MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *ANAPLASMA* HAEMOPARASITES ISOLATED FROM CATTLE AND SHEEP IN HOMABAY COUNTY, KENYA

BY

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DECLARATION

This thesis is my original work and has not been submitted for a degree award in any other University.

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DEDICATION

To God Almighty first, my parents: Mr and Mrs Metinou, my discipler and mentor Engineer and Mrs Lemnyuy, Pastor and Mrs Chumbow, my elder sisters: Kenyo Metinou Lambertine, Metinou Tchinda Esther Ladouce, Dr. Gabriel Oluga Aboge, Prof. Njeruh F. M., Dr. and Mrs Yunga, my brothers: Metinou Djou Firmin, Metinou Takenne Brice, Dr. and Mrs Pelle, Professor Lyna Mukwa Fama Tongo.

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LIST OF ABBREVIATIONS

A	**:**	Adenine
bp	**:**	Base pair
BLAST	**:**	Basic Local Alignment Search Tool
C	**:**	Cytosine
DNA	**:**	Deoxyribonucleic acid
dNTP	**:**	Deoxynucleotide triphosphates
EDTA	**:**	Ethylenediaminetetraacetic acid
G	**:**	Guanine
µl	**:**	Microliter
mg	**:**	Milligram
ml	**:**	Millilitre
mM	**:**	Milli molar
Min	**:**	Minute
ng	**:**	Nanogram
pmol	**:**	Picomole
PCR	**:**	Polymerase Chain Reaction
qPCR	**:**	Quantitative real time PCR
RLB	**:**	Reverse line blot
rRNA	**:**	Ribosomal ribonucleic acid
T	**:**	Thymine
U	**:**	Enzyme unit

ABSTRACT

Anaplasmosis is a cosmopolitan tick-borne disease of great importance in tropical and subtropical regions of sub-Saharan Africa and is caused by *Anapalsma* haemoparasites. The parasite infects a variety of hosts including cattle, sheep, dogs, humans as well as wildlife and affects the health of livestock and human population. The disease causes economic losses in livestock production. Some studies have been carried out on characterization of *Anaplasma* heamoparasites by using molecular techniques in Kenya. However, these studies covered limited regions in Kenya including Homabay County in Western Kenya where the disease is endemic. In addition, previous diagnostic techniques used in Homabay for identification included history, clinical signs, microscopy and serology. These were inadequate for identification of the exact species of *Anaplasma* currently present in Homabay County. Therefore the present study was conducted to identify and characterize of *Anaplasma* heamoparasites infecting cattle and sheep in Homabay County, Kenya. *Anaplasma marginale 16S rRNA* (*Am-16SrRNA*), *Anaplasma marginale msp1b* (*Am-msp1b*), *Anaplasma centrale msp2 (Ac-msp2)* and *Anaplasma centrale 16S rRNA* (*Ac-16S rRNA*) genes were analyzed using bioinformatics tools. The information obtained was used to design specific gene primers by using Primer Quest software of the Integrated DNA Technology (California, USA). The primers were tested using conventional and multiplex PCR reactions with positive and negative control DNA samples. For screening, whole blood samples were collected in vacutainer tubes containing EDTA from 180 animals which included 157 cattle and 23 sheep. The samples were transported to the laboratory facility located at the Department of Public Health, Pharmacology and Toxicology of the University of Nairobi. DNA was extracted using Qiagen's QIAamp DNA Mini kit following the manufacturer's instructions and stored at -20^{o}C.

PCR was conducted and the products were subsequently purified using ZR-96 DNA Clean-up Kit™ and sequenced using the Sanger method to characterize the *Anaplasma species*. The generated sequences were analysed by bioinformatics and were used to construct a phylogenetic tree to determine the genetic diversity of the *Anaplasma species*. As predicted, these primers yielded PCR amplicons of 835 bp for *Am-16S rRNA*, 436 bp for *Ac-16S rRNA*, and 576 bp for *Ac-msp*2. The amplification of *Am-16S rRNA*, *Ac-16S rRNA* and *Ac- msp2* in cattle revealed 9(5.7%), 11(7%) and 3(1.91%) were positive, while in sheep it was 1(4.3%), 9(39.1%) and 2(8.7%) respectively. The overall prevalence from both cattle and sheep was 10 (5.55%), 20 (11.1%) and 5 (2.7%) for the genes *Am-16S rRNA*, *Ac-16S rRNA* and *Ac-msp2* respectively. The *Ac-msp2* and *Ac-16S rRNA* genes were detected in five different isolates from samples labelled; 30A, 108A, 58A, 88A, and 171A. Blast analysis of the *Ac-16S rRNA* amplicons revealed that the gene was homologous to *Anaplasma phagoctophylum* having sequence identities of 99% for cattle and 99% identity to *Anaplasma ovis* in sheep. Three samples revealed homologous sequences to *Anaplasma ovis* in cattle with 99% nucleotide identity. The phylogenetic analysis revealed that the sequences of *Anaplasma centrale 16S rRNA* of Homabay samples clustered together, suggesting that they were genetically related. The Kenyan isolates were also grouped with other isolates from China and USA.

The findings indicated that the novel primer for *Ac-16S rRNA* could amplify specific conserved region of the *16S rRNA* of the *Anaplasma species.* Primers *Ac-msp2* yielded non-specific bands and needed further PCR optimization before use. The results also provided information on possible infection of cattle with *Anaplasma ovis* although further studies are required to confirm this. There was also a possible occurrence of *Anaplasma phagoctophylum* in cattle, necessitating further studies to confirm the role of this zoonotic pathogen in cattle in Homabay Counties.

1.0 INTRODUCTION

1.1 Background

Anaplasmosis is an important tick-borne disease caused by the rickettsia *Anaplasma* species, which are obligate intracellular pathogenic organisms. The main species that are known currently include *Anaplasma centrale*, *A. marginale*, *A. phagocytophilum*, *A. ovis A. bovis* and *A. platys*. However, there are reports of uncultured species of the organisms (Dumler *et al*., 2001; Gofton *et al*., 2015). These six species of *Anaplasma* are known to infect erythrocytes of a variety of mammalian hosts including cattle, sheep, goats, wild ruminants and human (Dumler *et al*., 2001; Dumler *et al*., 2005). *A. marginale* and *A. centrale* are mainly known to infect cattle although there are reports of infection of small ruminants by *A. marginale* (Yousefi *et al*., 2017). *A. bovis* is known to infect cattle whereas *A. ovis* has been reported to infect sheep and goats as well as wild ruminants (Hornok *et al*., 2011). *A. phagocytophilum* is a granulocytotropic bacterium-like organism, which causes non-specific febrile illness in human and a range of animals including ruminants (Ooshiro *et al*., 2008). *A. platys* haemoparasite infects canine species including dogs with the organism having preference for platelets. Therefore, the infection results in canine cyclic thrombocytopenia (Dumler *et al*., 2001; Zobba *et al*., 2015). This pathogen has been also reported in domesticated cats (Lima *et al.*, 2010).

The intracellular rickettsials are transmitted to the mammalian hosts by ticks such as *Ixodes*, *Dermacentor*, *Rhipicephalus* and *Amblyomma* (Paddock and Yabsley, 2007).There is also a possibility of mechanical transmission by fomites and bites from fleas (De la Fuente *et al*., 2001; Kocan *et al*., 2004). The major clinical signs of anaplasmosis include; anemia and jaundice seen as yellowing of the mucus membranes of the eyes and gums. The other signs are incoordinated movements, laboured breathing, abortions in females, decreased milk yield and deaths in some cases with surviving animals becoming persistently infected (Kocan *et al*., 2010; Aubry and Geale, 2011). The infections with *Anaplasma* organisms cause mortalities and morbidities

leading to reduction in yield of milk, meat, as well as other livestock by-products in tropical and sub-tropical regions (Kocan *et al*., 2003). Consequently, there are severe economic losses in dairy and beef industries in many parts of the world (Jongejan and Uilenberg, 2004). In Africa, 983 outbreaks of bovine anaplasmosis have been reported in 14 different countries including Kenya (AU-IBAR, 2011). Indeed, anaplasmosis is ranked among the most common causes of economic losses in dairy and beef industry in Kenya (Kanyari and Kagira, 2000; Wesonga *et al*., 2010).

The diagnosis including detection and molecular characterization of *Anaplasma species* infecting cattle and sheep is critical for the control as well as the prevention of the disease. In Kenya, the disease has been diagnosed in cattle mainly by clinical signs (Kanyari and Kagira, 2000), microscopic examination of blood smears and serology (Karanja *et al*., 2006; Okuthe *et al*., 2006; Kiara *et al*., 2014). A few studies in Kenya have employed molecular methods such as polymerase chain reaction (PCR), reverse line blot hybridization (RLB), quantitative real time PCR (qPCR) and DNA sequencing (Githaka *et al*., 2014; Odongo *et al*., 2003). Detection and molecular characterization of *Anaplasma species* infecting cattle and sheep is an important tool for understanding the nature of the *Anaplasma species* and thus helping in the control of the diseases caused by the bacteria-like microorganisms. In other regions of the world, many studies have reported the detection and molecular characterization of *Anaplasma* organisms infecting cattle and sheep using PCR followed by sequencing and phylogenetic analysis (Adjou *et al*., 2015; Zhang *et al.*, 2016; Noaman *et al*., 2016; Silaghi *et al*., 2011). The genetic characterization has been done using specific primers targeting fragments of genetic markers including *16S rRNA, major surface protein 1 (MSP1b), major surface protein 2 (MSP2), major surface protein 4 (MSP4), major surface protein 5 (MSP5), 18S rRNA, 23S rRNA* (Adjou *et al.*, 2015; Zhang *et al*., 2016; Silaghi *et al*., 2011). In Kenya, a few of these studies have used specific genetic

markers to detect and characterize *Anaplasma* pathogens infecting cattle (Adjou *et al*., 2015; Matei *et al*., 2016; Wamuyu *et al*., 2015). In the current study, various species of *Anaplasma* were detected using novel specific primers designed to target fragments of *16S rRNA*, *msp1b* and *msp2*. The amplified gene fragments were sequenced and the bioinformatics information used to characterize the *Anaplasma* isolates infecting cattle and sheep in Homabay County.

1.2 Problem statement

Anaplasma organisms cause serious disease in cattle and sheep leading to severe economic losses in livestock production systems worldwide. In Kenya, the disease is ranked among the most common causes of economic losses in dairy and beef industry (Kanyari and Kagira, 2000; Wesonga *et al.*, 2010). The economic losses are in terms of high morbidity and mortality of livestock as well as decreased meat and milk yield. In order to control and prevent anaplasmosis, it is important to detect and characterize the molecular profiles of *Anaplasma* species so that appropriate methods for molecular diagnosis of the infection can be developed. A number of studies have been done in other parts of the world for identification and characterization of the molecular profiles of *Anaplasma* organisms infecting cattle and sheep (Scharf *et al*., 2011). These studies have generated a range of information that has made it easy to understand the molecular epidemiology of these pathogens in other regions. Nevertheless, such studies are still limited or lacking in Kenya, meaning that the genetic profiles of *Anaplasma* species infecting cattle and sheep in Kenya, especially in Homabay County is still largely unknown. Therefore, this study was done to develop PCR test in conjunction with sequencing to identify and characterize the *Anaplasma* isolates infecting cattle and sheep reared in Homabay County.

1.3 Justification of the study

Anaplasmosis is ranked among the most common causes of economic losses in dairy and beef industry in Kenya (Kanyari and Kagira, 2000; Wesonga *et al*., 2010). However, the diagnosis of *Anaplasma* infection is still limited in Kenya or lacking in other parts such as Homabay. This is because the former diagnostic techniques employed in this regards focused mainly on history, clinical signs and serology. These couldn't give a precise account of the disease in the field, and the species incriminated. Furthermore its rapidely growing public health implications worldwide calls for concern, as the contact rate at Human-livestock interface in Homabay is high. This research focused on the identification and characterization of *Anaplasma species* isolated from cattle and sheep in Homabay County, Kenya. Therefore, the current study has provided valuable preliminary data on the detection and genetic diversity of *Anaplasma* haemoparasites. This may go a long way in assisting in the understanding of the molecular epidemiology of *Anaplasma* infections in cattle and sheep in the County. Additionally, the data generated from this study provides a basic knowledge about the actual *Anaplasma species* infecting cattle and sheep in these areas. The study also provides some basic information on the genetic diversities of *Anaplasma species* infecting cattle and sheep in the above County. This should provide a basis for further studies on molecular epidemiology of the disease in the current study areas. Moreover, the novel PCR primers pairs generated in this study have provided useful molecular tools that can be further optimized and validated for subsequent improvement of molecular diagnosis of bovine and ovine anaplasmosis. This will eventually contribute to accurate diagnosis resulting into improved control of the disease, thereby reducing economic losses in the livestock industry, ensuring food security and improving livelihoods of livestock farmers.

1.4 Research hypothesis

i. Cattle and sheep reared in Homabay County are not infected with various species of *Anaplasma* organisms.

ii. The *Anaplasma species* infecting cattle and sheep in Homabay County are not genetically identical to the other isolates infecting the cattle and sheep reared in the other regions of world.

1.5 General objective

To identify and characterize the molecular profiles of *Anaplasma species* infecting cattle and sheep in Homabay County, Kenya.

1.6 Specific objectives

i. To analyze selected genes and use them to design PCR primers (genetic markers) for identification of *Anaplasma species* isolated from cattle and sheep in Homabay County.

ii. To identify *Anaplasma species* that infect cattle and sheep in Homabay County.

iii. To analyse the genetic diversity of *Anaplasma species* from cattle and sheep in the above County.

2.0 LITERATURE REVIEW

2.1 *Anaplasma* haemoparasites

2.1.1 Taxonomic classification

The *Anaplasma* genus of the order *Rickettsiales* is an intracellular obligate organisms belonging to the *Anaplasmataceae* family, found exclusively in the membrane-bound vacuole within the cytoplasm of the host cell (Kocan *et al*., 2004). In the recent past, the *Anaplasma* organisms have been broadly reclassified to include the following species; *A. phagocytophilum* (previously known as *Ehrlichia phagocytophilum*, *E. equi)*, *A. centrale* (called a while back *A. marginale subspecies centrale*), *A. marginale* (type species), *A. bovis* (in the past called *Ehrlichia bovis*), *A. platys* (earlier known as *Ehrlichia platys*) *A. ovis*, (Dumler *et al*., 2001; Kocan *et al*., 2004). This reclassification led to the separation of *Erhlichia* organisms, from the genus *Anaplasma.* The current reclassification of *Anaplasma* organisms is summarized in the Table 1 below.

Table 1: Reclassification of *Anaplasma* organisms based on biological characteristics (Kocan *et al*., 2003).

Taxonomy
Family: *Anaplasmataceae*
Order: *Rickettsiales*
Genus: *Anapalsma marginale* (species types)
Anaplasma centrale
Anaplasma ovis
Anaplasma bovis (formerly *Erhlichia bovis*)
Anaplasma phagocytophilum (*E. phagocytophilum*, HE age)
Anaplasma platys (formerly *Erhlichia platys*)

2.1.2 Cellular morphology

Anaplasma are obligate intracellular bacteria like organisms the entire members of the family *Anaplasmataceae*. They have in common, identical characteristic morphology. Morphologically, the *Anaplasma genus* are small gram negative, often coccoid to ellipsoidal and pleomorphic organisms residing in vacuoles that are in the cytoplasm of erythrocytes of mammalian hosts (Dumler *et al*., 2001). The organisms occur independently, regularly in firm packed formations of cells termed morulae. When present in domestic ruminants or mammals, they either occur in haematopoetic cells as immature or mature haematopoietic cells, especially myeloid cells as well as red blood cells or mononuclear phagocyte system circulating in peripheral blood (Dumler *et al*., 2001). *A. centrale* and *A. marginale* appear roundish and as blue cytoplasmic inclusions when stained with Grünwald Giemsa (Ismail *et al*., 2010). These inclusions are seen in the monocytes, granulocytes and more impotantly the neutrophils (Rikihisa, 2011**)**. *A. centrale* is found in the center of erythrocytes whereas *A. marginale* is usually observed at the margin of the infected erythrocytes on microscopy (De Waal, 2000). *A. phagocytophilum* is a granulocytotropic bacterium-like organism that appears as roundish and purplish structures in granulocytes of human and animals (Ooshiro *et al.,* 2008).

2.1.3 Life cycle

The life cycles of *A. marginale* and *A. centrale* are maintained by tick (about 19 species of ticks) feeding cycle (Kocan *et al*., 2004). The arthropod tick is infected by feeding on blood from animals infected with *Anaplasma* organisms. Infection of the tick has a higher probability in acute infection in comparison the chronic phase (Wormser *et al*., 2006). Infected red blood cells are therefore consumed in the form of blood meal by the tick, which is an adequate source of *A. marginale* infection in the cells of the tick gut. Thereafter, massive proliferation occurs within other tissues and the salivary glands (kocan *et al*., 1992).

