

**SUSCEPTIBILITY OF INDIGENOUS CATTLE BREEDS TO CO-  
INFECTION WITH MULTIPLE TICK-BORNE PATHOGENS**

**BY**

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## DECLARATION

I, Carl Selorm Kwabla Beckley, hereby declare that this thesis which is submitted to the College of Agriculture and Consumer Sciences, University of Ghana, Department of Animal Science for the award of Master of Philosophy in Animal Science Degree is the result of my own investigation. This thesis has not been submitted or presented for another degree elsewhere, either in part or in whole, except for other people's work which was duly cited and acknowledged.



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## DEDICATION

I dedicate this thesis to the Lord Almighty for His infinite mercy and vision which imparted into my life the strength to endure the numerous challenges. To my mentor Senior Prophet Temitope Balogun Joshua for his spiritual guidance and inspiring message which said, and I quote, “Nothing can stop you from reaching your goal when you have a heavenly vision. No hardship, trouble, sickness, or even unrighteousness can stop you from reaching your goal” unquote. To my virtuous and God fearing wife who stood strongly beside me both spiritually and materially, to you I say God richly bless you.

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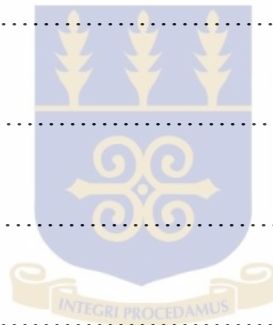
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**ABBREVIATIONS**

|          |   |
|----------|---|
| Am       | <i>Anaplasma marginale</i>  |
| BBG      | <i>Babesia bigemina</i>   |
| T        | <i>Theileria species</i>  |
| Am+BBG   | <i>Anaplasma marginale</i> and <i>Babesia bigemina</i> combined                               |
| Am+BBG+T | <i>Anaplasma marginale</i> , <i>Babesia bigemina</i> and <i>Theileria species</i><br>combined |
| Am+T     | <i>Anaplasma marginale</i> and <i>Theileria species</i> combined                              |
| ARI      | Animal Research Institute   |
| ECF      | East Coast Fever  |
| ELISA    | Enzyme-Linked Immunosorbent Assay   |
| HGA      | Human granulocytic Anaplasma  |
| IM       | Intra Muscular  |
| LB       | Luria-Bertani   |
| LIPREC   | Livestock and Poultry Research Centre   |
| MSP      | Major Surface Protein   |
| PCR      | Polymerase Chain Reaction   |

|      |                                 |
|------|---------------------------------|
| PCV  | Packed Cell Volume              |
| RBC  | Red Blood Cell                  |
| SC   | Subcutaneous                    |
| TBDs | Tick-Borne Diseases             |
| TBPs | Tick-borne pathogens            |
| UV   | Ultra Violet                    |
| WASH | West African Shorthorn          |
| WTSI | Wellcome Trust Sanger Institute |

## ABSTRACT

The control of tick-borne diseases in an endemic region is very challenging and costly due to the presence of multiple pathogens from the seven globally-important livestock intracellular pathogens. This problem is exacerbated by the availability of competent vector ticks for each pathogen strain, thus resulting in enhanced transmission efficiency and higher infection prevalence of disease among cattle from the tropical region than the temperate region. Unfortunately, these diseases are poorly controlled in sub-Saharan Africa largely due to lack of understanding of the infection biology of the pathogen species.

Understanding the species composition of pathogens of infected cattle in Ghana will allow for the formulation of cost effective interventions that will enable the development of applicable control strategies and improve the adaptive capacity of tick-borne diseases. In this thesis, *Anaplasma marginale*, *Babesia bigemina* and *Theileria* species were used as model bacterial and protozoan blood-feeding pathogens to examine whether genetic diversity in cattle correlates with quantitative differences in co-infection prevalence among indigenous breeds of cattle from different Agro-ecological zones in Ghana.

A low-cost molecular approach (multiplex PCR) was developed to assess the multi-pathogen complex situation in the indigenous cattle of four distinct breeds (Sanga, Gudali, West African Shorthorn (WASH) and White Fulani).

The overall pathogen co-infection prevalence in indigenous cattle breeds examined was 26.4% (105/397). Out of this total, only six cattle, representing 1.5% (6/397) of the population were co-infected with all three different tick-borne pathogens - *Theileria*

*species*, *Anaplasma marginale*, and *Babesia bigemina*. 95 cattle 23.9% (95/397) were co-infected with *Anaplasma marginale* and *Theileria* species, whereas four cattle 1.0% (4/397) were co-infected with *Anaplasma marginale* and *Babesia bigemina*. Surprisingly, no individual cattle were found to be co-infected with *Babesia bigemina* and *Theileria* species. The overall infection prevalence of cattle was 62.9% (250/397). Significant ( $p=0.00$ ) variation was observed in co-infection prevalence between breeds of indigenous cattle examined. Comparisons of quantitative differences in the number of different strains among cattle from diverse agro-ecological zones strongly support the significant ( $p=0.01$ ) effect of the type of ecological zone on the number of tick-borne pathogens that circulate in cattle. The data also demonstrated that 145 cattle 36.5% (145/397), harboured single pathogen species. Out of the total number of single infected cattle, *Anaplasma marginale* alone constituted 36.3% (144/397). Only one cattle 0.3% (1/397) was infected with *Theileria species* alone. Notably no cattle were infected with *Babesia bigemina* pathogen alone at the time of the study. Importantly high progression of the number of multiple-pathogen infected cattle, over the duration of infection, at both the level of the individual host and the population was found, suggesting the lack of immunity against co-infection of cattle in the endemic region.

## CHAPTER ONE

### 1.0 INTRODUCTION

#### 1.1 Background

Tick-borne bacterial and protozoan diseases of animals are poorly controlled in sub-Saharan Africa (Walker and Koney, 1999; Bell-Sakyi *et al.*, 2004). This is largely due to lack of understanding of the infection biology of the pathogen species. Arguably, these organisms are the most ubiquitous parasites that have adapted to intracellular lifestyle within the terminal host species including domestic ruminants and wild games (Walker and Koney, 1999; Bell-Sakyi *et al.*, 2004). While they rely on the host for survival, it remains a paradox why these organisms harm the host, and cause diseases that often result in death of the terminal host. Notably, tick-transmitted pathogens are different in taxonomy, virulence and competitive capacity in diverse populations of microbes but they are known to demonstrate unique ability to coexist in the face of within-host competition (Carter *et al.*, 1995). This feature is common among all parasitic organisms, reflecting the tremendous capacity to establish multiple infections in one host. Previous studies have shown that infection with multiple pathogen species would have detrimental effects on livestock productivity in the context of disease outcome and effectiveness of control strategies (Read and Taylor, 2001; Ben-Ami *et al.*, 2008). This disease burden has been shown to have a disproportionate impact on resource-poor farmers, who incidentally own > 70% of domestic ruminants in Ghana, due to the high cost of prevention using acaricides / insecticides and treatment of sick animals (Bell-Sakyi *et al.*, 2004).

Although all the major breeds of cattle worldwide are susceptible to infection, the severity of disease is highest among high-producing immunologically-naïve temperate breeds such as Friesian, Holstein, and the cross-breeds than the low producing indigenous breeds (Nadelman *et al.*, 1997; Thompson *et al.*, 2001; Belongia, 2002). Specifically, the imported cattle, with low immunity to infectious tick-borne pathogens, are extremely susceptible to each of the globally-important livestock hemoparasites - *Anaplasma marginale*, *Babesia bigemina*, *Babesia bovis*, *Theileria parva*, *Theileria annulata*, *Cowdria* and *trypanosoma* often resulting in severe morbidity with mortality approaching 100% (Baldwin *et al.*, 1988; Glass *et al.*, 2005; Bock *et al.*, 2008).

The control of tick-borne diseases in the tropical region is particularly more challenging and costly due to the presence of multiple pathogens from the seven globally important livestock intracellular pathogens (Kocan, 1995; Frisch *et al.*, 2000; Graf *et al.*, 2004; de la Fuente and Kocan, 2006; Sonenshine *et al.*, 2006; Willadsen, 2006). This problem is exacerbated by the availability of competent vector ticks for each pathogen strain (Walker *et al.*, 2004), thus resulting in enhanced transmission efficiency and higher infection prevalence of disease among cattle from the tropical region than the temperate region. Several factors may account for differences in disease prevalence. It could be due to seasonal variations, differences in the length of the transmission cycle of the associated vector species based on agro-ecological conditions, abundance of pathogen strains, availability and susceptibility of the terminal mammalian host to infection (Bekele, 2002; Stephen *et al.*, 2006).

In the temperate region, for example, where the duration of a favorable climatic condition such as the warm season is short, many vector species can only afford to

complete a single round of reproduction (Bekele, 2002). Continuous truncation of the organism's life cycle will most likely alter the pathogen's overall ability to acquire the host resources-blood, cells and tissues essential for growth. Over time, the evolutionary pathway to survival, growth, capacity to reproduce and compete with other parasites within the host) will be reduced or completely lost (Johnson and Hoverman, 2012). This may explain why majority of cattle in the western hemisphere harbor only one pathogen species (Palmer *et al*, 2002). In most of the tropical regions, however, the life cycle of the vector ticks continues uninterrupted. Where tick transmission of the pathogen is continuous, the inter-host transfer of multiple pathogen strains is highly likely, consistent with the intermittent feeding habit of ticks (de Roode *et al.*, 2005). Consequently, susceptible animals in endemic areas are likely repeatedly challenged with multiple strains by tick feeding (Swanson *et al.*, 2006). In this case, multiple infection of the mammalian host by both closely-related and distantly-related pathogenic organisms is the norm rather than the exception in the endemic tropical regions worldwide (Barthold, 1999; Pal *et al.*, 2001).

Co-infection of tick-borne pathogens is the simultaneous infection of a host with more than one pathogen and it may occur through single or multiple tick species carrying multiple pathogens that can transmit more than one organism to the same animal, and through multiple tick species which simultaneously feed on a host and infect it with multiple pathogens resulting in the serial transmission of pathogens from multiple ticks at different times (Adelson *et al.*, 2004; Adam *et al.*, 2006).

Co-infection with multiple organisms increases the likelihood of disease in cattle and it is predicted to have a disproportionate impact on disease risk (Alekseev *et al.* 2001; Nyarko

*et al.*, 2006). The key questions to address are whether genetic differences among indigenous cattle breeds reflect quantitative differences in the prevalence of multiple infections and whether co-infection prevalence of cattle from the three major agro-ecological zones in Ghana is sufficiently large in the reservoir population to indicate a real difference in the herds from which they were selected. It is common knowledge that these organisms feed directly on the host resources to derive nutriment and energy essential for survival. As the availability of resources from the host is limited, simultaneous exploitation by different types of parasite species will be expected to result in fierce in-host antagonistic interactions which can cause detrimental effects and death of the host (Johnson and Hoverman, 2012).

Surprisingly, studies of infected cattle have shown that majority of the co-infected animals are immune to their individual load of pathogenic species (Mahoney *et al.*, 1981). Notably, not all infected animals in the population were co-infected and some of the cattle were even not infected. One of the strongest factors that influence the outcome of this interaction is immunity in the mammalian host population (Mahoney *et al.*, 1981). Whether the resulting adaptive immunity results in the reduction of co-infection remains uncertain. For animals that were infected, there is evidence of widespread persistent infection with different types of organisms. Within an endemic region, this long standing interaction between the host and the pathogen will result in increased selective pressure for pathogen diversification, emergence of variant strains and widespread prevalence of infection and additional layers of population immunity (Futse *et al.*, 2008). There is evidence that high prevalence of tick-transmitted pathogens results in over 80% of the

world's cattle becoming infected with one or more diseases, with the possibility of death if not treated (Mc Cosker, 1979 ; Guglielmone, 1995; Minjauw and de Castro, 2004). .

The global cost attributed to tick-borne diseases (TBDs), in terms of mortality, production losses including cost of veterinary diagnosis, treatment and tick control are in excess of \$14 billion per year (McCosker, 1979; Young *et al.*, 1988; Bell-Sakyi *et al.*, 2004; Minjauw and de Castro, 2004; Rubaire-Akiiki *et al.*, 2004; Okuthe and Buyu, 2006; Swai *et al.*, 2007; Jonsson *et al.*, 2008). Incidentally, organisms that are responsible for causing each of the deadly infections are known to be widely prevalent in Africa (Walker and Koney, 1999). This is particularly true for tick-borne diseases of cattle and small ruminants that represent the most important asset within the household as they provide high quality protein, draft power, fertilizer and cash income particularly in resource-poor countries in sub-Saharan Africa (Bell-Sakyi *et al.*, 2004). In Ghana, species identification by genotyping indicates widespread prevalence and wide diversity of pathogen strains in infected cattle, including, all the six most deadly haemoparasitic organisms of cattle (Walker and Koney, 1999; Bell-Sakyi *et al.*, 2004). Most importantly, there appears to be competent tick vectors that can acquire and transmit the different strains that arise in an endemic area (Walker *et al.*, 2004). However, not all cattle become infected with multiple pathogen strains and some cattle are not infected at all. Where cattle from one breed could resist co-infection with multiple organisms, while all the other animals are susceptible within the same population suggest genetic diversity as an important force mediating organism co-infection of the host (Nadelman *et al.*, 1997).

To address the importance of effect of breed on co-infection prevalence among cattle, this study investigated if there is evidence of difference in the number of co-infecting

organisms between five indigenous cattle that previously demonstrated significant variations in the prevalence of tick-transmitted *Anaplasma marginale* (Shaban, 2012). If genetic differences between cattle breeds underlie variability in cattle susceptibility to co-infection, then in herds with many resistant breeds, there will be high infection prevalence maintained through time by high transmission resulting in little episode of disease due to herd immunity. The distribution and abundance of multiple-infected cattle may also vary by agro-ecological zones (Shaban, 2012).

Tick life cycles are regulated by the geographic differences in Agro-ecological region due primarily to the constant shifts in environmental conditions – rainfall, temperature and humidity (Hugh-Jones, 1991; Chaput *et al.*, 2002; Furlanello *et al.*, 2003; Daniel *et al.*, 2004). Such shifts in the habitats suitable for particular tick species and the overall greater variation in tick populations are likely to result in alterations in pathogen prevalence, herd immunity and disease susceptibility (Nadelman *et al.*, 1997). In biological terms, incidence of co-infection is predicted to rise significantly in regions that provided suitable tick habitat. Understanding the species composition of pathogens of infected cattle in Ghana will allow for the formulation of cost effective interventions that will enable the development of applicable control strategies and improve the adaptive capacity of tick-borne diseases.

## **1.2 Problem Statement**

The control of tick-borne diseases of cattle and other ruminants in the tropical region is particularly more challenging and costly due to the presence of multiple pathogens. This

problem is exacerbated by the availability of competent vector ticks for each pathogen strain and susceptibility of the mammalian host. Specifically, the epidemiology of tick-borne diseases in domestic ruminants is complex due to the interaction between the tick, the pathogen and the mammalian host. One of the strongest factors that influence the outcome of this interaction is immunity in the mammalian host population. For animals that were infected, there is evidence that they have established a long-lasting persistent infection with different types of organisms. Where tick transmission of the pathogen is continuous, the inter-host transfer of multiple pathogen strains is highly likely, consistent with the intermittent feeding habit of ticks. Consequently, susceptible animals in endemic areas are likely repeatedly challenged with multiple strains by tick feeding. The key questions to address are whether genetic differences among indigenous cattle breeds may lead to quantitative differences in the prevalence of multiple infections and whether co-infection prevalence of cattle from the three major agro-ecological zones in Ghana is sufficiently large in the reservoir population to indicate a real difference in the herds from which they were selected.

### **1.3 Justification**

Tick-borne diseases are production-limiting but, surprisingly, these diseases are rather poorly controlled in sub-Saharan Africa largely due to lack of understanding of the potential effects of multiple pathogen infection of livestock on productivity, disease outcome and effectiveness of control strategies.

In the tropical and sub-tropical regions, households that rely heavily on animal husbandry for their livelihood spend substantial amounts of their resources on ticks and tick-borne disease control. Tick-borne diseases are a major threat to livestock production, rendering farmers poor due to huge losses of livestock and expenses from veterinary diagnosis and treatment (McCosker, 1979; Young *et al.*, 1988; Minjauw and de Castro, 2004; Jonsson *et al.*, 2008). It is estimated that 40% of the livestock produced in Ghana is lost to disease annually (Aryee *et al.*, 1991).

