EFFECT OF TOLL–LIKE RECEPTOR7/8 AGONIST IMMUNISATION OF YOUNG CROSSBRED CATTLE ON INNATE IMMUNE STIMULATION AGAINST TICK–BORNE DISEASES

BY

SONGLIEDONG ZUMOH–BALIGI

10382401

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JULY, 2018
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.

SONGLIEDONG ZUMOH–BALIGI

DATE

DR. JAMES E. FUTSE

DATE

(PRINCIPAL SUPERVISOR)

PROF. GEORGE K. ANING

DATE

(CO–SUPERVISOR)
DEDICATION

I dedicate this work to the Almighty and most gracious God in whom I live and move and have my being, to my beloved parents; Naa Baligi and Mrs. Jane Baligi, my siblings, Joanita Selorm Akakpo and all my loved ones.
ACKNOWLEDGEMENT

My foremost gratitude goes to the Almighty God for the abundance of grace and mercy bestowed upon me throughout the entire study; thank you Lord from day one until now.

I want to express my sincere and profound appreciation to my supervisors, Dr. J.E. Futse and Prof. G.K. Aning for their guidance, counsel and relevant suggestions during the study. I am especially thankful to Dr. J.E. Futse for the essential equipment and resources he provided and made available in order for this study to be a success.

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God richly and abundantly bless you all.
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ABSTRACT

Infectious diseases of livestock are difficult to control in Africa due partly to lack of vaccines. In the absence of these vaccines, livestock production in endemic regions is constrained by the need for repeated acaricide use and/or the loss of opportunity to genetically improve the productivity of the indigenous breeds. Indigenous cattle have innate resistance to majority of diseases but are of low productivity. There is evidence that resistance to severe disease and death upon infection in indigenous breeds is linked to innate immunity and “adjuvant-only” experiments have shown that innate immunity and resistance to severe disease can be induced in susceptible higher productivity breeds. Consequently, if innate immunity can be stimulated in young calves, acute diseases and mortality can be prevented. This approach would allow genetic improvement of cattle while maintaining innate resistance.

The current study tested whether single inoculation with synthetic Toll-like Receptor 7/8 (TLR7/8) agonist would trigger discernible innate immune response in F1 Friesian-Sanga crossbred cattle. The goal of this study is to stimulate the innate immune response in young cattle with the goal to mitigate disease severity and reduce mortality in crossbred animals. Our strategy is to expose the immune system of calves to TLR7/8 agonist-fortified emulsions in a water-in-oil, slow release preparation. Whether the innate immune response of crossbred calves can be stimulated by injection with synthetic toll–like receptor7/8 agonist was previously unknown. This question has been resolved by data from the current study.

Three month old Friesian x Sanga (F1) calves with no evidence of prior infection were selected. Calves were conditioned for five days during which the normal rectal temperature and sizes of the pre–scapular lymph nodes were measured to establish reference dataset for cattle in the sub region. The normal rectal temperature of the Friesian x Sanga F1 cattle was 38.5°C representing the first empirically determined biomarker for crossbred cattle in the West African sub region.
Following the 5 day period of observation, the animals were randomly divided into two groups of 12 calves. One group was inoculated with the emulsion, referred to as the experimental group, but the second group was not inoculated and was referred to as the control group. Calves in the experimental group were injected, subcutaneously, with 1ml of the agonist preparation but the control cohorts (n=12) were not injected. The rectal temperature and sizes of the lymph node from individual calves were measured, at same time, (08:00 hrs) daily for additional 7 days post-inoculation. Blood samples were also collected daily for serology and genomic DNA analyses.

Calves in the experimental group recorded a significant (p = 0.013) rise in the rectal temperature and sizes of the pre-scapular lymphoid organs as compared to the control calves.

Also, the amount of IL-6 increased significantly (p < 0.05) from $10^2$ to $10^6$pg/ml during 48 hours of injection in experimental calves. TNF–α concentration appears to increase from 100pg/ml to 1000pg/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. The results from this study indicate that animals injected with the agonist have responded to stimulation and the corresponding innate responses would be expected to provide protection against multiple pathogens without the need for multiple antigen-specific vaccines. That actually is the innovation in this approach with the possibility to save cost of producing crossbred cattle in Ghana where infectious disease are widely prevalent. Success with this approach will guide development of a low-cost disease control strategy based on innate immunity stimulation that would be globally scalable for improvement of livestock productivity in the tropical and subtropical regions.
**LIST OF ACRONYMES AND ABBREVIATIONS**

<table>
<thead>
<tr>
<th>Acronym</th>
<th>Description</th>
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<tbody>
<tr>
<td>BCG</td>
<td>Bacillus Calmette – Guérin</td>
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<tr>
<td>CD</td>
<td>Cluster of differentiation</td>
</tr>
<tr>
<td>DRG</td>
<td>Dorsal root ganglion</td>
</tr>
<tr>
<td>ECF</td>
<td>East Coast fever</td>
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<tr>
<td>ELISA</td>
<td>Enzyme–linked immunosorbent assay</td>
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<tr>
<td>ER</td>
<td>Endoplasmic reticulum</td>
</tr>
<tr>
<td>GM–CSF</td>
<td>Granulocyte macrophage colony stimulating factor</td>
</tr>
<tr>
<td>INF α</td>
<td>Interferon alpha</td>
</tr>
<tr>
<td>Ig</td>
<td>Immunoglobulin</td>
</tr>
<tr>
<td>IgA</td>
<td>Immunoglobulin A</td>
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<tr>
<td>IL – 1β</td>
<td>Interleukin 1 beta</td>
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<tr>
<td>IL</td>
<td>Interleukin</td>
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<tr>
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<td>Interferon beta</td>
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<tr>
<td>INF γ</td>
<td>Interferon gamma</td>
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<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>INF τ</td>
<td>Interferon tau</td>
</tr>
<tr>
<td>INF ω</td>
<td>Interferon omega</td>
</tr>
<tr>
<td>LIF</td>
<td>Leukemia inhibitor factor</td>
</tr>
<tr>
<td>LT–β</td>
<td>Lymphotoxin beta</td>
</tr>
<tr>
<td>MCP–1</td>
<td>Monocyte-chemotactic protein-1</td>
</tr>
<tr>
<td>M–CSF</td>
<td>Macrophage colony stimulating factor</td>
</tr>
<tr>
<td>MHC</td>
<td>Major Histocompatibility Complex</td>
</tr>
<tr>
<td>NK–cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>NLRs</td>
<td>Nucleotide oligomerization domain–like receptors</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen – associated molecular patterns</td>
</tr>
<tr>
<td>PBS</td>
<td>Phosphate – buffered saline</td>
</tr>
<tr>
<td>PRRs</td>
<td>Pattern recognition receptors</td>
</tr>
<tr>
<td>RNA</td>
<td>Ribonucleic acid</td>
</tr>
<tr>
<td>TBDs</td>
<td>Tick – borne diseases</td>
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<tr>
<td>TBP(s)</td>
<td>Tick – borne pathogen(s)</td>
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<td>Th 1 cells</td>
<td>Type 1 helper T cells</td>
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<tr>
<td>TLRs</td>
<td>Toll – like receptors</td>
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<tr>
<td>TNF – α</td>
<td>Tumor necrosis factor alpha</td>
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<td>TNF – β</td>
<td>Tumor necrosis factor beta</td>
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CHAPTER ONE

1.0.0 INTRODUCTION

1.1.0 BACKGROUND

Livestock diseases transmitted by arthropod vectors impact a severe disease burden on small farmers in tropical countries where these diseases are endemic. In West Africa, the tick-borne diseases heartwater, babesiosis and anaplasmosis, together with the tick-associated skin disease dermatophilosis and tsetse-transmitted trypanosomiasis, have repeatedly frustrated attempts to increase animal productivity by genetic improvement of the indigenous cattle breeds through cross-breeding with exotic highly productive cattle (Aning, 1982; Uilenberg Gerrit, 1995; Bell-Sakyi et al., 1996; de Castro, 1997; Walker and Koney, 1999; Bell-Sakyi et al., 2004).