A. marginale level in mature tick male *Dermacentor andersoni* could approximately reach 10^5 in each of the salivary gland no matter the level of rickettsemia when feeding (Eriks *et al*., 1993). Subsequently, the *Rickettsiae* are disseminated to the vertebrate host through the salivary gland of the tick when the tick feeds on the host (Kocan *et al*., 1992). After infection of the tick, *A. marginale* grow inside the vacuoles, giving rise to colonies. The vegetative or reticulated cell is the first form observed in *A. marginale's* colonies which divides by binary fission (figure 1). This results in multiple large colonies with hundreds of pathogens. These forms (reticulated or vegetative) therefore are transformed as packed forms (0.5-0.8 µm) (Figure 1). These packed forms are the infective forms. They are able to subsist for a short while out of the cells (Kocan *et al*., 2008; Kocan *et al*., 2003). Domestic ruminants (cattle and sheep) get the infection when, during feeding, the dense form is transmitted to the host through the salivary glands (Kocan *et al*., 2004; Kocan *et al*., 2003) **(**Figure 2**)**.

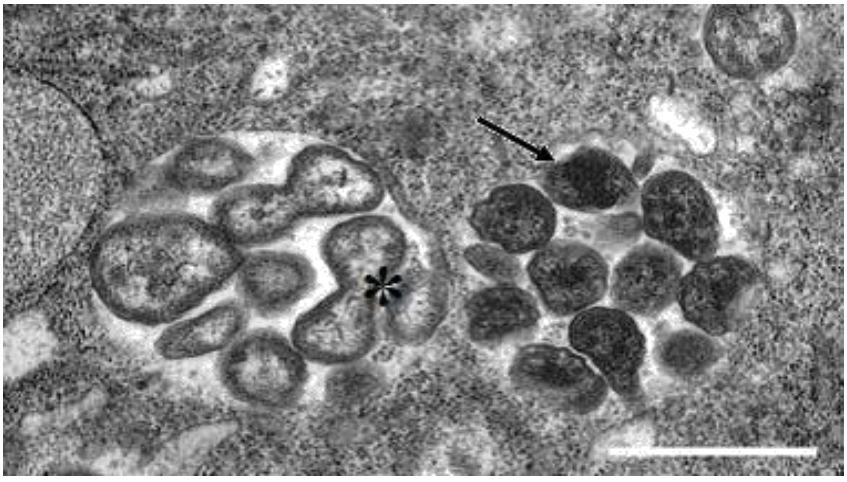

Figure 1: Electron micrograph of the developmental stages of *A. marginale* within colonies in tick cells. Reticulated forms within a colony divide by binary fission (asterisk), dense forms (arrow). Bar =1 mm taken from Kocan *et al.*, 2004**).**

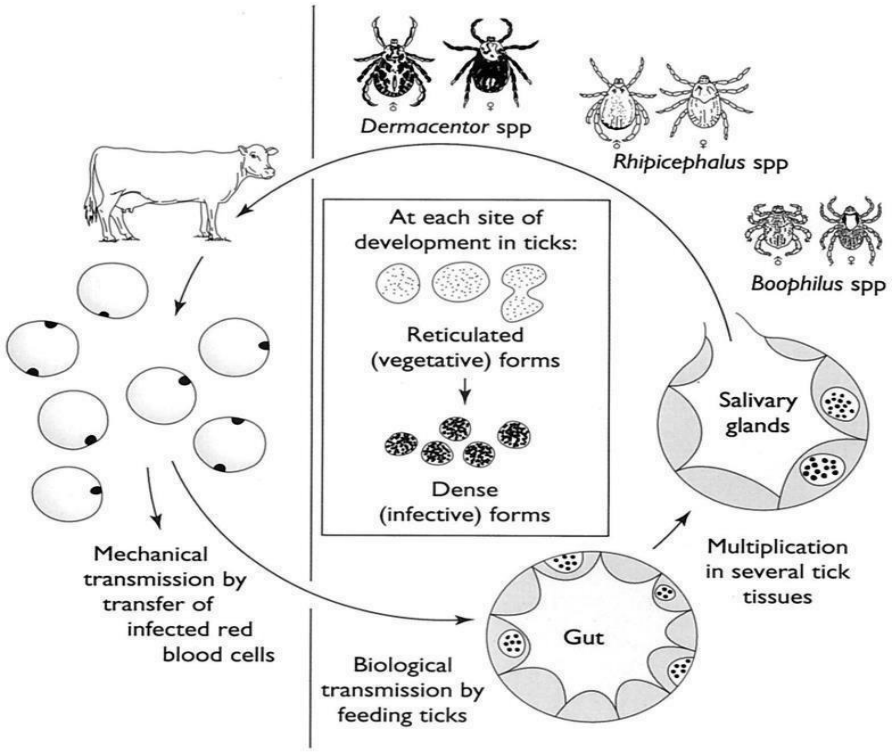

Figure 2: Developmental cycle of *Anaplasma species* in cattle and ticks (Kocan *et al*., 2003).

2.1.4 Ticks vectors

Various ticks-vectors are known to support the survival of the *Anaplasma* organisms. For example; *A. marginale* has been shown to be transmitted by 19 different species of ticks experimentally. These ticks include: *Argas persicus, Boophilus calcaratus, Ornithodoros lahorensis, Rhipicephalus decoloratus, Rhipicephalus annulatus, Rhipicephalus microplus, Dermacentor albipictus, Dermacentor hunteri, Dermacentor variabilis, Dermacentor occidentalis, Dermacentor andersoni, Hyalomma rufipes, Hyalomma excavatum, Ixodes ricinus, Ixodes scapularis, Rhipicephalus evertsi, and Rhipicephalus simus, Rhipicephalus sanguineus, Rhipicephalus bursa* (Kocan *et al*., 2004; Marchette and Stiller, 1982). In countries such as Australia and Africa, *Rhipicephalus* species are known as vectors of anaplasmosis whereas other species of *Dermacentor* ticks are vectors of anaplasmosis in USA (Aubry and Geale, 2011). The multihost ticks found are Africa is thought to be the biological vector of *A. centrale*.

2.1.5 Mammalian hosts

Cattle, sheep, goats, dogs, cats, human and a range of wild animals including buffalo, deer, leopard, raccoons and antelopes are mammalian hosts of *Anaplasma* organisms (Hapunik *et al*., 2011; Overzier *et al*., 2013). However, to date, knowledge about the specific hosts' reservoir of *A. phagocytophilum* in both humans and animals is still unclear. According to Baráková *et al*. (2014), there is a greater degree of host tropism existing in *A. phagocytophilum* isolates. *A. bovis* was firstly discovered in cattle (Donatien and Lestoquard, 1936). Since then, it has also been known to infect domestic and wild animals in Italy, Brazil, South Africa, India, China, Korea, Spain, Japan, America and Tunisia (Said *et al*., 2015). The major reservoir of *A. bovis* pathogen includes cattle and goats (Goethert and Telford, 2003). Other mammalian hosts of *Anaplasma species* include: Korean water deer (Kang *et al*., 2011), Brazilian brown brocket deer (Silveira *et al*., 2012), roe deer (Jin et al., 2012), dog (Sakamoto *et al*., 2010) and marsh deer (Silveira *et al*., 2012).

A. ovis infects a wide range of domestic animals including: sheep, goats as well as wild ruminants worldwide with the organism being mainly intraerythrocytic pathogen (Yang *et al*., 2015; Lee *et al*., 2015). Dairy cattle have been reported to host *A. ovis* in Greece (Giadinis *et al*., 2015). *A. marginale* occurs mostly in cattle, although buffaloes, deer, bighorn sheep, pronghorn antelope, elk, rocky mountain, elk, giraffes and bison also host the organism. *Anaplasma platys* has been found in dog, cat, cattle, camel (Eygelaar *et al*., 2015; Silveira *et al*., 2012) and wild ruminants (Dahmani *et al*., 2015; Lima *et al*., 2010; Qurollo *et al*., 2014). In carnivores such as wolves, jackals and foxes (Sainz *et al*., 2015), the organism has not been detected. There are also report of *Anaplasma platys* infection human by Maggi *et al*. (2013); Arraga-Alvarado *et al*. (2014) in United States and Venezuela

2.2 Anaplasmosis

2.2.1 Aetiology

Anaplasmosis is caused by *Anaplasma* organisms. In both animal and humans it is caused by *A. marginale*, *A. centrale* and *A. phagocytophilum, A. platys, A. bovis,* (Baráková *et al*., 2014; Eygelaar *et al*., 2015). *Anaplasma* species infect erythrocytes of cattle, sheep, goats, wild ruminants and human, resulting in a disease called anaplasmosis (Kang *et al*., 2011; Overzier *et al*., 2013). Recently, *A. phagocytophilum, A. platys* and *A. ovis* have been recovered from human (Arraga-Alvarado *et al*., 2014; Giadinis *et al*., 2015; **Hosseini**-Vasoukolaei *et al*., 2014). In cattle, *A. marginale* causes bovine anaplasmosis. In particular, the species involved here include *A. marginale* and *A. centrale* although the latter species more often results in mild disease (Aubry and Geale, 2011). Goethert and Telford (2003) established that cattle and small ruminants are also infected by *A. bovis* and it is thought that small ruminants may act as reservoir of this *Anaplasma* species (**Hosseini**-Vasoukolaei *et al*., 2014). In calves, the organism causes monocytic anaplasmosis, which involves infection of the circulating monocytes (Uilenberg, 1997; Munderloh *et al*., 2004). *A. phagocytophilum* causes non-specific febrile disease in humans and in a range of animals including cattle, sheep, goats and dogs (Ooshiro *et al*., 2008; Baráková *et al*., 2014). Infectious cyclic anaplasmosis is caused by *A. marginale subsp centrale*, *A. marginale,* and *A. platys* (Rymaszewska and Grenda, 2008). Which infects platelets principally.

Additionally, *A. ovis* (Gharbi *et al*., 2015) causes disease in sheep, goats and deer, but does not appear to be infectious to cattle (Aubry and Geale, 2011). The organism has been established in both wild and farm animals in many regions across the world (García Pérez *et al*., 2015; Said *et al*., 2015).

2.2.2 Transmission

Anaplasmosis is transmitted biologically by ticks, after ingesting infected erythrocytes. Thereafter, *Anaplasma* organism divide inside the tick's gut and then migrate to the salivary gland, where they are transmitted to non-infected animals via saliva after a tick bite. The transmission can also occur mechanically by biting flies or fomites contaminated with blood. It can also be transmitted transplacentally from cow to foetus (Costa *et al*., 2016; Molad *et al*., 2006). Biological transmission is the most common mode of transmission worldwide with about twenty tick species serving as vectors (De La Fuente *et al*., 2004; De La Fuente *et al*., 2005). However, mechanical transmission is considered to be more important in South America (Scoles *et al*., 2008). The one-host-tick *R. (B.) microplus* is the main vector of *A. marginale* in Brazil (Kessler and Schenk, 1998; Ribeiro and Passos, 2002).

Mechanical transmission of *Anaplasma* frequently occurs via fresh erythrocytes from contaminated needles, dehorning-saws, nose-tongs, tattooing equipment, ear-tagging devices and castration instruments. Biting flies also transmit *Anaplasma* organisms and they include: *Tabanus, Stomoxys, Culex* and *Aedes* (Ewing, 1981; Kocan *et al*., 2015). Transplacental transmission of *A. marginale* from cow to calf during gestation has been documented (Grau *et al*., 2013). Furthermore, transplacental transmission of *A. marginale* and *A. centrale* has been reported in South Africa during the first, second and third trimester of gestation (Potgieter and Van Rensburg, 1987). In this report, Kocan *et al*. (2003) suggested that transplacental transmission of *A. marginale* may contribute to the epidemiology of this disease in some regions.

2.2.3 Pathogenesis

Anaplasma species in cattle infect erythrocytes, propagating in a bovine endothelial cell line (Munderloh *et al*., 2004). *A. marginale* enters erythrocytes by endocytosis and resides within small membrane-bound inclusions, referred to as initial bodies, where it divides by binary fission

(Kocan *et al*., 1978). The membrane-bound vacuole is derived from the erythrocyte membrane and can contain four to eight organisms. In acute disease, multiple infections of single erythrocytes are observed. Upon invasion of red blood cells (RBC), *Anaplasma* species start dividing into six initial bodies and enlarges within its outer thin membrane (Figure 3). The multiplication of the organism causes the red blood cells to rupture and subsequently the membrane, releasing initial bodies into the blood stream in order to invade surrounding erythrocytes.

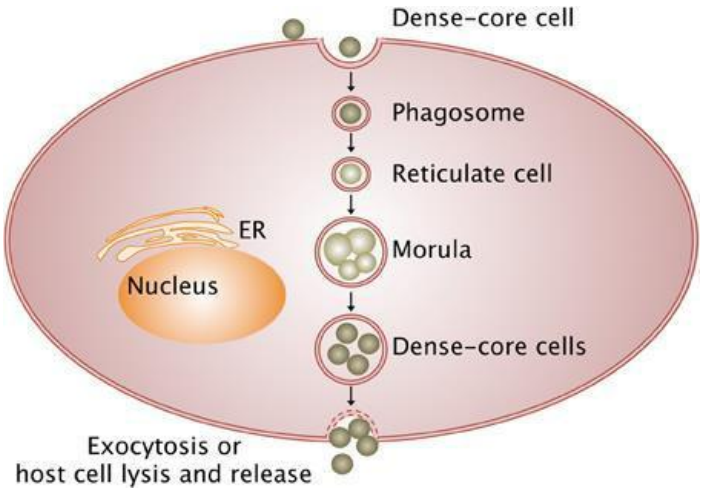

Figure 3: A diagram illustrating the pathogenesis of thev intracellular *Anaplasma* organisms (Pruneau *et al*., 2014).

2.2.4 Clinical signs

The incubation period of anaplasmosis ranges from 7 to 60 days (Kocan *et al.*, 2003). Thereafter, the following clinical symptoms appear; fever, weight loss, abortion, lethargy, anorexia, lower milk production, retardation of growth and death (Sanchez *et al*., 2016). The disease severity is associated with the degree of anemia and includes pallor of the mucous membranes and increased heart and respiratory rates. The packed cell volume drops accordingly with increasing parasitaemia (Kocan *et al*., 2010). For calves below six months, the disease is rare but between the ages of six to twelve months, they usually develop mild disease. Animals ranging from one to

two years of age suffer from acute but rarely fatal disease. Adults over two years suffer from acute disease usually causing death (Aubry and Geale, 2011).

2.2.5 Biochemical and haematological changes of anaplasmosis

Upon infection, the *Anaplasma* organisms multiply to a considerable level causing hemolytic crisis (Allison and Meınkoth, 2010). Thereafter, with the increased level of infected erythrocytes, phagocytosis sets in within endothelial cells. This eventually leads to haemolytic anaemia and icterus (De UK *et al*., 2012). The high levels of biochemical enzymes of serum such as aspartate aminotransferase (AST), gamma-glutamyl transferase (GGT) and alkaline phosphatase (ALP) are good indicators of hepatic disfunction (Coşkun *et al*., 2012). Haematological and biochemical alterations are the indicators of severity of disease in dogs, cats and other animals. The common hematological and biochemical findings of *Anaplasma* species include; thrombocytopenia, anemia and / or high level of liver enzymes, Leucopaenia with marked neutropenia, especially dogs and cats (Dondi *et al*., 2014; Savidge *et al*., 2016). In cattle, infection with *A. marginale* has been recently shown to be correlated with high levels of salicylic acid in the serum, especially during the acute phase response (Nazifi *et al*., 2012).

2.2.6 Diagnosis

Anaplasmosis diagnosis is based on history, the clinical signs, and necropsy. Confirmatory diagnosis is done by laboratory tests, which include: microscopic examination and molecular detection. Serological tests may be used for screening purposes (Aubry and Geale, 2011).

2.2.6.1 Direct microscopic examination

Acute anaplasmosis is best confirmed by direct microscopic examination, which includes preparation of blood or organ smears, staining with giemsa and then examination by microscopy (Ribeiro and Passos, 2002). Tail and jugular veins may be used to ascertain *Anaplasma* species, because the organism is evenly distributed throughout the blood (Böse *et al*., 1995).

Microscopic examination has also proved to be ideal for pathogen detection (Ribeiro and Passos, 2002). Impression smears prepared from organ during necropsy may be used for the detection of the organisms (Böse *et al*., 1995). On microscopic examination, *Anaplasma* species are observed as dense, roundish, intra-erythrocytic bodies seen either on or close to the margin of the red blood cells especially for *A. marginale* (OIE, 2008).

2.2.6.2 Serological tests

Various serological tests are used to detect specific antibodies in epidemiological studies, involving the screening of carrier animals. The complement fixation test (CFT test), card agglutination test (CAT), indirect fluorescent antibody test (IFAT), indirect ELISA, competitive ELISA (cELISA), (iELISA) and dot ELISA are examples of serological tests that have been used to screen *Anaplasma* antibodies in animals (Aubry and Geale, 2011). Two of the serological assay which have been adopted for identification of animals that are infected include; cELISA and the CAT (OIE, 2008; De La Fuente *et al*., 2005). The cELISA based on monoclonal antibody (MAb), which is specific for MSP5 has been used to detect antibodies against *Anaplasma* species in cattle (Knowles *et al*., 1996; Torioni *et al*., 1998; Strik *et al*., 2007). Nonetheless, this assay cannot differentiate other *Anaplasma* species from *A. marginale*.

cELISA has been documented as one of the most sensitive serological tests for detecting *Anaplasma* species (Aubry and Geale, 2011). However, this test suffers from problem of cross reactivity among species of *Anaplasma*. Therefore the test cannot be used to differentiate *A. marginale* from other *Anaplasma* species (OIE, 2008).

