COMPARISON OF REVERSE LINE BLOT AND β-TUBULIN TARGETED NESTED PCR TECHNIQUES IN THE DETECTION OF TICK-BORNE HEAMOPARASITES OF RUMINANTS

BY

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DECLARATION

I, **ATUHAIRE David Kalenzi**, declare that this is my own original work and it has not been submitted to any institution for any related purposes.

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DEDICATION

This work is dedicated to my parents Bob and Grace who lived a peasant's life to give their progeny the precious gift of education, and to my fiancée Pam and our son Matthew, who endured my long absence from home, missed my love and care. Our greatest days lie ahead.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS AND SYMBOLS

et al	and others
β	Beta
^{0}C	Degrees Centigrade
DNA	Deoxyribonucleic acid
ECF	East coast fever
µl	Micro liter
M	Microns
ml	millilitres
mM	millimolar
Min	Minutes
PCR	Polymerase Chain reaction
RLB	Reverse Line blot
RNA	Ribonucleic acid
rDNA	ribosomal DNA
rRNA	Ribosomal RNA
S	Seconds
SDS	Sodium Dodecyl Sulphate
SSU	Small sub unit
Spp	Species
TBDs	Tick borne diseases
UDG	Uracil DNA glycosylase
US$	United States dollars

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

In this study, Reverse Line Blot assay and Beta-tubulin targeted nested PCR were compared in detection of tick-borne piroplasms in areas around L.Mburo in Western Uganda. A total of 300 blood samples were collected from apparently normal cattle and examined for intra-erythrocytic forms of *Theileria* and *Babesia* parasites by Microscopy out of which 120 (40%) were positive for *Theileria* piroplasms. Eighty positive and 40 negative samples by microscopy were selected for comparison of the two tests. Out of the 80 samples positive by microscopy, 57 (71.25%) were positive for the individual species and 23 (28.75%) were negative by the RLB assay while 50 (62.5%) tested positive and 30 (37.5%) tested negative by beta-tubulin targeted nested PCR. Of the 57 samples positive by RLB assay, 8 (14.04%) were infected by *Theileria mutans* only, 47 (82.46%) were simultaneously infected with *Theileria parva* and *Theileria spp (buffalo)* but not with any other species, while one was simultaneously infected with *Theileria parva*, *Theileria spp (buffalo)* and *Babesia bovis*. Out of the 40 samples testing negative by microscopy, 17 (42.5%) samples were positive and 23 (57.5%) were negative by RLB assay while 11 (27.5%) were positive and 29 (72.5%) were negative by beta-tubulin nested PCR analysis. Eleven (64.7%) of the positive samples had mixed infection, while 6 (35.3%) had single infections. The RLB assay allowed the detection of individual species that simultaneously infected the cattle. However, it was not possible to identify the species with the beta-tubulin targeted nested PCR. The sensitivity of RLB technique for the detection of *Theileria* and *Babesia spp* was 71.25% (95% CI: 60.05 – 80.82%) and the specificity was 57.50% (95% CI: 40.89 - 72.96%) while the sensitivity of the beta-tubulin targeted nested PCR was 62.5% (95% CI: 50.96 – 73.08%) and the specificity was 72.5% (95% CI: 56.11 – 85.40). The Positive Predictive Value using RLB was 77.03 % and the Negative Predictive Value was

50 % while the Positive Predictive Value using beta-tubulin targeted nested PCR was 81.97 % and the Negative Predictive Value was 49.15%. The Kappa statistic for level of agreement in detection of tick-borne piroplasms between RLB assay and beta-tubulin targeted nested PCR was 0.7984 which indicated substantial agreement between the two tests. Sixty two of the samples positive by RLB were positive by beta-tubulin targeted nested PCR and 12 were negative, while none of the samples negative by RLB was detected as positive by beta-tubulin targeted nested PCR. The 46 samples negative by RLB were also negative by beta-tubulin targeted nested PCR. Although microscopy remains the most appropriate method for diagnosis in the field setting, its use as a gold standard may result into false negative result as observed in this study.

CHAPTER ONE
INTRODUCTION

1.0 Background

Tick-borne diseases (TBDs) are most prevalent and numerous and exert their greatest impact in the tropical and sub-tropical regions (Bram, 1983, Minjauw and Mcleod**,** 2003). Theileriosis alone was estimated to cause a loss of US$168 million in 11 African countries in 1989 (Mukhebi, 1992).TBDs constitute a major constraint on cattle production and the expansion of the dairy industry in many countries across Africa (Uilenberg, 1995). Tick-borne diseases affect 80% of the world's cattle population and are widely distributed throughout the world, particularly in the tropics and subtropics (de Castro, 1997); they represent an important proportion of all animal diseases affecting the livelihood of poor farmers in tropical countries. Tick-borne infections are known to exert the greatest limitations for improved cattle production by causing serious debility, morbidity, mortality and production losses in susceptible exotic cattle, hybrids, as well as in indigenous breeds of cattle raised in tick-borne disease free areas (Bram 1983, Yeoman 1966, Norval *et al*. 1992, Okello-Onen *et al*. 1994, Perry and Randolph 1999). Ticks are responsible for a variety of losses, caused by the direct effect of attachment ('tick worry'), by the injection of toxins, or through the morbidity and mortality associated with the diseases that they transmit. In a review by de Castro (1997), he estimated that the annual global costs associated with ticks and TBDs in cattle amounted to between US$ 13.9 billion and US$ 18.7 billion.