This disease burden has a disproportionate impact on resource-poor farmers due to the high cost of prevention using acaricides/insecticides and treatment of sick animals (De Castro, 1997; Minjauw and McLeod, 2003). However, the greatest impact results from the severity of disease among higher producing dairy and dual-purpose breeds and crosses between these higher productivity breeds and indigenous cattle breeds with consequent loss of capacity to improve productivity (Simuunza et al., 2011). The world cost of Tick–borne diseases, in relation to death of animals, losses in production, treatment and control of ticks are projected to be between $13.9 billion and $18.7 billion yearly.

Importantly, effective control of tick-borne diseases in endemic regions requires control of a complex of pathogens rather than a single pathogen-disease entity. A separate vaccine targeting each pathogen is not feasible for a variety of reasons. Therefore, the most effective means of control is to use one intervention to target multiple pathogens (Frisch et al., 2000; Graf et al., 2004; Estrada – Peña and Salman, 2013; de la Fuente et al., 2017). Multiple
pathogens infection is commonplace in most tropical regions. Here, the pathogen strains and the associated biological vector are widely prevalent. This condition allows for continuous pathogen challenge by intermittent feeding behaviour of tick species resulting in two or more pathogens circulating in individual cattle. However, not all cattle became infected with multiple pathogens. Notably the indigenous cattle have demonstrated high level of resistance to clinical disease without the need for treatment (Nadelman et al., 1997; Alekseev et al. 2001; Bock et al., 2004; Nyarko et al., 2006) and this could be due to the fact that, the innate immune responses of these calves have evolved to provide some sort of protection for the local animals. The exotic and cross breeds on the other hand appear to lack this innate capability and are therefore unable to withstand infectious diseases challenge. To prevent high mortality rates associated with the production of the crossbred in the endemic tropical requires intensive use of drugs and acaricides at a high cost to the farmer. This genetic difference between the indigenous and crossbred cattle raises a key question whether the innate immune responses of the exotic and cross breeds can be induced by stimulation to allow these breeds survive mortality and also withstand multiple pathogen infections.

One potential means to achieve this goal is through stimulation of the innate immune system using synthetic version of TLR7 and 8. It is predicted that when calves were stimulated at young age will reduce or prevent disease while allowing for infection with the pathogen and the subsequent development of long-term adaptive immunity required for lifetime protection against death.

Innate immunity is responsible for protection against severe disease upon hemoparasitic infection in indigenous cattle breeds (Aguilar–Delfin et al., 2001; Beutler et al., 2006; Ahmed et al., 2008; Bannerman et al., 2008a; Bannerman et al., 2008b). *Babesia bovis* is illustrative of this effect: in Friesian cattle, the parasite causes a high fever, severe anemia, neurologic disease (resembling cerebral malaria), and death within 10 days of infection, while
indigenous breeds show only mild disease. The time course (<10 days) points to innate immunity as the critical difference (Bock et al., 1997a; Bock et al; 1999a; Bock et al; 1999b; Goff et al., 2003; Bock et al; 2004; Brown et al., 2006; Bannerman et al., 2008a; Bannerman et al., 2008b; Carvalho et al., 2008;). This is supported by both laboratory studies showing induction of the innate immune response early in infection and, most importantly, by studies in which pre-treatment with mycobacterial-based adjuvants induce protection against severe disease (Aguilar-Delfin et al., 2001; Glass et al., 2005; Andersen et al., 2009). This innate immunity is reduced but not completely lost in exotic breeds, and that it can be induced is evident from studies with Babesia bovis (Goff et al., 2003). Cattle that survive the acute clinical disease phase remain persistently but asymptotically infected and resistant to subsequent challenge with homologous or heterologous strains. The establishment of persistent infection actually functions as a live vaccine similar to that used to protect cattle in Israel, Australia and South Africa (Brown et al., 1999; Shkap et al., 2007). This pattern of asymptomatic persistent infection and protection holds for the other tick-borne pathogens (Peter et al., 1998; Barbour and Restrepo, 2000; Palmer et al., 2000).

Pathogens stimulate the innate immune response through Toll-like receptors that serve as sensors for pathogen molecules, including glycoproteins and carbohydrates, and therefore represent key initiators of innate immunity (Hajjar et al., 2002; Ibeagha et al., 2008; Ishii et al., 2008; Uematsu and Akira, 2008; Kumar et al., 2009). There is evidence that innate receptors can be stimulated by direct interaction with the agonists in humans, mice, and cattle. The strategy in the present study was to target the bovine TLR-7 and TLR-8 by activation with the cognate agonists. TLR7 and TLR8 typically recognize pathogen RNA and synthetic agonists which are small amines (Diebold et al., 2004; Kawai and Akira, 2007; Severa and Fitzgerald, 2007; Miller et al., 2008) and activation with agonists induces secretion of both anti-microbial effectors and inflammatory cytokines (Severa and Fitzgerald,
2007; Miller et al., 2008). There is strong precedent for innate immunity stimulation that resulted in significant protection as compared to untreated cohorts based on studies using mycobacterial extracts in *Bos indicus* x *Bos taurus* calves (Tewari et al., 1996). However, whether protection can be induced by synthetic TLR agonists in the *Friesian* x *Sanga* F1 calves has not been tested but requires empirical data to resolve. One of the key questions regarding the effectiveness of our approach is whether injection with the synthetic TLR 7/8 agonist emulsion can stimulate the innate immune response in young crossbred calves. To answer this question, we evaluated the temperature, swelling of the draining pre-scapular lymph node, and secreted cytokines. Any significant shifts in these biologic indicators in response to immune stimulation are easy to measure on the field. Success with this approach will provide a low-cost disease control strategy that would be globally scalable for improvement of livestock productivity in tropical and subtropical regions.

### 1.2.0 PROBLEM STATEMENT

Exotic breeds of cattle and their crosses (Friesian X Sanga) are known to grow faster and also produce more milk and meat than the low productivity indigenous cohorts. In the endemic region, farmers therefore desire the rearing of these high productivity crossbred cattle and exotic breeds but are frustrated by their high susceptibility to diseases. Interestingly, the indigenous cattle co-habiting the same endemic environment, on the other hand, have demonstrated unique capability to withstand severe diseases with no need for treatment. Vaccination remains the most effective and dependable approach to tick-borne disease control. However, this classical approach to control of tick–borne diseases of cattle by vaccination is essentially futile due primarily to occurrence of multiple tick–borne pathogens circulating in cattle in the endemic tropical region. It appears that, in the absence of vaccines,
indigenous animals are dependent on their innate immune response to survive. Innate immunity is responsible for protecting the indigenous but low productivity breeds against severe disease. As innate immunity is reduced but not completely lost in higher productivity crossbred cattle, this raises the question whether this perceived dormant innate immune response of the crossbred cattle can be triggered by injecting young crossbred calves with synthetic Toll-like receptor agonist emulsified in an adjuvant. If successful, then long-term activation of the innate cellular receptors in young calves will mitigate disease severity and restore full disease resistance in crossbred animals and thus provide the opportunity to improve livestock productivity to ensure food and economic security.

1.3.0 JUSTIFICATION OF THE STUDY

Cattle are sources of protein thus, making them economically significant but diseases-related mortality represents one of the major problems to profitable cattle production, food and economic security in Ghana (Saunsoucy, 1995; De Castro, 1997; Kaewthamasorn and Wongsamee, 2006, Rajput et al., 2006).