2.2.6.3 Molecular diagnosis

The techniques using nucleic acid have been developed and are able to detect sub-optimal infection level in a carrier host and tick vectors (OIE, 2010). Molecular diagnostics are used for detection of *Anaplasma species* DNA (Böse *et al*., 1995). The technique however, requires

specialized laboratory equipments and well-trained personnel (Aubry and Geale, 2011; OIE, 2010). The nucleic acid assays based methods include real-time PCR (qPCR) and PCR-based hybridization assays. The *msp4* and *msp1a* genes have been used as molecular markers for detection of *A. marginale* isolates (Guillemi *et al*., 2016). These genetic markers have been used to distinguish the various species of *Anaplasma* such as *A. centrale* and *A. marginale* (De La Fuente *et al.,* 2001; Lew *et al*., 2002).

Quantitative real time PCR (qPCR) targeting msp1b gene of *A. marginale* has been used to identify naturally infected cattle (Carelli *et al*., 2007). This assay specifically detects *A. marginale* and does not result in cross-reactions with other species such as *A. phagocytophilum*, *A. centrale*, *A. platys*, *A. ovis* and *A. bovis* (Carelli *et al*., 2007).

Reverse line blot hybridization assay (RLB) is another molecular method that has been used to detect *Anaplasma species* DNA. This technique combines the use of PCR and hybridization, and has been employed in differentiating *Anaplasma* from *Theleiria* and *Babesia* (Gubbels *et al.,* 1999; Bekker *et al*., 2002). In this technique, PCR products from amplification are fixed on a nitrocellulose paper and probes that are specific to the species are used in hybridization to detect *Anaplasma species* DNA. The test has been broadly used to study the epidemiology of tick-borne haemoparasites for a long time (Matjila *et al*., 2008; 2009; Bhoora *et al*., 2010; Chaisi *et al*., 2011).

2.2.7 Treatment of anaplasmosis

The antimicrobial treatment of bovine anaplasmosis involves the use of tetracycline drugs at the dose rate of 8-11 mg/kg for three consecutive days by intramuscular route (Ribeiro and Passos, 2002). The use of oxytetracycline and chlortetracycline at their recommended chemotherapeutic dose rate can be effective in limiting clinical signs of the infection and therefore reduce the parasitaemia level. However, in the event of a persistent infection, the therapy may not eliminate the organism completely. Therefore there is no evidence that it can prevent cattle from becoming

infected with the pathogens (Aubry and Geale, 2011). Another drug is immidocarb; the drug is administered either subcutaneously or intramuscularly at dose rate of 3mg/kg body weight, and usually results in cure of infected cattle (Stuen *et al.,* 2013).

2.2.8 Control and prevention

Management of anaplamsosis involves the control of arthropods. Drugs use and the use of vaccines have been attempted to prevent anaplasmosis. The predominant antibiotics used in the control of bovine anaplasmosis are the tetracyclines. Other antibiotics which have also shown good results are enrofloxacin (Facury-Filho *et al*., 2012) and immidocarb (Stuen *et al*., 2013) although these are not frequently used (Coetzee *et al*., 2006). Swift and Thomas, (1983) demonstrated that constant administration of repeated doses of tetracycline may eradicate persistent *A. marginale's* infection, but total eradication is not most often attained. Kuttler *et al*. (1980) have reported that during the earlier stages of the disease tetracycline is most effective, but it could be quite a problem to diagnose the disease during early stage of the disease in a herd of cattle (Kocan *et al*., 2010). In the US, the drug is occasionally used for prophylaxis (Kocan *et al*., 2010). Lew-Taylor (2012) states that in antibiotic-treated cattle the withholding period before they can be used for meat or milk can be an issue for farmers, particularly when using long-lasting oxytetracycline preparations.

The 'infection-treatment' control method has been attempted in some regions. In this case, cattle inoculated with erythrocytes infected with *A. marginale* are treated with low loses of tetracyclines during the initial phase of the disease (Kocan *et al*., 2010). The objective is to initiate persistent infection without acute disease. Therefore, the opportunity is given to the animal to develop an immune response and thereafter to be immune to challenge by homologous strains.

Blood-based vaccine has been utilized in areas where there is no endemicity of the disease (Kocan *et al*., 2010). Additionally, prevention of the disease can also be achieved by the use of acaridae, which may play an important role in reducing transmission (Sainz et al., 2015). The use of inactivated or live vaccines has been reported to be one of the effective means for preventing the disease (Kocan *et al*., 2000). Current vaccines may be effective methods for controlling bovine anaplasmosis, though development of improved, safer and globally effective vaccines is still a priority. Although the current vaccines are promising, to date, there are no vaccines that can effectively produce sterile immunity (Bock and DeVos, 2001). Killed vaccines consisting of killed *A. marginale* such as a commercial vaccine known as 'Anaplaz' prepared from infected cattle have been used to prevent the disease (Fort Dodge Laboratories, Fort Dodge, Iowa). The killed vaccines confer lower degree of contamination with other pathogens, are easy to store, and manifest little reactions after inoculation. Also, they appear to offer cross-protection with regards to other strains from various regions (Kuttler *et al*., 1984).

Other vaccines candidates that have been evaluated include those based on outer membrane proteins (OMPs) (Grandi, 2010), recombinant proteins of OMPs (Albarrak *et al*., 2012) and DNA vaccines (Kano *et al*., 2008; Mwangi *et al*., 2007). Live vaccines of *Anaplasma* (Marcelino *et al.*, 2012) and attenuated *A. marginale* have been reported to produce promising results and thus could be used in future to prevent and control anaplasmosis. The use of naturally low pathogenic *A. marginale* strains as potential candidate vaccine has also been documented (Bastos *et al*., 2010). However, the vaccine failure, especially from highly pathogenic *A. marginale* strains has limited the use of this

vaccine (Bock and Vos, 2001). The use of tick cell culture as a source of vaccine against anaplasmosis is also in the pipeline although this is still limited to laboratory research (passos, 2012; Bastos *et al*., 2010).

2.3 Genome structure of *Anaplasma* parasites

The genome of *A. marginale* has been sequenced and found to consist of approximately 1.2 Mb in length (Brayton *et al*., 2005). The genome is known to contain genes encoding a member of OMPs to be 62 in number, with 49 of these products belonging to the major surface proteins 1 and 2 (MSP1 and MSP2) (Brayton *et al*., 2005; De la Fuente *et al*., 2005).The msp1 and msp2 genes have been reported to encode proteins implicated in parasite hosts interaction (Brayton *et al*., 2005; Kocan *et al*., 2004). Nine genomes of *A. phagocytophilum* have been sequenced with only three genomes done to completion (Brayton *et al*., 2002). *A. phagocytophilum* genomes also consist of genes MSPs, which are an ortholog of MSP2, known as MSP2 (P44) (Brayton *et al.,* 2005).

A. phagocytophilum has more msp2 pseudogene in its genome as compared to those present in the genome of *A. marginale* (Hotopp *et al*., 2006; Brayton *et al*., 2005). In particular, the P44 genes present in the genome of *A. phagocytophilum* consists of full length P44 genes, shorter P44 genes without a start codon and fragmented P44 genes with only 5' or 3' conserved region. The other regions of the genome consist of truncated P44 genes containing only the hypervariable region (Hotopp *et al*., 2006).

2.4 Primers and gene markers for *Anaplasma* characterization

The molecular characterization of *Anaplasma* infecting cattle has been done using specific genetic markers that have unique nucleotide sequence to the haemoparasite. The characterization is usually

based on specific genetic markers or whole genome sequence of the parasite, especially in cases where evolutionary relationship between the haemoparasite isolates is determined. Usually, when a specific region of the parasitic genome is used, primers specific to a target marker gene are used to amplify the gene by PCR. For example, primers targeting *msp5* and *msp1a* genes, which encodes MSP5 and MSP1a respectively, have been used for identification and characterization of *A. marginale* (Guillemi *et al*., 2016). The primers targeting *msp*5 amplified 548 bp and 345 bp amplicons by a nested PCR while those targeting *msp*1a gene amplified 84–87 bp tandem repeats (Torioni de Echaide, 1998; De la Fuente, 2001). In other studies, degenerate primers targeting the highly conserved regions of *ftsZ* genes have been used to amplify a fragment of the *ftsZ* gene from the genomic DNA of *A. phagocytophilum-ftsZ*$_{Ap}$ (Lee *et al*., 2003). In the same study, primers targeting a part of the variable carboxyl-terminal region of the *ftsZ* gene, yielded 278 bp amplicon and thus could differentiate *A. phagocytophilum* from *Ehrlichia chaffensis* and *Rickettsia.* TaqMan primers namely ESP-F and ESP-R as well as a probe, which specifically targets the *16S rRNA* gene of *Ehrlichia* and *Anaplasma* species has also been used to detect the parasites in ticks (Kim *et al*., 2003). Primers targeting heat-shock protein (groEL) and *16S rRNA* genes have also been used to detect and characterize *Anaplasma* species including *A. phagocytophilum* in ticks (Wei *et al*., 2016). In a recent study, primers targeting fragments of *msp*5*, 16S rRNA*, heat shock protein (groESL) and *msp*1*a* genes have also been used to detect and characterize *A. marginale* infection in cattle (Ybañez *et al*., 2016).

2.5 Detection of *Anaplasma* by molecular tools

2.5.1 Extraction of *Anaplasma* DNA from blood

Extraction of the parasite DNA from blood is crucial before detection of *Anaplasma*. It can be achieved by molecular methods. Different strategies employing both manual and commercial kits have been used to extract *Anaplasma* DNA from blood of infected animals and ticks. For example, the genomic DNA of *A. marginale* has been extracted from blood samples by phenol chloroform

method followed by a standard ethanol precipitation (Sambrook *et al*., 1989; Canever *et al*., (2014) have also extracted haemoparasite DNA from blood by using phenol-chloroform method. The DNA extraction using this method involves lyses of whole blood with lysing buffer (10 mM Tris-HCl pH 7.4, 10 mM NaCl, 25 mM EDTA and 1% SDS) followed by digestion using proteinase K at 42^{o}C. The lysed and digested blood is then washed with phenol-chloroform in a ratio of 1:1 and then chloroform, respectively. The DNA is precipitated with isopropanol, washed with 70% ethanol, air-dried and then eluted with DNase-free Milli-Q water.

However, many studies have nowadays reported the use of commercial kits for extraction of the parasitic genomic DNAs from blood and ticks. In one recent study, total DNA has been extracted from homogenized ticks using a TIANcombi DNA Lyse and Det-PCR Kit supplied by Tiangen Biotech, Co. Ltd, China. The DNA extract has been used to detect *Anaplasma* species and other haemoparasites by nested PCR (Wei *et al.*, 2016*).* Other researchers have used the NucleoSpin® Blood QuickPure kit, purchased from Macherey-Nagel, Bethlehem in USA, to extract *A. phagocytophilum* DNA from blood (Dugat *et al*., 2014). In another report, DNA was extracted from clinical blood samples of human for the PCR amplification of *A. phagocytophilum* DNA using the QIAamp DNA Blood Kit from Qiagen (Lee *et al*., 2003). In another research involving molecular diagnosis of *A. marginale* in carrier cattle, Noaman *et al.* (2009) extracted DNA using a DNA isolation kit supplied by MBST in Iran. To use this kit, a fixed blood sample is air dried and then lysed with lysis buffer. The proteins in lysed blood is then digested with proteinase K followed by addition of binding buffer and 96% ethanol. To recover the DNA, the mixture with binding buffer is passed through MBST-column where DNA binding occurs. The bound DNA is then eluted with elution buffer. Bilgic *et al*. (2013) extracted DNA of haemoparasites including *A. marginale* from bovine blood using the Promega Wizard genomic DNA extraction kit obtained from Madison, (USA).

Other commercial kits that have been used to extract haemoparasites DNA from whole blood for subsequent PCR assay includes AxyPrep-Blood Genomic DNA Miniprep Kit marketed by Axygen (Rufino *et al*., 2013) and QIAamp DNA Blood Mini-Kit manufactured by Qiagen in Germany.

2.5.2 Detection of DNA by conventional PCR

The detection of *Anaplasma* organism by conventional PCR is based on the amplification of a single fragment of the parasite DNA by one pair of primers. Though this method is not suitable for simultaneous detection of multiple haemoparasites in blood, it does not suffer from the multiple interactions of primers seen with multiplex PCR.

2.5.3 Detection of *Anaplasma* organism by multiplex PCR

A number of studies have used a multiplex PCR to detect *Anaplasma* in blood. The advantage of multiplex PCR over single detection (singleplex) is that it facilitates the simultaneous detection of more than one organism (Henegariu *et al.*, 1997; Markoulatos *et al.*, 2002). In combining both real-time PCR and multiplex abilities, a considerable potent method for detection of multiple pathogens in one assay's tube format has been reported (Courtney *et al*., 2004). It appears also to be a type of PCR reaction where two or many more loci coming from single or more organism are amplified using several specific pairs of primer in one (Edwards and Gibbs, 1994; Henegariu *et al*., 1997; Markoulatos *et al*., 2002).

2.5.4 Other *Anaplasma* detection techniques

Other genetic based detection techniques that have been used to screen *Anaplasma* species include nested-PCR, real-time PCR, MassTag PCR and the reverse line blot (RLB) that simultaneously allow detection of several species of organism in a single sample (Gubbels *et al*., 1999).

2.6 Genetic characterization and phylogenetic analysis

Bioinformatics analyses of sequences of *Anaplasma* isolates have been done using Basic Local Alignment Search Tool (BLAST), sequence alignment and phylogenetic analysis (pyron *et al*., 2013). Sequences of *Anaplasma* isolates have been used to identify the species of *Anaplasma* using Blast analysis (Said *et al*., 2015). This analysis also indicates the sequence identities and this may be used to compare closely related *Anaplasma* species or even strains. For example the blast analysis has been used to identify *A. marginale*, *A. centrale*, *A. phagocytophilum*, *A. bovis*, *A. ovis* and many other species (Laloy *et al*., 2009; Ozawa *et al*., 2009; Jafarbekloo *et al*., 2014). For sequence alignment, various nucleotides of *Anaplasma* species have been aligned to determine conserved and variable regions within respective genes (Zhan *et al*., 2010). This has been achieved for *A. marginale* (De la Fuente *et al*., 2007), *A. centrale* (Molad *et al*., 2009), *A. phagocytophilum* (poitout *et al*., 2005) and *A. ovis* (Said *et al*., 2015). For example *msp4* and *msp2* coding regions of *A. phagocytophilum* have been aligned (Mongruel *et al*., 2017). This has been used to generate phylogenetic tree. In the case of phylogenetic analysis, sequences of various *Anaplasma* species or even sub-species have been used to group related strains together (Mongruel *et al*., 2017). Various gene markers including *msp1, msp2, msp4* and *16S rRNA* have been used to generate phylogenetic tree for reclassification of the various *Anaplasma* species or even for differentiating *Anaplasma species* from *Erhlichia* species (Kocan, 2003; Guillemi *et al*., 2016; Silaghi *et al*., 2011).

2.7 Development of molecular tools for detection *Anaplasma*

A number of molecular tools based on PCR and sequencing have been developed for detection and differentiation of *Anaplasma* species (Silaghi *et al*., 2011; Njiiri *et al*. 2015). For example Torina *et al*. (2012) developed and validated PCR assays for detection and differentiation of *A. ovis* and *A. marginale*. In this case two sets of primers targeting msp4 gene were used to amplify the genes for detecting *A. ovis* and *A. marginale* in ticks, wildlife, domestic animals (Torina *et al*., 2012). In another study, Bilgic *et al*. (2013) developed a multiplex PCR that could simultaneously detect *T. annulata*, *B. bovis*, and *A. marginale* in cattle. In this case, *A. marginale msp1β* available in the genebank was used to design primers using oligonucleotides analysis software. The single and multiplex PCR could detect *A. marginale* and other tick borne diseases specifically without non-specific bands. Other molecular tools have been developed for detection and characterization of *A. ovis* (Chi *et al*., 2013), *A. phagocytophilum* (M'ghirbi *et al*., 2016), *A. platys* in dogs and ticks (Matei *et al*., 2016) and have produced promising results.

2.8 Molecular epidemiology of anaplasmosis

Molecular epidemiology of *Anapalsma* organisms have been studied by detecting the organisms by PCR, sequencing and generating phylogenetic profiles. This was in order to understand the genetic variation or relatedness of various *Anaplasma* isolates (Kocan, 2003; pyron *et al*., 2013). Whole genome or fragments of genome or genes have been used to characterize the variation in genetic profiles of *Anaplasma* species (Brayton *et al*., 2005; Silaghi *et al*., 2011). The genetic sequences of *Anaplasma* organisms have been used to confirm the identity of the isolates and establish phylogenetic trees, which are important for the understanding of the molecular epidemiology of the various *Anaplasma* species (Rar and Golovljova, 2011).