In Uganda among the major constraints to livestock productivity in pastoral and agro-pastoral systems of farming where domestic ruminants are kept under tethering, open grazing or communal grazing, are ticks and TBDs (Ocaido *et al*., 2005). The piroplasms, comprising mainly the genera *Babesia* and *Theileria* are tick-transmitted protozoa that are highly

pathogenic to ruminants, horses, pigs, dogs, cats and, in some cases, even to humans. East Coast Fever (ECF), caused by a protozoan parasite *Theileria parva*, is one of the most important livestock diseases in Africa (Lessard *et al*., 1988; Muraguri *et al*., 1999). It is transmitted by the tick *Rhipicephalus appendiculatus* (Neuman 1901), a three-host tick, which parasitizes mainly cattle, for its transmission and its distribution is directly related to the distribution of this tick species. The distribution range of ECF extends south from southern Sudan to eastern South Africa and as far west as the Democratic Republic of Congo (Minjauw and McLeod, 2003).

At present, there is a lack of a safe, practical and effective vaccine. Apart from regular use of acaricides to kill ticks, the only effective means of protecting cattle at risk in the field is by the "infection and treatment" method of immunization for ECF and chemotherapy for Babesiosis. Although drugs are available to treat these diseases, these are expensive and require an early diagnosis to be effective. The advent of the polymerase chain reaction (PCR) coupled with the specificity of deoxyribonucleic acid (DNA)-DNA hybridization has led to the development of specific and sensitive molecular diagnostic tests to detect and characterize the organisms that cause theileriosis and other tick-borne diseases. A PCR-based technology originally developed for bacterial pathogens, reverse line blotting (RLB), has been adapted for detection of TBDs in livestock (Gubbels *et a.,l* 1999, Schnittger *et al*., 2004). Although many useful species-specific PCR assays have been developed to detect a particular tick-borne pathogen, many of them occur together with other species transmitted by ticks within the same host. Reverse line blot (RLB) hybridization, wherein multiple samples can be analyzed against multiple probes to enable simultaneous detection, does fulfill these criteria.

Many investigations have shown that PCR assays performed equally well, or even better, than other diagnostic tools in terms of specificity and sensitivity. Moreover, a few PCR assays have been developed for the simultaneous detection of multiple parasites in cattle (Figueroa *et al.*1993 and Gubbels *et al*., 1999). More generally, it would be desirable to have a `universal' PCR test for the detection and identification of these parasites. This requires the analysis of a molecular target conserved among piroplasms, but that contains enough genetic variation to design a reliable species identification protocol. An informative molecular target has been identified in the ß-tubulin gene, a fragment of which has been amplified and sequenced from *Theileria* and *Babesia* species (Caccio *et al*., 2000). The presence of an intron within the amplified gene fragment that varied extensively both in length and in sequence, has allowed the development of an assay to differentiate the species directly on the basis of the specific size of the PCR products or by employing a simple PCR-restriction fragment length polymorphism (RFLP) protocol (Caccio *et al*., 2000).

1.1 Statement of the problem

In Africa and specifically in Uganda tick-borne diseases are considered to be the major constraints to livestock productivity (Young *et al.*, 1988; Ocaido *et al*., 2005). *Babesia* and *Theileria spp* are highly pathogenic to cattle and cause severe clinical infections (Friedhoff, 1997). In acute cases, bovine piroplasmosis can be diagnosed by microscopic examination of Giemsa-stained thin blood smears and by clinical symptoms. But, following acute infections, recovered animals frequently retain subclinical infections (carriers). Serological methods are employed in diagnosing subclinical infections, but false positive and false negative results are commonly observed due to cross-reaction. More so, the use of microscopy for diagnosis of subclinical infections is not conclusive in wildlife interface areas since multiple infections are common due to cattle-wildlife interaction. Therefore, a highly specific and sensitive method

for the diagnosis of piroplasms is required. Recently, species-specific Polymerase Chain Reaction (PCR) and PCR-based reverse line blot (RLB) hybridization methods have been developed and used (Schnittger *et al*., 2004; Aktas *et al*., 2005). This study compared the capacities of ß-tubulin targeted nested PCR and RLB techniques in detection and identification of *Theileria* and *Babesia* species in apparently healthy cattle and clinical samples so that one can be adopted in the screening and epidemiological studies of tick-borne infections.

1.2 Justification

Tick-borne diseases remain a challenge to farmers especially in the wildlife interface areas. At present, there is a lack of safe, practical and effective vaccine and in endemic areas, and the control of the disease relies on acaricides to prevent tick transmission and antibiotic treatment of clinical cases. There is lack of information on the epidemiology of the disease in most parts of Uganda and studies into the incidence and prevalence of infection have until recently been hampered by the lack of sensitive and specific diagnostic tools that are particularly suitable for use in countries in Africa. Thus the focus of the current research is on the search, validation and development of improved diagnostic tools. The molecular diagnostic tools available like RLB, is a laborious and expensive technique that has been used for screening of tick-borne infections. Therefore there is a need to adopt a less laborious and cheap molecular techniques that can also simultaneously detect multiple infections. The ß-tubulin targeted nested PCR falls into this category of simultaneously detecting multiple infections (Cassio *et al*., 2000) hence the necessity to compare it with RLB technique. The research would also help in providing valuable information to agencies (FAO, ICTTD, and Ministry of Agriculture, Animal Industry and Fisheries, Diagnostic Laboratories and Institutions) involved in making policy decisions about diagnostics and control of tick-borne diseases.

1.3 General objective

To compare reverse line blot and β-tubulin targeted nested PCR techniques for diagnosis of tick-borne haemoparasites in ruminants in wildlife interface areas in Uganda.