Throughout the world, tick–borne diseases have caused significant losses to cattle production in excess of $18.7 billion yearly. In Ghana, it is projected that 40% of livestock raised by farmers die of tick-borne diseases annually (Aryee et al., 1991; Bell – Sakyi et al., 2004; Jonsson et al., 2008; Estrada – Peña and Salman, 2013).

Exotic breeds are the most affected thereby making their production unsuccessful in resource-poor countries (Khan et al., 2004; Jongejan and Uilenberg, 2004). Currently, there is no effective vaccine for tick-borne infections and the near-term prospects for developing and deploying vaccines is not encouraging due to budgetary constraints. Disease control by way
of innate immune stimulation may provide the best alternative strategy for controlling animal pathogens that co-circulate in animals resident in the endemic tropical region. That this approach is applicable is supported by the evidence that TLR7/8 agonist does not target specific pathogen entity upon stimulation (Futse, unpublished). Subsequent encounter of the recipient cattle with challenges on the field from different pathogen strains will result in the development of pathogen-specific adaptive immune response. If this process is successful, it could be applied to the control of infections in other animals including small ruminants to reduce mortality and improve income of farmers. It could also provide research institutes/communities with substantial information on the normal temperature of cross bred (Friesian x Sanga) (F1) calves in West Africa and Ghana. It will be a novel discovery for researchers as the normal rectal temperature of Friesian x Sanga calves is not known. The present study therefore represents the first study establishing the baseline data of the rectal temperature of Friesian x Sanga (F1) calves.

Understanding how innate immune response can be induced and effectively stimulated to help host animals combat disease infections will provide evidence-based information critically needed by researchers to develop effective control against diseases of exotic and cross bred cattle in the endemic regions. Also, it will provide specific pathway to producing single vaccine to target multiple pathogens. The overall success of this approach will drive farmers’ interests to procure additional cross breed cattle to improve production of milk and meat.
1.4.0 HYPOTHESIS

Immunization with single dose of synthetic TLR7/8 agonist-fortified emulsions will trigger the innate immune response of the *Friesian x Sanga* F1 calves.

1.5.0 MAIN OBJECTIVES

The goal of this study is to determine whether the innate immune response of crossbred calves can be stimulated by injection with synthetic toll-like receptor7/8 agonist.

1.6.0 SPECIFIC OBJECTIVES

The specific objectives of the study are to determine:

1. Whether crossbred calves (Friesian X Sanga) injected with TLR7/8 agonist will show significant variations in some measurable biomarkers for innate immune response in cattle.

   These biomarkers include:

   I. Elevation of rectal temperature – fever.
   II. Increase in width of pre-scapular lymph node.

2. Whether injection-induced secretion of plasma proteins, associated with innate immune response, will increase in calves stimulated with TLR7/8 agonist immunization. The following cytokine readouts were analysed in all calves:

   I. Interleukin – 6 (IL – 6)
   II. Tumor Necrosis Factor – α (TNF – α)
CHAPTER TWO

2.0.0 LITERATURE REVIEW

2.1.0 MAJOR TICK – BORNE DISEASES IN AFRICA

2.1.1 ANAPLASMOSIS

Anaplasmosis is caused by *Anaplasma* species, a group of obligate bacteria, which reside within the cells of cattle. They are commonly found in the tropical, subtropical regions and some temperate zones (Yang *et al*., 2018). This disease has been recognized for over hundred years, nevertheless, it is of significant animal and human health concern (Battilani *et al*., 2017). Anaplasmosis causes crucial economic losses in both beef and dairy cattle in the tropics and subtropical zones (Gardiner *et al*., 1989; Guglielmone, 1995; Palmer *et al*., 1999; Riet – Correa, 2001). A number of *Anaplasma* species have been discovered in both domestic and wild animals. They include *Anaplasma marginale*, *A. ovis*, *A. phagocytophilum*, *A. bovis* and *A. capra* (Zhan *et al*., 2010; Liu *et al*., 2012; Li *et al*., 2015). *Anaplasma marginale* is the aetiologic agent of bovine anaplasmosis while *A. ovis* is the aetiologic agent of ovine anaplasmosis (Kocan *et al*., 2003; Ben Said *et al*., 2015; Battilani *et al*., 2017). *Anaplasma centrale* is related to *A. marginale*, however, it is less virulent, and used as live vaccines against anaplasmosis (Battilani *et al*., 2017). These *Anaplasma* species survive in red blood cells of cattle (Battilani *et al*., 2017). For instance *A. marginale* (the parasite) infects the red blood cells (erythrocytes) in mammalian hosts, forming a vacuole obtained from the erythrocyte membrane (Corona *et al*., 2005) and then settling in the bloodstream after infection (Richey, 1981). *Anaplasma phagocytophilum* is also a bacteria, however, it survives in granulocyte –rich cells. It is known to cause diseases of both veterinary and medical significance (Dumler *et al*., 2001; Kocan *et al*., 2015). *Anaplasma bovis* infects the
monocytes of animals (Ben Said et al., 2015). *Anaplasma platys* on the other hand, infects platelets and is the aetiological agent of dogs (Harvey et al., 1978). *Anaplasma capra* was discovered recently as a new zoonotic organism, but, the type of host cells this pathogen infects is not yet confirmed (Li et al., 2015).

The causative agents of anaplasmosis can be transmitted biologically by approximately 20 species of ticks (Richey, 1981) including *Rhipicephalus (Boophilus) microplus*, *R. decoloratus*, *R. simus*, *R. evertsi evertsi*, etc. (De Waal, 2000) or mechanically by blood sucking flies or arthropods which are less effectual carrier agents than ticks (Guglielmone, 1995; Valenzuela, 2004; Bowman, 2010). Also, it can be transmitted through iatrogenic or transplacental transmission (Riet – Correa, 2001) and contaminated fomites (Aubry and Geale, 2011) or surgical equipment associated with removal of horns, testes or tattooing (Inokuma, 2007; Aubry and Geale, 2011).

The acute phase of anaplasmosis is characterized by fever, weight loss, jaundice, progressive prostration, abortion (Jones et al., 2000), lowered milk production and often death of animals (Kocan et al., 2004). According to studies, dairy cows are most affected by anaplasmosis during lactation and the periparturient period (Aubry and Geale, 2011; Da Silva and Da Fonseca, 2014) and these may be related to associated temporary immunosuppression (Kehrli Jr et al., 1989). Adult animals exhibit severe clinical signs, unlike calves (up to about eight months), which develop subclinical disease due to innate resistance (Schalm et al., 1975; Madruga et al., 2001).

Evidence from infections of *Anaplasma* in animals suggests that severity of the disease is mediated by the immune system (Scorpio et al., 2005; Johns et al., 2009; Dumler, 2012).
Significant losses have been reported in imported cattle from temperate regions into Africa for the purposes of improving milk and meat production, leads to substantial losses if prevention methods are not taken (Lima, 1991).