The global cost attributed to TBDs, in terms of mortality, production losses including cost of veterinary diagnosis, treatment and tick control is in excess of US\$14 billion per year (McCosker, 1979; Young *et al.*, 1988; Minjauw and de Castro, 2004; Jonsson *et al.*, 2008). Incidentally, organisms that are responsible for causing each of the deadly infections are known to be widely prevalent in Ghana and most of the agro-ecological regions in Africa (Bell-Sakyi *et al.*, 2004; Rubaire-Akiiki *et al.*, 2004; Okuthe and Buyu, 2006; Swai *et al.*, 2007).

The level of susceptibility to co-infection in the indigenous cattle breeds to tick-borne diseases is not well known in Ghana and the sub-region. However, the overall impact of harbouring multiple infected individual cattle has dire immunologic implications from the standpoint of disease transmission within susceptible cattle populations. As tick-borne pathogens are production-limiting, high prevalence of co-infection will increase the cost of production and discourage potential investors from funding livestock agriculture. It will also have enormous impact on the development of livestock agriculture in that it increases the virulence of tick-borne pathogens, affects the therapeutic strategies for disease control or complicates disease diagnosis and treatment (Nadelman *et al.*, 1997).

Most importantly, it stymies all the efforts to develop vaccine by rendering vaccines ineffective. This is particularly true because the multi-pathogen complex situation mandates that vaccine production be directed at specific or single pathogen rather than a complex pathogen entity. This requirement exerts unusually high budgetary constraints approaching a budget for developing vaccine against the mosquito-vectored plasmodium parasite in humans (Susannah, 2008).

Understanding the co-infection status of the indigenous cattle breeds will inform the farmers and breeders to effectively select the genetically most suitable cattle for breeding purposes. It will also provide scientists and researchers guidance to develop effective control strategy against tick-borne diseases in the Sub-region and to develop multivalent or polyvalent vaccine against the co-infected tick-borne pathogens. This would also provide baseline data to guide vaccine development relevant for implementation.

#### **1.4 Hypothesis**

The co-infection of indigenous cattle breeds by different pathogen strains varies between breeds and location and increases over the duration of infection.

#### **1.5 Main objective**

To evaluate the degree of co-infection prevalence in indigenous cattle and assess whether genetic differences among indigenous cattle breeds from three major agro-ecological zones may lead to quantitative differences in the prevalence of multiple infections.

### **1.5.1 Specific objectives**

1. To determine if there are quantitative differences in co-infection among indigenous cattle breeds and location.
2. To determine if co-infection increases with duration of infection.

## CHAPTER TWO

### 2.0 LITERATURE REVIEW

#### 2.1 Importance of ruminant livestock in Ghana

Agriculture drives Ghana's economy, employing over 40% of its working population (MOFA, 1990). The livestock sub-sector is an important component of agriculture in Ghana and is a pivotal link in the Ghanaian farming and livelihood systems even though its contribution to the agricultural gross domestic product has been hovering around 7% (MTADP, 1991).

Prominent among the numerous contributions the livestock sub-sector makes to the economy of the country is food security and provision of animal protein to enhance the nutritional adequacy in the diets of the people (MOFA, 1990). Additionally, the livestock sub-sector provides employment opportunities for a large part of the population, particularly, in the rural areas and offers considerable prospects for wealth generation, income enhancement and improvement in rural livelihoods (MOFA, 1990).

The livestock industry in Ghana is largely composed of small-scale enterprises. People living in the rural areas of Ghana are smallholder livestock farmers raising chickens, guinea fowls, cattle, sheep, goats, pigs and donkeys (Baiden and Duncan, 2008). These farmers depend on their livestock not only as an important food source for their families, but also as a means of generating income (Baiden and Duncan, 2008).

## **2.2 Indigenous cattle breeds present in Ghana**

The indigenous cattle breeds present in Ghana are Ghana Shorthorn, also called West African Shorthorn, Sanga (which is a cross between the Ghana Shorthorn and White Fulani), Zebu, N'Dama, Sokoto Gudali, White Fulani, and Muturu (GVS/MoFA, 1975; Aboagye, 2002). They are widely distributed in the country (GVS/MoFA, 1975). As at 1975, the indigenous cattle breeds in Ghana were found in order of numbers and percentages as follow: Ghana Shorthorn (616 200; 79%), Sanga (122 300; 16%), Zebu (19 400; 2.6%), N'Dama (16 600; 2.1%), others (2 500; 0.3%), respectively (GVS/MoFA, 1975). Along the Northwestern border around Bole, Wa, Lawra and Tumu, there is a high concentration of relatively pure Ghana Shorthorns (GVS/MoFA, 1975).

The indigenous cattle breeds are hardy and can withstand the harsh weather condition of drought, high ambient temperature, shortage of feed. In addition, the local breeds are also known to be resistant to infectious diseases, including the most devastating haemoparasitic diseases transmitted by arthropod vectors (Bell-Sakyi *et al.*, 2004; Nadelman *et al.*, 1997).

## **2.3 Susceptibility and resistance of indigenous cattle breeds to vector-borne diseases**

The indigenous cattle breeds are known to be less susceptible to tick-borne pathogens and tsetse-borne infection than the exotic cattle breeds probably due to early exposure to tick-borne diseases in life (Taylor, 1998). They do not develop clinical signs of the disease and are subsequently proposed to be immune to subsequent infection (Taylor, 1998).

Over time a state of balanced pathogenicity evolves where the pathogen becomes less virulent while simultaneously the host becomes less susceptible (Latif, 1992). A typical example of this phenomenon is demonstrated by the N'dama cattle. N'dama is trypanotolerant (Olutogun, 1976; Adeniji, 1985) while Ghana Shorthorn cattle breed is known to be tolerant to trypanosomiasis, tsetse-borne disease, and dermatophilosis (Ngere *et al.*, 1975; Olutogun, 1976; Adeniji, 1985).

The Muturu cattle breed is tolerant to tsetse flies, tick-borne diseases and Trypanosomiasis and it has the ability to withstand mixed infections and it is also extremely susceptible to Rinderpest (Ferguson, 1967; Rouse, 1970; Fricke, 1979; ILCA, 1979; Adeniji, 1985).

Ghana Sanga is known to be tolerant to trypanosomiasis and dermatophilosis than N'dama and Muturu when they are reared in the same locality (Ngere *et al.*, 1975; Olutogun, 1976; Adeniji, 1985). White Fulani is tolerant to Trypanosomiasis and Dermatophilosis, similar to Gudali (Ngere *et al.*, 1975; Olutogun, 1976; Adeniji, 1985).

It is evident that some animals or breeds of animals continuously harbor fewer ticks than other animals kept in the same environment (Roberts, 1968a; Wagland, 1975). This distinction suggests a primary role of genetic variation in the ability of the animal to immunologically respond to tick infestation using either the innate mechanism or adaptive immunity (Roberts, 1968b; Hewetson, 1972; Seifert, 1984; Latif, 1992) and others are acquired (Riek, 1962; Roberts, 1968a).

It has also been shown that stress due to lactation or sickness causes decline in resistance to disease (Wharton *et al.*, 1970; Seifert, 1971; Utech *et al.*, 1978). Zebu cattle and their

crosses have elevated levels of resistance to tick infestation and tick-borne diseases as compared with other breeds of cattle (Riek, 1962; Wilkinson, 1962; Wharton *et al.*, 1969; Seifert, 1971; Hewetson, 1979; Trail and Gregory, 1981; Saeed *et al.*, 1987).

## **2.4 Tick-borne pathogens of cattle**

The tick-borne pathogens are responsible for the numerous haemoparasitic diseases of health and economic importance in the tropical and subtropical regions (Jongejan and Uilenberg, 1994). In Ghana, the major ticks of cattle include *Boophilus spp.*, *Hyalomma spp.*, *Amblyomma spp.*, and *Rhipicephalus spp.* (Bell-Sakyi *et al.*, 2004). *Boophilus spp.* transmits *Babesia spp.* (Babesiosis) and *Anaplasma marginale* (Anaplasmosis) which is the globally prevalent of the economically important haemoparasites (Bock *et al.*, 2008). It affects the exotic cattle breeds including cattle from Latin America, Oceania and Asia, Africa and the East (Bock *et al.*, 2008). *Hyalomma spp.* transmits *Theileria annulata* (Tropical Theileriosis) found in northern Africa, southern Europe, East and West Asia (OIE, 2009). *Amblyomma variegatum* transmits *Ehrlichia ruminantium*, the causative agent for *cowdria* (also commonly known as heartwater disease) (Barre' and Uilenberg, 2010). Cattle, sheep and goats are susceptible to heartwater disease and there is evidence that *Ehrlichia ruminantium* organisms preferably infect cattle, sheep and goats and are known to be present in Sub-Saharan Africa and the Caribbean (Ewing, 1981; Figueroa *et al.*, 1998). *Rhipicephalus spp.* transmits *Theileria parva*, East Coast fever (ECF), also known as Corridor Disease or January Disease (OIE, 2009).

### 2.4.1 Bovine Anaplasmosis

Bovine anaplasmosis is an arthropod-borne hemolytic disease of cattle that is caused by the rickettsia *Anaplasma marginale* (*Rickettsiales: Anaplasmataceae*) (Schmidt, 1937; Bram, 1975; Kocan *et al.*, 2000; Dumler *et al.*, 2001). The disease is endemic in tropical and subtropical areas of the world and causes considerable economic loss to both the dairy and beef cattle worldwide (Kocan *et al.*, 2003). Taxonomically, *Anaplasma* belongs to family *Anaplasmataceae*, Order *Rickettsiales* and now consists of four genera: *Anaplasma*, *Ehrlichia*, *Neorickettsia*, and *Wolbachia* (Dumler *et al.*, 2001).

Outbreaks of bovine anaplasmosis are usually attributable to infection with the highly virulent *Anaplasma marginale* strain (Bock *et al.*, 2008). However, *Anaplasma centrale* infection is usually sub-clinical producing a moderate degree of anaemia, but clinical outbreaks are extremely rare (Kocan *et al.*, 2003). *Anaplasma phagocytophilum*, which utilizes rodent as the primary reservoir, has been reported to infect cattle, but does not cause clinical disease (Hofmann-Lehmann *et al.*, 2004; Dreher *et al.*, 2005). The clinical disease however is most notable in cattle, but other ruminants including water buffalo, bison, African antelopes, and mule deer can become persistently infected with *Anaplasma marginale* (Kuttler, 1984). Sir Arnold Theiler (1910) first described *Anaplasma marginale* infection in erythrocytes of South African cattle as “marginal points”.

Morphologically, the body of *Anaplasma marginale* is located near the edge of the corpuscle (Salmon and Smith, 1896), whereas *Anaplasma centrale*, which appeared to be less pathogenic are found in the centre of erythrocytes rather than in a marginal location (Theiler, 1911).

#### **2.4.1.1 Signs of *Anaplasma marginale* infection**

Erythrocytes are the only known site of infection of *A. marginale* in cattle (Jonsson, 2006). Within these cells the membrane-bound inclusions also known as initial bodies contain four to eight rickettsias with 70% or more of the erythrocytes becoming infected during acute infection (Ristic *et al.*, 1968; Potgieter and Van Rensburg, 1982).

*Anaplasma marginale* causes severe anaemia during acute stage of the disease, and it is characterized by fever, weight loss, abortion, lethargy, icterus and often death in animals older than two years (Ristic, 1977). *Anaplasma* species are transmitted either mechanically or biologically by arthropod vectors (Kocan *et al.*, 2004). The mechanical transmission is by biting flies or blood-contaminated fomites while biological transmission is by ticks (Dikmans, 1950; Ewing, 1981; Kocan, 1986). Other mechanical transmission frequently occurs via blood-contaminated fomites, including needles, dehorning saws, nose tongs, tattooing instruments, ear-tagging devices, and castration instruments (Dikmans, 1950; Ewing, 1981; Kocan, 1986). Mechanical transmission by arthropods has been reported for blood sucking diptera of the genera *Tabanus*, *Stomoxys*, and mosquitoes (Ewing, 1981; Potgieter *et al.*, 1981; Foil, 1989).

This form of mechanical transmission is considered to be the major route of dissemination of *Anaplasma marginale* in areas of Central and South America and Africa where tick vectors are not highly prevalent (Ewing, 1981; Foil, 1989; Figueroa *et al.*, 1998; Coronado, 2001).

Biological transmission of *Anaplasma marginale* is mediated by feeding ticks (Radunz and Darwin, 2008). Approximately 20 species of ticks have been incriminated as vector-

borne disease worldwide (Dikmans, 1950; Ewing, 1981). Tick transmission can occur from stage to stage (transstadial) or within a stage (intrastadial), while transovarial transmission from one tick generation to the next does not appear to occur (Stich, *et al.*, 1989).

Incubation period of infection (pre-patent period) varies with the number of organisms in the infective dose and ranges from 7 to 60 days, with an average of 28 days (Ristic, 1977).

#### **2.4.1.2 Control, prevention and treatment of *Anaplasma marginale* infection**

Tetracycline antibiotics drugs (tetracycline, chlortetracycline, oxytetracycline, rolitetracycline, doxycycline and minocycline) and imidocarb are used for treatment of *Anaplasma marginale* (MVM, 2011). Anaplasmosis immunization, using either killed vaccine, attenuated live vaccine, or live *Anaplasma centrale* vaccine, has been practiced with varying results in many parts of the world (FAO, 1975) including Australia, South Africa and Israel.

#### **2.4.2 Bovine Babesiosis**

Bovine babesiosis is a tick-borne disease of cattle caused by protozoan of the genus *Babesia*, order Piroplasmida, phylum Apicomplexa with *Babesia* as the principal species that causes Bovine babesiosis (*Babesia bovis* and *Babesia bigemina*) in cattle (OIE, 2009). Bovine babesiosis is vectored by one-host *Rhipicephalus (Boophilus) microplus* tick, and it is widespread in tropical and subtropical regions i.e. Africa, Asia, Australia,

Central, South America, and in some parts of Europe (Friedhoff *et al.*, 1981; Young, 1988; Bock *et al.*, 2008).

The eradication of Bovine Babesiosis has been accomplished by elimination of tick vector in non-endemic areas and in endemic regions eradication of tick is not feasible or desirable; ticks are controlled by repellents and acaricides (Graf *et al.*, 2004; de la Fuente *et al.*, 2007). Reduce the exposure of cattle to tick and regular inspection of animals and premises. Cattle develop a durable, long-lasting immunity after a single infection with *B. bovis*, *B. divergens* or *B. bigemina*, a feature that has been exploited in some countries to immunize cattle against Babesiosis (OIE, 2009).

*Imidocarb (Imizol)* and the allied drug *amicarbalide* are effective babesiocides for cattle at the dose rate of 1-3 mg/kg and 5-10 mg/kg body weight respectively (Todorovic *et al.*, 1973; Kuttler *et al.*, 1975)

#### **2.4.2.1 *Babesia bovis***

*Babesia bovis* is a highly pathogenic tick-borne disease of cattle characterized by high fever, ataxia, anorexia, general circulatory shock and sometimes also nervous signs as a result of sequestration of infected erythrocytes in cerebral capillaries (Zintl *et al.*, 2003). Anaemia and haemoglobinuria may appear later in the course of the disease. In acute cases, the maximum parasitaemia in circulating blood is less than 1% (Zintl *et al.*, 2003). This is in contrast to *Babesia bigemina* infections, where the parasitaemia often exceeds 10% and may be as high as 30% (OIE, 2009).

Infected animals develop a life-long immunity against reinfection with the same species (Mahoney *et al.*, 1981). There is also evidence of a degree of cross-protection in *B. bigemina* immune animals against subsequent *Babesia bovis* infections. Calves rarely show clinical signs of disease after infection regardless of the *Babesia species* involved or the immune status of calves (Zintl *et al.*, 2003; Bock *et al.*, 2008).

#### **2.4.2.2 *Babesia bigemina***

*Babesia bigemina* is a non-pathogenic tick-borne disease of cattle (Walker and Koney, 1999). It has two-host life-cycle involving a tick and a mammal. In the mammalian host the organisms reproduces asexually (Callow, 1984). It is transmitted by *Boophilus species* as tick vectors. *B. bigemina* is distributed wherever *Boophilus* ticks are encountered, which includes North and South America, Southern Europe, Africa, Asia and Australia (Callow *et al.*, 1993).