1.4 Specific objectives

The specific objectives of this study were:

i. To detect *Theileria* and *Babesia* haemoparasites in ruminants using RLB and β-tubulin targeted nested PCR.
ii. To determine the sensitivity, specificity and level of agreement of the two techniques in detection of *Theileria* and *Babesia* haemoparasites.
iii. To determine the occurrence of mixed infection using the two techniques.

1.5 Hypothesis

Beta-tubulin targeted nested PCR is more sensitive and specific in detection of Tick-borne piroplasms of cattle than Reverse Line Blot analysis.

CHAPTER TWO
LITERATURE REVIEW

2.1Tick-borne diseases of ruminants

Tick-borne diseases affect 80% of the world's cattle population and are widely distributed throughout the world, particularly in the tropics and subtropics where they represent an important proportion of all animal diseases affecting the livelihood of poor farmers (Bram, 1983; de Castro, 1997; Minjauw and Mcleod, 2003).

The major tick-borne diseases of cattle can be classified into four groups according to the vector species (McCosker, 1979):

Boophilus spp. are responsible for the transmission of species of *Babesia* (protozoa) and *Anaplasma* (rickettsia). World-wide, anaplasmosis and babesiosis constitute the most widely distributed TBD complex. They have a severe effect on exotic dairy and beef cattle.

Hyalomma spp. are responsible for the transmission of *Theileria annulata*, which causes tropical theileriosis.

Amblyomma spp. are responsible for the transmission of the rickettsia *Cowdria ruminantium*, which causes heartwater, a fatal disease of sheep and goats, and exotic cattle, throughout sub-Saharan Africa. *Amblyomma* spp. also transmits the protozoan *Theileria mutans* and facilitates the introduction of the actinomycete *Dermatophilus congolensis*, which is responsible for significant livestock losses in West Africa.

Rhipicephalus spp. are responsible for transmitting the protozoan *Theileria parva*, which causes East Coast fever (ECF), a devastating disease in eastern, central and southern Africa which is responsible for major losses in both small- and large-scale production systems.

2.1.1 Babesiosis

Babesia was first identified by Babes (1888) in association with haemoglobinuria in cattle in South Africa, as intraerythrocytic, apicomplexan protozoan parasites widespread in both tropical and temperate regions affecting all livestock species (Gray and Murphy, 1985).

Babesiosis is characterized by haemolytic anaemia that may be fatal if not treated (Gutierrez, 2000), and is of great economic importance in countries where specific tick vectors occur. Bovine babesiosis is caused by *B. bovis* and *B. bigemina* both of which are transmitted trans-ovarially by one host *Boophilus* ticks which are distributed worldwide between latitudes 32°S and 40°N. These parasites are responsible for fever, haemolysis, haemoglobinuria or "redwater", anemia, and circulatory disorders and occasionally neurological signs (Ristic, 1988).

2.1.1.1 Life cycle and transmission of *Babesia spp.*

Babesia bigemina is more widespread in Africa than the more pathogenic *B. bovis*. *B. bigemina* is transmitted by several ticks of the genus Boophilus, including the wide spread *B. decoloratus* (the blue tick), while *B. bovis* is transmitted by *B. microplus* (the cattle tick) and *B. annulatus* (the texas fever tick) but not *B. decoloratus* (Mackenstedt *et al*., 1990). *B. bovis* generally causes a more acute and severe disease than *B. bigemina*. Transovarial transmission from one generation of ticks to the next is important, particularly as these vector species are one host ticks. A single engorged female tick may give rise to many thousands of infected eggs, and the parasite may be maintained in the tick population in the absence of a suitable bovine host (Gray and Murphy, 1985).

The incubation period of *B. bovis* (time from tick attachment to appearance of parasites in erythrocytes) is variable and depends on factors such as the inoculation dose but is usually around 8-16 days (Ristic, 1988). The incubation period for *B. bigemina* is at least 9 days longer as infection is not transmitted until the nymphal stages (Ristic, 1988). Following infection the parasites multiply within the host's erythrocytes. Escaping parasites destroy the infected erythrocytes and invade new erythrocytes. The release of parasites and host constituents from

destroyed erythrocytes is toxic resulting in various physiological disturbances and shock. The destruction of erythrocytes causes anemia and tissue anoxia and the resultant release of excessive amounts of hemoglobin results in icterus (jaundice) and haemoglobinuria (red water) (Ristic, 1988).

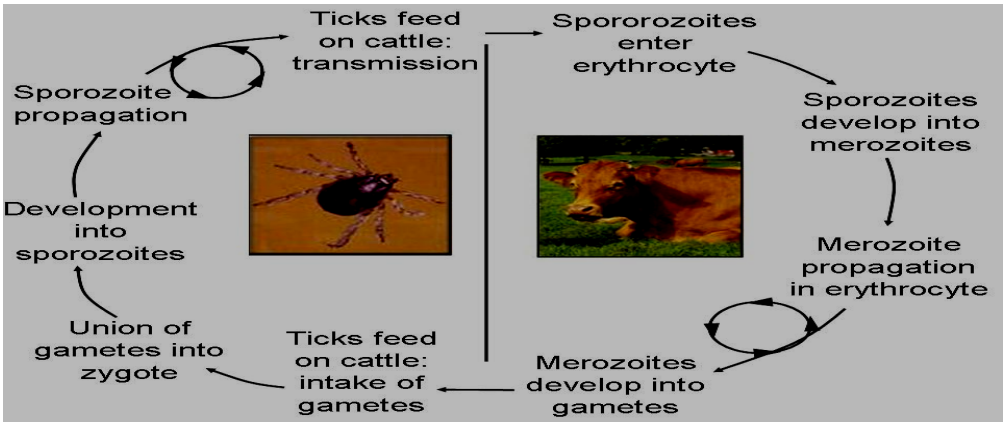

Figure 1: Life cycle of *Babesia* spp. Adopted from www.medlabab.net/medlabab.com/images/bovine%2...