2.1.2 BABESIOSIS

Bovine babesiosis is a TBD caused mainly by *Babesia bovis* and *B. bigemina*. Babesiosis is of major importance in Africa (Kirupananthan *et al.*, 2016). Together with other TBDs, it is responsible for over 50 percent losses in crossbred (Chaudhry *et al.*, 2010). *Babesia bovis* and *B. bigemina* are protozoan parasites that infect the erythrocytes of mammalian host (Chaudhry *et al.*, 2010), leading to anemia in the host (Khamesipour *et al.*, 2015). *Boophilus microplus*, the major vector of *Babesia*, is prevalent in the tropics and the subtropics (Chaudhry *et al.*, 2010). Generally, *B. bovis* is more pathogenic (Chaudhry *et al.*, 2010) than *B. bigemina* as it is less sensitive to some babesiacidal compounds (Khamesipour *et al.*, 2015). *Babesia bovis* infection can last for a minimum of four years in cattle while infection with *B. bigemina* generally lasts less than 6 months (Kirupananthan *et al.*, 2016). However, immune response to both pathogens last for a minimum of four years irrespective of the level of infection (de Vos *et al.*, 2004). Infections are characterized by high fever, loss of appetite, circulatory failure, etc. (Chaudhry *et al.*, 2010). Animals that survive infection usually become carriers of the parasites and act as a reservoirs for transmission (Chaudhry *et al.*, 2010). Carrier cattle infected with *Babesia* are challenging to notice because of the low amounts of parasites that occur in peripheral blood (Fahrimal *et al.*, 1992), however, parasites can be detected in the blood easily during acute infections (Chaudhry *et al.*, 2010).
2.1.3 EAST COAST FEVER (ECF)

East Coast fever, a TBD of cattle is caused by the protozoan parasite *Theleira parva* (Gachohi *et al.*, 2012). The disease has been reported to rank first in tick – borne disease constraints of cattle production in sub – Saharan Africa. It is principally transmitted by *Rhipicephalus appendiculatus* and is characterized by the multiplication of lymphoblasts infected with *Theileria* schizonts throughout the body, predominantly in the lymph nodes, spleen, kidneys, liver and lungs (Norval *et al.*, 1992; Minjauw and Mcleod, 2003).

2.2.0 CONTROL OF TICK – BORNE DISEASES

Numerous strategies have been employed to reduce the threat of Tick borne - diseases. These strategies involved the application of acaricides, which have been somewhat effective, but have usually been associated with severe limitations including the choice of acaricide – resistant ticks (de la Fuente *et al.*, 2017). Cattle breeders in Western Africa, depend on acaricides treatment and manual removal to lessen the effect of tick population and avoid the incidence of tick–borne diseases (Stachurski, 2000; Adjou Moumouni, 2012; Adehan *et al.*, 2016a). Nevertheless, the efficacy of the acaricides presently used is compromised by the rapid growth of acaricide–resistant *Rhipicephalus (Boophilus) microplus* ticks (Adehan *et al.*, 2016a). Ticks are greatly feared for their capability to invade and transmit *B. bovis, B. bigemina* and *A. marginale* (Walker *et al.*, 2003). Other drawbacks to the use of acaricides include pollution of the surroundings and products of animals with residuum (de la Fuente and Contreras, 2015; Eisen and Dolan, 2016). Integrated control approaches such as good habitation practices and selection of animals that exhibit higher genetic resistance to tick challenge have been recommended to minimize the usage of acaricides to limit the infestation of ticks (de la Fuente and Contreras, 2015; Eisen and Dolan, 2016). Recent developments
have also proposed the possibility of bringing together chemicals that possess repellency and parasiticidal effect to decrease the threat of tick–borne diseases (Schorderet – Weber et al., 2017). This strategy aims to avert both tick infestations and pathogen transmission (Schorderet – Weber et al., 2017). However, significant problems like long-term consequence and safety of human and animal usage support the generation of vaccines, which may possibly stimulate a lifelong protective immunity against tick infestations and pathogen infections and transmissions (de la Fuente and Contreras, 2015).

2.3.0 VACCINATION AGAINST TICK – BORNE DISEASES

Vaccines represent one of the ultimate developments in science with a very significant influence in enhancing human and animal health (de la Fuente et al., 2017). Vaccination represents about a quarter of the research on strategies to curb TBDs (de la Fuente et al., 2015) and its control of TBDs has been debatable because of the difficulties of averting tick – infestation among other limitation and subsequently the likelihood of pathogen transmission (de la Fuente et al., 2017). According to de la Fuente et al. (2007), the first vaccines produced to curb tick infestation became accessible in the early 1990s. These vaccines included the R. microplus BM86 or BM95 recombinant antigen and their utilization established that vaccine may possibly form part of an effective integrated program for the control of TBDs. These vaccines were not intended to avert the infestation of ticks, but to decrease the population of ticks and incidence of tick–borne pathogens (TBPs) by reducing feeding, reproduction and increase of ticks that feed on vaccinated cattle (de la Fuente and Kocan, 2003; de la Fuente et al., 2007; de la Fuente and Contreras, 2015).

Some factors must be taken into account when formulating potent vaccines to control TBDs. Factors such as tick species, ticks life cycle, differences in TBD–affected hosts, tick–borne
pathogen transmission pattern and tick–borne pathogen transmission stage after ticks feeding must be taken into account. Moreover, for tick–borne diseases in which pathogens are transmitted by a single host tick, it is feasible to concentrate on a particular host for vaccines distribution and efficiency. On the other hand, for several–host ticks, it could be essential to take into account several hosts to be vaccinated (de la Fuente et al., 2017). Additionally, the host's defenses to vaccination could vary between different hosts (Contreras and de la Fuente, 2017) and should therefore be taken into account during vaccine formulation and efficacy trials (de la Fuente et al., 2017).

Vaccines have remained successful in decreasing the population of ticks for a single host tick species such as *Rhipicephalus* species, which infects cattle and acts as a vector of highly economically significant tick–borne diseases (de la Fuente and Contreras, 2015). Vaccination remains the best strategy available to effectively control infectious diseases. However, it is difficult to develop cost–effective vaccines that has long-term protective immunity. (de la Fuente et al., 2017). For bovine anaplasmosis, which is spread by biting insects and contaminated fomites (Kocan et al., 2010), vaccines which affects tick pathogen infection, transmissions of tick–borne pathogen and infection of host tick–borne pathogen as well as lessening the infestation of ticks would be more effective in controlling diseases (de la Fuente et al., 2017).

### 2.4.0 THE INNATE IMMUNE SYSTEM

The Immune system offers a state of protection to the host against infectious and other harmful substances (Vu et al., 2017). It is induced by the infestation of ticks, infection with tick – borne pathogens and other foreign substances (Brossard and Wikel, 2004). The immune response in general, is divided into two arms, the innate (natural) and adaptive
(acquired) immunity (Medzhitov and Janeway, 2000). Innate immunity provides immediate protection (Christmas, 2010) because it is the first line of defense against foreign substances and it is rapid. Also, it lacks memory, and does not have the ability for a more aggressive reaction following subsequent exposure to a particular agent (Medzhitov and Janeway, 2000). Nevertheless, it is general in its mode of attack in microorganisms (Christmas, 2010). Conversely, adaptive immunity is more complex and happens over a prolonged period (i.e. over hours to days). It is specific and efficient at targeting pathogens (Christmas, 2010). The adaptive immune response is made up of two components; the humoral (antibody) and cellular immunity (Vu et al., 2017). The innate immune system has an important role in averting infections, but also operates to restore structural and functional integrity of the damaged tissues. Its key functions include stimulation of pro-inflammatory signalling cascades, opsonisation and apoptosis (Janeway and Medzhitov, 2002). Another important characteristic of the innate immunity is a set of recognition molecules called pattern recognition receptors (PRRs). These PRRs bind conserved molecular structures identified in large groups of pathogens, termed pathogen – associated molecular patterns (PAMPs) (Vu et al., 2017).

The immune system of the host triggers both the innate and adaptive responses against invasion with the latter comprising of both humoral and cell – mediated immune – regulatory and effector pathways (Wikel and Bergman, 1997). Tick feeding triggers a collection of host immune regulatory and effector reactions including APCs, cytokines, immunoglobulins, complement etc. (Wikel et al., 1997). The development of the adaptive immunity can lead to an inflow of cells into the dermis and the epidermis surrounding the tick’s mouthparts (Wikel and Bergman, 1997). Basophils and eosinophils are employed to the site of tick attachment and stimulate tick elimination by local basophil degranulation (Brown et al., 1982). Langerhans cells entrap salivary antigens and move to the lymph nodes where they operate as
APCs for specialized lymphocytes. Antibodies against tick – specific antigens are produced and contribute, alongside the complement (Allen et al., 1979; Nithiuthai and Allen, 1985).