##### **2.4.2.2.1 Signs of *Babesia bigemina* infection**

Cattle infected with *Babesia bigemina* may show signs of fever, haemoglobinuria and anaemia, production of dark red or brown-colored urine, nervous signs, minimal or non-existent as intravascular sequestration of infected erythrocytes does not occur and parasitaemia often exceeds 10% and may be as high as 30% depending on the genetic status of the animal (OIE, 2009).

#### **2.4.2.2.2 Control, prevention and treatment of *Babesia bigemina* infection**

*Babesia bigemina* is controlled by destroying ticks on cattle, pasture, and manual removal of ticks in individual cattle by picking, frequent combing or brushing, acaricide application using sprinkler or hand spray in small ranches while in large ranches acaricide is best applied by dipping or using a mechanical sprayer that saturates all of the animal's skin (Henning, 1956). Berenil is injected intramuscularly in a 7% solution at the rate of 3.5mg/ kg of body weight to treat sick animals in Ghana (Personal experience).

#### **2.4.3 Heartwater**

Heartwater disease, also known as Cowdriosis, is an infectious, non-contagious, rickettsial disease of domestic and wild ruminants (Walker and Koney, 1999; Bell-Sakyi *et al.*, 2004). It is vectored by *Amblyomma variegatum* (Walker and Koney, 1999; Bell-Sakyi *et al.*, 2004). This three-host tick becomes infected during either the larval or nymphal stages and transmits the infection during one of the subsequent stages (transstadial transmission) to mammals including cattle, sheep, goats, antelope and buffalo (Callow, 1984).

The disease has the biggest economic impact on cattle production in affected areas (Uilenberg, 1981; MVM, 2012). The cowdria organism is an obligate intracellular Gram-negative coccal-shaped bacterium parasite of cattle and other small ruminants (MVM, 2012). This disease is common in sub-Saharan Africa and some of the West Indian islands and was first identified in sheep in South Africa in the 1830s. In Ghana, infection is more severe in sheep and goats than in cattle because sheep and goats

compared to cattle have a small body index with corresponding number of erythrocytes which transport oxygen to cells and tissue (Henning, 1956).

#### **2.4.3.1 Signs of *Heartwater* infection**

The typical clinical signs of heartwater include pyrexia, fever, anaemia, incoordination or convulsion, hydrothorax, oedema of lungs and brain, jaundice, drop in milk production, abortion, depression, weakness and difficult breathing with rapid and shallow breaths and increased heart rate (OIE, 2009). The disease is seen primarily in exotic cattle, consistent with evidence that indigenous cattle have acquired considerable resistance through natural selection over centuries of co-adaptation between cowdria and local terminal mammalian host (Uilenberg, 1981).

#### **2.4.3.2 Control, prevention and treatment of *Heartwater* infection**

Heartwater disease in animals may be treated with sulphonamides and tetracyclines during early stage of the disease (MVM, 2011). Tetracyclines drug is used prophylactically when animals are introduced into an endemic area with Cowdriosis (Walker and Koney, 1999). Ectoparasiticides dips can be used to reduce exposure of animals to ticks (Dumler *et al.*, 2001).

#### **2.4.4 Theileriosis**

The Theileriosis is a complex disease caused by tick-borne apicomplexan parasites of the genus *Theileria* (MVM, 2011). Theileriosis in cattle in Africa has been considered to have more impact on the development of the beef and dairy industries, and on veterinary

infrastructure legislation, policies and on research in Africa than any other livestock disease complex (McCosker, 1979).

Tropical Theileriosis caused by *T. annulata* and *T. parva* have a far more extensive distribution in tropical and Sub-tropical region globally, including much of North Africa (Norval *et al.*, 1992) and as many as 200 million cattle are considered at risk worldwide (Purnell, 1978).

*Theileria mutans* affect cattle and Cape buffalo and it is widespread in Sub-Sahara Africa, where the distribution follows that of its tick vectors (Walker and Olwage, 1987), at least five species of Amblyoma tick (Norval *et al.*, 1992). As many as 200 million susceptible cattle are considered at risk worldwide (Purnell, 1978). Compared to *T. parva*, and *T.annulata*, *T.mutans* is generally regarded as only mildly pathogenic to cattle, although there are records of *T. mutans* being pathogenic and fatal to cattle in East Africa, and anaemia can become severe in case of infection with pathogenic strains (Brown *et al.*, 1990; Bell-Sakyi *et al.*, 2004). *T. parva* and *T. annulata* have not been reported to occur in the West African sub-region and in Ghana (Bell- Sakyi *et al.*, 2004). *Theileria species* prevalent in Ghana are *T. mutan* and *T. velifera* which is transmitted by *Amblyoma variegatum* and are normally non-virulent (Walker and Koney, 1999).

#### **2.4.4.1 Signs of Theileriosis infection**

Cattle infected with Theileriosis shows signs of fever, anaemia, jaundice, decreased milk production, abortion, depression, weakness and difficulty in breathing with rapid and shallow breaths and increased heart rate and less commonly seen clinical signs such as

excessive salivation, diarrhoea, constipation, swelling of lymph nodes, and brown urine (Brendan, 2013).

#### **2.4.4.2 Control, prevention and treatment of Theileriosis**

No practical therapy exists for the treatment of Theileriosis, however there are tick control strategies such as the frequent spraying or dipping of cattle using acaricide at intervals less than seven days depending on the vector species and its biology in different geographical areas and the immunization of cattle against Theileriosis (FAO, 1975).

### **2.5 Tick control measures**

Several tick control measures have been put in place to control the infection and transmission of tick-borne pathogens. The typical approach includes the use of naturally-resistant animal against ticks, use of pheromone impregnated decoys for attracting and killing ticks, use of vaccines, and genetic technique and integrated pest management (Kocan, 1995). The genetic technique involves the introduction of an isolate anti-tick gene into breeds of cattle with the intention to producing cattle with total tick resistance (Kocan, 1995; Frisch *et al.*, 2000; Graf *et al.*, 2004; de la Fuente and Kocan, 2006; Sonenshine *et al.*, 2006; Willadsen, 2006).

In Ghana, acaricide is commonly used for tick control. However, it is expensive and difficult to dispose off, and has limited effects on tick infestations (Walker and Koney, 1999). Some ticks may become resistant to acaricide due to excessive usage and also tends to pollute the environment if disposed of indiscriminately (Graf *et al.*, 2004; de la

Fuente *et al.*, 2007). Also is the contamination of milk and meat products with drug residues (Willadsen and Kemp, 1988; Nolan, 1990; Graf *et al.*, 2004).

The anti-tick vaccines show more promise for controlling ticks in the future (Kocan, 1995). Allen and Humphreys (1979) demonstrated that it is possible to control tick infestations through immunization of hosts with selected tick antigens. Of all the control measures established and in-use in the world, it is proven that vaccination is the most effective means of controlling ticks and tick-borne infections because it reduces intervention cost burden on farmers and reduces over reliance on acaricide (Graf *et al.*, 2004; de la Fuente *et al.*, 2007).

## **2.6 Impact of Pathogen on Host cattle**

The impact of pathogen on host populations depend on several factors, including pathogen virulence and the reduction in host fitness (survival or reproduction) caused by the pathogen (Anderson and May, 1981) The size of the impact also depends on whether the pathogen reduces host survival and reproduction (Torchin *et al.*, 2003). In general, for pathogens that lower host survival, those with intermediate virulence tend to have the largest negative impacts on host populations. This is because hosts infected with highly virulent pathogens tend to die quickly, thus cutting short the infectious period of parasite transmission (Anderson and May, 1981). Pathogens that have density-dependent transmission can regulate host populations (i.e., reduce and hold them to a lower density than without the pathogen present) in the absence of any other density-dependent factors influencing host abundance (Torchin *et al.*, 2003). This requires that the mortality rate

from the pathogen be greater than a value that depends on a combination of host survival, reproduction, recovery, and loss of immunity (Anderson and May, 1979).

One consequence of the reduced fitness caused by pathogens is that host species that can escape infection of a pathogen by immigrating to a new region may have significantly higher population growth rates and invasion potential (Torchin *et al.*, 2003). Introduced host species can also bring and introduce pathogens with them to new areas, with sometimes devastating effects on native host species (Bradley *et al.*, 2008). Pathogens can also impact host species interactions in other ways that increase host community diversity, including preventing competitive exclusion and altering predation pressure (Bradley *et al.*, 2008).

## **2.7 Susceptibility to co-infection with tick-borne pathogens**

Animals in endemic region are susceptible to co-infection with tick-borne pathogens (Kordick *et al.*, 1999). This observation is consistent with diverse clinical signs in some animals (Kordick *et al.*, 1999; Stephen *et al.*, 2006). A number of reports also describe co-infections in animals (Breitschwerdt *et al.*, 1998; Kordick *et al.*, 1999; Suksawat *et al.*, 2001; Tuttle *et al.*, 2003; Mylonakis *et al.*, 2004). Susceptibility to co-infection may be affected by several factors including geographic location, travel, husbandry, host immune status, use of preventive drugs and prophylactic therapy (Adam *et al.*, 2006).

Geographic location has a tremendous influence on the susceptibility to co-infection with multiple tick-borne pathogens, in that when host cattle or animal is moved to a non-endemic zone from an endemic area for slaughter, sale or for breeding purposes, it has the

ability to transmit the co-infected pathogens to another susceptible animal when bitten by a co-infected tick or ticks (Stephen *et al.*, 2006). Ticks tend to flourish more in geographic location or ecological areas with warm, humid climates, as they require certain amount of moisture in the air to undergo metamorphosis, and because low temperatures inhibit their development from egg to larva (Nuttall, 1905). Especially ticks of domestic animals are common and vary in ecological areas, where they cause considerable harm to livestock by transmission of many species of pathogens and also causing direct parasitic damage (de Castro, 1997). For an ecosystem to support ticks, it must satisfy two requirements: the population density of host species in the area must be high enough, and humidity must be high enough for ticks to remain hydrated (Wall and Shearer, 2001).

Depending on the system of production, cattle may be herded out in the early hours of the day for grazing and returned in the evening (semi-intensive system) or cattle may be kept for long period of years for breeding and research (State Research Institutions) purposes (Baiden and Duncan, 2008). These husbandry practices enormously affect the susceptibility to co-infection, in that animals tend to accumulate tick-borne pathogens over time (Adam *et al.*, 2006).

The immune status is an important innate and adaptive defense mechanism which determines the susceptibility or otherwise of a host to co-infection (Mahoney, 1969). This is however influenced by the genetic adaptation of the host, i.e. the exotic breeds (Holstein, Friesian) and the crosses between them and the indigenous cattle breeds being susceptible to co-infection (Mahoney, 1969).

An animal may become susceptible to co-infection due to excessive usage of preventive drugs like antibiotics, leading the pathogen to evolve over time (antigenic variation) and becoming resistant to the preventive drug and the host to become persistently infected (Adam *et al.*, 2006).

Ticks may become co-infected with pathogens while consuming a single blood meal containing multiple pathogens, or by transfer of pathogens between co-feeding ticks (Piesman and Happ, 2001). It is less likely that ticks become infected by a diversity of pathogens from sequential feeding on multiple animals given that the hard tick life cycle includes feeding on three blood meals (a practice known as hematophagy) well-separated in time (Adelson *et al.*, 2004). Ticks will initially become attracted to the host through breath, body odors, sensing body heat, moisture and vibrations of the host (CDC, 2010). Ticks upon climbing onto the host would then inflict wound on the host by piercing the epidermis where they insert their hypostome and excrete into the host an anesthetic property and an anticoagulant capable of preventing blood from clotting (Goddard, 2008). Since blood is an indispensable nutritional requirement for ticks, when they are unable to find a host to feed they will die (CDC, 2010).

Co-infections of vertebrates by tick-borne pathogens could easily result from sequential and independent tick bites given that a large animal host could have a tick burden in the hundreds or thousands (Ginsberg, 2008). Moreover, hosts may be bitten by ticks co-infected with multiple pathogens. Co-infection may direct the host immune response, induce immune suppression and impair antigen presentation (Pappalardo *et al.*, 1997). The immunologic changes caused by the tick-borne pathogens may increase the risk of

infection with other pathogens that includes tick-borne organisms and co-infection may have the greatest impact on the host than do single infection (Zeidner *et al.*, 2000).

## 2.8 Climate of Ghana

Ghana is a Coastal West African country bounded in the south by the Gulf of Guinea with Greenwich meridian running through. The climate of Ghana is influenced by dry, hot and dusty-laden air mass that moves from the northeast across the Sahara and by the tropical maritime air mass that moves from the south-west across the southern Atlantic Ocean (Benneh *et al.*, 1990). The climate varies from bimodal rainfall equatorial type in the south to the tropical unimodal monsoon type in the north (Dickson and Benneh, 1988; Benneh *et al.*, 1990).

The mean monthly temperature across the country is always above 25°C, as a result of the low latitude areas of Ghana. The average mean annual temperature is 27°C with the maximum approaching 40°C; especially in the north, and the minimum descending to about 15°C (Dickson and Benneh, 1988; Benneh *et al.*, 1990).

In the coastal areas, the sea breeze influences the annual range of temperature between 5°C and 6°C, while in the interior, the ranges are higher, and its influence is about 7°C to 9°C (Dickson and Benneh, 1988; Benneh *et al.*, 1990).

The rainfall decreases from the south to the north. The highly wet area in the country is the extreme southwest where the rainfall is over 2,000 mm per annum while in the extreme north, the annual rainfall is less than 1,100 mm. The driest area in the country is

the south-eastern coastal tip where the rainfall is about 750 mm. The annual mean relative humidity is about 80 percent in the south and 44 percent in the north (Dickson and Benneh, 1988; Benneh *et al.*, 1990).

### **2.8.1 Influence of climate on ticks and tick-borne pathogens**

Climate change affects the preferred habitat of ticks; therefore increase in maximum temperatures, increases the frequency and distribution of ticks (Sutherst and Maywald, 1985; Kitron *et al.*, 1997). A shift in climatic conditions, especially temperature and humidity most likely will alter the geographic distribution of ideal and marginal habitat (Estrada-Penna, 2003). The hotter the climate, the more prevalent are the tick-borne hemoparasitic diseases (Sedaghat *et al.*, 2013).

Increased temperatures, decreases the percentage of relative humidity and accelerates the development and shorten generation times, and as a result may lead to higher tick populations (Sutherst and Maywald, 1985; Kitron *et al.*, 1997). Since temperature determines rates of invertebrate development, reproduction and mortality, a decrease in temperature could accelerate the rates of population increase, especially in areas where these are currently limited by high temperatures (Nicholson and Mather, 1996; Kitron *et al.*, 1997).

A variety of biotic and abiotic environmental factors influences vector populations, a few parameter changes such as minimum and maximum temperature and rainfall, when considered together, are predictive of pathogen prevalence in the terminal host species (Hugh-Jones, 1991; Chaput *et al.*, 2002; Furlanello *et al.*, 2003; Daniel *et al.*, 2004).

The distribution and abundance of tick species in Ghana vary by vegetative zone (Walker and Koney, 1999; Ntiamoah-Baidu *et al.*, 2004). Differences in population density of tick in vegetative zones influences the epidemiology of tick-borne disease pathogens they transmit to cattle. Variations in the environmental conditions may further alter the geographical ranges of ticks (Sedaghat *et al.*, 2013).

In biological terms, the lifecycle of the vector tick are largely temperature-dependent and when not attached to the host, they are extremely sensitive to desiccation (Randolph, 2009), and the death of tick vector will undoubtedly reduce the likelihood of pathogen transmission. As successful pathogen-vector-host interaction is central to multiple infections of cattle, diminution of the reproductive cycle will result in corresponding reduction of transmission to the host (Randolph, 2009).

## CHAPTER THREE

### 3.0 MATERIALS AND METHODS

#### 3.1 Study sites with geo-coordinates

The study was carried out in three ecological zones in Ghana, namely the Guinea Savannah (Northern zone), Transitional (Middle zone), and the Coastal Savannah (Southern zone) from August, 2011 to July, 2013. Sampling of cattle was carried out at Animal Research Institute, Livestock and Poultry Research Centre and private farms (Juapong, Oyarifa, Obeyeyie, Shai Hills, Kasoa, Sunyani, Ejura, Beposo, Jetiase, Langugo, Pong-Tamale, Nyankpala, Zuro, and Masaka) located in three agro-ecological zones in Ghana (Table 1).

