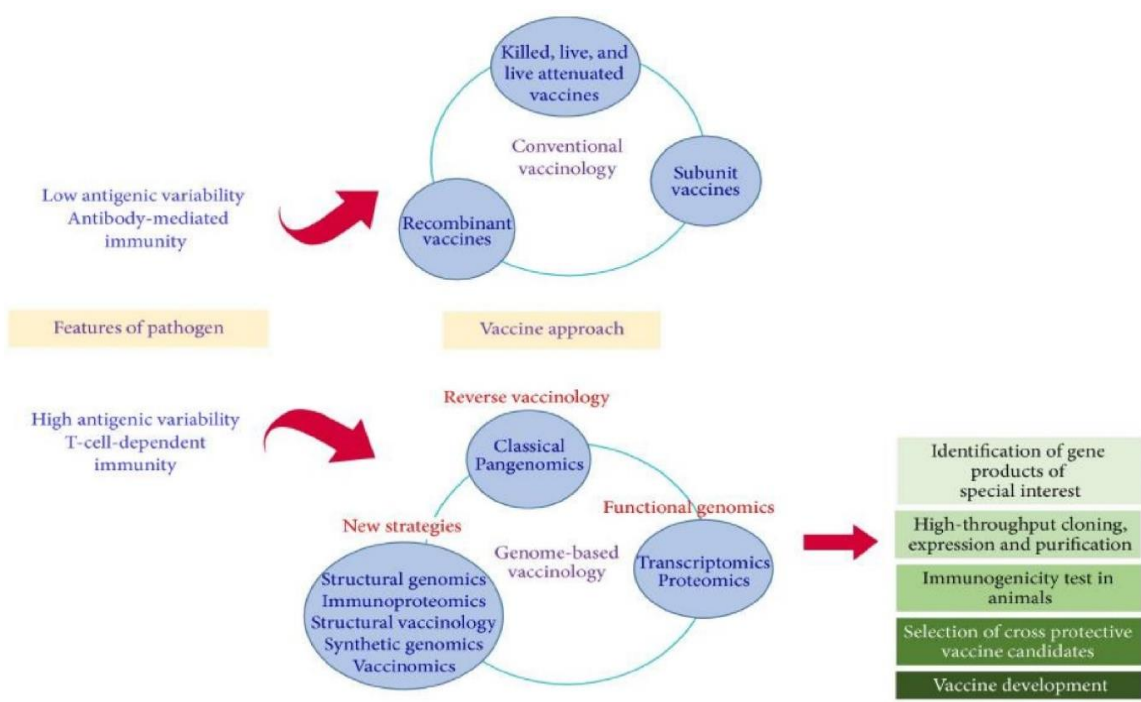

Figure 2: General scheme of *Anaplasma marginale* vaccine development (Quiroz-Castañeda et.al., 2016)

In spite of these challenges, there has been recent vaccine development strategies which include the Subunit Vaccines such as Outer membrane proteins (OMP) which are sub-dominant surface antigens (Figure 2). Evidence shows that Omps protect against *Anaplasma marginale*; Omp immunization partially protected cattle against Anaplasmosis (Noh *et.al.*, 2010). Protein complex *Omp1, Omp7–9, Msp1a, Msp2, Msp3, Msp4* used to immunize cattle indicated that a surface protein subset of the outer membrane is cross-linked and had the capacity of inducing protective immunity and tends to direct vaccine development (Lopez *et.al*.,2007; Quiroz-Castañeda et.al., 2016). Globally-conserved T cell epitope in *Omp 7-9* of *Anaplasma marginale* has been successfully identified and found to share sequence identity with the *Omp7* protein in the vaccine strain *A. marginale* subsp. *Centrale* (Deringer *et.al.,* 2017)

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Two outer membrane proteins, Major Surface Protein (MSP) *Msp2* and *Msp3*, are extremely abundant and immunodominant within the protective outer membrane and surface protein complexes. During infection, these MSPs exhibited sequential antigenic variation and there was convincing proof that these proteins were not accountable for protective immunity in induced immunization (Abbott *et.al*., 2005, Noh *et.al*., 2010). *Msp2* and *Msp3* were also variable between strain-specific alleles that encoded structurally and antigenically different proteins, making them poor candidates for vaccine development (Durken *et.al.,* 2015). As a result, the development of vaccines has concentrated more lately on subdominant OMP antigens. Even among subdominant antigens, an important issue appropriate to the development of an efficient vaccine is how best to recruit candidates for immunization and challenge trials. One of the largest super family of outer membrane protein in *A. marginale* protein is pfam01617, which involves both *Msp2* and *Msp3* and many subdominant proteins (Brayton *et.al*., 2005). Many of the proteins in pfam01617 are candidates for vaccines because some are invariant through an infection cycle (Noh *et.al*., 2006), are known or predicted to be exposed on the surface (Noh *et.al.,* 2008) and are detected by immune serum of protected cattle or are a component of the outer membrane protein complex that has been shown to be protective (Noh *et.al*., 2008).

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2.11 Outer membrane protein protection of cattle against anaplasmosis

The functions necessary for survival, replication and transmission are determined by the outer membranes of tick-transmitted intracellular bacterial pathogens. Therefore, the proteins expressed on the surfaces of these pathogens are potential candidates for vaccine development. This protein will target the induction of protective immune responses in the vertebrate hosts and/or stop the colonization of the tick vector. The identification of surface proteomes is vital to the development of vaccines and this process has been fast-tracked by genome sequencing (Noh *et.al.,* 2008; Junior *et.al.,*2010).

The roles of *Omps 4, 7, 10* and *14* are still not assigned in *A. marginale*. These proteins are classified as members of the *Msp2* superfamily according to their sequence identity with the surface antigens of the PFAM01617 family (Brayton *et.al*., 2005). However, these OMPs were the targets of the antibodies; specifically, IgG2 induced by the immunization of cattle with purified outer membranes (Lopez *et.al*., 2005) and, which is associated with protective immunity (Brown *et.al*., 1998).

Omps 7 – 9 of *A. marginale* are ideal vaccine candidates as they are surface exposed, can be found in the outer membrane immunogen and the protective cross-linked outer membrane proteins. These OMPs can be recognized by immune serum IgG2 and T cells in cattle immunized with OM of the *A. centrale* vaccine strain (Deringer *et.al.,*2017). OMPs are also relatively stable in the face of immune pressure, they can be expressed in bacterial cells and co-expression of 2-3 proteins is feasible (Durken *et.al*., 2015).

Anaplasmosis protective immunity can be stimulated by inoculating live *A. marginale* subspecies centrale, or by immunization of purified outer membranes or surface protein complexes (Tebele, *et.al.,* 1191; Potgieter *et.al.,* 1983; Noh *et.al.,* 2006; 2008; Shkap *et.al.,* 2009; Durken *et.al.,* 2015)

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There is indication that vaccine development based on Omps is achievable, yet, the outer membrane preparation contains over 20 proteins making it difficult to prepare and standardize. Nevertheless, the long- term objective is to develop a recombinant vaccine by the identification of proteins which are found within the outer membrane and protective. Durken *et.al.,* 2015

2.12 Interleukins

Interleukins (ILs) are proteins whose binding affinity is receptor specific and involved in intercellular interaction amongst white blood cells (Dinarello *et.al.,* 2010). IL-6 is a member of type I group of cytokines, including leukemia inhibitor factor (LIF), oncostatin M (OSM), and ciliary neurotrophic factor (Honda *et.al*., 1992). Majority of members in this family uses the glycoprotein 130 (gp 130) or CD 130 receptor (Cameron and Kelvin, 2000). As such, mice deficient in CD 130 demonstrate embryonic fatality, a finding which seems to be associated to a significant CD 130–dependent signalling in homeostasis (Ohtani *et.al*., 2000). IL-6 initially, was regarded as a differentiation factor of B cell hybridomas (Hirano *et.al*., 1986; Ferguson *et al*., 1988). The IL-6 cytokine is synthesized by endothelial cells, fibroblasts, monocytes, macrophages (Hirano *et.al*., 1985), T cells (Th 1) and B cells (Cameron and Kelvin, 2000) in response to various stimuli (as IL-1, IL-17 and TNF-α) during systemic inflammation (Hirano *et.al*., 1985).