2.4.1 LYMPH NODES

The lymphoid system consists of the primary, secondary and tertiary lymphoid tissues. The primary lymphoid tissues consist of the thymus and bone marrow while the secondary tissues are the lymph nodes, spleen and Peyer’s patches. The Tertiary tissues mature during inflammation and are highly adaptable structures (Buettner and Bode, 2012).

In mammals, lymph nodes are located throughout the body. They monitor the body fluids for pathogens or antigens (Junt et al., 2008) thus making them very effective in sampling and concentrating antigens (Randolph et al., 2008). Lymph nodes function mainly to sieve the lymph entering from draining areas and to scan these lymph for antigens (Sainte – Marie, 2010), for instance, the pre – scapular lymph node of the ear drains the skin and subcutaneous tissue of the caudal regions of the auricle (Rogers et al., 1993). Foreign antigens introduced into tissues by vaccination or immunization are transported either as soluble molecules or by antigen presenting cells to draining lymph node (Vrieling et al., 2013). Migration of these antigens is achieved when antigen– filled dendritic cells coming from the draining region present their antigens to T – lymphocytes in the T cell region (paracortex). T cells are stimulated, they differentiate and proliferate. T helper cells move into the B cells region (cortex) to help B cells. B cells then differentiate into plasma cells for effective antibody production. All stimulated effector cells (plasma cells, CD4+ or CD8+ T cells) move to the medulla, where they exit the lymph node to the endangered area of their particular draining region (Buettner and Bode, 2012). The structure of the lymph node changes dramatically following infection (Siegert, 2012). This dramatic change in lymph node structure is because,
during infection, antigen presenting cells and lymphocytes come together in the lymph node to initiate primary immune responses (Kaldjian et al., 2001).

2.4.2 CYTOKINES

Cytokines are minute proteins secreted by cells which have a specific effect on the interactions and communications amongst cells. They have other names such as lymphokine (cytokines made by lymphocytes), monokine (cytokines made by monocytes), interleukin (IL) (cytokines made by one white blood cell and acting on other white blood cells) and chemokine (cytokines with chemotactic actions) (Zhang and An, 2007). They help in bridging the innate and acquired immunity by coordinating the movement of white blood cells and other cells (Blackwell and Christman, 1996; Gogos et al., 2000; Ioannidis et al., 2014). Also, they can act on the cells that secreted them; this is termed as autocrine action, on neighbouring cells which is also termed as paracrine action or in certain cases on distant cells; endocrine action. Generally, it is usual for various cells to secrete the same cytokine or for a single cytokine to act on a number of different cell types but they are redundant in their action; suggesting that similar roles can be induced by several cytokines (Zhang and An, 2007).

Cytokines are produced by many cells, however, the key producers are helper T cells (Th) and macrophages (Zhang and An, 2007). For instance, Th1 cells produce Interferon gamma, which guards against intracellular organisms and plays a role in stimulation-induced cell death of mucosal epithelial cells, skin keratinocytes and T – cells (Akkoc et al., 2008; Rebane et al; 2012; Yoo et al., 2013), effector Th2 cells synthesize IL – 4, IL – 5, IL – 9 and IL – 13 (Akdis, 2006) which also have vital roles such as regulation of epithelial barrier integrity, as well as production of mucus, eosinophilia and allergen – specific IgE (Soyka et al., 2012;
Georas and Rezaee, 2014; Kubo et al., 2015; Seltmann et al., 2015). They could be synthesized in and by peripheral nerve tissues during physiological and pathological processes by resident and recruited macrophages, mast cells, endothelial cells (Xie et al., 2006) and basophils (Harvima et al., 2014). Recent studies have shown that basophils for instance contribute significantly to the expansion and advancement of Th2 cytokine – mediated inflammation (Siracusa et al., 2013; Harvima et al., 2014). Also, Mast cells produce many ILs and release them on stimulation over innate immune response receptors (Harvima et al., 2014; Munoz – Cano et al., 2016). Epithelial cells on the other hand can control both innate and acquired immune responses through the secretion of many cytokines, chemokines, and lipid mediators in reaction to external stimuli detected by intracellular sensors as TLRs, NOD – like receptors (NLRs) etc and these cytokines include IL – 1α, IL – 25, IL – 33 (Hammad and Lambrecht, 2015), type I interferons, INF – γ – induced protein etc (Vareille et al., 2011). These cytokines orchestrate Th2 immunity and Cytokines can also be produced and released from herniated nucleus pulposus, produced in the spinal cord (DeLeo et al., 1996), the Dorsal root ganglion (DRG) (Schafers et al., 2003), or the swollen skin (Heijmans – Antonissen et al., 2006). They are generally synthesized in a cascade, as one cytokine induces its target cells to produce additional cytokines. Also, they can operate synergistically or antagonistically (Zhang and An, 2007) but ultimately, the role of cytokines is to regulate the immune response (Liyings et al., 2012).

Cytokines can either be pro – inflammatory or anti – inflammatory. Pro – inflammatory cytokines are synthesized primarily by stimulated macrophages and are associated with the up – regulation of inflammatory responses (Zhang and An, 2007). Pro-inflammatory cytokines and chemokines, such as TNF-α, INF-γ, IL-1, IL-2, IL-6, IL-8, IL-12, IL-18 and monocyte-chemotactic protein-1 (MCP-1) are essential for starting an effective inflammatory reaction (Blackwell and Christman, 1996; Gogos et al., 2000; Fernandes et al., 2008) but an
extreme pro-inflammatory reaction, with high concentration levels of cytokines such as TNF-α, INF-γ, IL-6, IL-8, IL-18 and MCP-1 has been related with death (Malaguarnera and Musumeci, 2002; Artavanis-Tsakonas et al., 2003; Awandare et al., 2006; Fernandes et al., 2008; Deroost et al., 2013; Lourembam et al., 2013). The anti–inflammatory cytokines are a sequence of immune-regulatory molecules that regulate pro–inflammatory cytokine reaction. Anti–inflammatory cytokines include IL–4, IL–10, IL–11, IL–13 etc (Zhang and An, 2007).

2.4.3 INTERFERONS

Interferons (INFs) are a family of naturally occurring cytokines with significant immunomodulatory, antiviral, antiangiogenic, anti-proliferative and antitumor activities, which are released by cells upon exposure to various stimuli such as viruses, double-stranded RNA, and polypeptides (Pestka et al., 1987; Parmar and Platanias, 2006) hence are involved in various immune interactions as inducers, regulators and effectors of the innate and the acquired immunity (Priyanka and Muralidharan, 2014). The interferons are a group of cytokines that play a focal role in pathogen resistance (Cameron and Kelvin, 2000) and they are the first set of cytokines that exhibited efficiency in the treatment of malignancies and viral infections. Interferons were first identified in 1957 and were termed for its capacity to interfere with viral replication in treated cells (Isaacs and Lindenmann, 1957; Parmar and Platanias, 2006).