The Guinea Savannah (Northern) zone is characterized by continuous grass cover interspersed generally with fire-resistant, deciduous, broad-leaf trees showing a varying completeness of canopy. The minimum and maximum temperatures during the time of study are 23°C and 35.05 °C respectively. It also registered 3mm minimum and 82 mm annual mean rainfall with 45 % annual mean relative humidity (GMA, 2012).

The Transitional (Middle) zone is characterized by degraded vegetation forest with a wide range of tall grasses. During the period of study, it recorded 21.8 °C minimum and 31.7 °C maximum annual mean temperature, 4 mm minimum and 93.5 mm maximum annual mean rainfall with 62 % mean annual relative humidity (GMA, 2012).

The Coastal Savannah (Southern zone) is characterized by thicket grassland which consists of a dense scrub with relatively few trees with 24.2°C minimum and 31.9 °C

maximum annual mean temperature. The Coastal Savannah during the period recorded minimum rainfall of 6.5 and maximum rainfall of 142 mm. The relative humidity over the period of study (August, 2011 to July, 2013) was 76%.

The study sites were chosen based on the history of wide diversity of haemoparasitic organisms and the associated tick species coupled with availability of major breeds of indigenous cattle.

**Table 1: Study sites with geo-coordinates**

| <b>Agro-ecological zone</b> | <b>Number of cattle tested</b> | <b>Geo-co-ordinates</b>                 |
|-----------------------------|--------------------------------|---|
| Guinea Savannah             | 92                             | 9 <sup>0</sup> 26'N 0 <sup>0</sup> 49'W |
| Transitional                | 69                             | 6 <sup>0</sup> 45'N 1 <sup>0</sup> 37'W |
| Coastal Savannah            | 236                            | 6 <sup>0</sup> 15'N 0 <sup>0</sup> 10'E |
| <b>Total</b>                | <b>397</b>                     |   |

### **3.2 Animals and pathogen species**

The indigenous cattle from five distinct breeds [Sanga, White Fulani, Gudali and West African Shorthorn (WASH)] were selected. All sexes and age groups were randomly selected and examined. These indigenous cattle breeds were sampled from the University of Ghana Livestock and Poultry Research Centre at Legon, Animal Research Institute, Frafraha, where stringent management and health care is provided, and privately owned cattle farms spanning the breadth of the cattle species geographic distribution in Ghana from the South to the North (Table 1). These cattle are present in the three ecological

zones visited and are the mostly preferred breeds by farmers for rearing due to their resistant nature. The current study was focused on three of the most virulent of the haemoparasitic diseases of livestock - *Babesia*, *Theileria* and *Anaplasma* species. All these organisms are common in tropical and subtropical regions throughout the world and exhibit epidemiologic features similar to other tick-borne pathogens of cattle – fever, anaemia, reduced production and in some cases abortion and death.

### **3.3 Blood sampling, and genomic DNA extraction**

Five milliliters of peripheral blood samples were collected by jugular venipuncture from three hundred and ninety seven (397) indigenous cattle over a period spanning August, 2011 to July, 2013. The blood was transferred into 5ml EDTA-coated vacutainer tubes to prevent coagulation and samples were transported on wet ice to the Biotechnology Centre of the University of Ghana for genomic DNA extraction (Table 2).

**Table 2: Blood sampling from breeds of cattle**

| Agro-ecological zone | Breed of cattle | Number of cattle<br>sampled | Total      |
|----------------------|-----------------|-----------------------------|------------|
| Guinea Savannah      | Sanga           | 23                          | 92         |
|                      | White Fulani    | 20                          |            |
|                      | Gudali          | 23                          |            |
|                      | WASH            | 26                          |            |
| Transitional         | Sanga           | 17                          | 69         |
|                      | White Fulani    | 18                          |            |
|                      | Gudali          | 19                          |            |
|                      | WASH            | 15                          |            |
| Coastal Savannah     | Sanga           | 86                          | 236        |
|                      | White Fulani    | 50                          |            |
|                      | Gudali          | 30                          |            |
|                      | WASH            | 70                          |            |
| <b>Total</b>         |                 | <b>397</b>                  | <b>397</b> |

Genomic DNA was extracted according to manufacturer's specification, using QIAGEN blood kit (QIAGEN Inc, Valencia, U.S.A). Five milliliters of whole blood was decanted into 15ml of Red Blood Cell (RBC) lysis solution and allowed to incubate at room temperature for 10 min. During the incubation period, the sample was inverted several times to ensure complete lysing of erythrocytes. After incubation, the samples were centrifuged at 8,000 rpm for 10 minutes. The supernatant was discarded after centrifuging leaving behind the cell pellet of between 100-200  $\mu$ l residual liquid. The pellet in the tube was vigorously vortexed in the residual supernatant. Four milliliters of Cell Lysis solution was then added to the resuspended cells and pipetted up and down to

lyse the cells. The samples were then cooled to room temperature before 3ml of protein precipitation solution was added to the cell lysate. The tube was vortexed vigorously at high speeds for 20 seconds to mix the protein precipitation solution uniformly with the lysate and then centrifuged at 8,000 rpm for 10 minutes. This was done to precipitate the protein as visibly seen as a tight dark brown pellet.

The supernatant containing the DNA was decanted and transferred into a 15 ml tube containing 10 ml of isopropanol. The tube was gently inverted 50 times enabling the DNA to be visible as a white pellet. The visible DNA was carefully removed using a pipette and transferred into 1.5ml tube, air-dried for 10 minutes before it was reconstituted in 500 µl of DNA hydration solution. The DNA pellets from different samples were incubated at 65°C for 1 hour in a water bath to allow complete dissolution in the hydration buffer before they were stored at -20 °C.

### **3.4 Primer selection and determination of specificity**

PCR primers specific for *Anaplasma marginale*, *Babesia bigemina* and *Theileria spp.* were used to simultaneously amplify DNA fragments of 265 bp, 1125 bp and 462 bp respectively from genomic DNA templates obtained from individual cattle (Table 3). To determine whether each primer pair was specific for the gene of interest, equivalent amounts of the primer pairs were applied to 100ng of genomic DNA obtained from the organism. Using *A. marginale* infected DNA samples as example, the expected 265 bp fragment of the *m脾1b* primers was amplified from genomic DNA using forward (5'-GCTCTAGCAGGTTATGCGTC -3') and reverse (5'- CTGCTTGGGAGAATGCACCT -

3') primers (Table 3). PCR amplifications were performed in reaction volumes of 25  $\mu$ l using 0.5  $\mu$ M of each primer. Amplification reactions were performed using an initial denaturation step of 95  $^{\circ}$ C for 5 min, followed by 30 cycles of 94  $^{\circ}$ C for 5 sec, 55  $^{\circ}$ C for 30 sec, and the initial extension at 72  $^{\circ}$ C for 1 min. The products were finally extended at 72  $^{\circ}$ C for 7 min before holding at 10  $^{\circ}$ C. The amplicons were size-separated by agarose gel electrophoresis, excised from the gel and purified (QIAGEN), and the ends were extended at 72  $^{\circ}$ C for 20 min. The same primer pairs were applied, prospectively, to genomic DNA samples obtained from unrelated DNA samples from *Theileria* species and *Babesia bigemina* to confirm that there would not be amplification. In all cases, only genomic DNA from the cognate primer pairs resulted in PCR amplification. Using the non-specific primer of *Anaplasma marginale* against the genomic DNA of *Babesia bigemina* and, *Theileria spp.* resulted in no amplification. Similar procedure was carried out with the non-specific primer of *Babesia bigemina* against genomic DNA of *Anaplasma marginale* and *Theileria spp.* without amplification. *Theileria spp* non-specific primer was used against genomic DNA of *Anaplasma marginale* and *Babesia bigemina* without amplification. Non-infected bovine DNA was used as a control.

**Table 3: PCR primers**

| Pathogen species           | Gene(length)          | Forward primer( 5'-3') | Reverse primer ( 3'-5') | Reference                  |
|----------------------------|-----------------------|------------------------|-------------------------|----------------------------|
| <i>Anaplasma marginale</i> | msp1b (265bp)         | gctctagcagggttatgcgctc | ctgcttgggagaatgcacct    | Bilgic <i>et al.</i> ,2012 |
| <i>Theileria spp.</i>      | Cytochrome b (462bp)  | actttggccgtaatgtaaac   | ctctggaccaactgtttgg     | Bilgic <i>et al.</i> ,2012 |
| <i>Babesia bigemina</i>    | Cytochrome b (1125bp) | tggcggcgtttattagttcg   | ccacgctgaagcacagga      | Bilgic <i>et al.</i> ,2012 |

### 3.5 Verification of the identity of PCR fragments

Cloning was carried out to identify and confirm the identity of PCR amplicons as *Anaplasma marginale*, *Babesia bigemina* and *Theileria spp.* The corresponding amplicons of 265 bp, 462 bp and 1125 bp for *Anaplasma marginale*, *Theileria species* and *Babesia bigemina* respectively, were ligated into TOPO<sup>®</sup> cloning Plasmid vector and *Escherichia coli* cells were transformed, according to the manufacturer's protocol.

Briefly, 2µl of the cloning reaction was ligated into the TOPO 4 vector and allowed to incubate on ice for 30 min. Following ligation, cells were heat-shocked for 30 sec at 42 °C and transferred immediately back to ice. Approximately, 250 µl of SOC media was added and the cells allowed to incubate at 37 °C with shaking for 60 min. Following incubation, aliquots of 100 -150 µl cell cultures were plated on LB (Luria-Bertani) agar with 50 mg/ml of carbenicillin and incubated at 37°C overnight to develop colonies. Individual colonies were selected into 5ml broth and allowed to grow overnight. Plasmid DNA was isolated from the transformed colonies using Wizard plus Miniprep DNA purification system (QIAGEN Inc, Valencia, USA), and the presence of inserts was confirmed by *EcoRI* digestion. Briefly, the overnight cultures were centrifuged and prepared for plasmids DNA extraction using the QIAGEN plasmid purification kit (QIAGEN Inc, Valencia, U.S.A). To confirm if plasmids contained the desired insert, 3 µl of purified plasmid DNA was digested in *EcoR1* followed by electrophoresis on ethidium bromide-stained 0.9 % agarose gel. Plasmid DNA samples containing the desired inserts were sequenced in both directions using the ABI 3031xl sequencer.

### 3.6 Sensitivity of PCR assay

The sensitivity of PCR assay was determined using 10-fold dilutions of genomic DNA from *A. marginale*, *B. bigemina* and *Theileria spp.* Genomic DNA samples containing initial amounts of 100ng were serially diluted to 10ng, 1ng, 0.1ng, 0.01ng stocks. First, the lowest detection limit of each primer was determined by PCR using the serially diluted samples, starting from samples containing the highest concentration of pathogen DNA (100ng) to those that contained the least amount of the target DNA. Secondly, the sensitivity of detection of individual pathogens was determined in the background of one another to mirror the detection during mixed infection. Reciprocal PCR amplifications were performed using respective primers for each pathogen gene with reciprocal amounts (100ng of pathogen A, with 0.01 of pathogen B; 100ng of pathogen B with 0.01ng of pathogen A) of genomic DNA template. Briefly, the highest amount of genomic DNA (100ng) from one organism was paired with the lowest amount from the other organisms in a duplex PCR reaction. The PCR reaction mixture was performed in a total volume of 25  $\mu$ l as previously described and the amplicons were size-separated by ethidium bromide-stained agarose gel electrophoresis.

### 3.7 Multiplex PCR and determination of co-infection prevalence

The multiplex PCR were performed as previously described with specific modification to the annealing temperature and the time for initial extension step in order to optimize the reaction. Amplification reactions were performed using an initial denaturation step of 95  $^{\circ}$ C for 5 min, followed by 30 cycles of 94  $^{\circ}$ C for 5 sec, 55  $^{\circ}$ C for 30 sec, and the initial

extension at 72 °C for 45 sec. The products were finally extended at 72 °C for 7 min before holding at 10 °C. Individual DNA samples from cattle were subjected to multiplex PCR and the number of amplicons generated was noted. The total number of animals containing one, two and three pathogen species was recorded for all breeds of cattle based on the ecological zones.

### **3.8 Statistical analysis**

The data collected from the three ecological zones were analyzed using Chi-squared test (PH Stat: 2004 Prentice Hall, Inc., a Microsoft Excel Add in, Version 12.0) to determine if differences in co-infection prevalence between indigenous cattle is associated with differences in the type of breed and the ecological zones.

## CHAPTER FOUR

### 4.0. RESULTS

A total of 397 cattle representing four breeds were examined from sixteen cattle rearing locations from three agro-ecological zones in Ghana. The overall co-infection prevalence of cattle was 26.4% (105/397). The Coastal Savannah recorded the highest co-infection prevalence rate of 33.5% (80/239) followed by Guinea Savannah 17.4% (16/92) and Transitional zone 13.0% (9/69) respectively (Table 4).

**Table 4: Overall pathogen co-infection prevalence in agro-ecological zones**

| Agro-ecological zones   | Geo-coordinates                            | Number of cattle examined | Number of cattle un-infected | Number of cattle co-infected | Co-infection prevalence |
|-------------------------|--|---------------------------|------------------------------|------------------------------|-------------------------|
| <b>Guinea Savannah</b>  | 9 <sup>0</sup> 26'N<br>0 <sup>0</sup> 49'W | 92                        | 43                           | 16                           | 17.4%                   |
| <b>Transitional</b>     | 6 <sup>0</sup> 45'N<br>1 <sup>0</sup> 37'W | 69                        | 38                           | 9                            | 13.0%                   |
| <b>Coastal Savannah</b> | 6 <sup>0</sup> 15'N<br>0 <sup>0</sup> 10'E | 236                       | 66                           | 80                           | 33.5%                   |
| <b>Total</b>            |  | <b>397</b>                | <b>147</b>                   | <b>105</b>                   | <b>26.4%</b>            |

The difference is highly significant  $\chi^2$  cal 25.38, df =2, p = 0.00

The overall infection prevalence recorded during the study was 62.9% (250/397). The Coastal Savannah zone recorded the highest infection prevalence of 72% (170/236) followed by Guinea Savannah 53.3% (49/92) and Transitional zone 44.9% (31/69) respectively (Table 5).