IL-6 is a pleiotropic cytokine associated with regulation of immune responses, inflammation, etc. (Hirano *et al*., 1985) and can as well function as a co-factor in hematopoiesis by increasing granulocyte macrophage colony stimulating factor (GM-CSF) and macrophage colony stimulating factor (M-CSF) expression (Kopf *et.al*., 1998). In addition, the IL-6 can primarily induce fever, hormones and T and B cell development following cell damage and infection (Fattori *et al*., 1994). In innate immune response, the

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IL-6 regulates leukocyte trafficking and stimulation and triggers the production of acute-phase proteins by hepatocytes. Also, it stimulates T-cell proliferation, B-cell differentiation and survival and plasma cell production of IgG, IgA and IgM (Hirano *et al*., 1985); allergen–induced IL–6 stimulates type 2 and type 17 airway inflammation (Ullah *et al*., 2015).

2.13 IgG 2 reactivity

Protective immunity against *Anaplasma marginale* pathogen requires immunoglobulin G subclass 2 (IgG2) antibody to induce against outer membrane protein epitopes and coordinate macrophage activation for phagocytosis and killing. In a current research, cell-mediated immune responses were defined in calves immunized with purified exterior membranes of the Florida strain of *Anaplasma*, including induction of IgG isotype switching *marginale*. Importantly, these calves were eventually shown to be protected against experimental challenge with the Florida strain, and calves with the largest IgG2 titres were fully protected against infection. Peripheral mononuclear blood cells (PBMC) gathered after immunization proliferated heavily in reaction to both whole *A. marginale* homogenizes and purified exterior membranes, and this reactivity continued until the period of challenge (Brown *et.al.,* 1998).

During persistent infection of *A. marginale*, the development of variant-specific IgG2 responses signifies the requirement for T lymphocytes expressing interferon gamma IFN-γ. This plays a main role in activating bovine macrophages to generate toxic molecules, such as nitric oxide and its derivatives, as well as increasing the opsonizing IgG2 production. Studies with both bovine and murine models promote the induction of type 1 immune reaction leading to antigen-specific proliferation of CD4 + T-lymphocytes, IFN-γ

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manufacturing, and immunoglobulin G2 (IgG2) antibodies directed against the surface of the pathogen as required for protection.

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CHAPTER THREE

MATERIALS AND METHODS

3.1 Experimental Design:

3.1.1 Specific Aim:

Determine if vaccination with conserved Omp7-9 induces IgG and relevant immune components in immunologically naive calves

1.6.1 Strategy

Basic immunological studies have established that immunity can develop in animals either naturally or deliberately by immunisation with pathogen-derived immunogen. Guided by the results of the ongoing study in our laboratory indicating unequivocal high conservation of omp7/8/9 among *A. marginale* strains on a global scale, the recombinantly-expressed *Omp7-9* from Ghana strain of *A. marginale* was tested to find out whether immunisation provides significant IgG titres in recipient individuals than the unvaccinated control cohorts. Our laboratory had expressed and purified recombinant *Omp7-9* protein lacking the hydrophobic signal peptide sequence (which can be toxic) as His-tagged proteins in *E. coli*. Aliquots of this protein was emulsified in saponin and used to immunize calves. Serum from an animal immunized on an identical schedule and with the same adjuvant but with an unrelated antigen (*Babesia bovis* Msa-1) was used as a negative antibody control for serum samples from the outer membrane immunogen calves.

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3.1 Experimental Procedures

3.1.1 Location of study

The immunization trial was carried out among calves kept in the Wellcome Trust-funded tick-free facility at the Livestock and Poultry Research Centre (LIPREC), Legon. This structure contains holding pens for 16 calves of up to 150 kg and 4-5acres of pangola grass and panicum pasture maintained under irrigation.

3.1.2 Source of Plasmid DNA and confirmation of the presence of Omp7-9 Inserts

Our laboratory had expressed and purified recombinant Omp7 – 9 protein lacking the hydrophobic signal peptide sequence (which can be toxic) as His-tagged proteins in *E. coli*. These samples had been stored at -20 °C. EcoR1 digest of plasmid inserts were performed to confirm if the stored plasmids retained the desired inserts (Life technologies). First, samples were thawed on wet ice. Briefly, the Eco R1 restriction Digest was undertaken with 2 µl of OMP 7 – 9 plasmid DNA samples in 1 µl Eco R1 Enzyme and 3 µl of H Buffer. These were incubated at 65°C for one hour on a water bath followed by band size separation by ethidium bromide stained 1% agarose gel electrophoresis set at 70 V for 2 hours.

3.2 Expression of Omp 7 – 9 Proteins

Recombinant Omp7 – 9 (rOmp) was ligated into the pBAD/TOPO® ThioFusion™ Expression vector according to the manufacturer's protocol (Invitrogen by Life laboratories) and the One Shot® TOP10 Chemically Competent cells were transformed. Thereafter, 250 µl of pre-warmed S.O.C medium was added followed by incubation at 37°C with shaking at 225 rpm for an hour. Pre-warmed LB agar plates were inoculated and

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immediately incubated at 37°C overnight. Single colonies from overnight cultures were sub-cultured in 5 ml LB containing 50 µg/ml carbenicillin and incubated at 37°C with shaking at 225 rpm overnight. One millilitre of overnight culture was sub cultured into fresh 50 ml LB with carbenicillin and incubated at 37°C with shaking at 225rpm for 40 minutes after which 10 µl of 20% L–Arabinose was added to cultures to induce protein expression. The arabinose solution was not added to the control subculture to serve as the non–induced negative cells. Following the induction, samples were allowed to incubate for additional 90 minutes to ensure protein expression. The culture was transferred into 50 ml falcon tubes and centrifuged at 3000 X g for 10 minutes at 4°C. Supernatants were discarded and the pellets vortex to resuspend in residual fluids. Resuspended pellets were aliquoted into 2 ml cryotubes and stored at -20°C.

To visualise protein expression, pellets were thawed and 500 µl of cell lysis buffer was added to 50 µl of cell pellets followed by lysozyme and sonicated on ice using ultrasonic bath sonicator for 10 minutes. 500 µl SDS PAGE buffer was added and heated at 97°C for five minutes and allowed to cool. 15 µl was then loaded in 3.5% Tri-acetate SDS gel using 5 µl of Page ruler Protein Pre-stained ladder followed by electrophoresis at 50V for 90 mins. Gel was Coomassie Blue stained to visualize the expressed protein bands

To purify the expressed proteins, briefly, pellets were thawed and added to 8 ml of native binding buffer with 8 mg of lysozyme, thereafter incubated on ice for 30 minutes followed by sonication for ten 10 minutes on ice. After sonication, lysate was centrifuged at 3000 rpm for 15 minutes at 4°C. The supernatant was transferred to another tube and stored at -20°C. Molecular weight of lysate was determined using a Thermo Scientific NanoDrop 2000c Spectrophotometer and electrophoresed to visualize protein in lysate as previously described

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3.3 Protein Purification Under Native Conditions

Lysate was thawed and resuspended in 2 ml ProBond™ resin (NOVEX® by Life technologies) in a 10 ml Purification column. This was allowed to settle by gravity and the supernatant was aspirated gently followed by 6 ml of distilled water and then 6 ml native binding buffer. These were resuspended and allowed to settle to aspirate supernatant after each addition. Eight millilitres of cell lysate were added to the prepared purification column and allowed to bind for 60 minutes while agitating gently. Resin was then allowed to settle by gravity and the supernatant aspirated and stored at 4°C. Eight millilitres of Native wash buffer was used to wash bound resin three times and settled by gravitation, aspirating the supernatant carefully for 4°C storage. 12 ml of Native Elution buffer was added and eluted in 1ml factions for SDS PAGE electrophoresis.