There are three main classes of INFs that were initially known, based on their separation profiles in high – pressure liquid chromatography (HPLC) and they include alpha (α), beta (β) and gamma (γ) and subtypes. INF α is synthesized principally by white blood cells; INF β is also produced by fibroblasts ((Pestka et al., 1987; Pestka, 1997) and INF γ is produced by
cellular arm of the immune system (Pestka et al., 1987; Pestka, 1997; Parmar and Platanias 2006) i.e. cells from both the innate (such as Natural killer cells, macrophages etc.) and acquired (such as Th1 cells, cytotoxic T lymphocytes, and B cells) immunity (Zimmermann et al., 2011). Interferon alpha, Interferon beta and Interferon gamma were originally referred to as leukocyte interferon, fibroblast interferon (Derynck et al., 1980; Lawn et al., 1981; Owerbach et al., 1981; Shows et al., 1982; De Maeyer and Maeyer – Guignard, 1998) and immune interferon (Boehm et al., 1997) respectively. INFs are categorized into two main types, which are Type I and Type II (Pestka et al., 1987; Platanias, 1995; Pestka, 1997; Pfeffer et al., 1998; Stark et al., 1998). The Type I INF subclasses include INF Alpha, INF Beta (INFβ), INF Tau (INFτ), INF Omega (INFω) and the Type II include only INF Gamma (INFγ) (Pestka, 1986; Pestka et al., 1987; Pestka, 1997). Type I INFs are predominantly triggered in response to viral infection, while the Type II INF is principally stimulated by immune and inflammatory stimuli (Pestka et al., 2004).

Type I and Type II interferons transmit signals via diverse receptors to generate different, but corresponding, cellular effects (Boehm et al., 1997). Type I INF has a common receptor; For instance, INF α subclasses are structurally interrelated and bind to a common heterodimer receptor (Pestka et al., 1987; Stark et al., 1998; Platanias and Fish, 1999), the Type I INF receptor (CD 118) (Muller et al., 1994; Cousens et al., 1999; Dickensheets and Donnelly, 1999). Similarly, INF β (Pestka, 1986, Platanias and Fish, 1999) and INF ω (Adolf, 1987) bind to Type I INF receptor (CD 118) (Muller et al., 1994; Cousens et al., 1999; Dickensheets and Donnelly, 1999). INF α and INF β seem to be key actors in innate immunity (De Maeyer and Maeyer – Guignard, 1998). Moreover, they possess the capability to control acquired T cells (Cousens et al., 1999) or indirectly by hindering IL–4–inducible gene expression in monocytes (Dickensheets and Donnelly, 1999). These characteristics have been proved in knockout mice thus mice deficient in CD 118 demonstrate compromised
antiviral defenses and are deficient in stimulating INF γ production by T cells (Muller et al., 1994; Cousens et al., 1999; Dickensheets and Donnelly, 1999).

On the contrary, INF γ is structurally unrelated and so binds to a different cell – surface receptor (Pestka et al., 1987; Pestka et al., 1997; Stark et al., 1998; Platanias and Fish, 1999) known as CDw 119 (Cameron and Kelvin, 2000), thus, making INF γ an extremely distinct cytokine from the Type I INFs (Parmar and Platanias, 2006). INF γ which was originally identified as an antiviral agent has many biological functions (Cameron and Kelvin, 2000). For instance, it can activate macrophages, upsurge antigen processing and expression of Major Histocompatibility Complex (MHC) molecules, promote an immunoglobulin (Ig) class to IgG2a antibody secretion and regulate the proliferation of transmuted cells (Boehm et al., 1997). Thus, mice with mutations in interferon gamma (Dalton et al., 1993) or INF γ receptor (Huang et al., 1993) expression demonstrate reduced macrophage and NK cell action and amplified vulnerability to many intracellular bacteria, protozoan and viruses but cellular immunity can still mature in INF γ knockout mice (Graham et al., 1993; Wang et al., 1994).

2.4.4 INTERLEUKINS (IL)

Interleukins (ILs) are proteins that bind to specific receptors and involved in intercellular interaction amongst white blood cells (Dinarello et al., 2010).

IL – 6 is a member of the IL – 6 – type group of cytokines, which includes leukemia inhibitor factor (LIF), ciliary neurotrophic factor, and oncostatin M (OSM) (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 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).

2.4.5 TUMOR NECROSIS FACTOR (TNF)

The TNF family is a large group of interrelated cytokines (Gruss and Dower, 1995; Wallach et al., 1999) that has shared properties as cell death effectors (Ashkenazi and Dixit, 1995). The Tumor necrosis factor includes members such as TNF – α, TNF – β, lymphotoxin (LT) – β and Fas ligand (FasL)/CD 178 (Cameron and Kelvin, 2000). The members of TNF family seem to act in trimeric form (Gruss and Dower, 1995; Wallach et al., 1999).

It is a pro – inflammatory cytokine, which was first identified as a tumor cell killer (Pennica et al., 1984; Old, 1985; Nedospasov et al., 1986). TNF – α can be found in a membrane bound or soluble form following proteolytic processing. It shares a receptor with TNF – β
(CD 120a, b), which is expressed virtually on all cell types except red blood cells. The TNF – α cytokine is primarily synthesized by stimulated macrophages, NK cells and T cells primarily Th 1 cells (Gruss and Dower, 1995). TNF – α is a very significant pleiotropic cytokine associated in animal immunity, inflammation and apoptosis. It performs a twofold role in controlling immune responses, acting as both pro–inflammatory mediator, stimulating effective inflammatory responses and an immunosuppressive mediator, impeding the expansion of autoimmune diseases and tumor genesis and demonstrating a crucial role in maintenance of immune homeostasis by regulating the degree and duration of inflammatory process. TNF – α plays a key function in host defense pathogens (Brown et al., 2015). It can also circuitously change hormones and IL – 1 secretion to induce fever (Cameron and Kelvin, 2000). A shortcoming of TNF – α is that, high systemic levels can result in septic as well as local rises in TNF – α concentrations, which cause heat, swelling, redness, pain and loss of inflammation; the five cardinal signs of inflammation. Also, it is associated with the development of allergic diseases especially asthma (Brown et al., 2015) and atopic dermatitis (Zimmermann et al., 2011).
CHAPTER THREE

3.0.0 MATERIALS AND METHODS

3.1.0 LOCATION OF STUDY

The study was conducted at the Amrahia Dairy Farm (ADF) which is located (at latitude 05° 46' N and longitude 00° 08' W) on the Accra–Dodowa road about 27 km from Accra. The area has a bimodal rainfall pattern with the major rainy season occurring from April to July and the minor rainy season from September to November. The driest months are January–March, August and December. The annual rainfall and temperatures ranges between 600mm and 1000mm and 20°C and 34°C respectively (Obese et al., 2010, 2013).

3.2.0 TOLL–LIKE RECEPTOR AGONIST

Synthetic TLR agonist resiquimod/imiquimod stimulates TLR7 and 8 (Heil et al., 2004; Tomai et al., 2007). When emulsified in adjuvant, has been shown to bind and stimulate the cognate receptor on bovine cells. For our set of experiments, the agonist was delivered in a water-in-oil emulsion (squalane/mannide monooleate) prepared by the Infectious Diseases Research Institute (IDRI, Seattle, WA USA).

3.3.0 SOURCE OF CROSSBRED CALVES AND DRB3 HAPLOTYPES

A total of 24 three month old Friesian x Sanga (F1) calves, seronegative for A. marginale, were obtained from Amrahia Dairy Farm and Animal Research Institute of the Council for Scientific and Industrial Research of Ghana. To control for potential confounding effects of MHC haplotype on the immune responses, calves were MHC haplotyped and all were
confirmed to express DRB3*2404, DRB3*2711, DRB3*2405, DRB3*2406, and DRB3*2407 alleles unique to the Sanga from Ghana. Three-month old Friesian x Sanga calves were allocated randomly to two equal groups. Group A (calf ID: O554, 4NS26, 4NS22, 3777, 4NS21, 5NS6, 4533, R287, 0039, R288, 3936, 3937) was injected with the TLR agonist and Group B (calf ID: 5NS24, M9, 3NS51, 3NS2, 3NS53, 3768, 0096, N1299, R270, R286, 3932, 3933) was left untreated.