2.8.1 Molecular epidemiology of bovine anaplasmosis

The molecular epidemiology of bovine anaplasmosis have studied by a number of researchers including those caused by *A, marginale* and *A. centrale* (Belkahia *et al*., 2015; Lorusso *et al*., 2016). This has enabled the understanding of spread of *Anaplasma* species as well as other tick borne haemoparasites in the cattle population. As such, Lorusso *et al*. (2016) using PCR and reverse line blot (RLB) was able to identify and characterize *A. marginale*, *A. omatjenne*, *A. centrale* and *A. platys* in cattle population in Nigeria. This also helped ascertaining the rate of infection amongst cattle population in Nigeria. Additionally the genetic diversity and distribution of *Anaplasma* species has been studied. For example to study the spread or genetic diversity of *Anaplasma* species present in Tunisia, Belkahia *et al*. (2015) used the *msp4* gene of *A. marginale,* which revealed high genetic variation. In this report, the *16S rRNA* of *A. centrale* has been used to generate a phylogenetic three, which revealed the prevalence of *A. centrale*. In Turkey, the hypervariable region V1 of the 16S rRNA gene was amplified in sample from cattle. This led to the identification of *A. Phagocytophilum* (Munir Aktas and Sezayi Özübek, 2015).

2.8.2 Molecular epidemiology of ovine anaplasmosis

Unlike bovine anaplasmosis, there are few studies on molecular epidemiology of ovine anaplasmosis with most of molecular work focusing on detection of the *Anaplasma* organisms (Chi *et al*., 2013; Said *et al*., 2015). Nonetheless, some studies in China have documented the molecular epidemiology of *A. phagocytophylum* in sheep and other animals such as cattle goats (Zhan *et al*., 2010; Chahan *et al*., 2005). In a more recent past, *A. bovis* presence in sheep and other domestic ruminants have been reported (Zhou *et al*., 2010; Liu *et al*., 2012). In this report, molecular evidence for *A. bovis* infection in sheep revealed higher prevalence of about 49.6% in China (Zhou *et al*., 2010). Other studies related to molecular epidemiology of ovine anaplasmosis involved the analysis of *msp4* genes of *A. ovis* and *A. marginale* infecting sheep and cattle (Torina *et al*., 2008). Phylogenetic analysis of

msp4 sequences of *A. ovis* has also revealed variation in genotypes infecting sheep in the northern China. These genotypes were different from the genotypes infecting goats in other areas in China (Liu *et al*., 2012).

3.0 METHODOLOGY

3.1 Study area

Suba and Mbita sub-counties are located in Homa Bay County along the south shore of Lake Victoria. The sub-counties host smallholder cattle farmers who use the animals for their livelihoods and economic gains. The specific areas from which the samples were collected include: Gembe (00^{o} 30'S; 34^{o} 13'E), Lambwe West (00^{o} 40'S 34^{o} 17'E), Lambwe East (40^{o} 00'S; 34^{o} 17'E). The climatic condition in these areas is semi-arid with temperatures ranging from 26^{o}C to 34^{o}C with a rainfall pattern ranging between 250 mm and 1200 mm annually having an average of about 1,100 mm rainfall. The long rainy season is from April to May, whereas the short rainy season is from October to December. These areas were selected for this study because anaplasmosis is endemic in these areas and the genetic population of the causative agent is poorly understood in these localities. A map showing the actual areas where the study was done is shown in the Figure 4.

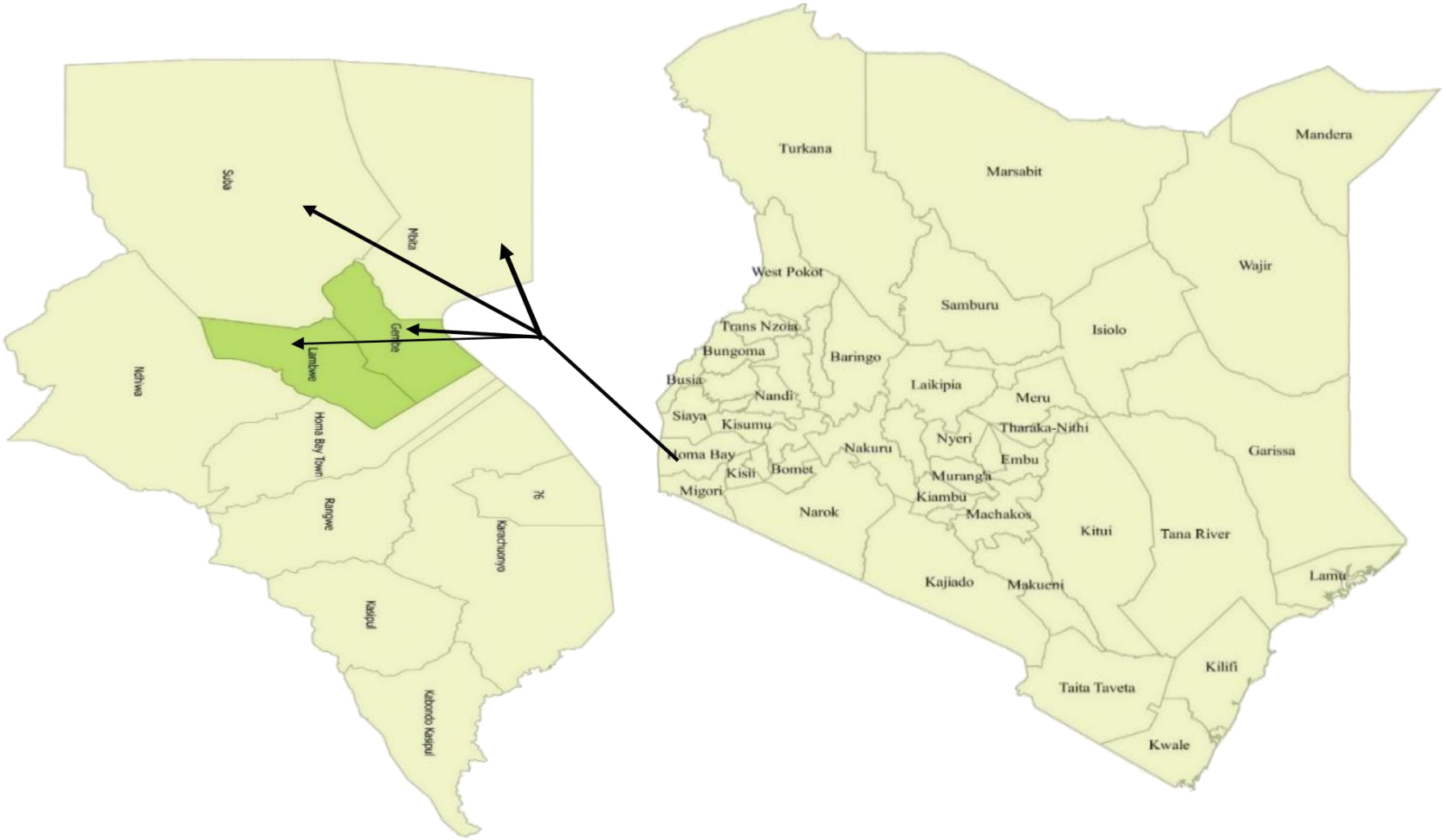

Figure 4: Map of Homabay County with the various sub-counties shown by the arrows (http://d- ps.com/m/africa/kenya/kenya74.gif).

3.2 Ethical statement

The smallholder farmers of the sampled homesteads in Suba and Mbita sub-counties were informed about the study, and thereafter gave their approval for the sampling of cattle and sheep. This study was done according to ethical guidelines for the use of animal samples permitted by Faculty of Veterinary Medicine of the University of Nairobi. The ethical approval number for this research work was **REF: FVM BAUEC/2017/127**.

3.3 Sample collection

This study was laboratory based. These samples were collected over a period of two months. A total of 180 samples of whole blood were collected from the jugular vein of local Zebu cattle and sheep using vacutainer tubes containing EDTA as anticoagulant. The samples were labeled and transported on ice to the molecular laboratory facility at the Department of Public Health, Pharmacology and Toxicology of the University of Nairobi. The blood samples were stored at -20^{o}C pending DNA extraction. Each sample was assigned a laboratory identification number. Positive control samples were obtained from (BecA-ILRI) Hub and were stored at -20^{o}C pending further analysis.

3.4 Extraction of DNA

The whole blood samples were retrieved from the freezer and allowed to thaw at room temperature. Extraction of DNA from each blood sample was carried out using the commercial QIAamp® DNA Mini Kit, following the manufacturer instructions (Qiagen, Germany). The buffer AE that was used for elution in the subsequent steps was equilibrated at room temperature (15-25^{o}C) and the water bath was heated to 56^{o}C. Buffers AW1, AW2, as well as QIAGEN protease were prepared before the procedure. To extract the genomic DNA of *Anaplasma species,* 20µl of proteinase K was pipetted into 1.5ml eppendorf tubes and then 200µl of blood samples was added into the same tube. Thereafter, 200µl of buffer AL was added to the samples with proteinase K and then mixed by pulse-vortexing for 15 seconds. This step was followed by incubation at

56^{O}C for 10 minutes. Then, 200µl of 100% ethanol was added to the homogenous lysed blood samples. This mixture was pulse-vortexed for 15 seconds to remove drops from the lid of eppendorf tubes. The QIAamp® DNA Mini Kit column was placed in a clean 2ml collection tube and the lysed blood sample was added into the spin column. To bind the DNA, the spin column with the collection tube was centrifuge at 5724g for 1 minute and the filtrate in the collection tube was discarded. After the DNA binding, the QIAamp® Minispin column was retained and 500µl of buffer AW1 was added into the spin column and then centrifuged at 5724g for 1 minute when the column cap was closed. The filtrate in the collection tube was discarded and 500 µl buffer AW2 was added into the retained spin column. The column was centrifuged at 17530g for 3 minutes and the filtrate was discarded again as outlined above. To elute the DNA, 200µl of buffer AE was added into the spin column and the mixture was allowed to stand for 1 minute at room temperature followed by centrifugation at 5724g for 1 minute. A second step of elution with 200µl of buffer AE was done to increase the DNA yield. The eluted DNA samples were stored at -30^{O}C pending further analysis.

3.5 Bioinformatic analysis analysis of selected genetic markers

Target genetic markers of *Anaplasma* parasites were selected and analyzed by bioinformatic tools for subsequent design of primers. The genes which had been previously used to detect *Anaplasma* haemoparasites were selected as follows: *A. marginale msp1b* (*Am-msp1b*), *A. marginale* (*Am-16S rRNA*), *A. centrale msp2* (*Ac-msp2*) and *A. centrale 16S rRNA* (*Ac-16S rRNA)*. Each of the genes was retrieved from the National Center for Biotechnology and Information (NCBI) Genebank https://www.ncbi.nlm.nih.gov/. The genes and their accession numbers (Appendix 2) were confirmed to be from *Anaplasma* species by basic alignment search tool (BLAST) analysis, and subsequently used for design of primers.

3.6 Primer design by PrimerQuest

The *Am-16S rRNA*, *Am-msp1b*, *Ac-msp2*, and *Ac-16S rRNA* gene markers were used to design specific primers using Primer Quest Tool of Integrated DNA Technology (California, USA). To design primers, the respective sequences of the target genes were entered into the input window of Primer Quest Tool Software interface. Then, the sequences were named and the software was run by clicking the appropriate primer design tool button which in this case was "PCR for 2 primers". The generated target amplicons and their respective primers were documented and reported in the results section (Table 2). These primers were then submitted to the International Livestock Research Institute (ILRI) for synthesis by Macrogen Inc Company, South Korea. The synthesized primers were used for PCR amplification as outlined in the subsequent sections 3.7 and 3.8.

3.7 Detection of *Anaplasma species* by multiplex PCR

This was carried out with a reaction volume of 12.5µl by using TaKaRa Ex Taq™ (TAKARA BIO INC, Seta 3-4-1, Otsu, Shiga, Japan) using the applied biosystem verti 96 well thermal cycler, model 9902, Singapore. Optimal conditions used included; 5ng of purified genomic DNA as template, 10X Taq PCR Takara buffer containing 2 mM MgCl2, 4 ng of each of the two pairs of primers targeting *Am-msp1b*, *Ac-16S rRNA*, *Ac-msp2* and *16S rRNA* 0.2 mM deoxynucleotide triphosphate (dNTP) mix, and 1.25 U of Takara Ex-Taq polymerase. The thermocycling conditions after preheating the reaction mixture were as follows: pre-denaturation at 94^{o}C for 5 minutes, followed by 35 cycles of denaturation at 94^{o}C for 5 minutes, annealing at 55^{o}C for 1 minute and extension at 72^{o}C for 1 minute. This was followed by one cycle of a final extension at 72^{o}C for 5 min. The PCR products were then electrophoresed using 1.5 % agarose gel in Tris–Borate–EDTA (TBE) buffer, pH 8, and thereafter stained with ethidium bromide. The stained DNA bands in the gels were visualized using UV-illuminator (GelMax® Imager Cambridge, UK). The sizes of the amplicons were determined using M1 GeneRuler™, 1 kb DNA ladder ranging from 75 to 20000 bp.

3.8 Detection of *Anaplasma species* by conventional PCR

A conventional PCR amplifying 576, 436, and 835 bp fragments of the respective *Ac-msp2*, *Ac-16S rRNA*, and *Am-16S rRNA* genes was used to identify *A. marginale* and *A. centrale* DNA. The final 20µl-PCR for each reaction volume consisted of 10 ng of purified genomic DNA, with a reaction buffer containing 1.5 mM $Mgcl_2$, 250 of µM dNTPs, 10 pmol of each primers (forward and reverse), and 2.5U of Taq DNA polymerase (BIONEER). The thermocycling conditions for the full-length amplicons after preheating were as follows: pre-denaturation at $94^{O}C$ for 5 minutes followed by 35 cycles of denaturation at $94^{O}C$ for 5 minutes, annealing at $55^{O}C$ for 1 minute and extension at $72^{O}C$ for 1 minute, one cycle of final extension at $72^{O}C$ for 5 minutes was done. The PCR products were then electrophoresed using 1.5 % agarose gel in the TBE buffer and thereafter stained with ethidium bromide. The stained DNAs were visualized using UV-illuminator (GelMax® Imager Cambridge, UK). The sizes of the amplicons were determined using M1 GeneRuler™, consisting of 1 kb DNA ladder ranging from 75 to 20000 bp. The positive results were determined by the appearance of distinct bands while negative results by the absence of a distinct bands.

3.9 Purification of PCR amplicons

The resulting positive PCR products were subjected to purification using ZR-96 DNA sequencing Clean-up Kit™ following the manufacturer's instructions (Zymo Research Corp Irvine17062 Murphy, Avenue, and Irvine, CA 92614, USA). Two hundred and forty microliters (240µ) of sequencing binding buffer was added to 20µl of PCR products. The mixture was then transferred into Zymo-Spin™ IB-96 Plate mounted onto a collection plate. Thereafter, the mixture was centrifuged at 2236g for 2 minutes. Subsequently, 300µl of sequencing wash buffer was added to each well of the plate and centrifuged at 805g for 5 minutes. Then, 20 µl of water was added directly to the matrix of the column of the filter plate. The Zymo-Spin™ IB-96 Plate was placed on top of the supplied 96-well PCR Plate

and mounted onto the collection plate. This was then centrifuged at 805g for 2 minutes to elute the DNA.

3.10 Sequencing of the purified PCR products

Twenty purified PCR amplicons of the samples amplified using primers targeting *Ac-16S rRNA* were sequenced with both the forward and reverse primers. The Sanger sequencing was performed using an ABI Prism1 Big Dye Terminator v3.1 Cycle Sequencing Kit (PerkinElmer, Applied Biosystems Division, and Foster City, CA, USA) in South-Africa according to the manufacturer's instructions. The obtained sequences were verified using chromatogram peaks, edited and assembled using CLC Main Workbench 7. The multiple alignments of the assembled sequences were performed using CLUSTAL Omega (http://www.genome.jp/tools/clustalw/) and then consensus sequences were obtained. BLASTn for nucleotide analysis accessed through GenBank of the NCBI database was then used to compare the consensus sequences with the correct *Anaplasma species* identity. The confirmation of the species was established as the nearest BLASTn match with an identity of between 99% and 100% to those homologues found in the GenBank (http://www.genome.jp/tools/clustalw/). The construction of the phylogenetic tree was done by the maximum likelihood method using MEGA version 6 software, tamura (Tamura *et al*., 2013). The statistical significance for the internal branches of the trees was estimated using the Bootstrapping with 1000 iterations. The obtained sequences were submitted to the GenBank and subsequently assigned accession numbers.

3.11 Data analysis

Data entry was done using the Microsoft excel 2013 for further statistical analyses. Thereafter, the proportions of samples positive for *Anaplasma* organisms in relation to genes detected were computed. Molecular data were analysed by bioinformatics tools using BLASTn, sequence aligments using CLUSTALW (http://www.genome.jp/tools/clustalw/), and phylogenetic analysis using MEGA version 6 software employing Tamura (Tamura *et al*., 2013).

4.0 RESULTS

4.1 In silico generated primers and predicted PCR products

Computer-based primer design using PrimerQuest tool of Integrated DNA Technology (IDT) generated a set of primer-pairs for subsequent amplification of the following four gene markers; the *Am-msp1b, Am-16S rRNA, Ac-16S msp2,* and *Ac-16S rRNA*. The specific pair of primer sequences generated for *Am- msp1b* gene produced a predicted PCR product of 716 bp. The %GC content and melting temperature of the computer generated primers were 50% and 62^{O}C for both forwards and reverse primers respectively. The second pair of primers was based on *Am-16S rRNA* gene to generate a predicted 835 bp DNA fragment. For both forward and reverse, these primers had a %GC of 50% and a melting temperature of 62^{O}C. The third pair of oligonucleotide primers based on *Ac-msp*2 gene had a %GC content of 52.4% for forward and 47.6% for reverse primer. The melting temperature for both oligonucleotide pair was 52^{O}C. These primers predicted a PCR fragment of 576 bp. For the *Ac-16S rRNA* gene, the PrimerQuest tool generated a DNA fragment of 436 bp from a forward primer with a %GC content of 50% and a reverse primer with a %GC content of 45%. The melting temperature for both primers was 62^{O}C. The nucleotide sequences of the computer generated primers including their lengths and their respective positions in the target genes are given in the Table 2 and Appendix 1. The positive control was used to detect *Anaplasma marginale.*

Table 2: The target genes and their respective computer generated pair of primers using PrimerQuest tool.