3.5 *Omp7-9* Immunisation of Cattle for increased IgG 2 Secretion.

3.5.1 Source of Calves and DRB3* Haplotypes

A total of sixteen 3-6-month-old Friesian x Sanga crossbred cattle purchased from the Amrahia Dairy Farm were recruited for the immunization trial. To control for potential confounding effects of MHC haplotype on the immune responses, calves were MHC haplotyped and were confirmed to express DRB3*2404, DRB3*2711, DRB3*2405, DRB3*2406, and DRB3*2407 alleles unique to cattle from Ghana. Selected calves were corralled under tick-free conditions using housing and routine application of acaricides.

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3.5.2 Verification of the Immune Status of Calves

The infection status was confirmed by *msp5* PCR and seronegative status (after decay of any passively transferred antibodies) by Msp5 C-ELISA. At 6 months of age, calves were allocated randomly to two equal groups with even distribution of haplotypes among groups. There were five calves per group; assuming homologous immune response to Omp immunisation is equivalent to that induced by sham-immunized calves (adjuvant only), this provides >95% confidence of rejecting the null hypothesis with a power of 0.98.

3.6 Blood Sampling, Serum and Genomic Extraction

To ensure that only disease-free and immunologically naïve calves were examined calves were subjected to primary PCR and ELISA tests prior to selection into groups and sampling. Peripheral blood from the jugular vein were taken into 5 ml EDTA and Serum Separator Gel tubes for genomic DNA extraction and Serum extraction respectively. Blood for serum was stored at 4°C for an hour and then centrifuged at 3500 rpm for 15 minutes at 4°C. Serum was transferred to cryovials and stored at -20°C. QIAGEN blood kit (QIAGEN Inc, Valencia, U.S.A) was used to extract genomic DNA with modification. 500 µl of whole blood from EDTA tubes was added to 1.5 ml of RBC lysis solution and incubated at room temperature for 10 minutes while inverting intermittently to enhance lysis. The lysate was centrifuged at 3500 rpm for 10 mins at 4°C and the supernatants were discarded leaving the pellet. Pellets were vortexed to resuspend in residual fluid and 400 µl of cell lysis solution was added and vortexed to lyse cells and incubated thereafter for 10 minutes at room temperature till the solution was homogenous. 250 ul of protein precipitation was then added to the cell lysates and vortexed to mix after which they were centrifuged at 3500 rpm for 10 minutes at 4°C to form tight dark brown pellets.

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Supernatants were transferred to 1.5 ml tubes with 1 ml of Isopropanol 2 propanol and inverted gently to mix till DNA formation of threadlike strand was observed. They were centrifuged at 3500 rpm for 3 minutes to form white pellets discarding the supernatants and allowing air dry for 5 minutes. 500 µl of DNA hydration was added to the dried pellets and placed on water bath at 65°C for 5 minutes. Hydrated DNA was allowed to cool and stored at -20°C.

3.7 Multiplex PCR and Infection Prevalence

The infection status and prevalence were determined by screening genomic DNA samples of calves using multiplex PCR with forward and reverse primers for *Anaplasma marginale* (msp1b-265 bp), *Theileria* spp (462 bp) and *Babesia bigemina* (1125 bp) in 25 µl reaction volumes for the amplification. The amplification began with an initial denaturing step of 94°C followed by 30 cycle of 94°C for 5 minutes, then, 55°C for 30 seconds and an initial extension at 72C for 1 minute. Products were finally extended at 72°C for 7 mins, holding at 4°C after which amplicons were electrophoresed using 1% agarose gel stained with ethidium bromide at 80V for 45 minutes.

3.8 Immunisation of Calves with Omp 7 – 9 Proteins

The immunogens consisted of 70 µg of purified recombinant proteins (Omp7-9) emulsified in 1ml of saponin as previously described (Noh *et.al*., 2008; 2010). Calves without infection after Multiplex PCR were selected for inoculation. Experimental calves were inoculated subcutaneously. Calves in the experimental group (Groups 1) were inoculated subcutaneously with 70 µg/ml of the immunogen. Group 2 animals, representing the control cohorts, were identically immunized with the adjuvant only.

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After immunization calves were enclosed in the tick-free shelter provided with feed and water *ad libitum.* Booster immunizations were given bi-weekly until the antibody titre correlated with the high IgG2 levels associated with the control of acute *A. marginale* infection (3 immunizations were predicted, only 1 was given). Blood samples were taken at weekly intervals post-inoculation for serology.

3.9 Measurement of the Induced Immune Response:

Blood samples were collected daily for serology and genomic DNA analyses. Initially (within 48 hrs of vaccination), the capacity of the immunogen to induce innate immune response were assessed by testing serum levels of IL-6 and TNF-α using capture cytokine-specific antibody ELISA (NEO Scientific Biolab, Cambridge, Massachusetts). For quantitative determination of the individual cytokines in calves, a standard curve was constructed using the SoftMax® Pro 6 Software standardized samples of each cytokine. One week after the immunization a second round of serum samples were obtained from calves to test for IgG anti-Anaplasma IgG2 secretion as surrogate marker for the development of immunization-induced adaptive immunity. Accordingly, the opsonizing antibody titres were determined in calves by competitive Enzyme-linked-immunosorbent assay - cELISA (VMRD, Inc, Pullman, WA 99163, USA) at weekly intervals. The levels were compared with immune sera from naturally-infected cattle to verify if the induced immune response in vaccinated calves attains the breath associated with vaccine-induced IgG titres to acute disease (Lopez *et.al*., 2005; Saleh *et.al*., 2012).

Opsonising antibody titres were determined by a commercially available (cELISA) used to measure any antibodies related to *Anaplasma*. The percentage inhibition was calculated using the manufacturer’s formulae. Reagents, serum samples and plates were allowed to attain room temperature (23 ± 2°C) before the start of test. Control samples (Positive and

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Negative) were loaded onto the plate in duplicates and triplicates respectively per the manufacturer's directive. 1X Antibody-peroxidase conjugate was prepared by diluting 1 part of 100X Antibody–Peroxidase conjugate with 99 parts of conjugate diluting buffer. For example, for 96 wells, mix 60 µl of 100X Antibody–Peroxidase conjugate with 5.490 ml of conjugate diluting buffer. Where 50 µl are needed per well, 1X wash solution was prepared by diluting 1 part of wash solution concentrate with 9 parts deionized or distilled water. About 1.5 ml was needed for each well. 50 µl of controls and test serum samples were carefully loaded into each antigen coated well and marked for easy identification. The sides of the plates were tapped gently to ensure the loaded samples had coated the bottom of the plates and incubated for an hour. After one hour, plates were washed manually by removing sera and sharply striking the inverted plate 4 times on a clean paper towel. 1X Wash solution was immediately pipetted into each well using a multichannel pipette. The wells were emptied and the plate was struck sharply on a clean paper towel 4 times to ensure there were no sera residue. The wells were filled using the same method with the 1X Wash solution one additional time for a total of two washes. 50 µl of the diluted (1X) Antibody-Peroxidase conjugate was added to each well and the side of the plate was tapped gently to ensure that the bottom was completely coated and incubate for 20 minutes at room temperature (23 ± 2˚C). After 20 minutes, the plate was washed 4 times. 50 µl of substrate solution was pipetted into each well on the plate and the plate was tapped gently to ensure the bottom of the wells are coated and incubated for 20 minutes at room temperature (23 ± 2◦C). After 20 minutes of incubation, 50 µl stop solution was added to the wells containing the substrate solution and optical densities were read on a microplate absorbance spectrometer at a wavelength of 630nm.

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Significance between vaccinated and the control groups was determined by using the Student's t-Test statistical tool with Holm-Sidak correction for multiple comparisons (in order to retain power).

3.10 Ethical Statement

In this study the cattle used were handled in stringent agreement to guidelines set by University of Ghana Institutional Animal Care and Use Committee. This procedure was approved to be used in sampling blood from cattle by the Noguchi Memorial Institute for Medical Research's NIACUC Board with reference to NIACUC protocol number 2015-01-5X.