3.4.0 MEASUREMENT OF PHYSIOLOGICAL PARAMETERS ASSOCIATED WITH THE INDUCTION OF INNATE IMMUNE RESPONSE

Prior to injecting calves with the agonist, the rectal temperature and width of pre–scapular lymph nodes were measured (0 hour) for individual Friesian x Sanga calves for five consecutive days. The mean values were computed to establish baseline data for cattle in the sub region. To determine rectal temperatures, calves were briefly restrained and an electronic thermometer was inserted into the rectum as previously described (Gaughan et al., 1999). Similarly, the pre–scapular lymph nodes around the neck region were measured using a tape measure.

Following the 5 day period of observation, the calves were divided into two groups of 12 calves. Calves in Group A represented the experimental group (n=12) and were injected, subcutaneously, with 1ml of the agonist preparation in the right side of the neck. Calves (n=12) in Group B represented the control group (n=12) were not injected. The height of their rectal temperatures (i.e. the level or rise in temperature) and width of the lymph node for both experimental and control groups were measured, at same time, (08:00 hrs) daily for 7 additional days post-inoculation.
3.5.0 DETERMINATION OF THE SECRETED CYTOKINES

Blood samples were collected daily for serology and genomic DNA analyses. The blood samples were transported on ice to the Microbiology Laboratory and left overnight at room temperature to coagulate. Serum was prepared from the coagulated blood samples and stored in 15 ml Falcon tubes at -20°C. Using these samples, we also tested for serum levels of IL-6 and TNF-α by capture cytokine-specific antibody ELISA (NEO Scientific Biolab, Cambridge, Massachusetts). Briefly, 100 µl of serum samples and equivalent standardized samples were added to the pre-coated ELISA plate in duplicate wells. Similarly, 100 µl of Phosphate buffered saline (PBS) at pH 7.0–7.2 was added to the blank wells. Enzyme solution of 50 µl was added to each well except the blank wells and mixed thoroughly. The plates were covered with parafilm and incubated for 1 hour at 37°C. The plate was then washed 5 times with 400 µl 1X wash solution. Substrate A of 50 µl was added to each well followed by 50 µl of substrate B. Each well was covered and incubated for 15 minutes at room temperature during which there was no visible colour change. Stop solution of 50 µl was added to each well and mixed thoroughly. This terminated the reaction resulting in a colour change from blue to yellow. The plates were then read immediately at optical density of 450nm using the VersaMax absorbance microplate reader (California, U.S.A) (Appendix 1).

3.6.0 STATISTICAL ANALYSIS

For quantitative determination of the individual cytokines in calves, a standard curve was constructed using the SoftMax® Pro 6 Software based on standardized samples of each cytokine. Significance between treated and untreated control groups was determined by the
Student’s $t$-test. Mean values were considered to be statistically different when P < 0.05 and considered not different when P > 0.05.

3.7.0 ETHICS STATEMENT

The cattle used in this study were treated in strict accordance to guidelines set by University of Ghana Institutional Animal Care and Use Committee. This protocol was approved for use in sampling blood from cattle by the Noguchi Memorial Institute for Medical Research’s NIACUC (protocol number: 2015-01-5X).
CHAPTER FOUR

4.0.0 RESULTS

4.1.0 PHYSIOLOGICAL BIOMARKERS

4.1.1 EFFECT OF TLR7/8 INJECTION ON RECTAL TEMPERATURE OF CALVES

One of the key questions regarding the effectiveness of our approach is whether injection with the synthetic TLR 7/8 agonist emulsion can stimulate the innate immune response in young crossbred calves. Any treatment-induced changes in the height of temperature or the sizes of the secondary lymphoid organ can easily be discerned by farmers. It is therefore essential to establish the normal values and sizes of these indicators to allow comparisons between experimental and control groups. The mean rectal temperature of calves (n = 24) as measured for five consecutive days without any stimulation was 38.5°C ± 0.10. This value represents the “normal” rectal temperature for Friesian x Sanga calves in Ghana and could be applied to early disease diagnosis for effective treatment of cattle across the West Africa sub region.

After calves in the experimental group were injected with the TLR7/8 agonist emulsion, the mean rectal temperature rose from 38.5°C ± 0.10 to 39.4°C ± 0.21 (Figure 1). This elevation is significantly higher (p = 0.013) than the normal rectal temperature of Friesian x Sanga F1 calves from Ghana. Similarly, the mean rectal temperature of the TLR7/8-stimulated calves was significantly higher (p = 0.013) than 38.56 ± 0.15 recorded for the control cohorts.

Notably, the normal temperature of calves from the control group, not stimulated with the agonist, was essentially the same (p = 0.09) as the empirically-determined normal temperature of the crossbred calves.
Figure 1. Rectal temperatures of crossbred calves. The blue bar represents the normal rectal temperature of the Friesian x Sanga FI calves (Conditioned calves, n = 24 which represented the baseline measurement). The black bar represents the mean rectal temperature of calves injected with TLR7/8 agonist (Experimental group). The grey bar represents the mean rectal temperature of calves that were not injected with TLR7/8 agonist (Control group). Testing was carried out daily for seven consecutive days. There was no significant difference in the rectal temperature between animals from the control and conditioned group (p = 0.29). The daily rectal temperatures were significantly higher in experimental calves than the control cohorts.

4.1.2 EFFECT OF TLR7/8 INJECTION ON SWELLING OF THE PRE–SCAPULAR LYMPH NODES OF CALVES

Lymph nodes are central to effective sampling and provision of the platform for immune cells to interact with foreign antigens. The pre–scapular lymph nodes (right and left) of the conditioned calves (n = 24) were measured to determine the normal width of pre–scapular lymph nodes of Friesian x Sanga calves.
The results (Table 1) have demonstrated that cross-bred calves injected with the TLR agonist emulsion developed significantly (p = 0.009) higher width of pre–scapular lymph nodes as compared to control cohorts. Similarly, there were significant differences (p = 0.009) in the injection-induced increases in the diameter of the pre-scapular lymph node (Table 1) in all experimental calves.

The mean width of the right and left pre–scapular lymph nodes of the experimental calves were 72 mm ± 6.63 and 86 mm ± 8.13, respectively. These increases were significantly different (p = 0.009) from the pre–scapular lymph node widths of 48 mm ± 4.90 and 46 mm ± 8.13 from the control cohorts. Notably, the pre–scapular lymph nodes of control calves were similar to the normal values determined among crossbred calves during the 5-day preconditioning period.

Interestingly, the maximum swelling of 86 ± 8.13 occurred in the left pre–scapular lymph nodes as compared with 72 ± 6.63 in right pre–scapular lymph nodes of experimental calves.
Table 1: The mean width (mm) ± S.E of pre–scapular lymph nodes Friesian x Sanga calves.

<table>
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<th>FRIESIAN x SANGA (F1)</th>
<th>WIDTH OF PRE–SCAPULAR LYMPH NODE</th>
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<td>RIGHT</td>
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<tr>
<td>Conditioned (n = 24)</td>
<td>46 ± 5.10b</td>
</tr>
<tr>
<td>Experimental (n = 12)</td>
<td>72 ± 6.63a</td>
</tr>
<tr>
<td>Control (n = 12)</td>
<td>48 ± 4.90b</td>
</tr>
</tbody>
</table>

Means in the same column that have the same alphabet are not significantly different.

4.2.0 CYTOKINE SECRETION

4.2.1 IL – 6

The amount of IL-6 increased significantly (p = 0.001) from $10^2$ to $10^6$ pg/ml during 48 hours (Figure 2) of injection in experimental calves after stimulating the innate immune response with TLR7/8 agonist emulsion. This sudden increase was followed by precipitous decline to levels < $10^2$ pg/ml within 48hrs of stimulation. There was however, no significant increases in the secretion of same in the control cohorts that did not receive any immune stimulation.
Figure 2. Mean 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 black and grey bars represents 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}.