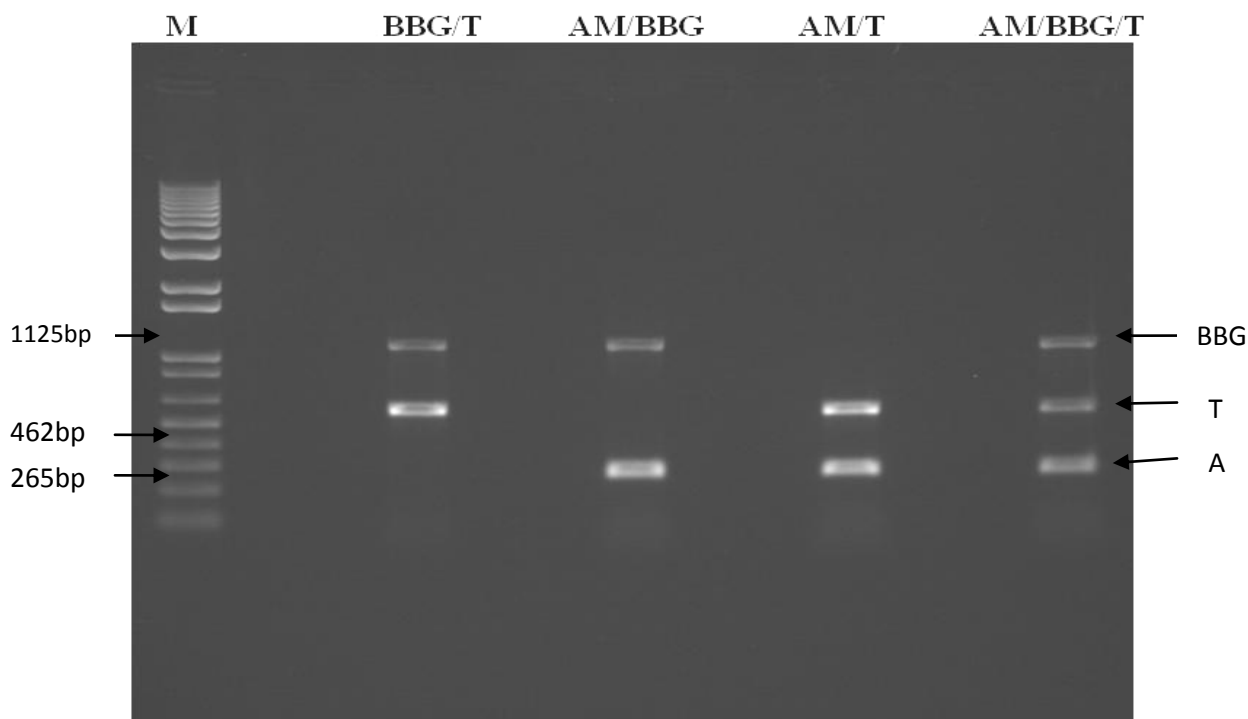
**Table 5: Overall infection prevalence of cattle within agro-ecological zones**

| Ecological zones | Geo-co-ordinates                             | Number of cattle examined | Number of cattle un-infected | Number of cattle infected | Infection prevalence |
|------------------|--|---------------------------|------------------------------|---------------------------|----------------------|
| Guinea Savannah  | 9 <sup>o</sup> 26'N<br>0 <sup>o</sup> 49'W   | 92                        | 43                           | 49                        | <b>53.3%</b>         |
| Transitional     | 6 <sup>o</sup> 45'N<br>1 <sup>o</sup> 37'W   | 69                        | 38                           | 31                        | <b>44.9%</b>         |
| Coastal Savannah | 6 <sup>o</sup> 15' N<br>0 <sup>o</sup> 10' E | 236                       | 66                           | 170                       | <b>72%</b>           |
| <b>Total</b>     |  | <b>397</b>                | <b>147</b>                   | <b>250</b>                | <b>62.9%</b>         |

The difference is highly significant  $\chi^2$  cal 21.6, df =2, p = 0.001

#### 4.1 Sensitivity of multiplex PCR and detection of co-infected cattle

The minimum detection limit for each of the three pathogen-specific primers in primary PCR was 0.01ng. Using the multiplex PCR method, the target DNA of all the three pathogen species in the multiplex PCR reactions were successfully amplified (Fig. 1).



## Figure 1: Detection of co-infected cattle by multiplex PCR

### Legend

|                 |  |
|-----------------|--|
| <b>M</b>        | 1Kb plus ladder  |
| <b>BBG+T</b>    | <i>Babesia bigemina</i> and <i>Theileria species</i> combined                              |
| <b>AM+BBG</b>   | <i>Anaplasma marginale</i> and <i>Babesia bigemina</i> combined                            |
| <b>AM+T</b>     | <i>Anaplasma marginale</i> and <i>Theileria species</i> combined                           |
| <b>AM+BBG+T</b> | <i>Anaplasma marginale</i> , <i>Babesia bigemina</i> and <i>Theileria species</i> combined |

### 4.2 Pathogen co-infection status of cattle examined

The overall pathogen co-infection prevalence in indigenous cattle breeds examined was 26.4% (105/397). Out of this total, only six cattle, representing 1.5% (6/397) of the population were co-infected with all three different tick-borne pathogens - *Theileria species*, *Anaplasma marginale*, and *Babesia bigemina*. 95 cattle 23.9% (95/397) were co-infected with *Anaplasma marginale* and *Theileria species*, whereas four cattle 1.0% (4/397) were co-infected with *Anaplasma marginale* and *Babesia bigemina*. Surprisingly, no cattle were found to be co-infected with *Babesia bigemina* and *Theileria species* (Table 6). The data also demonstrated that 145 cattle 36.5% (145/397), harboured single pathogen species. Out of this number *Anaplasma marginale* alone constituted 36.3% (144/397). Only one cattle 0.3% (1/397) was infected with *Theileria species* alone. Notably, no cattle were infected with *Babesia bigemina* pathogen alone at the time of the study (Table 6).

**Table 6: Pathogen co-infection status of cattle**

| ECOLOGICAL ZONES               | NUMBER OF CATTLE INFECTED/ TOTAL SAMPLED | AM         | BBG      | T        | AM+BBG   | AM+ T     | BBG+T    | AM+ BBG+T |
|--------------------------------|--|------------|----------|----------|----------|-----------|----------|-----------|
| Northern<br>(Guinea Savannah)  | 49/92                                    | 33         | 0        | 0        | 2        | 14        | 0        | 0         |
| Middle<br>(Transitional zone)  | 31/69                                    | 22         | 0        | 0        | 0        | 9         | 0        | 0         |
| Southern<br>(Coastal Savannah) | 170/236                                  | 89         | 0        | 1        | 2        | 72        | 0        | 6         |
| <b>Total</b>                   | <b>250/397</b>                           | <b>144</b> | <b>0</b> | <b>1</b> | <b>4</b> | <b>95</b> | <b>0</b> | <b>6</b>  |

**Legend**

|          |  |
|----------|--|
| AM       | <i>Anaplasma marginale</i>   |
| AM+T     | <i>Anaplasma marginale</i> and <i>Theileria</i> species combined                           |
| BBG      | <i>Babesia bigemina</i>  |
| AM+BBG   | <i>Anaplasma marginale</i> and <i>Babesia bigemina</i> combined                            |
| T        | <i>Theileria</i> Species   |
| AM+BBG+T | <i>Anaplasma marginale</i> , <i>Babesia bigemina</i> and <i>Theileria</i> species combined |

#### **4.3 Effect of differences in agro-ecological zone and genetic diversity between cattle on co-infection prevalence**

The Coastal Savannah recorded a significantly ( $p=0.00$ ) higher number of cattle 33.5% (80/236) infected with multiple-pathogen species than cattle from Guinea Savannah 17.4% (16/92) and Transitional zones 13.0% (9/69) based on the Chi-squared Goodness

of fit test. The Chi-squared analysis of co-infection prevalence data from 397 indigenous cattle randomly selected from each of four breeds - Sanga, White Fulani, Gudali and West African Shorthorn (WASH) supports the significant association ( $P= 0.01$ ) between differences in co-infection prevalence and type of breed (Table 7).

**Table 7: Effect of breed of cattle on co-infection prevalence**

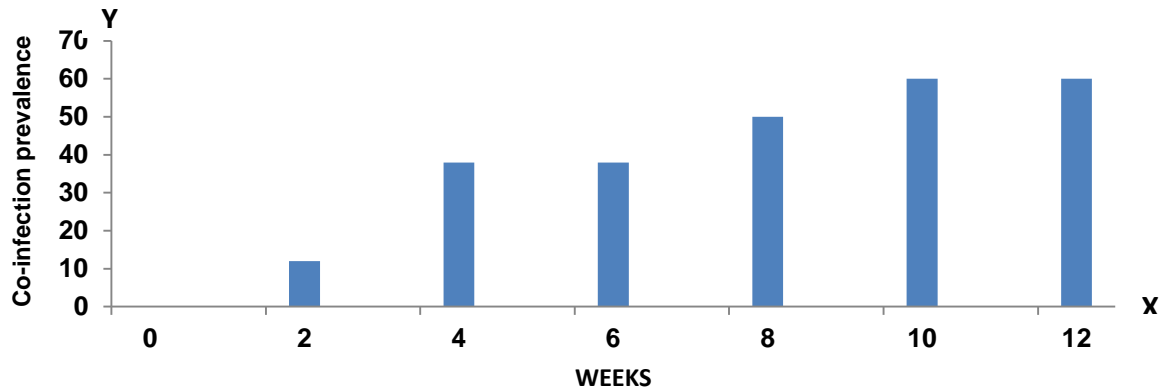
| Breeds of cattle          | Number examined | Number infected with single pathogen | Number infected with multiple pathogen |
|---------------------------|-----------------|--------------------------------------|--|
| Sanga                     | 151             | 59                                   | 37                                     |
| White Fulani              | 57              | 13                                   | 22                                     |
| Gudali                    | 76              | 34                                   | 14                                     |
| WASH                      | 113             | 39                                   | 32                                     |
| <b>Total</b>              | <b>397</b>      | <b>145</b>                           | <b>105</b>                             |
| $\chi^2$ (3df) calculated |                 | <b>29.54</b>                         | <b>12.07</b>                           |
| <i>p-value</i>            |                 | <b>0.00</b>                          | <b>0.01</b>                            |

#### 4.4 Duration of infection on prevalence of co-infection in individual cattle

As noted in Table 7, 36.5% (145/397) of cattle were infected with single pathogen species. To determine whether this was related to differences in the duration of infection between cattle, the correlation between increasing time of infection and the likelihood of cattle becoming infected with multiple pathogen species was tested using eight cattle from LIPREC because not all cattle that were initially found to be singly infected were found. These cattle were recorded to have harbored single pathogen species infections at the time of sampling. To determine whether co-infection prevalence increases over time in individual cattle, we examined the infection status of each of the eight cattle at bi-

weekly intervals for 12 additional weeks and found increased progression of prevalence of co-infection over the duration of infection in individual calf (Figure 2).

**Figure 2: Increasing co-infection prevalence rate in individual cattle**

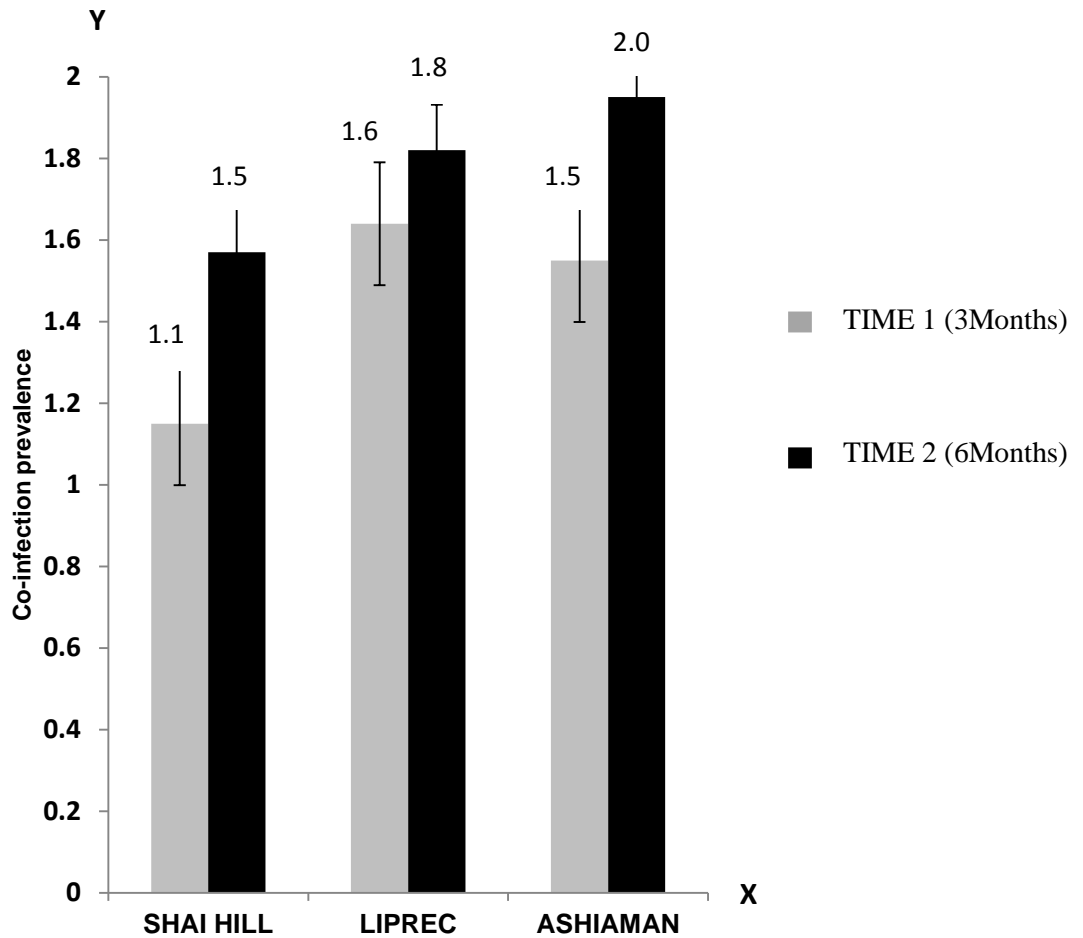


**Figure 2: Increasing co-infection prevalence rate within individual cattle**

The y- axis represents co-infection prevalence expressed in percentage. The x- axis represents duration of infection at bi-weekly intervals in individual cattle.

#### **4.5 Duration of infection on prevalence of co-infection at herd level**

For the remaining single-pathogen infected cattle that could not be tracked at individual level, the complete herd was tracked at bi-weekly intervals as previously described using three populations (cattle farms) located at LIPREC, Shai Hills and Ashaiman. The mean co-infection prevalence for herds of cattle from LIPREC 1.7, Shai Hills 1.3 and Ashiaman 1.8 were determined for 6 months. Figure 3 indicates an increasing rate of herd co-infection prevalence at Shai Hills, LIPREC and Ashaiman at same time points. The mean co-infection prevalence at the time point one, representing three months following the initial blood sampling from these herds from Shai Hills, LIPREC and Ashiaman were 1.1, 1.6, and 1.5 respectively. These values increased progressively to 1.5, 1.8 and 2.0 respectively over the duration of 3 months. This finding is consistent with the result at individual levels (Figure 3).



**Figure 3: Duration of infection on prevalence of co-infection at herd level**

The y- axis represents levels of co-infection prevalence and the x- axis represents duration of infection. The black bars represent the mean co-infection prevalence for the respective study herds at the time point 1, representing 12 additional weeks following the initial testing of the infectious status of cattle. The white bars represent the mean co-infection prevalence at time point 2, representing 24 additional weeks following the initial testing of the infection status of cattle

## CHAPTER FIVE

### 5.0 DISCUSSION

In sub-Saharan Africa, there are many tick vectors and associated tick-borne pathogens of cattle and the high prevalence are production-limiting (Walker and Koney, 1999). Disease control is particularly challenging and costly due to the presence of multiple pathogens from the seven globally important livestock intracellular pathogens (Nadelman *et al.*, 1997). The current study focused on three of the most virulent of the haemoparasitic diseases of livestock – *Babesia bigemina*, *Theileria species* and *Anaplasma marginale*. All these organisms are common in tropical and subtropical regions throughout the world and exhibit epidemiologic features similar to other tick-borne pathogens of cattle - fever, anaemia, reduced production and in some cases abortion and death (Bell-Sakyi *et al.*, 2004). Several factors may account for differences in disease prevalence. It could be due to seasonal variations, differences in the length of the transmission cycle of the associated vector species based on agro-ecological conditions, abundance of pathogen strains, and availability and susceptibility of the terminal mammalian host to infection (Andr n, 1994; Stafford *et al.*, 1998; Jongejan and Uilenberg, 2004; Cumming and Van Vuuren, 2006; Wimberly *et al.*, 2008).

#### 5.1. Effect of Agro-ecological zone on co-infection prevalence

The analysis of variation in co-infection prevalence revealed very significant ( $p=000$ ) variations across the agro-ecological zones (Table 4). The Coastal Savannah (Southern zone) recorded the highest co-infection prevalence of 33.5% (80/239), followed by

Guinea Savannah (Northern zone) with 17.4% (16/92) and Transition (Middle) zone with 13.0% (9/69), (Table 4). The perceived differences in the infection prevalence between the Southern belt and the Northern regions may be attributed to a shift in climatic conditions, especially temperature and humidity that will most likely alter geographic distribution of ideal and marginal habitat (Estrada-Penna, 2003). Though a variety of biotic and abiotic environmental factors influence vector populations, a few parameter changes such as minimum and maximum temperature and rainfall, when considered together, are predictive of pathogen prevalence in the terminal host species (Sedaghat *et al.*, 2013).

The distribution and abundance of tick species in Ghana varies by agro-ecological zone (Walker and Koney, 1999; Ntiamoah-Baidu *et al.*, 2004). The simple prediction is that differences in the population density of the ticks between the three agro-ecological zones will strongly influence the epidemiology of tick-borne disease pathogens they transmit to cattle. Variations in the environmental conditions may further alter the geographical ranges of ticks.