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CHAPTER FOUR

4.0 RESULTS

4.1 Eco RI digest of omp 7-9 in plasmid preps

From the restriction digest of five individual vaccine-relevant omp7-9 tested, all save one contained the desired product (Figure 3). All four positive samples were therefore selected for the protein expression analysis.

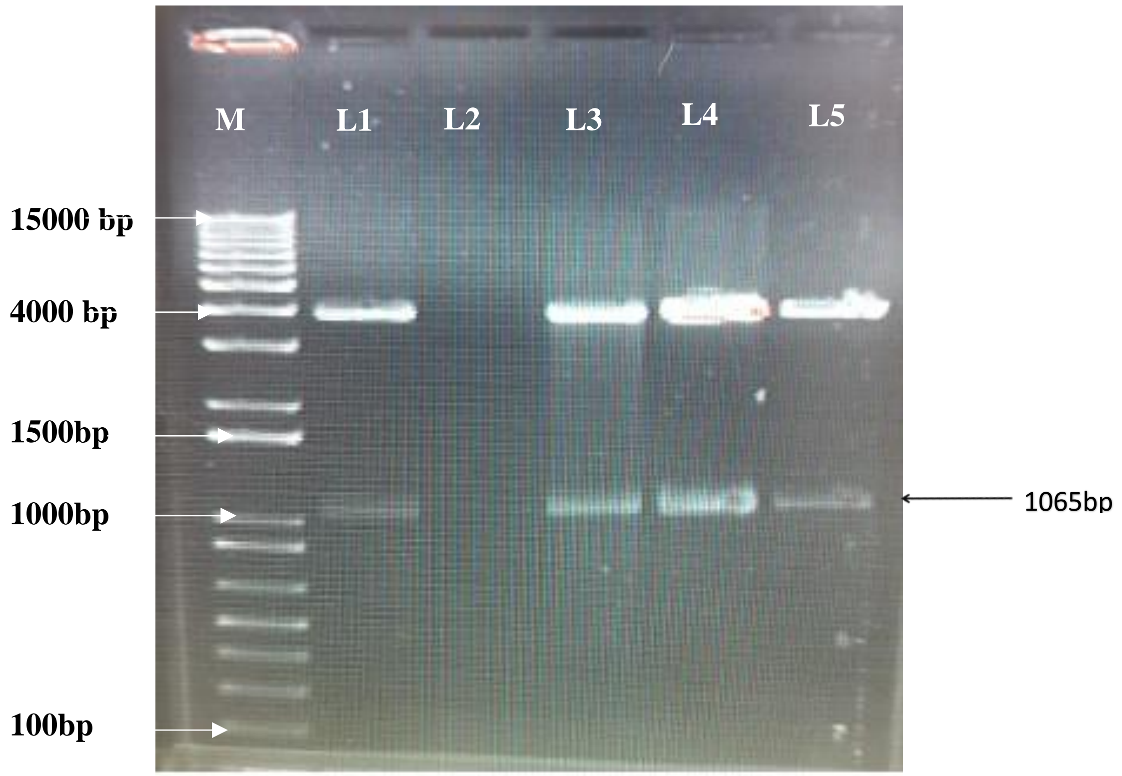

Figure 3. The digest of stored plasmids with DNA inserts of omp 7 – 9 of Anaplasma marginale.

M represents 1kb plus ladder in lane 1. **L1, L2, L3 and L4** represent plasmid prep samples in lanes 2, 3, 4, 5 and 6 respectively. The presence of DNA inserts of omp 7-9 was determined by the presence of a double band in the lanes **2, 4, 5** and **6. L2** in lane 3 **did not** contain plasmid with DNA insert. The upper band of **4000 bp** represents the plasmid. The lower band represent the **1065** bp of omp7-9 of Anaplasma marginale.

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4.2 Successful expression of rOmp 7-9 in bacterial cells

Recombinant proteins lacking the hydrophobic signal peptide sequence (which can be toxic) was expressed as His-tagged proteins in *E. coli.* Protein expression in plasmid transformed *E. coli* TOP 10® cells and was induced using arabinose and protein isolated from lysed cells using Ni^{++} columns [Probond, Invitrogen]. The purity of the *Omp 7 – 9* proteins was confirmed by SDS-PAGE with Coomassie brilliant blue staining (Figure 4).

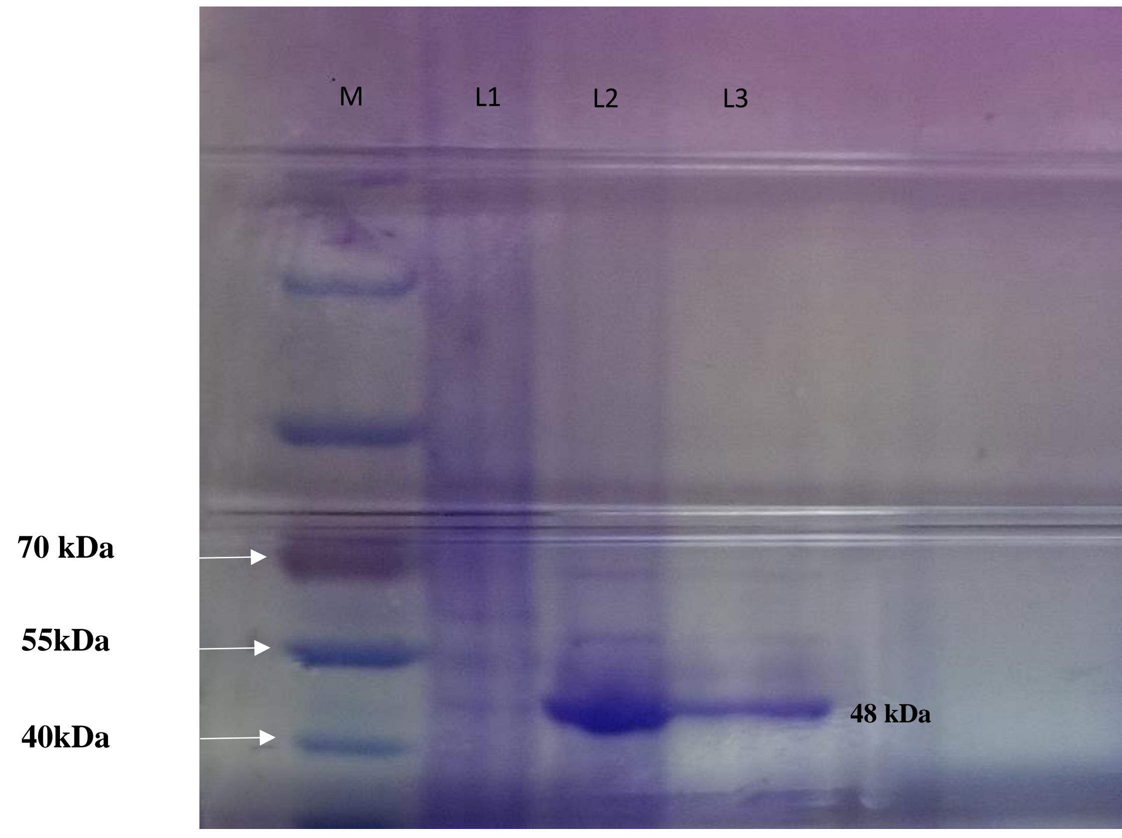

Figure 4: **Coomassie Stained SDS Gel of Expressed *rOmp 7,8* and *9* of Anaplasma marginale.**

M1 represents 180kDa Pre-stained protein marker in lane 1 showing the orientation band with a magnitude of 70 kDa in red. **L1** represents non induced bacterial cells with no expression in lane 2. **L3** represents expressed rOmp 7, 8 and 9 protein with a magnitude of 48 kDa in lane 3.

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4.3 Molecular concentration of *rOmp 7 – 9*

To determine the dosage of inoculum per millilitre of adjuvant needed to inoculate cattle, the purified r*Omp 7-9* proteins of Anaplasma marginale was quantified using Thermo Scientific Nanodrop 2000c Spectrophotometer-A280. The purified rOmp 7 – 9 harvested from a single colony of cloned *E. coli* through to induction of protein expression had a high concentration of 511.394 mg/ml (Figure 5).