Target genes	predicted primers generated	predicted PCR amplicons	Codons Start	Codons Stop	GC%(Tm°C)
Am-msp1b	CTA GAC TTG GTG CAG GGT TAT G	716 bp	587	609	50 (62)
	GCC AAA TGC ATC GCC AAT AG		83	1303	50 (62)
Am-16S rRNA	ACTAGAGTCCGGAAGAGGATAG	835 bp	554	576	50 (62)
	GTCACCGACCCAACCTTAAA		1369	1389	50 (62)
Ac-MSP2	GGC AGC ACT AGA GGT GTA TTC	576 bp	223	244	52.4 (62)
	TCC CTC CTT ATC GAG GCT ATT		778	799	47.6 (62)
Ac-16S rRNA	CAG ACG GGT GAG TAA TGC AT	436 BP	78	100	50 (62)
	ATG CCC TTT ACG CCC AAT AA		494	514	45 (62)

4.2. *Anaplasma species* detected by PCR using the computer generated primers

The conventional PCR performed using primers targeting *Am-16S rRNA* gene amplified a specific band of approximately 835 bp as predicted for both positive (*A. marginale*) control and field blood samples of cattle and sheep (Figure 5). A multiplex PCR employing the pairs of primers for *Ac-16S rRNA* and *Am-msp1b* genes of *Anaplasma species* amplified a specific band of approximately 436 bp for *Ac-16S rRNA*; the other primer-pairs targeting *Am-msp1b* gene did not yield an expected PCR-band of 716 bp (Figure 6). A conventional PCR using the same primers targeting *Ac-16S rRNA* gene also yielded a specific band of approximately 436 bp (Figure 7). A PCR amplification using primers targeting the *Ac- msp2* of *Anaplasma* parasites produced a band of 576 bp and other numerous non-specific bands (figure 8).

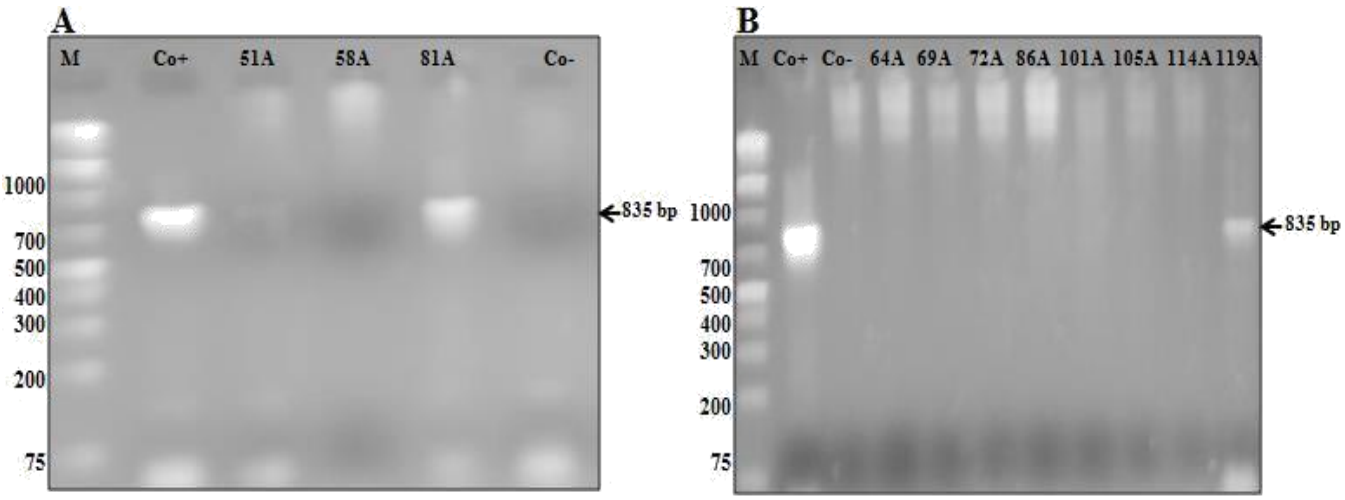

Figure 5: Conventional PCR amplification of *Anaplasma Am-16S rRNA* gene fragment, using TaKaRa Ex TaqTM. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Lane M is DNA marker; the first lane (Co+) is the positive control from cattle (A and B) and Co- the negative control. In A, Co+ and 81A are positive samples; the remaining samples are negative. In B, Co+ and 119A are positive samples; the remaining sample are negative samples. The arrow indicates the position of the amplicons.

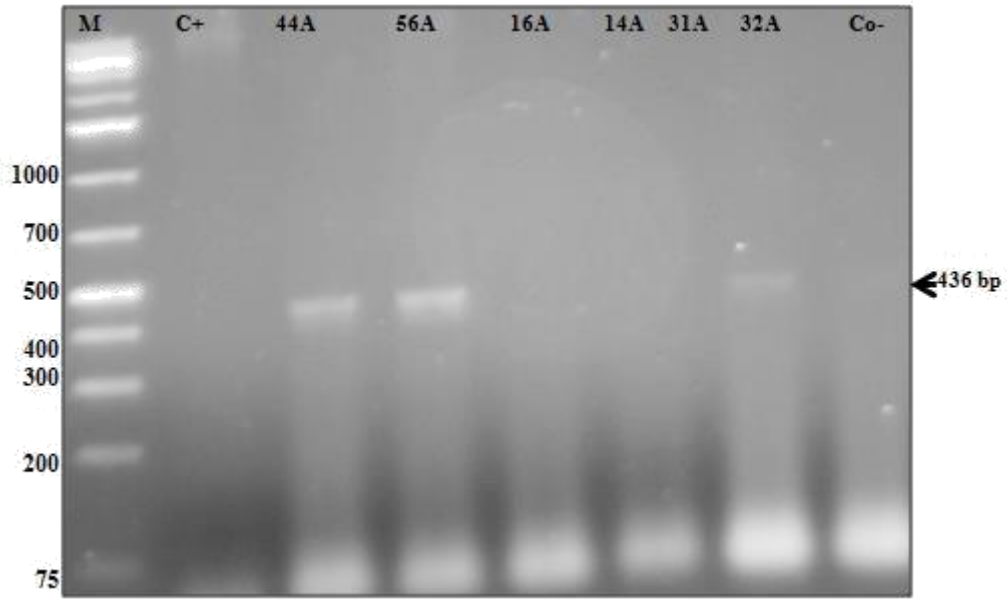

Figure 6: Multiplex PCR amplification of *Anaplasma Am-16S rRNA* and msp1b genes, using TaKaRa Ex TaqTM. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Lane M is DNA marker, the first lane (Co+) is positive control from cattle. In the above figure, 44A, 56A, 32A are positive samples and the remaining are negative samples. However, *msp1b* did no yield any positive band. The arrow indicates the position of the amplicons.

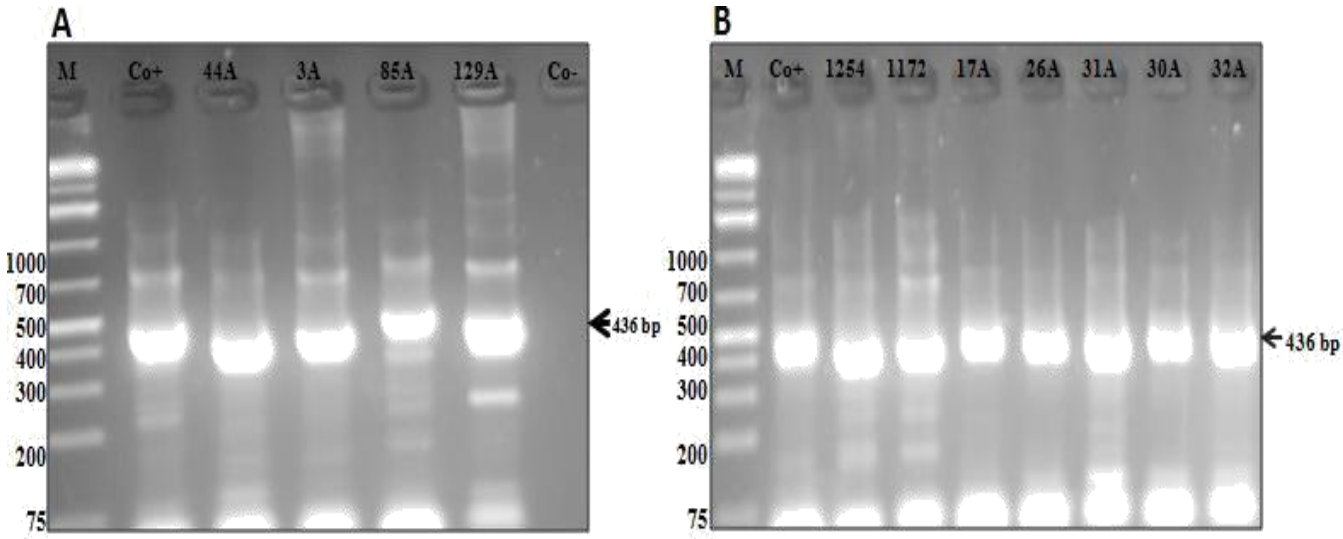

Figure 7: Conventional PCR amplification of *Anaplasma Am-16S rRNA* gene fragment using AccuPower® PCR PreMix from Bioneer. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Lane M is DNA marker, the first lane (Co+) is positive control from cattle (A and B). In A and B 44A, 3A, 30A, 32A (from sheep) 85A, 129A, 1254, 1172, 17A, 31A (from sheep) are positive samples from cattle and sheep. The arrow indicates the position of the amplicons.

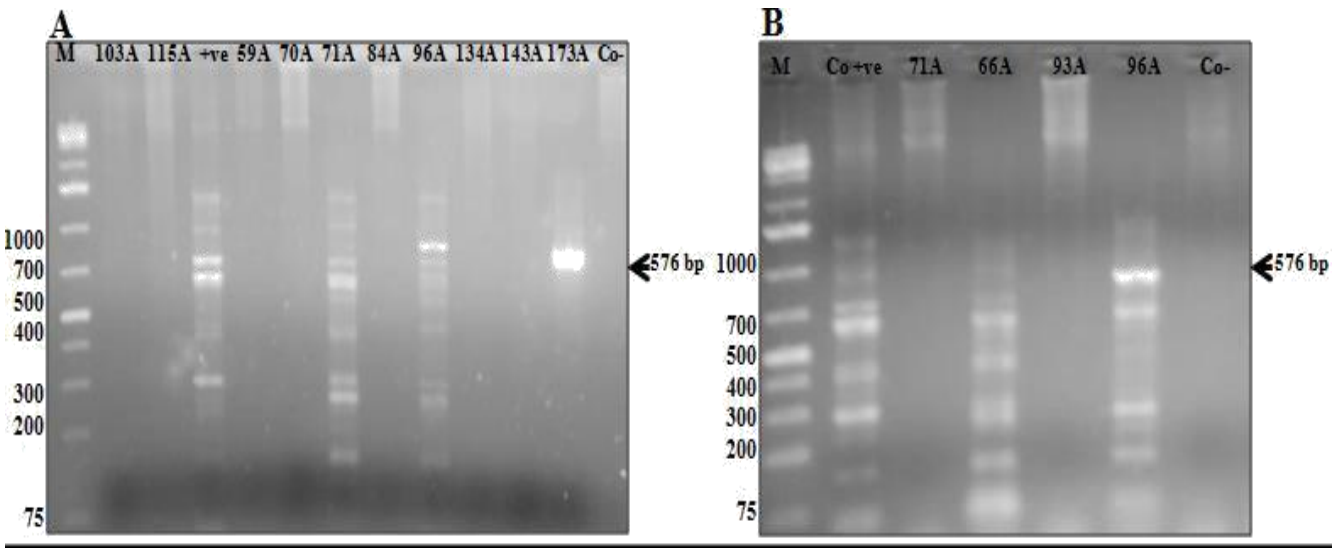

Figure 8: Conventional PCR amplification of *Anaplasma Ac-msp2* gene using AccuPower® PCR PreMix from Bioneer. The PCR products were analyzed by gel electrophoresis on 1.5% agarose gel, stained with ethidium bromide. Lane M is the DNA marker, the third and first lane are positive controls for A and B respectively. Panel A, 71A, 96A, 73A are positive samples and the remaining are negative samples. Panel B, 66A and 96A are positive samples. Other lanes are non specific bands. The arrow indicates the position of the amplicons.

4.3 *Anaplasma species* detected in cattle and sheep by PCR.

A total of 180 blood samples comprising of 157 samples from cattle and 23 samples from sheep were screened by PCR for the presence of *Anaplasma* parasites using primers targeting *Am-msp1b, Am-16S rRNA, Ac-msp2* and *Ac-16S rRNA* genes. The overall average positive rates from amplification of *Am-16S rRNA, Ac-16S rRNA* and *Ac-msp2* genes in both cattle and sheep blood samples were 10 (5.55%), 20 (11.1%) and 5 (2.7%) samples, respectively (Table 3). *Anaplasma* organism was detected in 9 (5.7%) samples from cattle when primers targeting *Am-16S rRNA* marker gene were used whereas 1 (4.3%) sample from sheep tested positive. For primers targeting *Ac-16S rRNA* gene, *Anaplasma* DNA was detected in 11 (7%) samples from cattle whereas 9 (39.1%) samples from sheep had the *Anaplasma* parasite. Three (1.91%) samples collected from cattle had *Anaplasma* organism and 2 (8.7%) samples from sheep were positive when primers targeting *Ac-msp2 gene* were used for the PCR-amplification. The *Ac-msp2* and *Ac-16S rRNA* genes were both uniquely amplified in five samples, namely 30A, 58A, from sheep and 108A, 88A, 171A from cattle. Primers targeting *Am-msp1b* gene failed to amplify the *Anaplasma* DNA in all the 180 samples analyzed.

Table 3: Cattle and sheep samples in which *Anaplasma* DNA was detected.

SPECIES	*Am*-16S rRNA (835bp	*Am-msp1* (716 bp)	*Ac*-16S rRNA (436 bp)	*Ac-msp2* (576 bp)	N
Cattle	9 (5.7%)	0 (0)	11(7%)	3 (1.91%)	157
Sheep	1 (4.3)	0 (0)	9 (39.1%)	2 (8.7%)	23
Total	10 (5.5%)	0 (0)	20 (11.1%)	5 (2.7%)	180

4.4 DNA sequencing of positive samples

The PCR products from 20 samples positive with primer set *Ac-16S rRNA* for *Anaplasma* parasites were sequenced. Out of the 20 samples, 19 generated nucleotide sequences were suitable for further analysis. Bioinformatic analysis by BLAST method of three sequences of *Ac-16S rRNA* gene from cattle, samples revealed homologues that were identical to *A. phagocytophilum* (03 sequences) *Ac-16S rRNA* gene. Ten sequences revealed homologues identical to *A. ovis* with 07 sequences from samples of ovine origin and 03 from samples from bovine origin. The other 02 sequences were from both cattle and sheep and corresponded to be uncultured bacteria. Two samples from cattle (positive control) yielded sequence homologues identical to *A. platys*. Two sequences that were not of good quality were not analyzed. The E-values and nucleotide sequence identities of the homologous sequences are outlined in Table 4.

Table 4: Results of *Anaplasma* species identified by BLASTn analysis using *16S rRNA* sequences of the isolates from cattle and sheep in Homabay County. The positive controls used in this study corresponded to *A. platys* homologues (with numbers 1254 and 1172).

Samples	Animal species	Homologous sequences	E.values	ID (%)
10A	Cattle	*A. phagocytophilum*	0.0	98
53A	Cattle	*A. phagocytophilum*	0.0	99
108A	Cattle	*A. phagocytophilum*	0.0	98
85A	Cattle	*A. ovis*	0.0	99
58A	Cattle	*A. ovis*	0.0	99
88A	Cattle	*A. ovis*	0.0	99
26A	Sheep	*A. ovis*	0.0	99
31A	Sheep	*A. ovis*	0.0	99
32A	Sheep	*A. ovis*	9e-160	100
30A	Sheep	*A. ovis*	0.0	98
46A	Sheep	*A. ovis*	0.0	99
56A	Sheep	*A. ovis*	0.0	99
58A	Sheep	*A. ovis*	0.0	99
44A	Sheep	*Uncultured bacterium*	0.0	97
129A	Cattle	*Uncultured bacterium*	0.0	97
1254	Cattle	*A. platys*	0.0	100
1172	Cattle	*A. platys*	0.0	100

4.5 Results of nucleotides sequence alignment

4.5.1 Multiple sequence alignment of *Anaplasma ovis* isolates with others from other regions.

Multiple alignment of *Anaplasma* nucleotide sequences of the *A. ovis* isolates revealed that all the sequences of samples from sheep were conserved except for the isolate 44A. The sequences of the *A. ovis* isolates from China, Mongolia and Netherland were also identical to the Kenyan isolates. However, the sequences of two isolates from South Africa and USA were different from the Kenyan *Anaplasma* isolates (Table 9).