2.1.2 Theileriosis

Theilerioses are a complex of diseases caused by tick-borne apicomplexan parasites of the genus *Theileria* (Norval *et al*., 1992*).*Theilerioses are tick-borne protozoan diseases of cattle, sheep and goats caused by pathogenic species of *Theileria* which are transmitted transtadially by two and three host ticks (Lounsbury, 1904). Theileriosis of cattle in Africa has been considered to have had more negative impact on the development of the beef and dairy industries, veterinary infrastructure, legislation, policies and research in Africa than any other livestock disease complex (McCosker, 1991). Most important of the theilerioses in sub-Saharan Africa is East Coast fever which is caused by infection with *Theileria parva* transmitted by three-host ticks of the genus Rhipicephalus, notably *R. appendiculatus*, the brown ear tick (Lounsbury, 1904). The African buffalo (*Syncerus caffer*) is the natural host of *T. parva* (Norval *et al*., 1992).

Theileria parva was first described in Southern Rhodesia by Robert Koch (1889). Koch (1903; 1905; 1906) described the distinctive schizont stage of the parasite in lymphoid cells. Bruce *et al.* (1910) first described the enzootic form of theileriosis in Uganda as a disease of calves known locally as Amakebe and which had long been recognized by local people (Mettam and Carmichael, 1936). Theileriosis caused by *T. parva* currently affects cattle in Burundi, Kenya, Malawi, Mozambique, Rwanda, Sudan, Tanzania, Uganda, Zaire, Zambia and Zimbabwe. The total regional loss in 1989 was estimated by Mukhebi *et al.* (1992) at US$168 million, including estimated mortality of 1.1 million cattle.

2.1.2.1 Life cycle and transmission of *Theileria parva*

Following infection from a tick bite, the *Theileria* multiply as schizonts in lymphocytes, initially in the drainage lymph nodes nearest the site of the infective tick bite. Following schizont proliferation, merozoites are released which invade erythrocytes to become piroplasms. The life cycle is completed when ticks ingest the piroplasms in blood meals and transmit the parasite in their next stage following development of the *Theileria* to the infective form (sporozoites) in the tick salivary glands. Domestic cattle become readily infected by inoculation of sporozoite stages of *T. parva* in the saliva of attached infected ticks. The

sporozoites invade bovine leucocytes to form multinucleate intracellular macroschizont stages, the cause of an acute lymphoproliferative-degenerative disease that results in significant morbidity and mortality in cattle. Macroschizonts later undergo merogony to produce intracellular microschizonts, which are released from host leucocytes into the bloodstream where they invade erythrocytes to become piroplasms. Engorging ticks are infected by ingestion of these parasitized host erythrocytes (Cowdry and Danks, 1933).

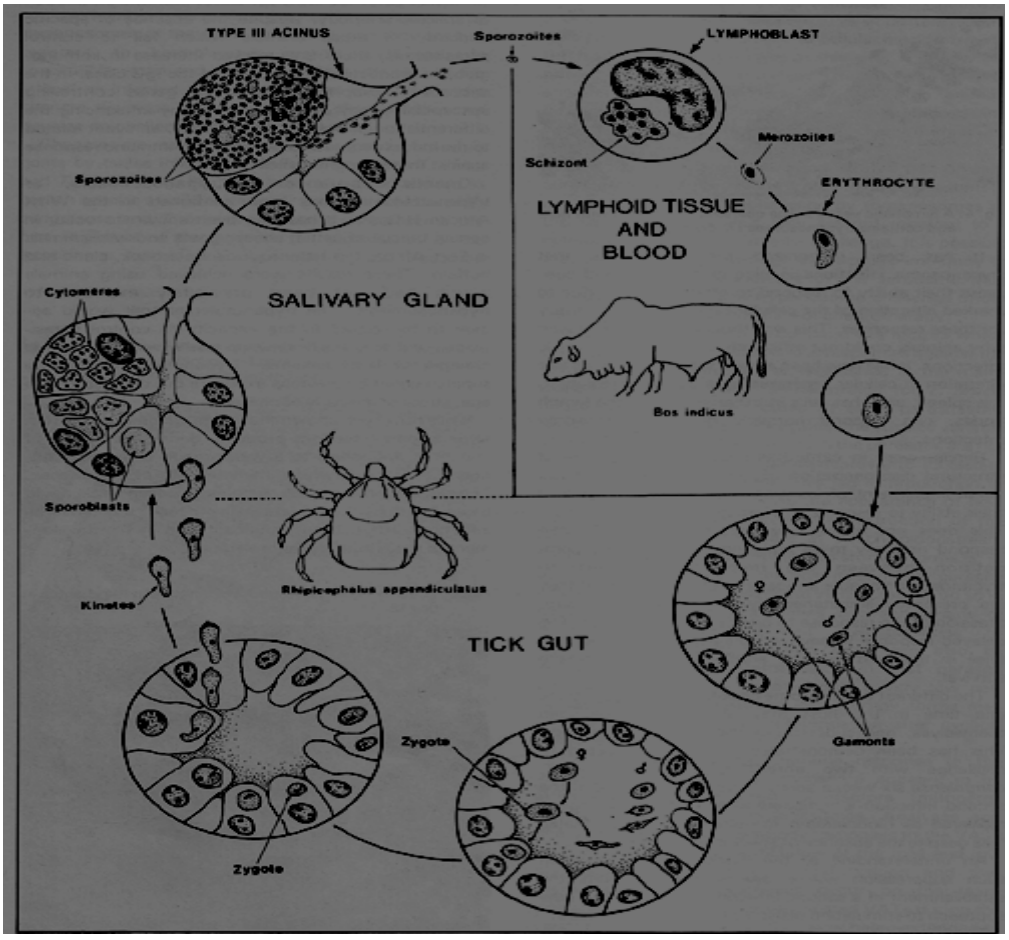

Figure 2: Life cycle of *Theileria parva*. Adopted from www.ilri.org/.../11rad88/images/FIG1-_P12.gif