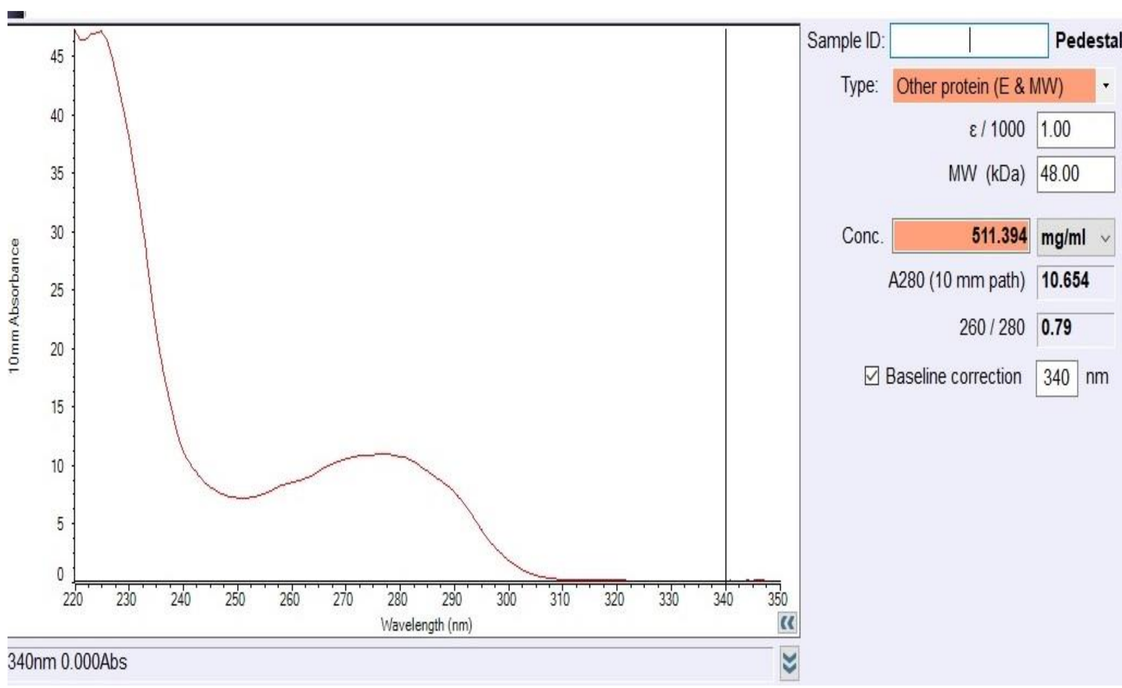

Figure 5: The display of protein concentration of *rOmp 7 – 9* of Anaplasma marginale using A280 Protein parameter of NanoDrop 2000c Spectrophotometer.

The y-axis of the graph represents 10 mm path Absorbance readings. The x-axis of the graph represents the wavelength readings in nanometres with a baseline correction of 340nm wavelength. The right panel represents the recorded molecular concentration of protein in milligram per millilitre of a 48kDa protein.

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4.4 Infection status of calves

Calves were screened for the presence of *A. marginale* using PCR with Genomic DNA from peripheral blood (Figure 6). It was observed that Anaplasma marginale was highly prevalent in the calf herd.

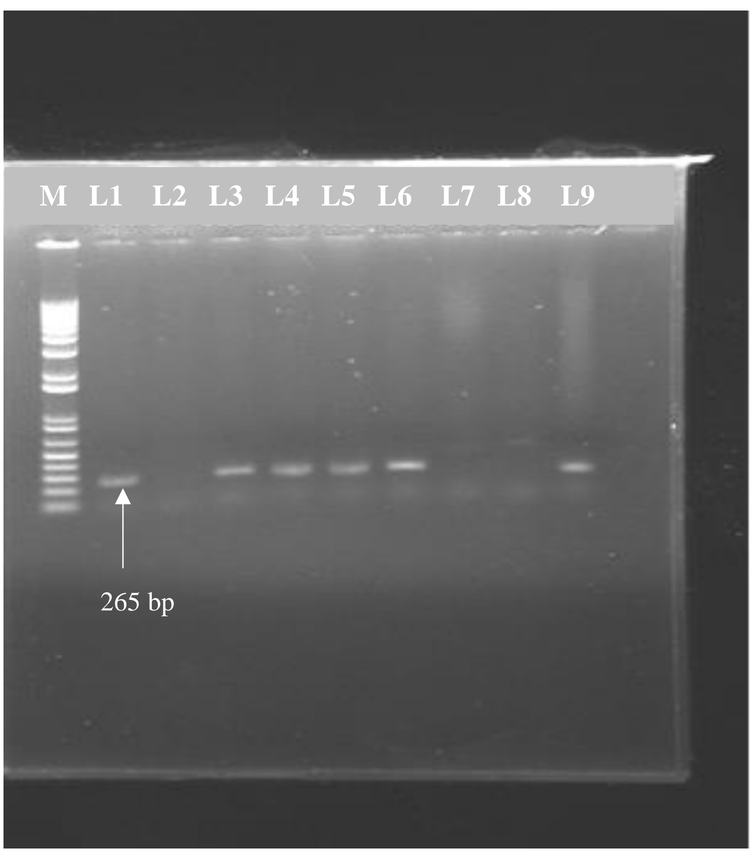

Figure 6: PCR Screening of calve herd for Anaplasma marginale infection status

M represents 1KB plus ladder in lane 1. **L1** represents **St. Maries strain of *Anaplasma marginale*** as PCR Positive control with **265bp** amplicon band size in lane 2. **L2, L7** and **L8** represent genomic DNA sample from calves 1, 6 and 7 showing no amplification as PCR negative in lanes 3, 8 and 9 respectively. **L3, L4, L5, L6** and **L8** represents a PCR positive amplicon with 265 bp band size equivalent to that on the positive control (**St. Maries Strain**) in lanes 4, 5, 6, 7 and 9 for calves 2, 3, 4 5 and 8 respectively.

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4.5 Prevalence of *Anaplasma marginale* Infection in calves

The infection prevalence of calves was 73% for *Anaplasma marginale* (Figure 7). However, a total of sixteen (16) calves were selected from the 27% tested negative for *Anaplasma marginale* in immunisation trial study.