Name	A	A	C	G	T	G	C	C	T	G	T	A	A	G	A	C	C	G	G	G	A	T	A	A	C	A	T
1. 44A Uncultured bacterium	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
2. 26A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
3. 31A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
4. 30A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
5. 32A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
6. 46A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
7. 56A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
8. 58A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
9. 88A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
10. 85A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
11. 171A A.ovis Kenya	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
12. LC194134.1 Anaplasma ovis Mongolia	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
13. KJ410246.1 Anaplasma ovis China	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
14. KJ410244.1 Anaplasma ovis China	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
15. AF318945.1 Anaplasma ovis Netherland	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	A	.	.	.	.	.	G	.	C	A
16. AF414870.1 Anaplasma ovis SOUTH AFRIC	.	.	T	C	.	.	.	.	.	A	G	T	.	.	T	A	T	.	.	.	.	.	.	G	.	C	A
17. AF309865.1 Anaplasma ovis USA	.	.	T	C	.	A	.	.	.	A	G	T	.	.	T	A	T	.	.	.	.	.	.	G	.	C	A

Table 9: A multiple sequence alignment of *16S rRNA* gene fragments for comparison of *A. ovis* isolates isolated from sheep and cattle. The *A. ovis* isolates 171A, 85A and 88A were found in cattle while other *Anaplama* organisms were isolated from sheep. The conserved regions are represented by the dots (.) while the variable regions are indicated by the letters representing the nucleotide A, C, G and T. The accession numbers AF309865.1, AF318945.1, AF414870.1, KJ410246.1 and LC194134.1, obtained from GenBank, are for *A. ovis 16S rRNA* sequences of isolates from USA, Netherlands, South Africa, China and Mongolia.

4.5.2 Comparison of bovine *Anaplasma* detected with isolates from other regions

In this study, the *Anaplasma* gene fragment sequences of the Kenyan isolates obtained from cattle were homologous to *A. phagocytophilum, A. marginale* and *A. centrale*. However, the sequences of *A. marginale* and *A. centrale* revealed lower nucleotide sequence identities and hits on blasting (Appendix 3). Multiple alignments of *A. phagocytophilum* nucleotide sequences of the bovine isolates were done in order to compare the Kenyan isolates with those from the other regions of the world. The sequences of all the Kenyan *A. phagocytophilum* were identical (99%) except for isolate 53A. The *A. phagocytophilum* isolates from Japan, Spain, Italy, North Korea, USA and Germany showed nucleotide sequence variations and appeared to be genetically diverse (Table 10).

Name	T A C C C A A T A G T A T A G G A T A G C C T T T G G A A A C G A A G
1. 10A A. phagocytophilum Kenya	. . . . .
2. 108A A. phagocytophilum Kenya	. . . . .
3. 53A A. phagocytophilum Kenya	. . . . . G .
4. KU686783.1 A. centrale Uganda	. . . T . G . . . . . A C . A . . . T . G T
5. KU686784.1 A. centrale Uganda	. . . T . G . . . . . A C . A . . . T . G T
6. JQ839010.1 A. centrale phillipine	. . . T . G . . . . . A C . A . . . T . G T
7. KU686793.1 A. marginale Uganda	. . . T . G . . . . G . . . . . A C . A . . . T . G T
8. KU686792.1 A. marginale Uganda	. . . T . G . . . . G . . . . . A C . A . . . T . G T
9. KU686785.1 A. marginale Uganda	. . . T . G . . . . G . . . . . A C . A . . . T . G T
10. AB196720.1 A. phagocytophilum JAPAN	. . . T . G . . . . . A C . A . . . T . G T
11. GU111742.1 A. phagocytophilum SPAIN	. . . T . G . . . . G . . . . . A C . A . . . T . G T
12. KP877313.1 A. phagocytophilum ITALY	. . . T . G . . . . G . . . . . A C . A . . . T . G T
13. KC422267.1 A. phagocytophilum NORTH KORE	. . . T . G . . . . . A C . A . . . T . G T
14. DQ426992.1 A. phagocytophilum USA	. . . . . C . . . . . G T
15. KU510431.1 A. phagocytophilum GERMANY	. . . T . . . . . G . . . . . A C . A . . . T . G T

Table 10: A multiple sequence alignment of *16S rRNA* gene fragments for comparison of *A. phagocytophilum, A. marginale* and *A. centrale* isolates obtained from cattle. The *A. Phagocytophilum* isolates 10A, 108A and 53A are Kenyan isolates showing conserved region represent as dots (.).The accession numbers KU686783.1, KU686784.1, JQ839010.1, KU686793.1, KU686792.1, KU686785.1, AB196720.1, GU111742.1, KP877313.1, KC422267.1, DQ426992.1, KU510431.1 obtained from GenBank, are for *A. phagocytophilum, A. marginale, A. centrale 16S rRNA* gene sequences of isolates from Uganda, Phillipine, Japan, Spain, Italy, North Korea, USA and Germany. The variable regions are indicted by the letters representing the nucleotides A, C, G and T.

4.6 Phylogenetic analysis

4.6.1 Phylogenetic analysis of the Kenyan *Anaplasma ovis* isolates

A phylogenetic tree was constructed in order to understand the genetic relationship of the Kenyan *A. ovis* as compared with the isolates from USA, Netherlands, South Africa, China and Mongolia. The *A. ovis* isolates clustered into one clade I except isolate 44A, which appeared in a separate clade V and represented a kind of outgroup. The *A. ovis* isolates from Mongolia, China and Netherland belonged to clade 2 but have recent common ancestor with the Kenyan isolates in clade I. The other isolates from South Africa and USA belonged to two separate individual clades (III and IV). These findings indicate that the some Kenyan *A. ovis* isolates are genetically close to isolates from Mongolia, China and the Netherland whereas other like 44A is not and may be specific to Kenya **(**Figure 11**).**

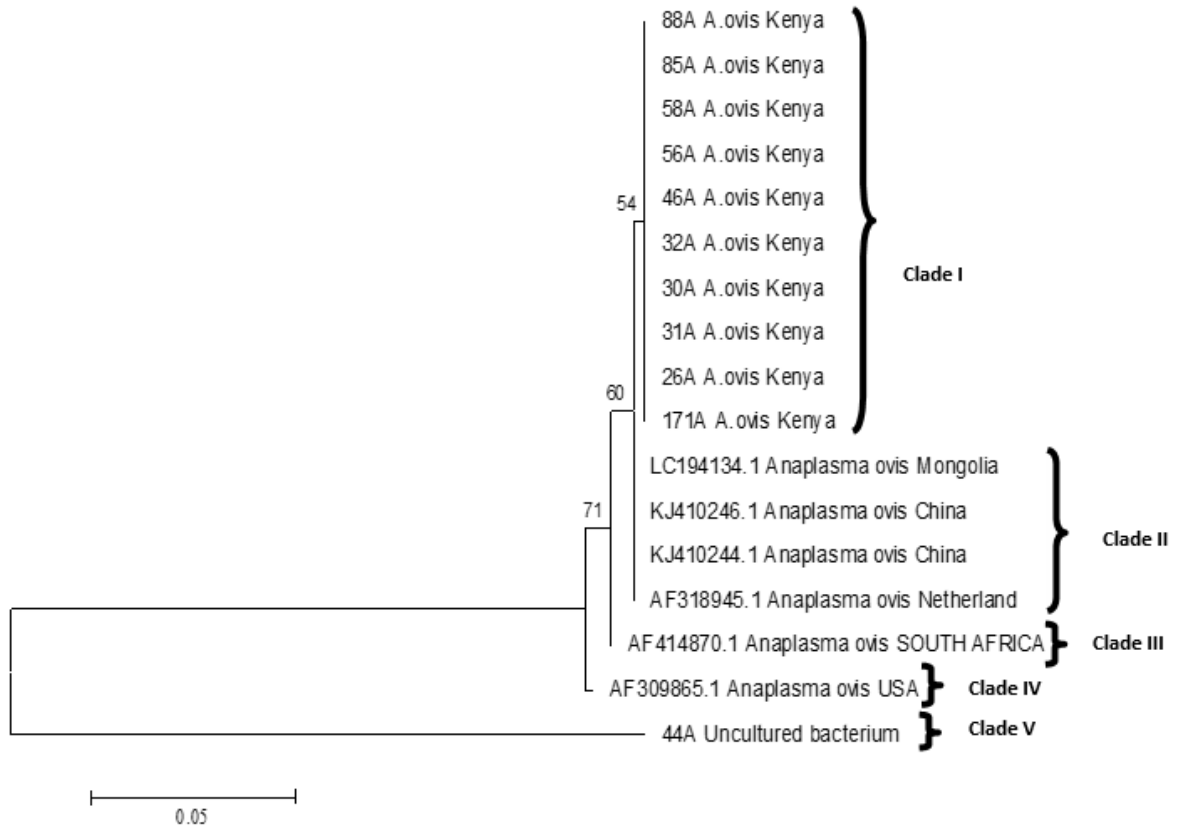

Figure 11: Phylogenetic analysis based on the *16S rRNA* fragment of the *A. ovis* isolated from sheep and cattle in Homabay and those from other regions of the world. The phylogenetic tree was constructed by the maximum likelihood method using MEGA 6 software; Bootstrap analysis was performed with 1000 replicates with Kimura 2-parameter (K2) model. The accession numbers AF309865.1, AF318945.1, AF414870.1, KJ410244.1 KJ410246.1 and LC194134.1, obtained from GenBank, are for *A. ovis 16S rRNA* gene sequences of isolates from USA, Netherlands, South Africa, China and Mongolia.

4.6.2 Phylogenetic analysis of the Kenyan *Anaplasma bovis* isolates

A phylogenetic tree was constructed in order to understand the genetic evolutionary relation of the Kenyan *Anaplasma species* and compare them with the isolates from Uganda, Spain, Italy Germany, Japan, North Korea and Kenya. The *A. centrale* isolates from Uganda belonged to clade I, *A. marginale* from Uganda isolates belonged to clade II. The other isolates of *A. phagocytophilum* from Spain, Italy, Germany, Japan, and North Korea clustered into different clades III, IV, V. The Kenyan's *A. phagoctophilum* isolates grouped into clade VI. The bootstrap value of 100 strongly indicates that the single USA isolate in the clade IV is significantly different from the Kenyan isolates, although evolutionarily, the Kenyans and USA clades descended from a recent common ancestor and were distantly related to all the other isolates in clades I to IV clustered in different monophyletic group. These findings indicate that the Kenyan *A. phagocytophilum* isolates display mixed genetic relationship with other isolates from the above listed regions (Figure 12).

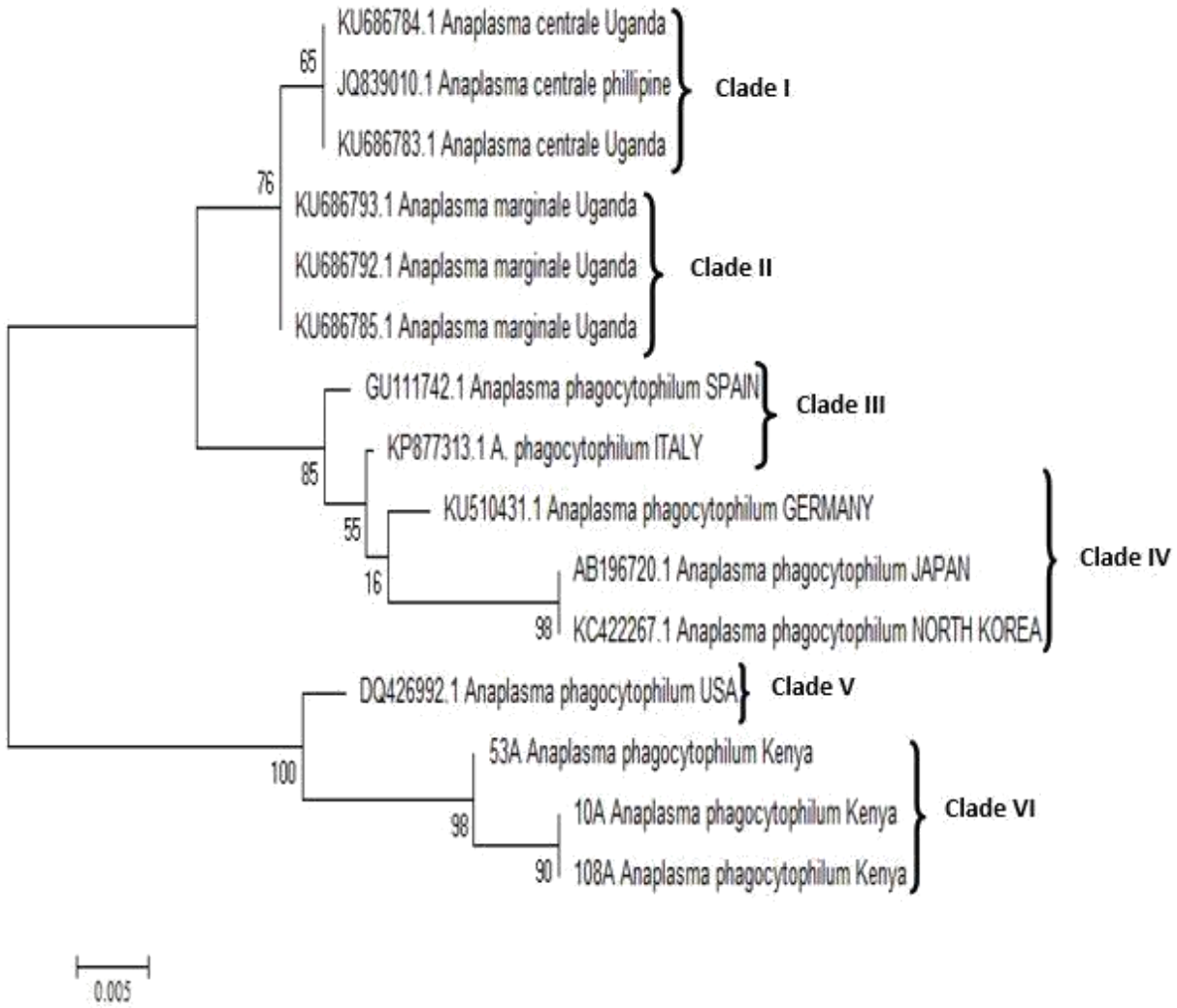

Figure 12: Phylogenetic tree of the *16S rRNA* gene from members of the genus Anaplasma in cattle. Phylogenetic analyses were conducted using Mega6. The tree provided nucleotide sequences from of *A. centrale, A. marginale*, *A. phagocytophilum* (performed with 1000 replicates) values are represented by the numbers above and below the internal branches with a model Kimura -2parameter (K2+G).The accession numbers KU686783.1, KU686784.1, JQ839010.1, KU686793.1, KU686792.1, KU686785.1, AB196720.1, GU111742.1, KP877313.1, KC422267.1, DQ426992.1 and KU510431.1, obtained from GenBank are for *A. centrale, A. marginale* and *A. phagocytophilum 16S rRNA* gene sequences of isolates from Uganda, Phillipine, Spain, Italy, USA, Germany, Japan, North Korea and Kenya.

5.0 DISCUSSION

*Anaplasma o*rganisms are known to infect humans and animals resulting in public health and economic consequences (Stuen *et al*., 2013; Kocan *et al*., 2003; Kocan *et al*., 2010). In this regard, *Anaplasma* organisms have been identified and characterized by molecular methods to be able to differentiate the various species of *Anaplasma* organisms and thus ensuring effective control of the disease (Woldehiwet, 2010; Dumler *et al*., 2007). In this study, novel primers were designed and used to detect as well as to characterize *Anaplasma* species infecting sheep and cattle in Homabay, Kenya.

The current study has generated pairs of primers using PrimerQuest software. The generated primers targeted and amplified the *16S rRNA* and *msp2* genes of *Anaplasma* organisms. This approach is consistent with that of previous studies that used the same software to design and synthesize primers for detecting plant pathogen by PCR (Haudenshield *et al*., 2017). Therefore, the fact that this software could be used to design primers for detection of both plant and animal pathogens by PCR indicates that it could be used widely to develop molecular techniques for detection of other pathogens of veterinary and medical importance. Indeed, from the ongoing literature, this appears to be one of the first study in which primers are designed using PrimerQuest tool for detection of *Anaplasma* organisms. Perhaps this software could also be useful for designing primers for detection of a range of *Anaplasma species* including other tickborne pathogens.

The generated primers could detect *Anaplasma 16S rRNA* gene fragment seen as specific and distinct bands of approximately 436 bp, which indicated the presence of *Anaplasma species* infection in cattle and sheep. These results are in agreement with previous studies that reported detection of *Anaplasma species* using primers targeting *16S rRNA* gene (Noaman and shayan, 2009). This indicates that the *16S rRNA* gene may be a good marker for the genetic detection of *Anaplasma* species by PCR.

This marker gene has therefore been used for understanding of the molecular epidemiology of bovine anaplasmosis (Wei *et al*., 2016; Ybañez *et al*., 2016). Furthermore, Jafarbekloo *et al*. (2014) also used the *16S rRNA* gene as a marker to detect *Anaplasma* DNA in tick collected from sheep.