T. parva is transmitted almost exclusively by the three host tick, *Rhipicephalus appendiculatus*. Immature, larval or nymphal ticks engorging on an infected bovid pick up the intraerythrocyte

piroplasms drop off after completion of feeding, moult, and the parasite develops in the salivary glands of the next stage. Unattached infected ticks can live for more than 1 year but infection dies out after about 11 months. If picked up by a new susceptible host, the infected *R. appendiculatus* nymph or adult attaches (usually to the ears) and starts feeding. Over the first three days after attachment, the *Theileria* parasites in the salivary glands complete their development to infective sporozoites and the tick transmits the infection between the third and fifth days after attachment (Lounsbury, 1904).

2.1.2.2 Other Theilerioses

- ***Theileria mutans* infection**

T. mutans a parasite of cattle and Cape buffalo is very widespread in sub-Saharan Africa, where the distribution follows that of its tick vectors (Walker and Olwage, 1987), at least five species of African *Amblyomma* ticks (Norval *et al.*, 1992). Compared to *T. parva* or *T. annulata*, *T. mutans* is generally regarded as only mildly pathogenic to cattle, although there are records of *T. mutans* being pathogenic and even fatal in East Africa, and anemia can become severe in cases of pathogenic strains (Brown *et al.*, 1990). *Theileria mutans* can be responsible for productivity losses in cattle, especially when present as a concurrent infection with other tick-borne parasites or stress caused by poor nutrition or other infections (Brown *et al.*, 1990).

- **Tropical theileriosis**

Tropical theileriosis caused by *T. annulata*, although of limited distribution in sub-Saharan Africa where it may have a small overlap with *T. parva* in southern Sudan, has a far more extensive distribution in tropical and sub-tropical regions globally, including much of

North Africa (Norval *et al.*, 1992). As many as 200 million cattle are considered at risk worldwide (Purnell, 1978). In contrast to *T. parva* infection, the pathogenesis of the disease is dominated by the hematological effects of the intra-erythrocytic piroplasm stages rather than the effects of the schizonts on the lymphoid system (Sergeant *et al.*, 1924; Neitz, 1957; Barnet, 1968; Eisler, 1988).

2.3 Microscopic diagnosis of haemoparasites

The traditional method of identifying pathogenic agents in infected animals is by microscopic examination of blood smears stained with Giemsa. This technique is usually adequate for detection of acute infections, but not for detection of carrier animals, where parasitaemias may be low (Friedhoff and Bose, 1994).

2.4 Integrated molecular diagnosis of tick-borne pathogens

Tick-borne diseases e.g. babesiosis, theilerioses and anaplasmoses, ehrlichioses and heartwater, affect the health of livestock, humans and/or companion animals worldwide (Jongejan and Uilenberg, 2004). RLB was originally developed for the identification of *Streptococci* serotypes by Kaufhold *et al* (1994). The first application of RLB for the detection and differentiation of pathogens in ticks was developed for *Borrelia* spirochetes by Rijpkema *et al* (1995) and was subsequently combined with *Ehrlichia* spp (Schouls *et al*, 1999). Successful application of RLB to detect and differentiate all known *Theileria* and *Babesia* species was carried out by Gubbels *et al* (1999). The subsequent development of an RLB suitable to detect and differentiate *Ehrlichia* and *Anaplasma* species provided a further basis for the present RLB hybridization kit, wherein 36 probes for *Anaplasma*, *Ehrlichia*, *Babesia* and *Theileria* species are included. RLB is quickly becoming a standard molecular diagnostic tool and

epidemiological studies in an increasing number of laboratories all over the world. For instance, Centeno-Lima *et al* (2003) used RLB for the characterization of *Babesia divergens* in a human case, whereas Nijhof *et al* (2003) discovered novel *Theileria* and *Babesia* species through the application of RLB. Furthermore, detection and differentiation by using RLB for many *Babesia* and *Theileria* spp occurring in small ruminants has been reported by Schittger *et al* (2004). Finally, RLB was also successfully applied to the study of protozoan haemoparasites in Uganda and in Portugal (Oura *et al*, 2004a; Oura *et al*, 2004; Brigido *et al*, 2004; Muhanguzi, 2009). Amplification of parasite DNA using PCR has advantages over the conventional methods of detecting animals infected with *Theileria* and *Babesia* species. It is more sensitive and specific than parasite detection by thin blood smears (d'Oliveira *et al*., 1995; Almeria *et al*., 2001; Aktas *et al*., 2002).