In biological terms, the lifecycle of the vector tick is largely temperature-dependent and when not attached to the host, ticks are extremely sensitive to desiccation (Randolph, 2009), and the death of tick vector will undoubtedly reduce the likelihood of pathogen transmission. As successful pathogen-vector-host interaction is central to multiple infections of cattle, diminution of the reproductive cycle will result in corresponding reduction of transmission to the host (Randolph, 2009).

The Coastal Savannah also represents the region with the largest concentration of cattle markets and abattoirs suggesting the high likelihood of continuous influx of pathogen infected cattle from neighboring countries, including Burkina Faso, Mali and Niger. Together, these factors would facilitate introduction of new pathogen strains to which the indigenous cattle have no pre-existing immunity.

In this event, the newly introduced pathogen strain will have a wider range of susceptible host population as it can establish co-infection not only in the naive susceptible host population but also all the cattle that have already established immunity to pathogen strains extant in the population due to lack of cross protection. The overall effect would be an increased efficiency of transmission and high prevalence of infected cattle with multiple-pathogen co-infection.

## **5.2 Effect of the genetic diversity between cattle on co-infection prevalence**

Successful pathogen-host interaction is central to the establishment of infection. One of the strongest factors that influence the outcome of this interaction is the immunity in the mammalian host population (Mahoney *et al.*, 1981). Notably, the capacity to mount a solid immunity to disease is driven by the genetic make-up of the individual that varies between different breeds of cattle (Nadelman *et al.*, 1997).

Where cattle from one breed can resist co-infection with multiple organisms, while all the other animals are susceptible within the same population suggest genetic diversity as an important force directing organism co-infection of the host (Nadelman *et al.*, 1997). To address if differences between breeds of cattle affect co-infection prevalence among

cattle, the evidence of differences in the number of co-infecting organisms between four indigenous cattle that previously demonstrated significant variations in the prevalence of tick-transmitted *Anaplasma marginale* (Shaban, 2012) was investigated.

If genetic differences between cattle breeds underlie variability in cattle susceptibility to co-infection, then in herds with many resistant breeds, there will be high infection prevalence maintained through time by high transmission resulting in little episode of disease due to herd immunity. There is significant ( $p < 0.05$ ) variations in the co-infection prevalence between the four genetically distinct breeds of cattle examined in the studies (Table 7).

There is certainly evidence that quantitative differences in the infection prevalence has occurred between individual cattle within every breed on the basis of analysis of the number of pathogenic organisms harbored at the time of sampling. Possible explanations for the observed, lack of variation between breeds are that either the level of immunity to infectious pathogen is similar, or genetic difference between the five breeds alone is not sufficient to support clustering of their responses to pathogen infection. It is also possible that the development of adaptive immunity is not required for the prevention of multiple-pathogen infection.

### **5.3 Effect of duration of infection on prevalence of co-infection in individual cattle and at herd level**

When cattle become infected as calves, they will suffer minor disease episodes, maintain lifelong persistent infections, and are protected from disease upon encounter with the

same or related pathogen strain in the future (Kieser *et al.*, 1990; French *et al.*, 1998; French *et al.*, 1999). In herds with high infection prevalence that is maintained through time by high transmission, little or no disease occurs due to herd immunity (Mahoney *et al.*, 1981). As expected, the incidence of co-infection was evident in all breeds of cattle examined.

This result suggests that although breed-dependent immunity can protect cattle against disease, the immune cattle are not protected from infection with new pathogen strains over time. This assumption was tested by tracking the co-infection prevalence rate in eight individual calves that were previously infected with single pathogen strains.

The data demonstrated that the co-infection prevalence rate, in individual calf increased progressively over the duration of infection (Fig. 2), suggesting the lack of correlation between adaptive immunity and the reduced likelihood of a new pathogen penetrating into the immune host. This finding emphasizes the epidemiologic importance of the presence of the reservoir individual cattle in the transmission efficiency of vector-borne organisms and the need to develop control strategy against infection. Based on this result, the hypothesis that co-infection prevalence increases over the duration of infection was accepted. However, the attempt to track the changes in single-pathogen infected cattle from the other farms was unsuccessful because either the animals were sold out or their identity got mixed up due to lack of good record keeping.

To confirm this observation at the host population level, the co-infection prevalence rates for cattle in three herds, located at the Shai Hills, LIPREC and Ashaiman were tracked. Historically, the three herds had comparable infection prevalence (>90%) (Shaban, 2012)

perhaps resulting in stable herd immunity with little opportunity for penetrance of new pathogen strains.

In contrast, the data demonstrated considerable support for an increasing rate of accumulation of new infection, over time, in three herds, resulting in corresponding increases in the mean co-infection prevalence between herds of cattle located at Shai Hills, LIPREC and Ashaiman at same time points. The mean co-infection prevalence at the time points 1, representing 3 months following the initial blood sampling from these herds from Shai Hills, LIPREC and Ashaiman were 1.1, 1.6, and 1.5 respectively. These values increased progressively to 1.5, 1.8, and 2.0 respectively, over the duration of 3 months (Fig. 3).

This finding is consistent with the result at individual levels, and strongly supports the lack of restriction in the number of different pathogen species that can infect susceptible cattle in the endemic region. Finally, the findings from the current study indicate that differences in the breed resistance to disease are not entirely adequate as a biologic indicator to precisely predict co-infection rates and the epidemiologic implications on indigenous cattle. Additional biomarkers should be investigated, and a more comprehensive assessment of type of vector strains and climate change may be needed to fully clarify relationships within indigenous cattle breeds.

## CHAPTER SIX

### 6.0. CONCLUSION AND RECOMMENDATION

#### 6.1 Conclusion

- Co-infection prevalence increased with the duration of infection among the indigenous cattle breeds at both individual and herds levels.
- There is evidence of association between co-infection prevalence of indigenous cattle breeds from different ecological zones.
- There is evidence that the type of breed of cattle influences the ability of indigenous cattle to resist co-infection with multiple pathogens.
- All the indigenous cattle breeds examined were susceptible to co-infection with multiple tick-borne pathogens.

#### 6.1. Recommendation

- Co-infection prevalence was determined in the indigenous cattle breeds. However, there is the need to conduct similar studies in ticks to determine the co-infection prevalence and its effect on invertebrate tick parasite.
- The study was carried out in three ecological zones; hence the need to extend it, to cover all the ecological zones in the country to encourage mapping of all multi-pathogen co-infected zones in the country.

- A more comprehensive assessment of the type of vector strains and the effect of the shift in climate, specifically, temperature and humidity, may be needed to fully clarify relationships within indigenous cattle breeds.

## REFERENCES

- Aboagye, G.S. (2002). Phenotypic and genetic parameters in cattle populations in Ghana. AGTR Case Study. Nairobi, Kenya. *Int. Livest. Res. Inst.*, (2):216-220.
- Adam, M., Erick, S., and Rance, K. S. (2006). Veterinary Medicine Supplement. Department of Veterinary Clinical Sciences, College of Veterinary Medicine, Washington State University, Pullman, WA. pp. 7060 - 99164.
- Adelson, M.E., Rao, R.V., and Tilton, R.C. (2004). Prevalence of *Borrelia burgdorferi*, *Bartonella* spp., *Babesia microti*, and *Anaplasma phagocytophila* in *Ixodes scapularis* ticks collected in Northern New Jersey. *J. Clin. Microbiol.*, 42:2799-2801.
- Adeniji K.O. (1985). Review of endangered cattle breeds of Africa. In: Hodges J. (ed), Animal genetic resources in Africa: High potential and endangered livestock. Second OAU Expert Committee Meeting on Animal Genetic Resources in Africa held in Bulawayo, Zimbabwe, 24–28 November 1983. OAU–IBAR (Organization of African Unity/Inter-African Bureau for Animal Resources), Nairobi, Kenya. pp. 20–32.
- Alekseev, A. N., Dubinina, H. V. , Van De Pol,, I. and Schouls, L. M. (2001). Identification of Ehrlichia spp, and Borrelia burgdorferi in Ixodes ticks in the Baltic regions of Russia. *J. Clin. Microbiol.*, 39 (6):2237-2242.
- Allen, J.R., and Humphreys, S.J. (1979). Immunisation of guinea pigs and cattle against ticks. *Nature*, 280: 491-493.

- Anderson, R. M., and May, R. M. (1979). Population Biology of Infectious Diseases I. *Nature*, 280: 361–367.
- Anderson, R. M., and May, R. M. (1981). The population dynamics of microparasites and their invertebrate hosts. *Phil. Transac. Royal Soc. London Series, B* 291: 451–524.
- Andrén, H. 1994. Effects of habitat fragmentation on birds and mammals in landscapes with different proportions of suitable habitat—a review. *Oikos*. 71:355-366.
- Aryee, A.K., Vorsogbe, S.O., Allotey, J.A., Kutawe, K., and Obuobi, E. (1991). Livestock Development Project Phase II. Preparation project paper submitted to the Ministry of Food and Agriculture/World Bank/Government of Ghana.
- Baiden, R. Y., and Duncan, L. (2008). Performance of indigenous beef cattle under two management systems at Pokuase, Animal Research Institute, Ghana. *Livest. Res. Rural Dev.*, 20 (6).
- Baldwin, C.L., Black, S.J., Brown, W.C., Conrad, P.A., Goddeeris, B.M., Kinuthia, S.W., Lalor, P.A., MacHugh, N.D., Morrison, W.I., Morzaria, S.P., Naessen, S.J., and Newson, J. (1988). Bovine T. cells, B cells and null cells are transformed by the protozoan parasites *Theileria parva*, *Infect. Immun.*, 56: 462-467.
- Barré, N., and Uilenberg, G. (2010). Spread of parasites transported with their hosts: case study of two species of cattle tick. *Rev. Sci. Tech. Off. Int. Epiz.*, 29 (1): 149-160.

- Barthold, S. W. (1999). Specificity of infection-induced immunity among *Borrelia burgdorferi* sensu lato species. *Infect. Immun.*, 67:36 - 42.
- Bekele, T. (2002). Studies on seasonal dynamics of ticks of Ogaden cattle and individual variation in resistance to ticks in eastern Ethiopia. *J. Vet. Med.*, 49: 285-288.
- Bell-Sakyi, L., Koney, E. B., Dogbey, O., and Walker, A. R. (2004). Incidence and prevalence of tick-borne haemoparasites in domestic ruminants in Ghana. *Vet. Parasitol.*, 124:25-42.
- Belongia, E.A. (2002). Review Epidemiology and Impact of Coinfections Acquired from Ixodes Ticks. Vector Borne and Zoonotic Diseases. *Mary Ann Liebert, Inc. Wisconsin*. 2(4): 265-273.
- Ben-Ami, F., Mouton, L., and Ebert, D. (2008). The effects of multiple infections on the expression and evolution of virulence in a *Daphnia*-endoparasite system. *Evolution*, 62: 1700-1711.
- Benneh, G., Agyepong, G. T., and Allotey, J. A. (1990). Land degradation in Ghana. Food Production and Rural Development Division. Commonwealth Secretariat, London.
- Bilgic, H. B., Karagenc, T., Simuunza, M., Shiels, B., Tait, A., Eren, H., and Weir, W. (2012). Development of a multiplex PCR assay for simultaneous detection of *Theileria annulata*, *Babesia bovis* and *Anaplasma marginale* in cattle. *Exp. Parasitol.*, 133: 222 - 229.

- Bock, R., Jackson, L., de Vos, A.J., Jorgensen, W., Bowman A.S., and Nuttall, P.A. (2008). Babesiosis of cattle. *In: Ticks: Biology, Disease and Control, eds. Cambr. Univ., Press, Cambridge, UK.* pp. 281–307.
- Bradley, D., J., and Gilbert, G. S. (2008). Pathogens promote plant diversity through a compensatory response. *Eco. Letters*, 11: 461–469.
- Bram, R. A. (1975). Tick-borne livestock diseases and their vectors. The global problem. *World Anim. Rev.*, 6:1–5.
- Breitschwerdt, E.B., Hegarty, B.C., and Hancock, S.I. (1998). Sequential evaluation of dogs naturally infected with *Ehrlichia canis*, *Ehrlichia chaffeensis*, *Ehrlichia equi*, *Ehrlichia ewingii*, or *Bartonella vinsonii*. *J. Clin. Microbiol.*, 36: 2645-2651.
- Brendan, S. (2013). Theileriosis in cattle. Tamworth Veterinary Hospital 106 Brisbane Street, Tamworth, NSW. pp. 2340.
- Brown, C.G.D., Hunter, A.G., Luckins, A.G., Sewell, M.M.H., and Brocklesby, D.W. (1990). Diseases caused by protozoa: Handbook on animal diseases in the Tropics, 4<sup>th</sup> (eds.), Bailliere Tindall, London. pp.161- 226.
- Callow, L. L. (1984). Piroplasms. In *Animal Health in Australia, Protozoal and Rickettsial Diseases, vol. 5. Animal Health in Australia*, Canberra, Australian Bureau of Animal Health, AGPS. pp. 121–160.
- Callow, L. L., Rogers, R. J., and de Vos, A. J. (1993). Tick-borne diseases: cattle pathology and serology. In *Australian Standard Diagnostic Techniques for Animal*

- Diseases (ed. Corner, L. A. & Bagust, T. J.), *East Melbourne, CSIRO Inf. Serv.*, pp. 1–16.
- Carter, G.R., Chengappa, M.M., Roberts, A.W., Claus, G.W., and Rikihisa, Y. (1995). *Essentials of Veterinary Microbiology*. 5<sup>th</sup> Ed., Williams and Wilkins, Media, USA. 394:73-80.
- CDC (2010) Centre for disease control and prevention. Life cycle of Hard Ticks that Spread Disease". *Centers for Disease Control and Prevention*. Retrieved 22 June 2013.
- Chaput, E.K., Meek, J.I., and Heimer, R. (2002). Spatial analysis of human granulocytic ehrlichiosis near Lyme, connecticut. *Emerg. Infect. Dis.*, 8(9): 943-948.
- Coronado, A. (2001). Is *Boophilus microplus* the main vector of *Anaplasma marginale*? *Technical note. Rev. Cient.* pp. 408 - 411.
- Cumming, G. S., and D. P. van Vuuren. 2006. Will climate change affect ectoparasite species ranges? *Global Ecol. Biogeog.* 15:486-497.
- Daniel, M., Kola, J.R., Zeman, P. (2004). Geographic Information Systems tools for tick and tickborne disease occurrence. *Parasitol.*, 129: S329-S352.
- de la Fuente, J., and Kocan, K.M. (2006). Strategies for development of vaccines for control of ixodid tick species. *Parasite Immunol.*, 28:275-283.
- de la Fuente, J., Almazan, C., Canales, M., de la Lastra, J.M.P., Katherine, Kocan, M., and Willadsen, P. (2007). A ten-year review of commercial vaccine performance for control of tick infestations on cattle. Cambridge University Press. *Anim. Hlth. Res. Rev.*, 8(1): 23–28.

- de Roode, J.C., Pansini, R., Cheesman, S.J., Helinski, M.E.H., and Huijben, S. (2005). Virulence and competitive ability in genetically diverse malaria infections. *Proc. Natl. Acad. Sci., USA.* 102:7624–7628.
- Dickson, K. B., and Benneh, G. (1988). *A new geography of Ghana*. Longman Group UK Limited. Essex, England. Retrieved 23 June 2013.
- Dikmans, G. (1950). The transmission of anaplasmosis. *Am. J. Vet. Res.*, 11:5–16.
- Dreher, U.M., de La Fuente J., Hofmann-Lehmann R., Meli, M.K., Pusteria, N., Kocan, K.M., Woldehiwet, A., Regula, G., and Staerk, K.D.C. (2005). Serologic cross reactivity between *Anaplasma marginale* and *Anaplasma phagocytophilum*. *Clin. Diagn. Lab. Immunol.*, 12:1177–1183.
- Dumler, J. S., Barbet, A. F. , Bekker, C. P. J., Dasch, G. A. , Palmer, G. H., Ray, S. C. , Rikihisa, Y., and Rurangirwa, F. R. (2001). Reorganization of the genera in the families Rickettsiaceae and Anaplasmataceae in the order Rickettsiales: unification of some species of Ehrlichia with Anaplasma, Cowdria with Ehrlichia and Ehrlichia with Neorickettsia, descriptions of six new species combinations and designation of Ehrlichia equi and “HGEagent” as subjective synonyms of Ehrlichia phagocytophila. *Int. J. Syst. Evol. Microbiol.*, 51:2145–2165.
- Estrada-Pena, A. (2003). Climate change decreases habitat suitability for some tick species (Acari: Ixodidae) in South Africa. *Onderstepoort J. Vet. Res.*, 70:79 – 93.