4.2.2 TNF – α

TNF–α concentration appears to increase from 100pg/ml to 1000pg/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 3). This level has declined to near the normal levels in all calves after 72hrs.
Figure 3. 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 black and grey bars represent mean amount of TNF–α in the experimental group and the control cohorts, respectively.
CHAPTER FIVE

5.0.0. DISCUSSION

In this study, we tested whether the innate immune response of crossbred calves could be stimulated by using a Toll-Like receptor 7/8 agonist (TLR7/8) with the goal to protect these animals against tick borne diseases. The results from this study have demonstrated that cross-bred calves injected with the TLR agonist emulsion developed significantly higher temperatures as compared to control cohorts. Similarly, there were significant differences in the injection-induced increases in the diameter of the pre-scapular lymph node (Table 1) in all experimental calves. Our data have provided empirical evidence that TLR 7/8 agonist is a potent innate stimulator in crossbred cattle. These findings support the hypothesis that TLR7/8 stimulates functional innate response in crossbred calves.

Synthetic TLR7/8 agonist emulsion stimulates the innate immunity of crossbred calves:

Pathogens stimulate the innate immune response through Toll-like receptors that serve as sensors for pathogen molecules and initiators of innate immunity (Hajjar et al., 2002; Ibeagha et al., 2008; Ishii et al., 2008; Uematsu and Akira, 2008; Kumar et al., 2009). Although this capability has been confirmed in the murine models, the effectiveness has not been verified in cattle and, specifically, in cross-bred African breeds. This perceived unknown terrain of the possibility to deploy innate immunity infectious disease control has been largely resolved by empirical biologic and immunologic data from cattle. The present data provided the first hand empirical evidence that TLR 7/8 agonist is a potent innate stimulator in crossbred cattle corralled in endemic region setting. These findings support the stimulation of an innate response in crossbred calves as effective alternative to use of classical vaccines for disease control in the endemic region typified by multiple pathogen infection of livestock.
Specifically, the study evaluated variabilities in the injection-induced rise in temperature and swelling of the draining pre-scapular lymph node. These biomarkers represent observable and measurable physiological parameters associated with the progression of innate immune response that farmers could use for rapid detection of disease to be able to figure out effective control strategy. Findings from the current work agree with the results of Sarfo (2016) who reported a sharp increase in rectal temperature (38.5°C to 40.7°C) within 24 hours after injecting cross–bred (Zebu x Sahiwal) calves with TLR7 agonist. Sarfo (2016) work was conducted on Zebu x Sahiwal cross–bred in southern Africa, however this study was conducted on Friesian x Sanga (F1) calves in Ghana, West Africa thus making the findings in this study the first in the West African sub region. The short duration of temperature rise is consistent with the rapid nature of the innate immune response (Medzhitov and Janeway, 2000) capable of protecting real time temporary protection against clinical disease (Christmas, 2010). Elevated temperature is known to be associated with infection and also helps a range of immune cells such as dendritic cells, macrophages to work efficiently (Sarfo, 2016). Also, elevated temperature is associated with increased phagocytic activity of dendritic cells (Sarfo, 2016).

The significantly higher enlargement of pre – scapular lymph node width in experimental calves upon innate immune stimulation and as compared with the control cohorts further supports the argument that lymph node is one of the immunologic sequestered sites of antigens during infection of a host animal (Siegert, 2012). This dramatic change in lymph node structure may derive from increased infiltration and cellular activity upon stimulation. According to pioneering studies, the lymph node filters lymph entering from draining areas and also scans the lymph for antigens (Sainte – Marie, 2010). The lymph node therefore represents an effective immune organ responsible for sampling and concentrating antigens (Randolph et al., 2008).
TLR7/8 agonist increases IL-6 secretion in calves:

The amounts of selected type 1 cytokines secreted during the induction of innate immunity were 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 was no significant difference in the amount of TNF-α (p = 0.26) in the serum samples between the control and experimental groups. IL – 6 secretion was significantly higher in all calves stimulated with the agonist emulsion but remain stable in all control calves. According to Bourquin et al. (2011) it was possible to increase IL – 6 levels within 24 to 48hrs with resiquimod (TLR7 agonist R848) stimulation consistent with the previous study (Bourquin et al. 2011). IL – 6 induces fever resulting in elevated temperature in infected animals (Fattori et al., 1994). This fact is consistent with the significant elevation in the height of the rectal temperature in calves stimulated with the agonist indicating the potential to trigger innate response by way of stimulation with synthetic TLR7/8 formulations. This is not simply elevation in temperature because according to Kubkomawa et al. (2015) an increase in body temperature just by 1 °F above the normal body temperature is considered as fever.

TNF – α was higher in inoculated calves after 48 hours of inoculation with the TLR7/8 agonist as compared to calves that did not receive the agonist inoculation but this increase was not significantly different between calves that did not receive the inoculum and the experimental cohorts. Ilyinski et al. (2014) reported a significant increase of TNF – α secretion within 4 hours of inoculation. This difference could be due to the fact that Ilyinski et al. (2014) measured amount of TNF – α secretion after 4 hours of inoculation.
CHAPTER SIX

6.0.0 CONCLUSION AND RECOMMENDATIONS

6.1.0 CONCLUSION

Innate immune responses following inoculation with TLR7/8 agonist initiated an influx of leukocytes and release of vasoactive mediators—resulting in local inflammation with edema and cellular infiltrates. This results in measurable swelling of the draining lymph node, skin thickness as well as an increase in temperature. All these are indicators of an innate response. In the current work the results indicate that the animals have responded to stimulation with the agonist. Unlike the classical vaccination that target immune system with specific antigen, the current approach is not antigen-specific. The corresponding innate response can therefore provide protection against multiple pathogens without the need for multiple antigen-specific vaccines. That actually is the innovation in this approach with the possibility to save cost of producing crossbred cattle in Ghana where infectious disease are widely prevalent. Consequently, if innate immunity can be stimulated for a long duration, during which infection occurs in the endemic regions, disease can be prevented at a much lower cost than the use of drugs. This approach would allow genetic improvement of cattle while maintaining innate resistance.

6.2.0 RECOMMENDATIONS

The long-term expectation is that the innate immune stimulation afforded by the TLR7/8 agonist will provide protection against severe disease upon pathogen challenge. However, the duration of immunity conferred by the agonist is unknown. In most disease endemic areas, challenge will occur within 6 months, and as shown in Ghana, often much sooner. Whether
the current formulation and single dose provides innate immune stimulation over this window is a critical question that will require data to resolve.

Also, further studies are needed to investigate if TLR7/8 agonist could also induce an effective adaptive immune response in crossbred calves.
REFERENCES


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APPENDICES

Appendix 1

ELISA ASSAY PROTOCOL

1. Add 100 µl of serum samples or Standards to the appropriate number of wells in the supplied ELISA plate. Wells have been pre–blocked and no additional blocking steps are required. Add 100 µl of PBS (pH 7.0–7.2) to the blank well.

2. Add 50 µl of Enzyme Solution to each well (NOT blank well) supplied ELISA plate and mix well.

3. Cover and incubate 1 hour at 37°C in a humid chamber.

4. Wash each well 5 times with 300 – 400 µl 1X Wash Solution per well. After the last wash invert the plate and blot dry by tapping on absorbent paper. Note: Hold the sides of the plate frame firmly when washing to assure that all strips remain securely in the frame. Complete removal of the liquid at each step is essential for good performance.

5. Add 50 µl of Substrate A to each well followed by addition of 50 µl of Substrate B. Cover and incubate 10–15 minutes at room temperature. Substrate is light sensitive. Keep out of direct sunlight or cover with foil.

6. Add 50 µl of Stop Solution to each well and mix well.

7. Immediately read the optical density (O.D.) at 450nm.

8. Subtract the mean blank value from each sample or standard value and calculate the mean for duplicate (or greater) wells.

Note: Blank well must NOT have Enzyme Solution.