2.5 Reverse Line Blot assay

The Reverse Line Blot (RLB) hybridization assay is a versatile diagnostic tool for sensitive and simultaneous detection and differentiation of haemoparasites in blood, tissue or ticks. RLB is based on simultaneous PCR amplification of related species; the *Ehrlichia*/*Anaplasma* cluster of species and the cluster of *Theileria*/*Babesia* species, making specific PCR reactions for each individual species obsolete. Each species can be identified by a species-specific oligonucleotide probe using a line-blotter apparatus. RLB thus combines PCR amplification followed by a hybridization step resulting in sensitivity up to 1000 fold or higher than PCR only. Moreover, detection is based on chemiluminescence instead of radioactivity making it more user-friendly. Due to the possibility of re-using the blot containing the oligonucleotides for 10 to 20 times and the limited number of PCR amplifications required, makes RLB cheap.

The first step in RLB is PCR amplification of a variable region in the 16S ribosomal RNA gene (*Ehrlichia* and *Anaplasma*) or 18S ribosomal RNA gene (*Theileria* and *Babesia*) using PCR-primers targeting conserved parts of the rRNA gene. The primers are designed for specific amplification of the rRNA gene of the target organisms and they are not complementary to the hosts or ticks rRNA genes resulting in a high specificity of the PCR reaction. Two sets of PCR-primers are required for the amplification of either *Ehrlichia*/*Anaplasma* or *Theileria*/*Babesia* rRNA genes.

However, both PCR-primer sets have matching melting temperatures and thus the same PCR-program can be used for both reactions. In the second step the PCR products are hybridized on a blot on which for each (known) *Ehrlichia, Anaplasma, Theileria* and *Babesia* species a specific oligonucleotide has been covalently linked.

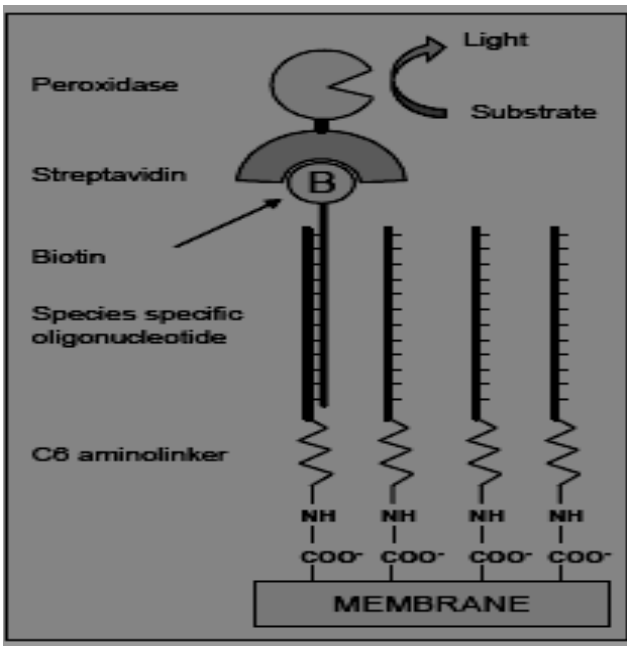

Figure 3: Schematic representation of RLB hybridization principles
Adopted from www.biotech-online.com/uploads/pics/10644c.jpg

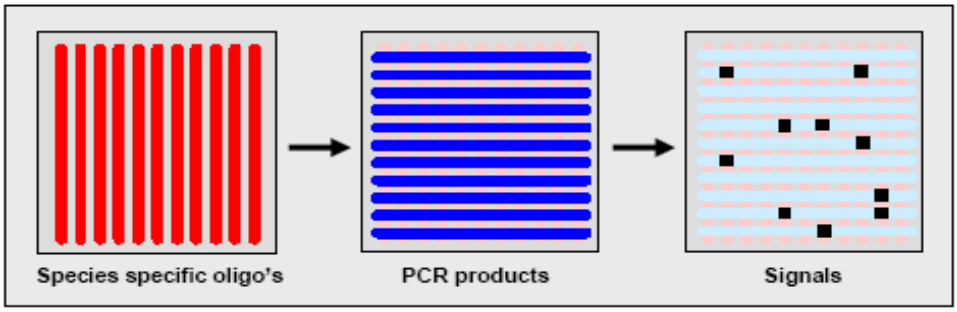

Figure 4: Schematic representation of the RLB assay
Adopted from www.biotech-online.com/uploads/pics

The species-specific oligonucleotides are deduced in the hyper variable region that is amplified in the first step by PCR. The species-specific oligonucleotides are applied in lines using a miniblotter and are covalently linked to the membrane by a 5' terminal amino linker (Fig.3). The PCR-products are applied on the membrane, using the miniblotter so that the direction of the PCR-products is perpendicular to the direction of the species-specific oligonucleotides (Fig.4). In this way the different pathogen species simultaneously amplified by PCR can each hybridize specifically at the cross-sections of the line containing the specific oligonucleotide and the line containing the PCR product. A control oligonucleotide for either the *Ehrlichia/Anaplasma* or the *Theileria/Babesia* species deduced from a region conserved in the amplified PCR product ensures detection of a species for which no specific oligonucleotide is incorporated.

2.6 Beta-tubulin as a diagnostic marker

Microtubules are composed of two major proteins, α and β-tubulin, and a third minor species the γ-tubulin (Little and Seehaus, 1988; Oakley and Oakley, 1989). Microtubules are hollow proteinaceous organelles present in all eukaryotic cells.

The β-tubulin gene is one of the few apicomplexan genes that are interrupted by one or more introns (Nagel *et al.*, 1988). In addition, the position of the first intron is conserved in all the species investigated so far, allowing for a rational design of primers around this region. Introns associated with the β-tubulin gene show extensive variations both in length and in sequence. These features make this region of the genome a good candidate for the development of informative markers, as it has been shown previously for several protozoan parasites (Costa *et al.*, 1997).