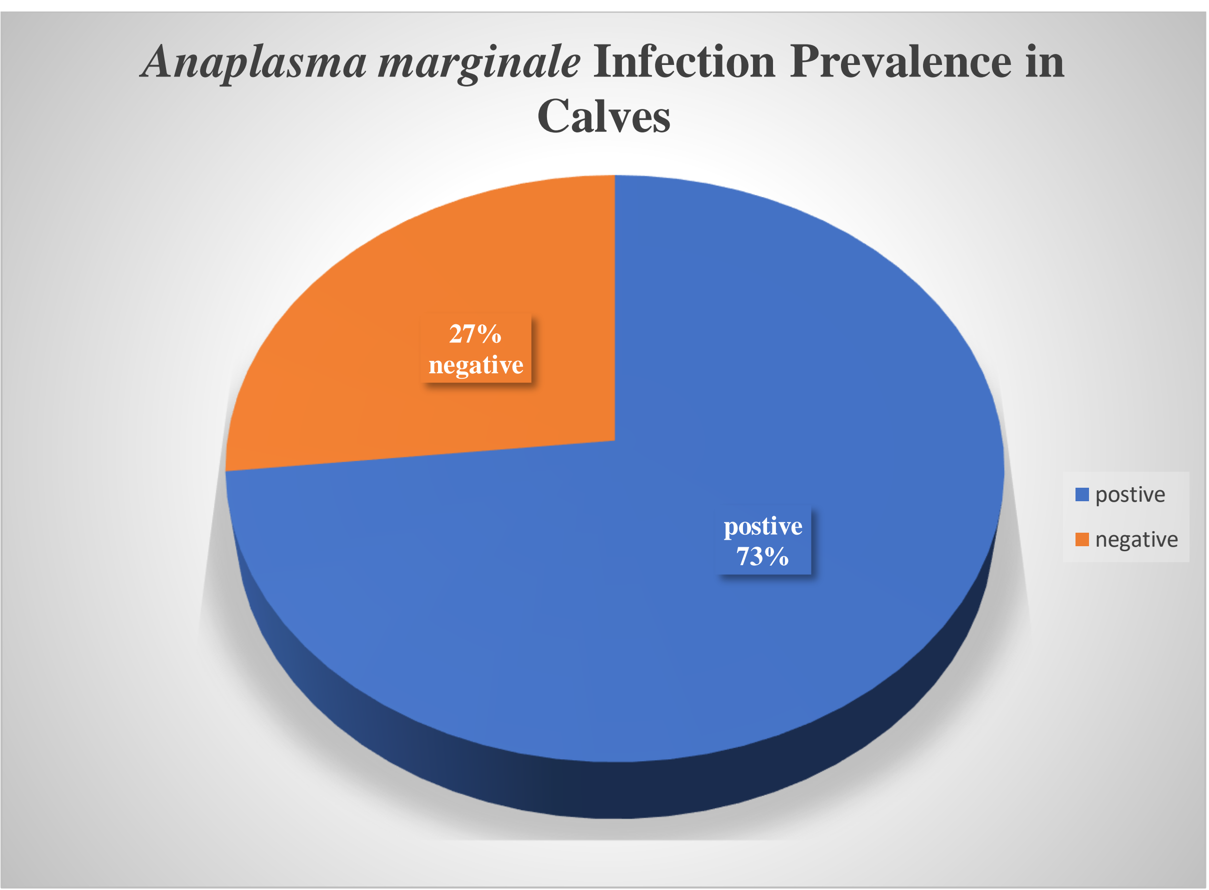

Figure 7: Anaplasma marginale Infection prevalence in Calves using PCR

The **blue section** of the pie chart represents positive PCR verification for the presence of *Anaplasma marginale* in calf herd. The **orange section** of the pie chart represents negative PCR verification for the presence of *Anaplasma marginale* in calf herd.

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4.6 Immune status of calves

The seronegative status (after decay of any passively transferred antibodies) was confirmed by *Msp5* C-ELISA. Percentage inhibitions less than thirty (< 30%) indicates a seronegative status while a percentage inhibition more or equal to thirty (≥ 30%) indicates a seropositive status (Figure 8).

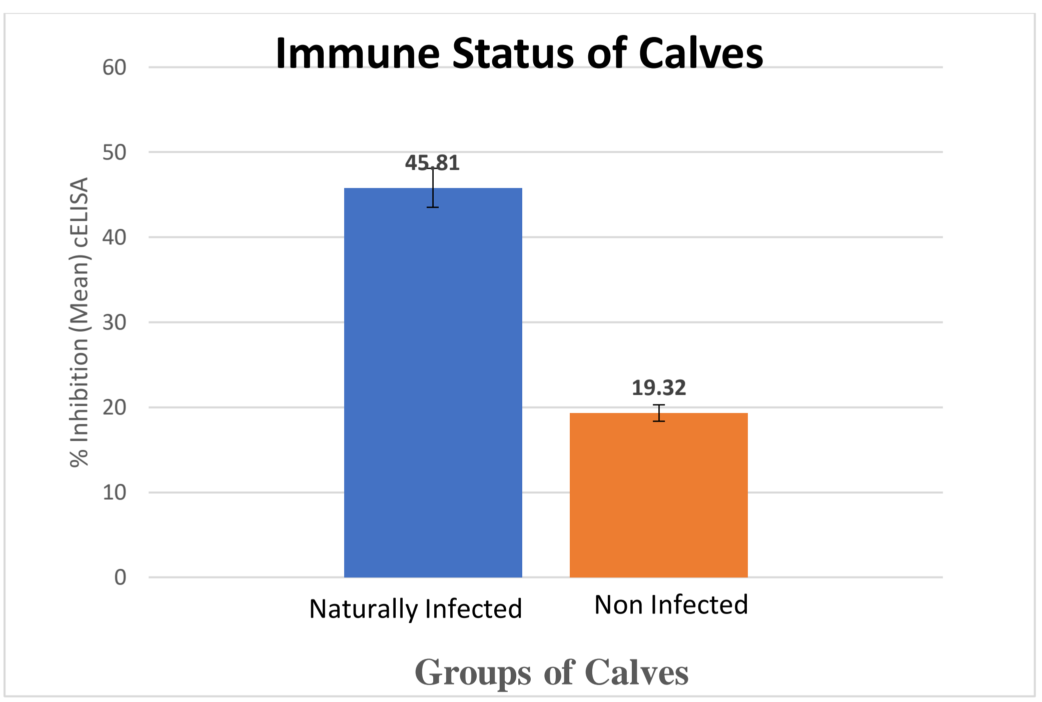

Figure 8: Immune status of Calf Herd

The y-axis represents the means of percentage inhibitions of **cELISA**. The x-axis represents the groups of calves in reference to their immune status prior selection to participate in immunisation trial. The blue bar represents the mean percentage inhibition (**45.81%**) of calves naturally infected with Anaplasma marginale used as an internal positive control. The orange bar represents the mean percentage inhibition (**19.32%**) of calves not infected with Anaplasma marginale. The difference (p=0.006) between the naturally infected group and non-infected groups of calves was highly significant.

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4.7 Cytokine secretion - IL 6

Within the 48 hours after injection of experimental test group calves with rOmp 7, 8 and 9 emulsified in saponin (Figure 9), the amount of IL-6 increased significantly ($p < 0.05$) from 10^2 to 10^6 pg/ml This abrupt increase was followed by precipitous decline to levels < 10^2 pg/ml also within 48 hrs of stimulation. Nevertheless, there were no significant increases in the secretion of IL-6 in the control cohorts that did not receive any immune stimulation.

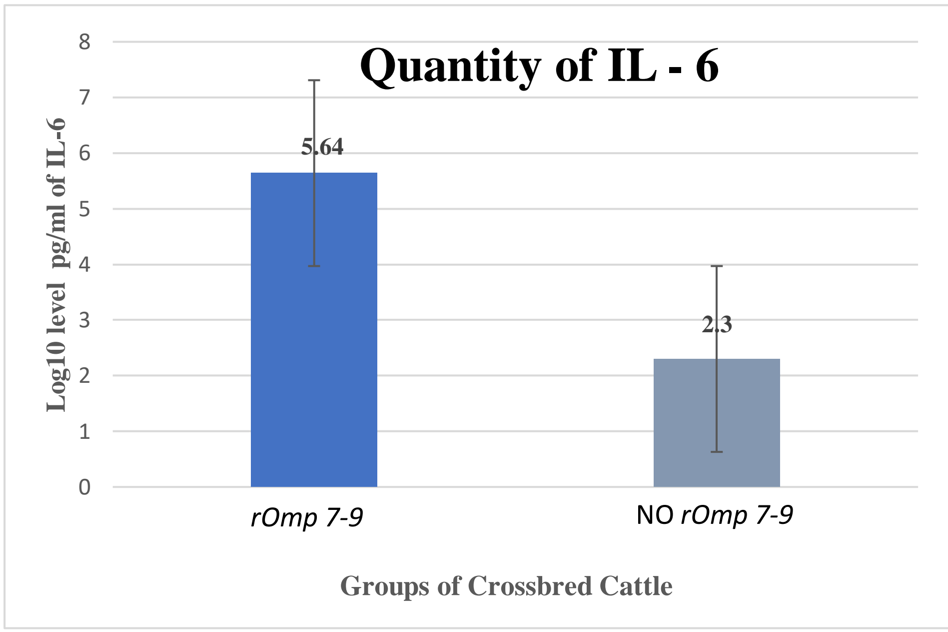

Figure 9: Quantity of IL-6 in pg/ml

The x-axis represents groups of crossbred cattle. The y-axis represents the amount of IL-6 secreted over 48hrs. The blue and grey bars represent mean amount of IL-6 in the experimental group and the control cohorts, respectively. *Student's t*-test (α 0.05, df 22) 9.51; $p = 10^{-4}$.