- Ewing, S. A. (1981). Transmission of *Anaplasma marginale* by arthropods. In R. J. Hidalgo and E. W. Jones (ed.), Proceedings of the 7th National Anaplasmosis Conference. Missi. State Univ., Mississippi State. pp. 395-423.
- FAO (1975). Report of the FAO Expert Consultation on Research on Tick-borne Diseases and their Vectors, Rome, Italy, Rome. Retrieved 23 June 2013.
- Ferguson, W. (1967). Muturu cattle of Western Nigeria. II. Survivability, reproduction and growth performance in an area of light *Glossina palpalis* density. *J. West Afr. Sci. Assoc.*, 12: 3744.
- Figueroa, J. V., Alvarez, J. A. , Ramos, J. A., Rojas, E. E., Santiago, C., Mosqueda, J. J., Vega, C. A., and Buening, G. M. (1998). Bovine babesiosis and anaplasmosis follow-up on cattle relocated in an endemic area for hemoparasitic diseases. *Ann. N. Y. Acad. Sci.*, 849:1–10.
- Foil, L. D. (1989). Tabanids as vectors of disease agents. *Parasitol. Today*, 5:88-96.
- French, D. M., Brown, W. C., and Palmer. G. H. (1999). Emergence of *Anaplasma marginale* antigenic variants during persistent rickettsemia. *Infect. Immun.*, 67:5834-5840.
- French, D. M., McElwain, T. F., McGuire, T. C., and Palmer, G. H. (1998). Expression of *Anaplasma marginale* major surface protein 2 variants during persistent cyclic rickettsemia. *Infect. Immun.*, 66:1200-1207.

- Fricke, W. (1979). Cattle Husbandry in Nigeria: A study of its ecological conditions and social geographical differentiations. Heidelberg Geographische Arbeiten, Germany. pp.330.
- Friedhoff, K.T. and Smith, R.D. (1981).Transmission of *Babesia* by ticks. In: *Babesiosis* by M. Ristic and J.P. Kreier (eds), *Academic Press*, New York. pp. 267-321.
- Frisch, J.E., O'Neil, C.J., and Kelly, M.J. (2000). Using genetics to control cattle parasites- the Rockhampton experience. *Int. J. Parasitol.*, pp.253.
- Furlanello, C., Neteler, M. and Merler, S. (2003). Geographic Information Systems and the random forest predictor: Integration in R for tick-borne disease risk assessment. Proceedings of the 3rd international workshop on Distributed Statistical Computing. Vienna; Vienna University of Technology. Retrieved 23 June 2013.
- Futse, J. E., Brayton, K. A., Dark, M. J., Knowles, Jr., and Palmer, G. H. (2008). Superinfection as a driver of genomic diversification in antigenically variant pathogens. Proceedings of the National Academy of Sciences USA 105. Retrieved 23 June 2013.
- Glass, R. I., Bresee, J. S., Turcios, R., Fischer, T. K., Parashar, U. D., and Steele, D. A. (2005). Rotavirus Vaccines: Targeting the Developing World. Viral Gastroenteritis Section, Centers for Disease Control and Prevention, Atlanta, Georgia; Initiative for Vaccine Research, World Health,Organization, Geneva, Switzerland, *J. infect. Dis.*, 192( 1):160–6.

GMA (2012). Ghana Meteorological Agency. Accra, Ghana.

Ginsberg, H.S. (2008). Potential effects of mixed infections in ticks on transmission dynamics of pathogens: comparative analysis of published records. *Exp. Appl. Acarol.*, 46:29–41.

Goddard, J. (2008). "Tick-borne diseases". *Infectious Diseases and Arthropods*. Springer. Retrieved 24 June 2013.

Graf, J.F., Gogolewski, R., Leach-Bing, N., Sabatini, G.A., Molento, M.B., Bordin, E.L., and Arantes, G.J. (2004). Tick control: an industry point of view. *Parasitology*, 129: S427–S442.

Guglielmone, A.A. (1995). Epidemiology of babesiosis and anaplasmosis in South and Central America. *Vet. Parasitol.*, 57: 109–119.

GVS/ MoFA. (1975). Veterinary Services/Ministry of Agriculture. 'Livestock Census' Accra, Ghana. Retrieved 25 June 2013.

Henning, M. W. (1956). Heartwater. In: Animal Disease of South Africa, 3<sup>rd</sup> (ed). *Central News Agency Ltd*, South Africa. pp. 1155-1178.

Hewetson, R. W. (1972). The inheritance of resistance by cattle to cattle tick. *Aust. Vet. J.*, Australia. 48:299-303.

Hewetson, R. W. (1979). Results of selection for cattle tick resistance in cattle. *Proceedings of the 56th Annual Conference of Australian Veterinary Association*. pp. 91-93.

- Hofmann-Lehmann, R., Meli, M.L., Dreher, U.M., Gönczi, E., Deplazes, P., Braun, U., Engel, S. M., Schüpbach, J., Jörgen, K., Thomas, R., Griot, C., Stärk, K.D.C., Willi, B., Schmidt, J., Kocan, K.M., and Lutz, H. (2004). Concurrent infections with vector-borne pathogens associated with fatal haemolytic anemia in a cattle herd in Switzerland. *J. Clin. Microbiol.*, 42: 3775–3780.
- Hugh-Jones, M. (1991). The remote recognition of tick habitats. *J. Agric. Entomol.*, 8 (4): 309-315.
- ILCA. (1979). Trypanotolerant Livestock in West and Central Africa. Monograph No. 2. ILCA, Addis Ababa, Ethiopia. Retrieved 25 June 2013.
- Johnson, P.T.J., and Hoverman, J.T. (2012) Parasite diversity and co-infection determine pathogen infection success and host fitness. *Ecology and Evolutionary Biology*, University of Colorado, Boulder. Retrieved 25 June 2013.
- Johnston, L. A., Leatch, G., and Jones, P. N. (1978). The duration of latent infection and functional immunity in droughtmaster and hereford cattle following natural infection with *Babesia argentina* and *Babesia bigemina*. *Aust. Vet. J.*, Australia. 54: 14–18.
- Jongejan, F., and Uilenberg, G. (1994). Ticks and control methods. *Rev. Sci. Tech.*, 13: 1201–1226.
- Jongejan, F., and Uilenberg, G. (2004). The global importance of ticks. *Parasitol.*, 129:S3-S14.

- Jonsson, N.N., Bock, R.E., and Jorgensen, W.K. (2008). Productivity and health effects of anaplasmosis and babesiosis on *Bos indicus* cattle and their crosses, and the effects of differing intensity of tick control in Australia. *Vet. Parasitol.*, 155: 1-2.
- Kieser, S. T., Eriks, I. E., and Palmer, G. H. (1990). Cyclic rickettsemia during persistent *Anaplasma marginale* infection in cattle. *Infect. Immun.*, 58:1117-1119.
- Kitron, U., and Kazmierczak, J.J. (1997). Spatial analysis of the distribution of Lyme disease in Wisconsin. *Am. J. Epidemiol.*, 145(6): 558-66.
- Kocan, K. M. (1995). Targetting ticks for control of selected haemoparasitic diseases of cattle. *Vet. Parasitol.*, 57: 121-151.
- Kocan, K.M. (1986). Development of *Anaplasma marginale* in ixodid ticks: coordinated development of a rickettsial organism and its tick host. In: Sauer J, Hair JA (eds) Morphology, physiology and behavioral ecology of ticks. *Ellis Horwood Ltd, Chichester*. pp. 472–505.
- Kocan, K. M., Blouin, E. F., and Barbet, A. F. (2000). Anaplasmosis control: past, present and future. *Ann. N. Y. Acad. Sci.*, 916:501–509.
- Kocan, K.M., de la Fuente, J. Guglielmo, A.A., and Meléndez, R.D. (2003). Antigens and alternatives for control of *Anaplasma marginale* infection in cattle. *Clin. Microbiol. Rev.*, 16:698–712.

- Kocan, K.M., de la Fuente, J., Blouin, E.F., and Garcia-Garcia, J.C. (2004). *Anaplasma marginale* (Rickettsiales: Anaplasmataceae): recent advances in defining host-pathogen adaptations of a tick-borne rickettsia. *Parasitology*. 129:S285–S300.
- Kordick, S.K., Breitschwerdt, E.B., and Hegarty, B.C. (1999). Co-infection with multiple tick-borne pathogens in a Walker Hound kennel in North Carolina. *J. Clin. Microbiol.*, 37:2631-2638.
- Kuttler, K.L., Graham, O.H., and Trevino, J.L. (1975). The effect of imidocarb treatment of babesia in the bovine and the tick (*Boophilus microplus*). *Res. Vet. Sci.*, 18: 198-200.
- Kuttler, K. L. (1984). *Anaplasma* infections in wild and domestic ruminants: a review. *J. Wildlife Dis.*, 20:12–20.
- Latif, A., 1992. Sustainable control methods for ticks and tick-borne diseases in Africa. In Kategile, A. and Mubi, S. (eds) (1992). Future of livestock industries in East and Southern Africa. Proceedings of the Workshop held at Kadoma Ranch Hotel, Zimbabwe. 20-23 July 1992.
- Mahoney, D. F. (1969). Bovine babesiosis: a study of factors concerned in transmission. *Ann. Trop. Med. Parasitol.*, 63: 1–14.
- McCosker, P.J. (1979). Global aspects to the management and control of ticks of veterinary importance. J.G. Rodriguez (ed.), Recent Advances in Acarology. *Academic Press Inc.*, London. pp. 45–53.

- Minjauw, B., and de Castro, J.J. (2004). *Bos taurus* and *Bos indicus* (Sahiwal) Host resistance to ticks and tick-borne diseases: its role in integrated control. R.F.E. Axford. Retrieved 25 June 2013.
- MOFA. (1990). Ministry of Food and Agriculture, Ghana Medium Term Agricultural Development program. Accra. Ghana.
- MTADP. (1991). Ghana Medium Term Agricultural Development Programme, Accra, Ghana.
- MVM. (2011). Merck Veterinary Manual, 10th ed. USA. Overview of heartwater disease. *Ame. J. Vet. Res.*, 25 June 2013.
- MVM. (2012). Merck Veterinary Manual, 11<sup>th</sup> ed. USA. Overview of heartwater disease. *Ame. J. Vet. Res.*, Retrieved 25 June 2013.
- Mylonakis, M.E., Koutinas, A.F., and Baneth, G. (2004). Mixed *Ehrlichia canis*, *Hepatozoon canis*, and presumptive *Anaplasma phagocytophilum* infection in a dog. *Vet. Clin. Pathol.*, 33:249-251.
- Nadelman, R.B, Horowitz, H.W., Hsieh, T.C., Wu, J.M., and Agüero-Rosenfeld, M.E. (1997). Simultaneous human granulocytic ehrlichiosis and Lyme borreliosis. *N Engl. J. Med.*, 337: 27–30.
- Ngere, L.O. (1975). The improvement of livestock production in Nigeria through breeding. *Nigerian J. Anim. Prod.*, 2(1):36-43.

- Nicholson, M.C., and Mather, T.N. (1996). Methods for evaluating Lyme disease risks using spatial geographic information systems and geospatial analysis. *J. Med. Entomol.*, 33(5): 20 – 711.
- Nolan, J. (1990). Acaricide resistance in single and multi-host ticks and strategies for control. *Parasitol.*, 32 : 145–153.
- Norval, R.A.I., Perry, B.D., and Young, A.S. (1992). The epidemiology of theileriosis in Africa. *Academic press*, London. Retrieved 20 June 2012.
- Ntiamo-Baidu, Y., Carr-Saunders, C., Matthews, B. E., Preston, P.M., and Walker, A. R. (2004). An updated list of ticks of Ghana and an assessment of the distribution of the ticks of Ghanaian wild mammals in vegetation zones. *B. Entomol. Res.*, 94:245-260.
- Nuttall, G.H. F. (1905). "Ticks and tick-transmitted diseases". *Transac. Epidemio. Soc., London*. 24: 12–26.
- Nyarko, E., Grab, D. J. and Dumler, J. S. (2006). *Anaplasma phagocytophilum* – infected neutrophils enhance transmigration of *Borrelia burgdorferi* across the human blood brain barrier in vitro. *Int. J. Parasitol.*, 36 (5):601-605.
- OIE (2009). World Organisation for Animal Health. - Terrestrial Animal Health Code. OIE, Paris. Retrieved 25 June 2013.
- Okuthe, O .S., and Buyu, G. E. (2006). Prevalence and incidence of tick-borne diseases in smallholder farming systems in the western-Kenya highlands. *Vet. Parasitol.*, 141: 307 – 312.

- Olutogun, O. (1976). Reproductive performance and growth of N'Dama and Keteku cattle under ranching conditions in the Guinea Savannah of Nigeria. *Ph.D. Thesis, Univ. Ibadan, Nigeria*. pp. 126.
- Pal, U., Montgomery, R. R., Lusitani, D., Voet, P., Weynants, V., Malawista, S.E., Lobet, Y., and Fikrig, E. (2001). Inhibition of *Borrelia burgdorferi*-tick interactions in vivo by outer surface protein A antibody. *J. Immunol.*, 166 (12):7398-7403.
- Palmer, R.E., Inoue, T., Sherwood, D.R., Jiang, L.I., and Sternberg, P.W. (2002). *Caenorhabditis elegans cog-1* locus encodes GTX/Nkx6.1 homeodomain proteins and regulates multiple aspects of reproductive system development. *Dev. Biol.*, 252: 202–213.
- Pappalardo, B.L., Correa, M.T., and York, C.C. (1997). Epidemiologic evaluation of the risk factors associated with exposure and seroreactivity to *Bartonella vinsonii* in dogs. *Am. J. Vet. Res.*, 58:467-471.
- Piesman, J., and Happ, C. M. (2001). The efficacy of co-feeding as a means of maintaining Pathogens: comparative analysis of published records. *Exp. Appl. Acarol.*, 46 (1-4):29-41.
- Potgieter, F. T., Sutherland, B., and Biggs, H. C. (1981). Attempts to transmit *Anaplasma marginale* with *Hippobosca rufipes* and *Stomoxys calcitrans*. *Onderstepoort J. Vet. Res.*, 48:119-122.

- Potgieter, F. T., and Van Rensburg, L. (1982). The effect of incubation and prefeeding of infected *Rhipicephalus simus* nymphae and adults on the transmission of *Anaplasma marginale*. *Onderstepoort J. Vet. Res.*, 49:99-101.
- Purnell, R.E. (1978). Theileria annulata as a hazard to cattle in countries in the northern Mediterranean littoral. *Vet. Sci. Commun.*, 2: 3–10.
- Randolph, S.E. (2009). Epidemiological Consequences of the Ecological Physiology of Ticks. *Adv. Insect. Physiol.*, 37:297-339
- Read, A. F., and L. H. Taylor. (2001). The ecology of genetically diverse infections. *Science*, 292:1099–1102.
- Riek, R. F. (1962). Studies on the reactions of animals to infestation with ticks. VI Resistance of cattle to infestation with the tick *Boophilus microplus*. *Australian J. Agric. Res.*, 13:532-551.
- Ristic, M. (1977). Bovine anaplasmosis. In J. Kreier (ed.), *Parasitic Protozoa*, vol. 4. *Academic Press, Inc.*, New York. pp. 235-249.
- Ristic, M., Sibinovic, S., and Welter, C. J. (1968). An attenuated *Anaplasma marginale* vaccine. In *Proceedings of the 72nd Annual Meeting of the United States Livestock Sanitary Association*, USA. pp. 56-69.
- Roberts, J. A. (1968a). Acquisition by the host of resistance to the cattle tick, *Boophilus microplus* (Canestrini). *J. Parasitol.*, 54:657-662.