CHAPTER THREE
MATERIALS AND METHODS

3.1 Study design

Blood samples were collected from areas around Lake Mburo National Park found in the western region of Uganda. This area was used in this study to compare RLB and β-tubulin targeted nested PCR techniques in the detection of tick-borne haemoparasites in apparently normal cattle. Simple random sampling was used in this study. With the help of area Veterinarians, ranches/kraals in the respective wildlife interface areas were identified and those used in the study were selected at random. Only adult free ranging Ankole cattle were used in this study.

3.2 Sample collection

Blood was collected from cattle by jugular venipuncture into EDTA coated vacutainers, kept on ice packs for transportation to the Molecular Biology laboratory, Faculty of Veterinary Medicine, Makerere University. In the laboratory, blood was aliquoted in 1.5ml eppendorf tubes and stored at -20ºC until DNA was extracted. Thin smears were made in the field and carried to the laboratory for microscopic analysis. Samples found positive for *Theileria* and *Babesia* by microscopy were selected for further analysis.

3.3 Sample size determination

For the cross-sectional study, the sample size for cattle that achieved 5% level of precision at 95% confidence level was calculated using the formula described by Pfeiffer (2002).

$$n = \frac{Z^2 \times P (1-P)}{d^2}$$

Where:

n= Sample size.

Z= 1.96 at 95% confidence level.

P= Probable prevalence of infection in cattle in the two places (taken as 19.8% from previous studies).

d= Level of precision (taken as 5%).

This gave a figure of 244 cattle blood samples. However, 300 blood samples in the cross-sectional study were successfully collected for analysis in the laboratory.

3.4 Microscopic analysis of samples

In the laboratory, the blood smears were fixed in methanol for 5 min and stained for 30 min in Giemsa stain diluted with 5% buffer. Slides were examined for intra-erythrocytic forms of *Theileria* and *Babesia spp*. piroplasms at 100X objective magnification. The smears were recorded as negative for piroplasms if no parasites were detected in 50 oil-immersion fields.

3.5 DNA extraction from blood

Blood was processed for DNA extraction as described by d' Oliviera *et al*. (1995) with some modifications. Briefly, 200µl of thawed blood in an eppendorf tube was washed 3-5 times by mixing with 0.5ml PBS (137mM NaCl, 2.6mM, KCl, 8.1mM Na2HPO4, 1.5mM KH2PO4, pH 7.4), each time followed by centrifugation at maximum speed (13,000 rpm) for 5 minutes. After the final wash, the cell pellet was resuspended in 100µl of lysis mixture (10mM Tris-HCl, pH 8.0, 50mM KCl, 0.5% Tween 20, 100µg/ml of proteinase K). This mixture was incubated over night at 56ºC, followed by 10 minutes of boiling to inactivate proteinase K. The mixture was then kept at -20ºC until needed for PCR.

3.6 PCR amplification of the 18S rRNA gene

One set of primers was used to amplify a 390-430 bp fragment of the 18S rRNA gene spanning the V4 region of *Theileria* and *Babesia* organisms. The forward primer used was RLB-F2 5'-GAC ACA GGG AGG TAG TGA CAA G-3' and the reverse primer was RLB-R2 5'-Biotin-CTA AGA ATT TCA CCT CTG ACA GT-3', as described by Georges *et al*. (2001).The primers were manufactured by Isogen, Maarssen, The Netherlands.

After optimization, the 1x PCR reaction constituents in a final volume of 25µl were as follows: 1xPCR buffer (Invitrogen), 3.0mM $MgCl_2$ (Invitrogen), 200µM each dATP, dCTP, dGTP, 100µM dTTP (ABgene) and 100µM dUTP (Amersham), 1.25 U of Taq DNA polymerase (Invitrogen), 0.1U of UDG (Amersham), 25 pmol of each primer, 2.5µl of template DNA and sufficient distilled water to top up the reaction (Bekker *et al*., 2002). Each time, positive control (*Theileria parva* or *Babesia bovis* DNA) and negative control (reaction constituents without DNA) tests were included. The reactions were performed using the following three phase touch-down program as described by Bekker *et al.* (2002): The first phase consisted of 3 minutes incubation at 37°C to allow degradation of any contaminating DNA template from previous PCR amplification by the UDG, followed by 10 minutes incubation at 94°C to inactivate the UDG and activate the DNA polymerase. The second phase consisted of 2 cycles of a touch down program of denaturation for 20 seconds at 94°C, annealing (touch down from 67°C to 57°C in steps of 2°C) for 30 seconds and extension for 30 seconds at 72°C. The third phase consisted of 40 cycles of denaturation for 20 seconds at 94°C, annealing for 30 seconds at 57°C and extension for 30 seconds at 72°C. After the cycle phase, final extension was at 65°C for 1 hour. Thereafter, PCR products were kept at -20°C until needed for reverse line blot

hybridization with *Theileria* and *Babesia* species-specific oligonucleotide probes bound onto the Biodyne C membrane.