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4.8 Purified Omp 7-9 triggers TNF – α secretion in cattle

Serum TNF–α from calves injected with the purified protein appeared to have increased from 100 pg/ml to 1000 pg/ml within 48 hours of inoculation. However, the rise was not significantly different ($p > 0.05$) between the amount circulating in cattle from the control and experimental groups (Figure 10). This level declined to near the normal levels in all calves after 72hrs.

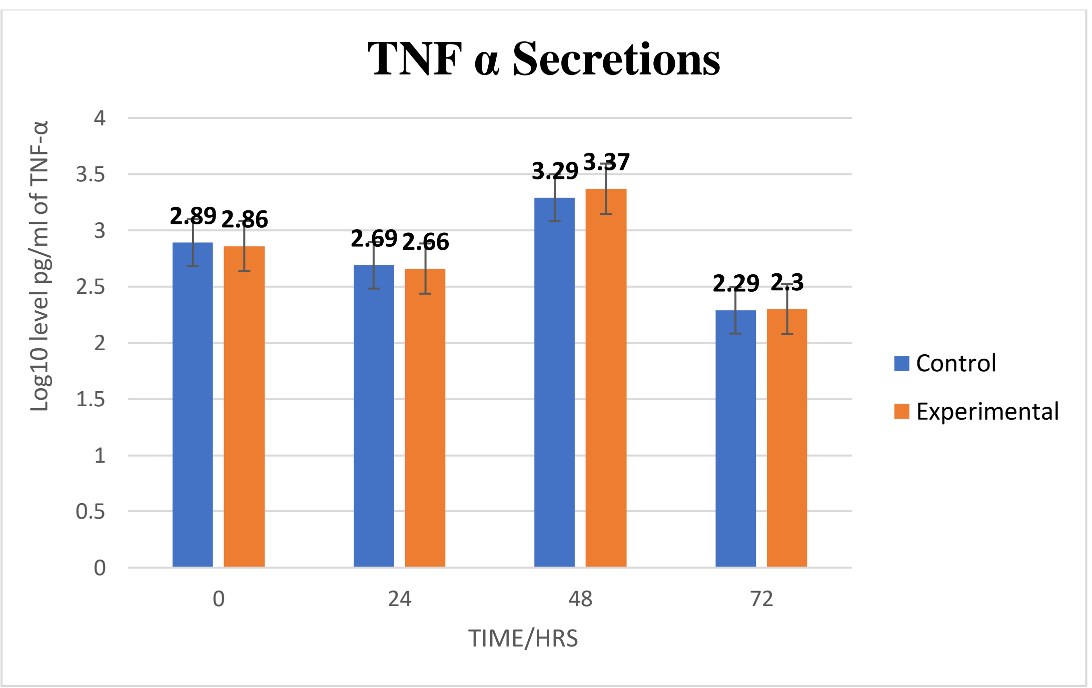

Figure 10. Mean quantity of TNF–α in pg/ml

The x-axis represents groups of crossbred cattle. The y-axis represents the amount of TNF–α secreted over 72hrs. The orange and blue bars represent mean amount of TNF – α in the experimental group and the control cohorts, respectively.

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4.9 Purified *rOmp 7-9* triggers IgG 2 Production in cattle

There were significant increases in IgG 2 production in calves immunized with *rOmp 7-9* from day zero (before immunisation) with mean percentage inhibition of (21.82%) to a mean percentage inhibition of (49.51%) one week after immunisation and IgG 2 secretions continued to rise steadily two weeks after immunisation with a mean percentage inhibition of (53.54%) compared with control cohorts mean percentage inhibitions of (19.14%, 19.15% and 19.32%) respectively (Figure 11).

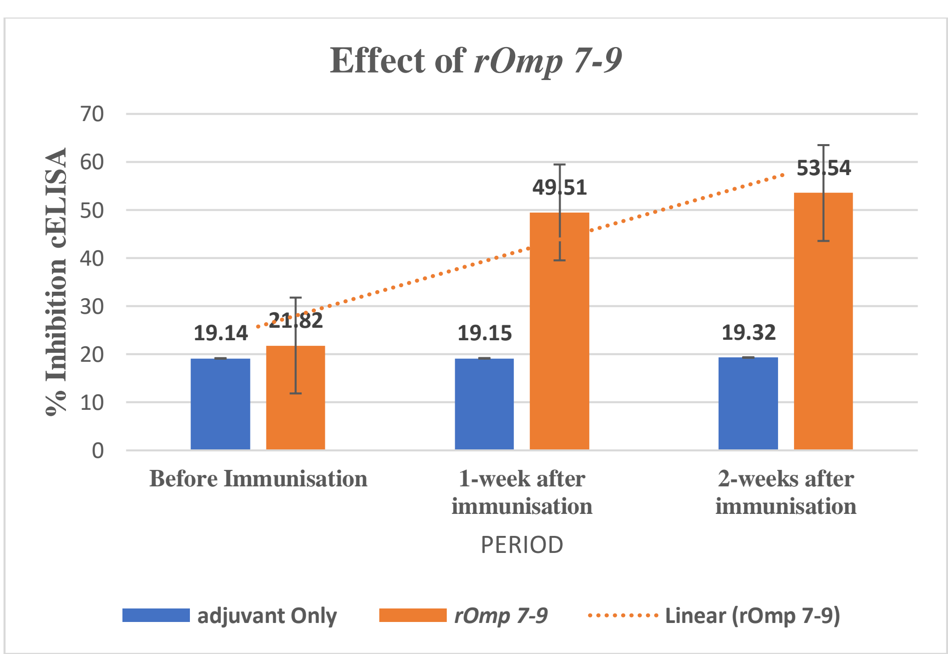

Figure 11: Effect of *rOmp 7-9* on IgG 2 production

The y-axis represents the means of percentage inhibitions of **cELISA**. The x- axis represents the periods before immunisation of *rOmp7-9*, one – week after the immunisation of *rOmp7-9* and two – weeks after immunisation of *rOmp7-9*. The blue bars represent control cohorts that received only the adjuvant. The -orange bars represent the test group immunised with *rOmp7-9*. The orange trendline represents the linear relationship of increasing IgG 2 production with time. The rise in IgG 2 production one week after

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immunisation was significant (p = 0.024), nevertheless, the steady rise at two weeks after immunisation was also significant (p= 0.02).

4.10 Comparison Between Artificially Stimulated and Naturally Infected

The observed differences in percentage inhibitions between naturally infected (**47.25%**) and artificially stimulated at one week after immunisation (**49.51%**) was not significant (*p* = **0.253**). Similarly, differences observed between percentage inhibitions between naturally infected (**47.25%)** and artificially stimulated at two weeks after immunisation (**53.54%**) was not significant (*p* = **0.10**).