The primers designed for the amplification of the *msp2* gene in the samples showed a positive band of 835 bp together with other nons-pecific bands. The presence of the positive band indicates of the infection of cattle and sheep by *Anaplasma* species and this is consistent with the results from other related studies, which used *msp2* as a marker gene (Wang *et al*., 2013; Rikihisa, 2011; Hotopp *et al*., 2006). However, the non-specific bands that resulted from this reaction suggested that this PCR assay required more optimization to achieve a specific PCR band corresponding to the *Anaplasma* organisms. It should be noted that the reagents available were limited and therefore further optimization of the PCR assay using *msp2* gene could not be achieved. Nevertheless, the above data confirm the relevance of the role of domestic ruminants as hosts and reservoirs for *Anaplasma* organisms in Homabay County. Previous studies have revealed that adequate optimization can yield *msp2* specific bands without other non-specific bands and thus could be used to detect *Anaplasma* pathogens in sheep and cattle (Hapunik *et al*., 2011; Overzier *et al*., 2013). Therefore, further optimization studies using the primers designed in this study and the *msp2* gene are suggested in order to develop a more specific and sensitive PCR for confirmation of the anaplasmosis in cattle and sheep.

It is noteworthy that the primers designed to specifically amplify the *msp1b* gene of *Anaplasma* species did not yield any positive bands on PCR. This was suggestive of either inadequate optimization of the PCR reactions or primers that were not able to bind specifically to the target *msp1b* gene of the *Anaplasma* isolates. A lack of optimization of the PCR assays using the primers targeting the gene could have been a possible cause of the negative results. The possibility of a lack of primer binding to the target DNA template was ruled out because primer blast analysis performed during primer design yielded sequences unique to the *Anaplasma* DNA. The chances of DNA samples being negative were also ruled out since the same samples

yielded positive bands when the other primers targeting *16S rRNA* gene. Therefore, the PCR assay using *msp1b* will require further optimization of the reaction in order to achieve better results.

Previous studies using *16S rRNA* and *msp2* genes has detected *Anaplasma* organisms in both cattle and sheep from China by PCR (Liu *et al*., 2012; Zhang *et al.*, 2016; Yang *et al*., 2016) consistent with the findings of this study. However, in the current study, most of the positive samples for sheep and cattle were detected by a PCR for *16S rRNA* gene as compared to an assay system using *msp2* marker. Subsequently, the detection of DNA was indicative of the *Anaplasma* presence in both cattle and sheep from Homabay County. The results implied that domestic ruminants may have been exposed to ticks that are infected by the various *Anaplasma* species. The relatively higher detection rate with *16S rRNA* gene as compared to *msp2* suggested that the former marker was more sensitive for *Anaplasma* detection whereas the later was highly polymorphic preventing primers to bind to the target. However, further studies are required to validate this claim since the lower positive rates seen when *msp2* was used could have been due to a lack of assay optimization. Furthermore, there were many other studies that had successfully reported the detection of *Anaplasma* DNA using *msp2* gene in cattle and sheep (M'ghirbi *et al*., 2016; Ybañez *et al*., 2013; Barbet *et al*., 2006). Therefore, the theory of lower detection rates of *Anaplasma* DNA by PCR when *msp2* is used as a marker gene may not hold in this case.

Many previous studies had confirmed the presence of *Anaplasma species* in cattle and sheep by BlASTn analysis after the sequencing of the *16S rRNA* gene (M'ghirbi *et al*., 2016; Ybañez *et al*., 2013; Barbet *et al*., 2006). Blastn analysis of the *16S rRNA* gene fragments sequenced in this study revealed nucleotides homologous to those of *A. phagocytophilum* and *A. ovis* for sample from cattle and *A. ovis* only for sheep samples. These findings indicated the infection of cattle with *A. phagocytophilum* and *A. ovis,* as well as infection of sheep with *A. ovis* in Homabay County. The infection of these animals with other *Anaplasma* species

such as *A. marginale*, *A. centrale* and other unknown *uncultured bacterium* could not be ruled out since blastn results also yielded homologues unique to these species although the sequence identities (95%) were relatively lower. For a long time, it has been known that *A. centrale* and *A. marginale* are the main *Anaplasma* species that infect cattle in Kenya, including Homabay County. However, the findings of this study revealed that infection of cattle in the sub-counties in Kenya can also involve a range of *Anaplasma* species including the zoonotic ones such as *A. phagocytophilum*. These results are consistent with those of a number of studies done in other countries in which *A. phagocytophilum* was found to infect cattle (M'ghirbi *et al*., 2016; Noaman and Shayan, 2009). These studies did not report severe clinical disease in cattle. It would be important to conduct studies to further confirm the extent of *A. phagocytophilum* infection of cattle in the study areas as this was the first study to report the pathogen in cattle in Kenya.

The presence of the various *Anaplasma* species infections in cattle and sheep suggested the presence of other ticks that may transmit the infection associated with *A. phagocytophilum* (Cao *et al*., 2003). *A. phagocytophilum* may be transmitted transtadially and transovarially indicating the tick *Dermacentor albipictus* was also a potential vector (Baldridge *et al.,* 2009). Nonetheless, whether *D. albipictus* has a role in the transmission of the pathogen in the study areas is yet to be documented. The detection of *A. phagocytophilum* in cattle warrants further study to determine its public health implications since it is a potentially zoonotic pathogen (Dumler *et al*., 2005; Zhang *et al*., 2014).

In this same study, *A. ovis* was detected in cattle, suggesting that the zebu cattle could be potential hosts for various species of *Anaplasma* isolates, contrary to the previous belief that cattle in Kenya are only infected by *A. marginale* and *A. centrale*. Although this was the first report of cattle being infected with *A. ovis* in Kenya, the infection of cattle was not new as there is a previous report of cattle infection with the *A. ovis* in Greece (Giadinis *et al*., 2015). Small ruminants such as sheep are susceptible to *A. ovis*, where it was found to cause disease (Renneker *et al*., 2013).

The organism was isolated from human samples by Chochlakis *et al*. (2010). This suggested that there was a possibility of infection with *A. ovis*-human variant. In the current study, *A. ovis* was detected in sheep in Homabay County and it is not clear whether these isolates could cause ovine anaplasmosis.

Indeed, previous studies documented infection of sheep with *A. ovis* where they cause clinical disease (Yasini *et al*., 2012). The results of this study appeared to be the first to detect *A. ovis* in cattle and sheep in Kenya and therefore, it would be necessary to do further studies to unravel the extent of ovine anaplasmosis in Kenya. Most farmers graze sheep goats and cattle together in the sub-counties in Homabay; therefore, infection of cattle with *A. ovis* isolates suggest that interactions between cattle and sheep may have resulted into the cross-infection possibly by tick transmission through bite. Although *A. ovis* was detected in cattle, it is not clear whether this *Anaplasma* species can cause clinical disease in cattle and it would be interesting to know their role in causing the disease in cattle.

Most of Kenyan *A. ovis* isolates infecting sheep and cattle appeared to have conserved genetic region and were identical (99%) to isolates from China, Mongolian, and Netherland (Bekker *et al*., 2002, Lew *et al*., 2003). It therefore appeared that the Kenyan *A. ovis* isolates were genetically similar to the isolates from these countries. One *A. ovis* isolate (44A) was genetically distant from the other isolates suggesting that it may have been introduced in Kenya long ago from a different ancestor. More investigations should be carried out with longer fragments of the genes to ascertain the existence of the genetic variations within the *A. ovis* isolates. Nevertheless, case of conserved and non-conserved *16S rRNA* gene sequences is not unique to this study since it has been have been documented for other *A. ovis* isolates (Bekker *et al*., 2002, Lew *et al*., 2003).

Previous studies had documented the genetic relatedness and variations of *A. phagocytophilum* infecting cattle and humans using *16S rRNA* gene (Michalski *et al*., 2006; Ybañez *et al*., 2013). Like in the current study, the nucleotide sequences of the Kenyan *A.*

phagocytophilum isolates infecting cattle appeared to be genetically closely related as most of the isolates revealed conserved gene regions.

Most of the *A. phagocytophilum* isolated from cattle in this study appeared to be genetically different from those from other regions (Michalski *et al*., 2006; Ybañez *et al*., 2013). Nevertheless, further studies are required to understand the extent of genetic relatedness or variations across geographical regions.

Previous studies using phylogenetic analyses based on *16S rRNA* gene showed that *A. ovis* isolates from the same geographical areas tended to cluster within the same clade (Bekker *et al*., 2002; Lew *et al*., 2003). Indeed, similar observations were made with the Kenyan *A. ovis* isolates infecting sheep and cattle. These isolates appeared to cluster in the same clade suggesting a genetic relationship amongst them. However, the Kenyan isolates did not cluster in the same phylogenetic clade as the *A. ovis* isolates from the other regions of the world (Bekker *et al*., 2002; Lew *et al*., 2003). This indicated that they are distantly related to isolates from other regions. The genetic variations seen in various isolates across different geographic regions may be as a result of many factors including climate variability, mutation or even selection pressure as a result of other environmental factors.

The phylogenetic analysis of one of the sequences (44A) of an uncultured bacterium isolates showed that it belonged to a separate clade (Figure 11). Although this wasn't expected in this study, this showed a clear difference between these isolates in sheep in comparison to the others found in both cattle and sheep from other regions of the world (Bekker *et al*., 2002; Lew *et al*., 2003).These differences could be suggestive of a novel different strain of *A. ovis* isolate but further studies should be carried out to validate this claim.

The Kenyan *A. phagocytophilum* isolates from cattle belonged in a unique and single clade compared to other isolates from USA, Germany, Italy and Spain (Michalski *et al*., 2006; Ybañez *et al*., 2013). It was possible that differences in climatic conditions and other geographical conditions could have resulted in *Anaplasma* isolates selection and the genetic

variations. Therefore, extensive molecular epidemiology studies are required to understand the genetic profiles of the *Anaplasma* isolates in Homabay County.

The current study has detected and characterised new species of *Anaplasma* infecting cattle and sheep in Homabay County in Kenya. The molecular based findings have enabled the understanding of the genetic difference that exists between the different species of *Anaplasma* isolates infecting animals in Homabay County and in other parts of the world. Also, the current study established that the *16S rRNA* gene may be an appropriate marker for characterising the prevalence and genetic diversity of *Anaplasma* species in the field. Nevertheless, this study encountered some setbacks including failure of primers targeting msp 1b gene to amplify the gene and a lack of optimization of some PCR assays based on *msp2* gene. The fragments of all genetic markers used were less than 1.0 kbp and therefore may have to not been able to give an ideal sequence variations for the phylogenetic characterization. Perhaps future studies should involve more samples and whole genome sequencing of the *Anaplasma* isolate representatives to be able to understand their detailed molecular epidemiology. In summary, this study provided basic information on molecular characteristics of *Anaplasma* isolates that may go a long way in promoting future research and understanding the molecular epidemiology of anaplasmosis.

The study carried out provided a basis for developing a PCR kit for molecular diagnosis and characterization of *Anaplasma* isolates in various geographical regions.

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

Based on the findings of this study, the following conclusions were made;

1. The Primerquest software is appropriate for use in primer design for the detection and characterization of *Anaplasma species* infecting cattle and sheep.
2. The PCR assay developed in this study could be used to detect *Anaplasma* organisms in the blood of cattle and sheep and thus may be validated for use in molecular diagnosis of anaplasmosis.
3. Cattle and sheep in Homabay County are infected with various *Anaplasma* species including *A. phagocytophilum, A. ovis*.
4. The *Anaplasma* organisms characterized in this study appear to be genetically distinct as compared with those from the other regions of the world.

6.2 RECOMMENDATIONS

The following recommendations were made;

1. There is need to conduct further research to establish the role of the zoonotic *A. phagoctophylum* infection in both domestic animals and humans in the Suba and Mbita sub-counties.
2. The role of ticks in the transmission of *Anaplasma* organisms should be investigated to be able to establish the public health implications of the zoonotic *A. phagocytophilum* isolates.
3. There is need to further optimize and validate the PCR assay developed in this study with the possibility of using it for molecular diagnosis of anaplasmosis in animals and human.
4. Optimization of other PCR assays based on *msp1b* and *msp*2 genes of *Anaplasma* organisms is recommended for sensitive and specific detection of the tickborne pathogen.

5. Further research should focus on the characterizations of other bacteria-like organisms related to *Anaplasma* to understand the nature and role of these organisms in disease causation.
6. Whole genome sequencing of the *Anaplasma* species isolated in the study areas is recommended to be able to further understand the molecular epidemiology of anaplasmosis at the intra and interspecies level.

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APPENDICES

Appendix 1: The output of computer gerenerated primers and their positions in the target genes.

1.1 Primer pair targeting the *Ac-16S rRNA* gene.

Parameter Set: General PCR (Primers only)
Sequence Name: 16SRNA
Amplicon Length: 436

		Start	Stop	Length	Tm	GC%
Forward	CAGACGGGTGAGTAATGCATAG (Sense)	78	100	22	62	50
Reverse	ATGCCCTTTACGCCCAATAA (AntiSense)	494	514	20	62	45

Base	Sequence
1	ATGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAGTAATGCATAGG
101	AATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAG
201	ATTAGCTAGTTGGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCC
301	TACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCCAGCTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAAAACTCTTTCAG
401	TAGGGAAGATAATGACGGTACCTACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCAAGCGTTGTTCGGAATTATTGG
501	GCGTAAAGGGCATGTAGGCGGTTTGGTAAGTTAAAGGTGAAATACCAGGGCTTAACCCTGGGGCTGCTTTTAATACTGCAGGACTAGAGTCCGGAAGAGG
601	ATAGCGGAATTCCTAGTGTAGAGGTGAAATTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTACTGACGCTGAGGTGCGAA
701	AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGCTGAATGTGGGGGCTTTTGCCTCTGTGTTGTAGCTAACGCGT
801	TAAGCACTCCGCCTGGGGACTACGGTCGCAAGACTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAAC
901	GCGAAAAACCTTACCACTTCTTGACATGGAGGCTAGATCCTTCTTAACGGAAGGGCGCAGTTCGGCTGGGCCTCGCACAGGTGCTGCATGGCTGTCGTCA
1001	GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCATCCTTAGTTACCAGCGGGTAATGCCGGGCACTTTAAGGAAACTGCCAGTG
1101	ATAAACTGGAGGAAGGTGGGGATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGGCGACTACAATAGGTTGCAACGTCG
1201	CAAGGCTGAGCTAATCCGTAAAAGTCGTCTCAGTTCGGATTGTCCTCTGTAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCATGCC
1301	ACGGTGAATACGTTCTCGGGTCTTGTACACACTGCCCGTCACGCCATGGGAATTGGCTTAACTCGAAGCTGGTGCGCCAACCGTAAGGAGGCAGCCATTT
1401	AAGGTTGGGTCGGTGACTGGGGTGAAGTCGTAA

1.2 Primer pair targeting the *Am-16S rRNA* gene.

Parameter Set: General PCR (Primers only)
Sequence Name: Anaplasma-16S RNA
Amplicon Length: 835

		Start	Stop	Length	Tm	GC%
Forward	ACTAGAGTCCGGAAGAGGATAG (Sense)	554	576	22	62	50
Reverse	GTCACCGACCCAACCTTAAA (Anti$ense)	1369	1389	20	62	50

Base	Sequence
1	AAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGGTTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACT
101	AGAAATGGTGGGTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAGATTAGCTAGTTGGTGGGGTAATGGCCTAC
201	CAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGA
301	CAATGGGCGCAAGCCTGATCCAGCTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAAAACTCTTTCAGTAGGGAAGATAATGACGGTACCTACAGAA
401	GAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCAAGCGTTGTTCGGAATTATTGGGCGTAAAGGGCATGTAGGCGGTTTGGTAA
501	GTTAAAGGTGAAATACCAGGGCTTAACCCTGGGGCTGCTTTTAATACTGCAGGACTAGAGTCCGGAAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAA
601	TTCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTACTGACGCTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACC
701	CTGGTAGTCCACGCTGTAAACGATGAGTGCTGAATGTGGGGGCTTTTGCCTCTGTGTTGTAGCTAACGCGTTAAGCACTCCGCCTGGGGACTACGGTCGC
801	AAGACTAAAACTCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAAAACCTTACCACTTCTTGACATGG
901	AGGCTAGATCCTTCTTAACGGAAGGCGCAGTTCGGCTGGGCCTCGCACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
1001	CCGCAACGAGCGCAACCCTCATCCTTAGTTACCAGCGGGTAATGCCGGGCACTTTAAGGAAACTGCCAGTGATAAACTGGAGGAAGGTGGGGATGATGTC
1101	AAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGGCGACTACAATAGGTTGCAACGTCGCAAGGCTGAGCTAATCCGTAAAAGTCGTC
1201	TCAGTTCGGATTGTCCTCTGTAACTCGAGGGCATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGTACA
1301	CACTGCCCGTCACGCCATGGGAATTGGCTTAACTCGAAGCTGGTGCGCCAACCGTAAGGAGGCAGCCATTTAAGGTTGGGTCGGTGACTGGGGTGAAGGC
1401	AG

1.3 Primer pair targeting the *msp1b* gene.

Parameter Set: General PCR (Primers only)
Sequence Name: Sequence 1
Amplicon Length: 716

		Start	Stop	Length	Tm	GC%
Forward	CTAGACTTGGTGCAGGGTTATG (Sense)	587	609	22	62	50
Reverse	GCCAAATGCATCGCCAATAG (AntiSense)	1283	1303	20	62	50