- Roberts, J. A. (1968b). Resistance of cattle to the tick *Boophilus microplus* (Canestrini). II. Stages of the parasite against which resistance is manifest. *J. Parasitol.*, 54:667-673.
- Rouse, J.E. (1970). *World cattle*. II: Cattle of Africa and Asia. Oklahoma City, OK, USA, *Univ. of Oklahoma Press*. Pp. 1046.
- Rubaire-Akiiki, C., Okello-Onen, J., Nasinyama, G. W., Vaarst, M., Kabagambe, E. K., Mwayi, W., Musunga, D., and Wandukwa, W. (2004). The prevalence of serum antibodies to tick-borne infections in Mbale district, Uganda: the effect of agro-ecological zone, grazing management and age of cattle. *J. Insect. Sci.*, 4:8. Retrieved May 22, 2009 <http://www.insectscience.org/4.8>.
- Saeed, A. M., Ward, P. N., Light, D., Durkin, J. W., and Wilson, R. T. (1987). *Characterization of Kenana cattle at Um Banein, Sudan. ILCA Research Report* 16. ILCA (International Livestock Centre for Africa), Addis Ababa, Ethiopia. pp. 46.
- Salmon, D. E., and Smith, T. (1896). Infectious diseases of cattle: southern cattle fever (Texas fever) *In* Special report on diseases of cattle and on cattle feeding. USDA Bureau of Animal Industry. *Gov. Printing Office*, Washington. pp. 428-438.
- Schmidt, H. E. (1937). Anaplasmosis in cattle. *J. Am. Vet. Med. Assoc.*, 90:723-736.

- Sedaghat, M.M., Shemshad, K., Kamali, K., Charrahy, Z., Biglarian, A. and Rafinejad, J. (2013). The Eco-Epidemiology of Tick-borne Hemoparasitic Diseases Using Geographic Information Systems. *Zahedan J. Res. Med. Sci.*, 15 (12) 17-22.
- Seifert, G. W. (1971). Variations between and within breeds of cattle in resistance to field infestation of the cattle tick *Boophilus microplus*. *Aust. J. Agric. Res.*, Australia. 22:159-168.
- Seifert, G. W. (1984). Selection of beef cattle in Northern Australia for resistance to the cattle tick (*Boophilus microplus*); research and application. *Prev. Vet. Med.*, 2: 553-558.
- Shaban.S. (2012). Molecular detection of *Anaplasma marginale* infection and vector ticks in some locations in Ghana. *MPhil. Anim. Sci. Thesis*, Univ. of Ghana, Legon.
- Sonenshine, D.E., Kocan, K.M., and de la Fuente, J. (2006). Tick control: further thoughts on a research agenda. *Trends Parasitol.*, 22: 550–551.
- Stafford, K. C., Cartter, M. L., Magnarelli, L. A., Ertel, S.H. and Mshar, P. A. (1998). Temporal correlations between tick abundance and prevalence of ticks infected with *Borrelia burgdorferi* and increasing incidence of Lyme disease. *J. Clin. Microbiol.*, 36:1240-1244.
- Stephen, J., Swanson, Neitzel, D., Kurt, D., Reed and Belongia, E.A. (2006). Co-infections Acquired from Ixodes Ticks. *Clin. Microbiol. Rev.*, 19(4):708-727.

- Stich, R. W., Kocan, K. M., Palmer, G. H., Ewing, S. A., Hair, J. A., and Barron, S. J. (1989). Transstadial and attempted transovarial transmission of *Anaplasma marginale* by *Dermacentor variabilis*. *Am. J. Vet. Res.*, 50:1377-1380.
- Suksawat, J., Pitulle, C., and Arraga-Alvarado, C. (2001). Coinfection with three *Ehrlichia* species in dogs from Thailand and Venezuela with emphasis on consideration of 16S ribosomal DNA secondary structure. *J. Clin. Microbiol.*, 39:90-93.
- Susannah, F. L. (2008). "Bug vs Bug: How do mosquitoes survive deadly viruses unscathed?" US Center for disease control, U.S.A. *Scie. Ame. Mag.*, pp.3-5.
- Sutherst, R.W., and Maywald, G.F. (1985). A computerized system for matching climates in ecology. *Agric. Ecosyst. Environ.*, 13(3-4): 281-299.
- Swai, E.S., Karimuribo, E.D., French, N.P., Fitzpatrick, J.L. Bryant, M.J., Kambarage, D.M., and Ogden, N.H. (2007). Seroprevalence of *Babesia bigemina* in smallholder dairy cattle in Tanzania and associated risk factors. *J. South Afr. Vet. Assoc.*, 78: 15–20.
- Swanson, S.J., Neitzel, D., Reed, K.D., and Belongia, E.A. (2006). "Coinfections acquired from ixodes ticks". *Clin. Microbiol. Rev.*, 19 (4): 27–708.
- Taylor, K.A. (1998). Immune responses of cattle to African trypanosomes: protective or pathogenic? International Livestock Research Institute. Nairobi, Kenya. *Int. J. parasitol.*, 28: 219 - 240.

- Theiler, A. (1910). *Anaplasma marginale* (gen. and spec. nov.). The marginal points in the blood of cattle suffering from a specific disease. In: Report to the Government, Transvaal, South Africa, *Vet. Bact. Dept. Agric.*, pp. 7–64.
- Theiler, A. (1911). Further investigations into anaplasmosis of South African cattle . In 1st Report of the Director of Veterinary Research, Department of Agriculture of the Union of South Africa. pp. 7-46.
- Thompson, F. L., Hoste, B., Vandemeulebroecke, K., and Swings, J. (2001). Genomic diversity amongst *Vibrio* isolates from different sources determined by fluorescent amplified fragment length polymorphism. *Syst. Appl. Microbiol.*, 24: 520–538.
- Todorovic, R.A., Vizcaino, O.G., Gonzalez, E.F., and Adams, L.G. (1973). Chemoprophylaxis (Imidocarb) against *Babesia bigemina* and *Babesia argentina* infections, *Am. J. Vet. Res.*, 39:1153-1161.
- Torchin, M. E., and Lafferty, K. D. (2003). Introduced species and their missing parasites. *Nature*, 421: 628–630.
- Trail, J .C. M., and Gregory, K. E. (1981). Characterization of the Boran and Sahiwal breeds of cattle for economic characters. *J. Ani. Sci.*, 52:1286-1293.
- Tuttle, A.D., Birkenheuer, A.J., and Juopperi ,T. (2003). Concurrent bartonellosis and babesiosis in a dog with persistent thrombocytopenia. *J. Am. Vet. Med. Assoc.*, 223:1306-1310, 1280-1281.

- Uilenberg, G. (1981). Heartwater disease. In: Diseases of cattle in the tropics, eds. Miodrag Ristic and Ian McIntyre. The Hague, Netherlands. *Martinus Nijhoff publishers*, pp. 345-360.
- Utech, K. B., Wharton, R. H., and Kerr, J. D. (1978). Resistance to *Boophilus microplus* in different breeds of cattle. *Aust. J. Agric. Res.*, Australia. 29:885-895.
- Wagland, B. M. (1975). Host resistance to cattle tick *Boophilus microplus* in Brahman (*Bos indicus*) cattle. I. Responses of previously unexposed cattle to four infestations with 20,000 larvae. *Aust. J. Agric. Res.* Australia. 26:1073-1080.
- Walker, J.B., and Olwage, A. (1987). The tick vectors of *Cowdria ruminantium* (Ixodoidea, Ixodidae, genus *Amblyomma*) and their distribution. *Onderstepoort J. Vet. Res.*, 54: 353–379.
- Walker, A. R., and Koney, E. B. M. (1999). Distribution of ticks (Acari: Ixodida) infesting domestic ruminants in Ghana. *Bull. Entomol. Res.*, 89:473-479.
- Walker, B.H, Holling, C.S., Carpenter, S.R., and Kinzig, A. (2004). Resilience, adaptability and transformability in social–ecological systems. *Ecology and Society* 9(2):5.
- Wall, R. and Shearer, D. (2001). "Ticks (Acari)". *Veterinary Ectoparasites: Biology, Pathology, and Control*. John Wiley & Sons. pp. 55–82.
- Wharton, R .H., Hary, K. R. S., Wilkinson, P. R., Utech, K. B., and Kelley, B. M. (1969). A comparison of cattle tick control by pasture spelling, planned dipping, and tick resistant cattle. *Aust. J. Agric. Res.*, Australia. 20:783-793.

- Wharton, R .H., Utech, K. B. W., and Turner, H. G. (1970). Resistance to the cattle tick, *Boophilus microplus* in a herd of Australian Illawarra Shorthorn cattle: Its assessment and heritability. *Aust. J. Agric. Res.*, Australia. 21:163-181.
- Wilkinson, P. R. (1962). Selection of cattle for tick resistance, and the effects of herds of different susceptibility on *Boophilus* populations. *Aust. J. Agric. Res.*, Australia. Retrieved 25 June 2013.
- Willadsen, P., and Kemp, D.H. (1988). Vaccination with concealed antigens for tick control. *Parasitol. Today*. 4(7):196–198.
- Willadsen, P. (2006). Tick control: thoughts on a research agenda. *Vet. Parasitol.*, 138: 161–168.
- Wimberly, M. C., Yabsley, M. J. , Baer, A. D. , Dugan, V. G. and Davidson, W. R. ( 2008). Spatial heterogeneity of climate and land-cover constraints on distributions of tick-borne pathogens. *Global Ecol. Biogeog*, 17:189-202.
- WTSI (2013). Wellcome Trust Sanger Institute, Genome Research Limited (reg no. 2742969) is a charity registered in England with number 1021457. Retrieved 25 June 2013.
- Young, A.S. (1988). Epidemiology of Babesiosis: In Ristic, M. (ed): Babesiosis of domestic animals and man, *CRC Press*, Boca Raton, Florida. pp: 82-88.
- Young, A.S., Grocock, C.M., and Kariuki, D.P. (1988). Integrated control of ticks and tick-borne diseases of cattle in Africa. *Parasitology*, 96: 403–432.

Zeidner, N.S., Dolan, M.C., and Massung, R. (2000). Coinfection with *Borrelia burgdorferi* and the agent of human granulocytic ehrlichiosis suppresses IL-2 and IFN gamma production and promotes an IL-4 response in C3H/HeJ mice. *Parasite Immunol.*, 22:581-588.

Zintl, A., Mulcahy, G., Skerrett, H.E., Taylor, S.M., and Gray, J.S. (2003). *Babesia divergens*: A Bovine Blood Parasite of Veterinary and Zoonotic Importance. *Clin. Microbiol. Rev.*, 16: 622–636.

## **APPENDIX**

### **APPENDIX 1**

#### **DNA ISOLATION FROM WHOLE BLOOD (QIAGEN PROTOCOL)**

1. Add 4 ml whole blood to 12 ml RBC lysis solution in a 15 ml tube. Invert to mix and incubate at room temperature for 10 mins. Invert again at least once during incubation.
2. Centrifuge at 8,000 rpm for 10 mins. Discard supernatant, leaving behind cell pellet and 100-200µl residual liquid.
3. Vortex the tube vigorously to resuspend the cells in the residual liquid.
4. Add 4 ml of Cell Lysis Solution to the resuspended cells and pipette up and down to lyse the cells.
5. Cool the sample to room temperature.
6. Add 2 ml of Protein Precipitation Solution to cell lysate.
7. Vortex vigorously at high speeds for 20 secs to mix the Protein Precipitation Solution uniformly with the lysate.
8. Centrifuge at 8,000 rpm for 10 mins. The precipitated proteins will form a tight dark brown pellet.
9. Transfer the supernatant containing the DNA (leaving behind the protein pellet) into a 15 ml tube containing 10 ml of 100% Isopropanol.
10. Mix the sample by inverting gently for about 50 times.
11. Centrifuge at 8,000 rpm for 3 mins; the DNA will be visible as a white pellet.

12. Discard the supernatant and drain tube briefly on clean absorbent paper. Add 1 ml of 70% Ethanol and invert tube several times to wash the DNA.
13. Centrifuge at 8,000 rpm for 1 min. carefully use a pipette to remove the DNA (white pellet) from the ethanol, and transfer into another clean tube.
14. Air dry the tube containing DNA in a speed-vac for about 10 mins.
15. Add 500µl of DNA Hydration Solution.
16. Allow DNA to rehydrate overnight at room temperature or incubate at 65°C for 1 hr.
17. Store DNA between -20 °C and -80 °C.

## APPENDIX 2

### CLONING REACTION PROTOCOL

1. Take 1  $\mu$ l salt solution into PCR tube, add 1  $\mu$ l of Topo vector, add 4  $\mu$ l of purified DNA or amplicon and incubate at room temperature for 15 minutes.
2. Add 2  $\mu$ l of the TOPO Clonal reaction into 50 $\mu$ l Competent E.coli cell and incubate on ice for 30 minutes.
3. Heat-shock the cells for 30 seconds at 42°C without shaking.
4. Immediately transfer the tubes to ice.
5. Add 250  $\mu$ l of room temperature S.O.C medium.
6. Cap the tube tightly and shake the tube horizontally (200 rpm) at 37°C for 1 hour.
7. Spread 10-50  $\mu$ l from each transformation on a pre-warmed selective plate. To ensure even spreading of small volumes, add 20  $\mu$ l of S.O.C. medium. We recommend that you plate two different volumes to ensure that at least one plate will have well-spaced colonies.
8. Incubate plates at 37°C overnight.
9. Pick 1 white or light blue colony into LB (Luria-Bertani) broth with 50mg/ml of Cabanicillin and incubate at 37°C overnight to grow in the Stuart Orbital Incubator.
10. Centrifuge at 8500 rpm for 2 minutes to form a pellet.
11. Remove gently and discard the supernatant leaving the pellets.
12. Vortex to dissolve the pellet.
13. Add 250  $\mu$ l of Cell lysis solution or P2 and gently mix.

14. Incubate at room temperature for 5 minutes.
15. Add 350  $\mu$ l of N3 buffer and gently mix.
16. Centrifuge at 1300 rpm for 10 minutes.
17. Discard the supernatant and centrifuge again at 1300 rpm for 1 minute.
18. Discard the solution and keep the spin column.
19. Add 500  $\mu$ l of PB Solution and centrifuge at 1300 rpm for 1 minute.
20. Discard the solution and keep the spin column.
21. Add 750  $\mu$ l of PE buffer and centrifuge at 1300 rpm for 1 minute.
22. Discard the solution.
23. Centrifuge the spin column.
24. Discard the tube containing the solution and transfer the spin column to a newly labeled 1.5 tube.
25. Add 50  $\mu$ l of PCR water.
26. Incubate at room temperature for 1 minute.
27. Centrifuge at 1300 rpm for 1 minute.
28. Discard the spin column and store the plasmid DNA at  $-20^{\circ}\text{C}$ .
29. Take PCR tubes and labeled it accordingly.
30. Pipette 17  $\mu$ l of Master Mix (Buffer 2 $\mu$ l + Enzyme 1 $\mu$ l + Water 14  $\mu$ l) in PCR tubes.
31. Add 3  $\mu$ l of Plasmid DNA.
32. Put it into water bath for 1 hour.
33. Load the Plasmid DNA on gel.
34. View on UV machine.

**APPENDIX 3****ELECTROPHORESIS (TRIS-BORATE 5 X TBE) BUFFER**

1. Concentrated stock solution preparation per litre.
2. Take 700ml of distilled water.
3. Add 54 g of Tris base.
4. Add 27.5 g of Boric acid.
5. Add 20ml of 0.5 M EDTA (pH 8.0).
6. Top it up to get to 1000 ml or 1 litre.

**APPENDIX 4****0.5 M EDTA (pH 8)**

1. Add 186.1g of disodium ethylenediaminetetra acetate .2H<sub>2</sub>O to 800 ml of H<sub>2</sub>O.
2. Stir vigorously on a magnetic stirrer.
3. Adjust the pH to 8.0 with NaOH (20 g of NaOH pellets).
4. Dispense into aliquots and sterilize by autoclaving.

Note: The disodium salt of EDTA will not go into solution until the pH of the solution is adjusted to approximately 8.0 by the addition of NaOH.

**APPENDIX 5****ETHIDIUM BROMIDE (10 mg/ml)**

1. Add 1 g of Ethidium bromide to 100 ml of H<sub>2</sub>O.
2. Stir on a magnetic stirrer for several hours to ensure that the dye has dissolved.
3. Wrap the container in aluminum foil or transfer the solution to a dark bottle.
4. Store at room temperature.

CAUTION: The Ethidium bromide is a powerful mutagen and is moderately toxic.

Gloves should be worn when working with solutions that contain this dye, and a mask should be worn when weighing it out. After use, these solutions should be decontaminated.