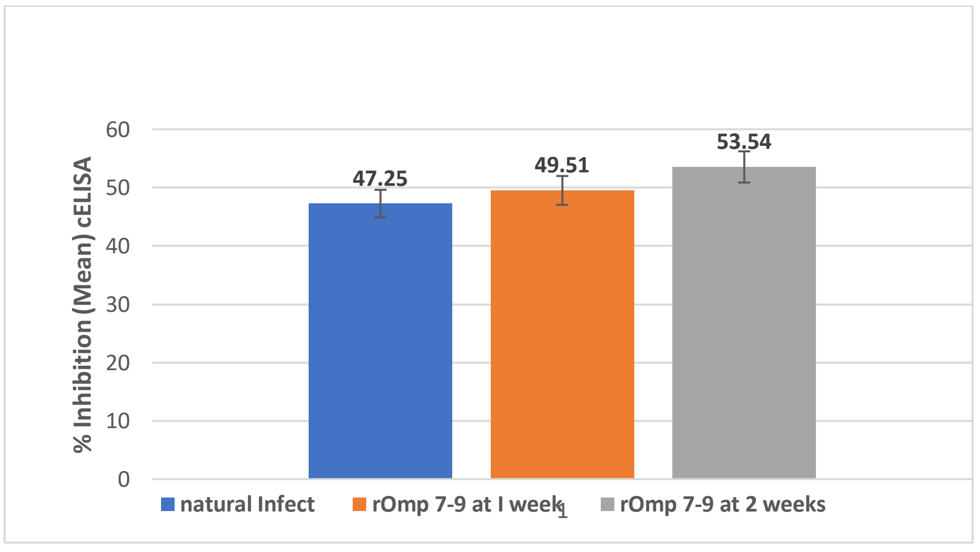

Figure 12: Comparison between Naturally Infected and Artificially stimulated

The y-axis represents the means of percentage inhibitions of **cELISA**. The x-axis represents the groups under comparison. The **blue** bar represents the mean percentage inhibition of calves naturally infected with Anaplasma marginale used as an internal control. The **orange** bar represents the mean percentage inhibition of calves one week after artificially

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stimulated with *rOmp 7-9* of *Anaplasma marginale*. The **grey** bar represents the mean percentage inhibition of calves two weeks after artificially stimulated with *rOmp 7*-9 of *Anaplasma marginale*.

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CHAPTER FIVE

5.0 DISCUSSION

In this study, we expressed and purified *rOmp 7-9* proteins of *Anaplasma marginale* which are structurally and immunologically conserved in endemic strains circulating in Ghana and other countries in West Africa in bacterial cells as a recombinant protein immunogen. These results have demonstrated for the first time in Ghana that omp 7-9 of *A. marginale* can be expressed and purified as a recombinant protein (*rOmp 7-9*) with a magnitude of 45kDa.

The prevalence of *A. marginale* in this study (73%) in calves is consistent with 45 – 75% previously reported (Beckley, *et.al,* 2016).

Macrophages and dendritic cells are immune cells of both the innate and adaptive immunity. With the invasion of foreign antigens, alarmins released activate these immune cells to prompt the release of a mixture of molecules including IL-6 and TNF-α that trigger inflammation, hinder microbial growth and initiate the first steps in adaptive immunity. As activated immune cells eliminate these antigens by innate defences, they at the same time process antigens into peptides to be carried by Major histocompatibility complex (MHC) molecules to the cell surface. Adaptive immunity is then triggered when MHC bound peptide is recognised by specific receptors on T-lymphocyte cells. Correspondingly, when administered subcutaneously to calves for the first time in Ghana, *rOmp 7-9* stimulated innate immune responses resulting in an adaptive immune response. This was confirmed by the significant increase in IL-6 ($p < 0.05$) and the increase from 100 pg/ml to 1000 pg/ml of TNF-α within 48 hours. The agonist-induced innate immune responses progressed to adaptive immunity as evident by the significant rise in IgG 2 secretion ($p = 0.02$) as compared to control cohorts. It was also observed that IgG 2 secretions in calves administered with *rOmp 7-9* which is an artificial immune stimulation was highly

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comparable to IgG 2 secretions of naturally infected calves as there were significant differences between them (p = 0.10) within the first seven days of immunisation. The potential for further increases in the IgG production is reflected in progression of IgG titres over time.

5.1 rOmp 7-9 stimulates innate immunity in calves

Whenever antigen gains entry through the barriers of an organism, the innate immune system provides the primary protection by means of diverse immune-modulatory mechanisms. These activities trigger the inflammatory response that activate immune cells like Natural Killer (NK) cells, lymphocytes and macrophages. The Lymphocytes as well as macrophages secrete IL-6 which stimulates the T lymphocytes and the B lymphocytes needed to mount adaptive immune response. The amounts of selected Type 1 cytokines secreted in the induction of innate immunity was evaluated to confirm production of soluble mediators critically required to trigger adaptive immune response.

Although the amount of IL-6 increased significantly from 10^2 to 10^6 pg/ml during 48 hours of injection in experimental calves, there were no significant differences in the amounts of TNF-α (p = 0.26) and INF- α (p = 0.39) in the serum samples between the control and experimental groups. IL - 6 secretions was significantly higher in all calves inoculated with rOmp7-9 but remain stable in all control calves. According to Bourquin *et al.* (2011) it was possible to increase in IL-6 levels within 24 to 48 hrs with resiquimod stimulation consistent with this study indicating the potential to trigger innate response by way of inoculating with synthetic immune stimulators such as Toll-Like Receptor formulations.

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5.2 rOmp 7-9 Increases IgG 2 secretions in calves

The specific antigens that cause *A. marginale* protective immunity are unknown. Up to now, immunization with individual OMPs has failed to provide protection in the natural bovine host against homologous strain challenges equivalent to that achieved by immunizing with OM. Therefore, the key to inducing protective immunity with a subunit vaccine may be to provide a constellation of immunogenic surface proteins. The globally conserved T- cell epitope *omp 7-9* of *Anaplasma marginale* was successfully expressed, purified and used to inoculate naïve calves less than six-month-old although it was difficult to replicate, solubilize and purify membrane-associated recombinant proteins (Boddicker *et.al*., 2016).

In this study, a relatively high amplification of IgG 2 production in inoculated calves compared with non-inoculated calves (***p* = 0.014**) signifies the potential of *rOmp 7 – 9* in inducing protective immunity and to serve as targets for vaccine development against worldwide prevalent *Anaplasma marginale*. Also, initial high-level IgG 2 production in immunized calves was comparable to levels existing in cattle naturally infected by *A. marginale* on the field (***p* = 0.10**). This level was therefore used as internal positive control for IgG secretion in calves. This can be attributed to the conserved T-cell epitopes of *rOmp 7 – 9* (Deringer *et.al.*, 2017) recognisable by T-cell to further stimulate B-cell to secrete IgG 2 in the calves. During persistent infection of *A. marginale*, the development of variant-specific IgG2 responses signifies the requirement for T lymphocytes expressing IFN-γ. This plays a main role in activating bovine macrophages to generate toxic molecules, such as nitric oxide and its derivatives, as well as increasing the opsonizing IgG2 production. Studies with both bovine and murine models promote the induction of type 1 immune reaction leading to antigen-specific proliferation of CD4 + T-lymphocytes, gamma

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interferon (IFN-γ) manufacturing, and immunoglobulin G2 (IgG2) antibodies directed against the surface of the pathogen as required for protection (Brown *et.al.,* 1998)

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CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATION

6.1 CONCLUSION

The long-term goal of this current study is to develop a broadly protective vaccine that specifically targets *Anaplasma marginale* strains currently endemic in Ghana using vaccine-relevant outer membrane proteins (Omps). To clarify the dilemma in the certainty of expressing and purifying the *omp*7-9 isolates circulating in cattle from Ghana as a recombinant protein, > 500 mg/ml of *Omp 7- 9* of *A. marginale* was expressed and purified as a 45 kDa recombinant protein (*rOmp 7-9*). Results from this study have demonstrated that for the first of its kind in Ghana purified *rOmp7-9* induces significant IgG 2 production in immunized calves which were comparable to calves naturally infected by *A. marginale* on the field. With this result the hypothesis that, inoculating cattle with recombinant *Omp 7-9* will amplify the magnitude of IgG 2 activity against *Anaplasma marginale* challenge on the field is accepted. The question of whether immunisation with purified Omp would protect against disease remains unresolved.

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6.2 RECOMMENDATION

Building on recent advances in understanding the conservation of a defined CD4+ T cell epitope in Omps 7-9 among all *A. marginale* strains, I propose vaccination based on an "epitope-defined" approach where full-length Omps can be combined with an epitope-specific immunogen. This approach could be realized using recombinant proteins either expressed individually or linked to the T cell epitope or unique chimeras.

The next logical step is to verify if vaccination with recombinant Omp7-9 will provide significant protection against anaplasmosis and prevent mortality of calves. If successfully implemented, this strategy will significantly advance Ghana's capacity for vector-borne disease control and provide the basis for future vaccine development and deployment.

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