Base	Sequence
1	ATGACAGAAGACGACAAGCAACAACAACAGAATCAAAGCGATGTAGTACAAGCCATCTCGGCCGTATTCCAGCGCAAGAGTGCAGAGCTGCAGCGGCTGA
101	ATGACTTCATAAAAGGCGCTGATGGTACACTCAAGAACGTCCATCCCCACATGAAGTCACTGGAAGCGCTTTCTAAGCAACTATCAGAAAAGATTGCAGC
201	TGAGGCAGCAGCGAAGGCAGATGCTAAATACGAGAGCGTGGGACTACGTGCTAAAGCAGCTGCAGCATTAGGTAATCTCGGGCGGCTTGTCGCCCGTGGT
301	AAACTCAAGAGCTCAGATGCACCCAAGGACCTTGACCAGAGCATTGACGCACTACCGTTCATGGATGAAGCACCTGACACTGGTGAGAAGATTGAAGTAC
401	CAGCGGGTGAGGAGCAAGAATTTGGCAAGGCAGCAGCTTGGGGTCTAGCAGGCTTCAAGCGTACAGTGGATGAAAGCCTGGAGATGTTAGACCGAGGCAT
501	GCACATGCTCGCGGAAGGCCAGGCACAGATATCACAGGGGATTGACGCCAAGGATACTGCACTAGTTAGGGAAGGTCTGGAAACATCTAGACTTGGTGCA
601	GGGTTATGTCGCAATGGCTTGGTAGAGGCCTCCTACGGCGTTGGTTATGCCAATGAGACCATGGGCAAGTATGCCGGCAAGGGTCTAGACAAGTGTAAAA
701	ACAAACTCGACAATGCATGCCACAAGTGGAGCAAGGCTCTCGAAGAGATTGAAAGCCTGCGCACAGCAATCGACGCGAAGGCAGAACAGCAAGTTGAAGG
801	TGAAGCATGGTCTCCTGAAGGGGTCAGTGCTAACACATTCTACAAAGGACTGCATAAAATTGGCACCGCAATTGCAGTAGCAGCTCAAGCTACCTGGGAA
901	GGCTTGGCTATGACCGGTAAGTTCATGGGTGCTGTAGCTAAACTAGCTGGTGCAGTATCCATGTGCGTTGCAGCATACACCGCAGCTATCGTGGGTATGG
1001	CCGCAGCTACACCTGCGACGCTGCTGCTGACAGCTATGGACAATCAATCCGTAAACAATGCCGTAGTTAAAGTCAGTGAGTACCTTCACAGTAACGTAGA
1101	ACAAGCAACTAAAGACCTCATGGCTTCAGAGTTTGCCATGATGACATTTGGTGGCATCATGACGTGTGCCAAGCTTATGAAGGGCTCCTTCGCAGCAATC
1201	AATCAGAAGTTTGAAGAAATCAACGCCACCCTCACACGGGAGGCCACAGACATCGCTCAAGGGGTCAAGGAGACTTACCAGTCTATTGGCGATGCATTTG
1301	GCAATGCATTCAAGTCTGTTGGCGATGCATTCAAGTCTATTGGCGATGCATTCAAGTCAGCTAATGATGGCATAGCTAAGTGGACAGCAGCTCTAGCAGG
1401	TTATGCGTCAGTTGAACAGCTAGAAGAAGCAAAGGAAGCAGACAGGGTACAGGCTGAGCAGCGAGCTGAAGCACAAGCAATGACCGAGCGTGTGGCAGGG
1501	GAGCGTGCAGCAACAGTTGCTGCAGGGACTGAAACCATTAAGACCATCGTCAGCGATATGCGGAATGAGCTTGCTAAAGGGCATGAACAGCTTCAGCTCG
1601	TCATCACCGATATGTGTAATGAGCTTGCACAAATAGGTGCATTCTCCCAAGCAGAGCGCGATGCACTTGTGAAGTCCTTCACGCCTAAACCTCCTGCTAG
1701	GACAACCAAGGAGCTTATCTCACATATGCATTCGGGCCTAGAATCCGTGATGTTCCGTATGGCACGTAGTCTTGGGATCATGAGCAAAGCTAGTATAGAG
1801	GCAAACTCGCAGGACAATAGTGTAGAGGTGGCAGAGATCAGCCCAGAAACGCAGAACATGAGCGACGCTATACCTGTAGAAGAAGCCCAAATTGTCGAAA
1901	CTGCCTTACTTGCAGCAGTAAATGACACTAGTAAGGACGACCAAGCAATTGTTACTGACCTTATAAACGCTACAATAGAGGTGTGCACAGAGCAGACTAA
2001	TACACTTGCGGGGCATACTGCCGAGGTCCAAGCAGGGCTGGAAGCTGCGGGTATTAGATTCGACGATGCACAGGGACTACAAGAAGCTACCCCTGAAGCC
2101	AAGGGCGTGGAAGGCATTAATCAAGAGGAACTCGAGCAGGCAGCTGAAGGTCTTGCTGCTGCTGTAAATGAGGCTTCTGCAGATGGGAAGATGCAGTCCC
2201	TCAATCAGCAGGAGACCCAGATTGCACAGGGAGAACAGCAGCAACAGCAGTCTTCTGGTTGGTCTAGGTAA

1.4 Primer pair targeting the *msp2* gene.

Parameter Set: General PCR (Primers only)
Sequence Name: MSP2
Amplicon Length: 576

				Start	Stop	Length	Tm	GC%
Forward	GGCAGCACTAGAGGTGTATTC (Sense)	Hairpin	Blast	223	244	21	62	52.4
Reverse	TCCCTCCTTATCGAGGCTATT (AntiSense)			778	799	21	62	47.6

Base	Sequence
1	ATGAGTGCTGTAAGTAATAGGAAGCTTCCCCTGGGAGGCGTGTTGATGGCTCTGGTCATAGGAGCTGCTACGCCGGGTTCTTTGTCTGCTGCCCCCGCAA
101	CTGGAGGCGGTGCCAGTAGTGACGGCCTGTTTTCAGGTGCAGGGGCTGGAAGTTTTTACGTAGGGTTGGATTACAGCCCAGCATTCGGCAGCATCAAAGA
201	CTTCAAAGTTGGGGAGGCCGGTGGCAGCACTAGAGGTGTATTCCCGTACAACGGAGACACTACCGGAAGGGTGGACTTCAAAGTCCAGAACTTCGACTGG
301	AGCGCCCCAGAACCTAGGATCAGCTTCAAGGACAGCATGGTTACTGCTGCAGAAGGAAGCATTGGGTATAGTATTGGAGGAGCCAGGGTTGAAGTTGAAG
401	TAGGGTATGAGAGGTTTGTTATTAAGGGAGGTAAGAAGTCTAATGAGGATACAGCTTCAGTATTCTTATTAGGAAAGGAGTTAGCATATGATACAGCTAG
501	AGGTCAGGTAGAGCGTCTTACTACTCATTTAGGTAAGATAACTAAGAGTGATGCCAAGAGGTGGGGTACTGCAGTAGAGGCTGCCACTAATGGTCAAACA
601	GTGAGCCAGAAGGTGTGTGGTGCCAATGGTACTAGTGGTGGTAGCACTGGCACCTGTGGTAAGAACACTGACGGCGCTAACAATGGCAACAAGATTAGTG
701	TGGTGTTTACTGAAGATGCGACACAACTCTCTGCTGAGAGTAACACCATCAGCTTGCAGGGGATGGCGAACAACATCAATAGCCTCGATAAGGAGGGAAA
801	GGCTGTTGTTGCTGGGGCTTTTGCCAAAGCTGTTGAAGGTGCGGAGGTGATAGAGGTTAGGGCTATTGGGTCTACTTCTGTAATGCTCAATGCTTGCTAT
901	GACCTCCTGACTGATGGTATTGGGGTTGTGCCTTATGCTTGTGCTGGTATCGGTGGTAACTTCATCAGTGTTGTAGACGGGCATATAAATCCTAAGTTCG
1001	CCTACAGAGTGAAGGCTGGTCTGAGTTATGCTCTGACTCCTGAAATTTCCGCTTTCGCTGGGGCTTTCTACCATAAGGTGCTAGGTGATGGTGACTATGA
1101	TGAGCTACCTCTTAGCCCTATTGCTGACTATACAGGCCCAGCTGGGAGAAATAAGGAAACTGGTGTAGCTTCCTTCACCATGGCTTACTTCGGTGGTGAG
1201	TTCGGTGTTAGGTTTGCCTTCTAGCT

Appendix 2: The target genes obtained from GenBank and used for primer design together with their respective accession number.

2.1. *Anaplasma marginale* strain Uganda MT34 16S ribosomal RNA gene, partial sequence

GenBank: KU686792.1

ATGAACGCTGGCGGCAAGCTTAACACATGCAAGTCGAACGGACCGTATACGCAGCTTGCTGCGTGTATGG
TTAGTGGCAGACGGGTGAGTAATGCATAGGAATCTACCTAGTAGTATGGGATAGCCACTAGAAATGGTGG
GTAATACTGTATAATCCCTGCGGGGGAAAGATTTATCGCTATTAGATGAGCCTATGTCAGATTAGCTAGT
TGGTGGGGTAATGGCCTACCAAGGCGGTGATCTGTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAAC
TGAGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGGGCGCAAGCCTGATCC
AGCTATGCCGCGTGAGTGAGGAAGGCCTTAGGGTTGTAAAACTCTTTCAGTAGGGAAGATAATGACGGTA
CCTACAGAAGAAGTCCCGGCAAACTCCGTGCCAGCAGCCGCGGTAATACGGAGGGGGCAAGCGTTGTTCG
GAATTATTGGGCGTAAAGGGCATGTAGGCGGTTTGGTAAGTTAAAGGTGAAATACCAGGGCTTAACCCTG
GGGCTGCTTTTAATACTGCAGGACTAGAGTCCGGAAGAGGATAGCGGAATTCCTAGTGTAGAGGTGAAAT
TCGTAGATATTAGGAGGAACACCAGTGGCGAAGGCGGCTGTCTGGTCCGGTACTGACGCTGAGGTGCGAA
AGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCTGTAAACGATGAGTGCTGAATGTGGGG
GCTTTTGCCTCTGTGTTGTAGCTAACGCGTTAAGCACTCCGCCTGGGGACTACGGTCGCAAGACTAAAAC
TCAAAGGAATTGACGGGGACCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAAAACC
TTACCACTTCTTGACATGGAGGCTAGATCCTTCTTAACGGAAGGGCGCAGTTCGGCTGGGCCTCGCACAG
GTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTCA
TCCTTAGTTACCAGCGGGTAATGCCGGGCACTTTAAGGAAACTGCCAGTGATAAACTGGAGGAAGGTGGG
GATGATGTCAAGTCAGCACGGCCCTTATGGGGTGGGCTACACACGTGCTACAATGGCGACTACAATAGGT
TGCAACGTCGCAAGGCTGAGCTAATCCGTAAAAGTCGTCTCAGTTCGGATTGTCCTCTGTAACTCGAGGG
CATGAAGTCGGAATCGCTAGTAATCGTGGATCAGCATGCCACGGTGAATACGTTCTCGGGTCTTGTACAC
ACTGCCCGTCACGCCATGGGAATTGGCTTAACTCGAAGCTGGTGCGCCAACCGTAAGGAGGCAGCCATTT
AAGGTTGGGTCGGTGACTGGGGTGAAGTCGTAA

2.2. *Anaplasma centrale* major surface protein 2 *(msp2)* gene, complete cds

GenBank: DQ357199.1

ATGAGTGCTGTAAGTAATAGGAAGCTTCCCCTGGGAGGCGTGTTGATGGCTCTGGTCATAGGAGCTGCTA
CGCCGGGTTCTTTGTCTGCTGCCCCCGCAACTGGAGGCGGTGCCAGTAGTGACGGCCTGTTTTCAGGTGC
AGGGGCTGGAAGTTTTTACGTAGGGTTGGATTACAGCCCAGCATTCGGCAGCATCAAAGACTTCAAAGTT
GGGGAGGCCGGTGGCAGCACTAGAGGTGTATTCCCGTACAACGGAGACACTACCGGAAGGGTGGACTTCA
AAGTCCAGAACTTCGACTGGAGCGCCCCAGAACCTAGGATCAGCTTCAAGGACAGCATGGTTACTGCTGC
AGAAGGAAGCATTGGGTATAGTATTGGAGGAGCCAGGGTTGAAGTTGAAGTAGGGTATGAGAGGTTTGTT
ATTAAGGGAGGTAAGAAGTCTAATGAGGATACAGCTTCAGTATTCTTATTAGGAAAGGAGTTAGCATATG
ATACAGCTAGAGGTCAGGTAGAGCGTCTTACTACTCATTTAGGTAAGATAACTAAGAGTGATGCCAAGAG
GTGGGGTACTGCAGTAGAGGCTGCCACTAATGGTCAAACAGTGAGCCAGAAGGTGTGTGGTGCCAATGGT
ACTAGTGGTGGTAGCACTGGCACCTGTGGTAAGAACACTGACGGCGCTAACAATGGCAACAAGATTAGTG
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2.3. *Anaplasma marginale* surface protein (*msp1b*) gene, complete cds GenBank: M59845.1

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2.4. *Anaplasma marginale* strain Uganda MT27 16S ribosomal RNA gene, partial sequence.
GenBank: KU686794.1

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AG

Appendix 3: Blast results

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenBank Graphics Distance tree of results

Description	Max score	Total score	Query cover	E value	Ident	Accession
Uncultured Anaplasma sp. clone Saso 16S ribosomal RNA gene, partial sequence	704	704	100%	0.0	99%	KY924885.1
Uncultured Anaplasma sp. clone Hadesa 16S ribosomal RNA gene, partial sequence	704	704	100%	0.0	99%	KY924884.1
Anaplasma phagocytophilum strain Teh00-1 16S ribosomal RNA gene, partial sequence	693	693	100%	0.0	99%	KT870142.1
Anaplasma phagocytophilum strain K47 16S ribosomal RNA gene, partial sequence	693	693	100%	0.0	99%	KT870141.1
Anaplasma phagocytophilum strain K26 16S ribosomal RNA gene, partial sequence	693	693	100%	0.0	99%	KT870140.1
Anaplasma phagocytophilum strain DA01-12 16S ribosomal RNA gene, partial sequence	688	688	100%	0.0	99%	KT870133.1

Appendix 4: Consensus sequences deposited in the genbank.

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Appendix 5: Ethical statement

UNIVERSITY OF NAIROBI
FACULTY OF VETERINARY MEDICINE
DEPARTMENT OF VETERINARY ANATOMY AND PHYSIOLOGY

P.O. Box 30197,
00100 Nairobi,

Tel: 4449004/4442014/ 6
Ext. 2300
Direct Line. 4448648

Dr Metinou Koumetio Sidouin

REF:FVM BAUEC/2017/127

c/o Dept of Public Health Pharmacology & Toxicology

Dear Dr Sidouin

14/06/2017

RE: Approval of Proposal by Biosafety, Animal use and Ethics committee

Molecular Epidemiology of Anaplasmosis affecting cattle and sheep in Homabay County, Kenya

By Metinou Koumetio Sidouin (J56/75748/2014)

We refer to the above proposal that you re-submitted to our committee for review and approval. We have now reviewed the proposal and note that you have addressed the issues that had been raised concerning your proposal including: change of title of the proposal to include sheep, details on restraint of animals and inclusion of details on biosafety, occupational health and safe disposal of materials and chemicals that will be used.

We hereby approve your study as detailed in your revised proposal.

Rodi O. Ojoo BVM MSc PhD
Chairman,
Biosafety, Animal Use and Ethics Committee,
Faculty of Veterinary Medicine

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Appendix 6: Turnitin Originality Report

MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *ANAPLASMA* HAEMOPARASITES ISOLATED FROM CATTLE AND SHEEP IN HOMABAY COUNTY, KENYA (MLIS) by **Metinou Sidouin**.

MOLECULAR IDENTIFICATION AND CHARACTERIZATION OF *ANAPLASMA* HAEMOPARASITES ISOLATED FROM CATTLE AND SHEEP IN HOMABAY COUNTY, KENYA (MLIS).

- Processed on 15-Jun-2017 13:55 EAT
- ID: 825180936
- Word Count: 12456

Similarity Index
14%
Similarity by Source
Internet Sources:
6%
Publications:
9%
Student Papers:
3%
sources:
1

2% match (Internet from 06-Dec-2014)

http://edoc.ub.uni-muenchen.de/16100/1/Pohl_Anna_E.pdf
2

1% match (publications)

ATIF, FARHAN AHMAD. "Alpha proteobacteria of genus *Anaplasma* (Rickettsiales: Anaplasmataceae): Epidemiology and characteristics of Anaplasma species related to veterinary and public health importance", Parasitology, 2016.
3

1% match (publications) has enabled the understanding of spread of Anaplasma species as well as other tick borne haemoparasites in the cattle population. As such, Vincenzo Lorusso et al. (2016) using PCR and reverse line blot (RLB) was able to identify and characterize A. marginale, A. omatjenne, A. centrale and A. platys in cattle population in Nigeria. This also helped ascertaining the rate of infection amongst cattle population in Nigeria. Additionally the genetic diversity and distribution.