3.7 Hybridization of species-specific oligonucleotide probes on to the Biodyne C membrane

The species-specific probes were applied to the membrane as described by Gubbels *et al*. (1999). The species-specific oligonucleotide probes for the different *Theileria* and *Babesia* species, and *Theileria/Babesia* catch-all are synthesized with a 5'-terminal amino group (N-terminal N-(trifluoracetamidohexyl-cyanoethyle, N,N-diisopropyl phosphoramidite [TFA]-C6 amino linker (Isogen), which covalently links the oligonucleotide probes to the activated negatively charged Biodyne C membrane. The required concentration of each oligonucleotide was obtained by adding a given volume of the 100 pmol/µl optimized concentration to 150µl of 500mM $NaHCO_3$, pH 8.4 at room temperature. Then the Biodyne C membrane was activated during a 10 minute incubation at room temperature in freshly prepared 16% (w/v) 1-ethyl-3-(3-dimethylamino propyl) carbodiimide, EDAC (Sigma). The membrane was rinsed with distilled water and placed on a support cushion in a clean miniblotter (MN45, Immunetics) and the screws turned hand-tight. Residual water was removed from the slots of the miniblotter by aspiration. Slots were filled with 150µl of the diluted appropriate oligonucleotide solutions. After adding the last oligonucleotide solution, at least 1 minute incubation at room temperature was allowed. The oligonucleotide solutions were removed by aspiration in the same order in which they were applied. The membrane was then removed from the miniblotter system and incubated in 100ml of 100mM freshly prepared NaOH solution for 10 minutes at room temperature under gentle shaking, to inactivate the membrane. The membrane was then washed in a plastic container under gentle shaking in 250ml 2XSSPE/0.1%SDS for 5 minutes

at 60°C and washed in 100ml 20mM EDTA, pH 8.0 for 15 minutes at room temperature. The membrane was sealed in a plastic bag containing 4ml of 20mM EDTA and stored at 4°C until required.

Table 1: Species-specific probe sequences on the RLB membrane

Species	**Probe sequence from 5' to 3' all with 5'-C6-TFA- amino linker**	**Reference**
Babesia bovis	CAGGTTTCGCLTGTATAATTGA	
Babesia bigemina	CGTTTTTTCCCTTTTGTTGG	
Theileria buffeli	GGCTTATTTCGGWTTGATTTT	
Theileria Velifera	CCTATTCTCCTTTACGAGT	
Theileria taurotragi	TCTTGGCACGTGGCTTTT	
Theileria mutans	CTTGCGTCTCCGAATGTT	Gubbels *et al.* (1999)
Theileria parva	TTCGGGGTCTCTGCATGT	
Theileria annulata	CCTCTGGGGTCTGTGCA	Georges *et al*.(2001)
Theileria spp(buffalo)	CAGACGGAGTTTACTTTGT	
Theileria/Babesia catchall	TAATGGTTAATAGGARCRGTTG	Gubbels *et al.* (1999)

3.8 Reverse line blot hybridization of PCR products

Hybridization of PCR products to the species-specific probes was performed as described by Gubbels *et al.* (1999). At least 15µl of each PCR product was mixed with 150µl 2XSSPE/0.1%SDS, heat denatured for 10 minutes at 100°C and chilled on ice. The diluted PCR products were span for a few seconds to collect the contents and kept on ice until needed. The Biodyne C membrane blotted with species-specific oligonucleotide probes was then rinsed for 5 minutes at room temperature in 100ml 2XSSPE/0.1%SDS. The membrane was placed on the support cushions in the miniblotter system in such a way that the slots were perpendicular to the line pattern of the applied oligonucleotides (rotated at 90°C). The residual fluid was then removed from the slots by aspiration. The slots were then filled with the diluted PCR products, avoiding air bubbles and hybridized for 60 minutes at 42°C in hybridization oven without shaking. The samples were removed from the slots by aspiration and the membrane taken out of the miniblotter. The membrane was washed twice in 100ml pre-heated 2XSSPE/0.5%SDS for 10 minutes at 52°C to remove any non-specific products that had hybridized onto the membrane. The membrane was then incubated for 30 minutes at 42°C with 10ml of pre-heated 2XSSPE/0.5% SDS containing peroxidase labeled streptavidin conjugate (dilution 4000:1). The membrane was washed twice with 100ml pre-heated 2XSSPE/0.5% SDS for 10 minutes at 42° and rinsed twice in 100ml 2XSSPE for 5 minutes at room temperature. The membrane was then incubated for 1 minute at room temperature in 10ml of ECL detection liquids 1 and 2 (Amersham).The membrane was placed between two overhead sheets, placed on the intensifying screen in an exposure cassette with the DNA side up and exposed to an ECL-hyper film (Amersham) for 20-25 minutes in the dark room.

3.9 Developing the X-ray film

After exposure, the film was removed from the exposure cassette in the dark room and placed in the developing solution diluted 1:5 with water. The film was agitated gently for about 2 minutes. The film was washed in tap water and placed in the fixer for about 2 minutes. The film was then washed in tap water, hanged to dry and image reviewed.

3.10 Stripping the RLB membrane for re-use

The membrane was stripped for re-use as described by Gubbels *et al.* (1999). The membrane was washed twice in pre-heated 100ml 1% SDS at 80°C for 30 minutes. The membrane was then washed once with 100ml of 20mM EDTA, pH 8 for 15 minutes at room temperature and was stored at 4°C in a plastic bag containing a small volume (4ml) of 20mM EDTA, pH 8 until needed for another round of reverse line blot hybridization.

3.11 PCR amplification of the β-tubulin gene

A fragment of the β-tubulin gene was amplified using the forward primer F34 (5'-TGTGGTAACCAGAT(t/c)GG(a/t)GCCAA-3'), and the reverse primer R323 (5'-TCnGT(a/g)TA(a/g)TGnCC(t/c)TT(a/g)GCCCA-3').

The reaction mixture consisted of 1X PCR buffer, 1.5mM MgCl2, 200mM each deoxynucleotide triphosphate, 40pmol each primer, 1.5U of Taq polymerase and approximately 1 ng of DNA, in a final volume of 50 µl. Amplification consisted of an initial denaturation step (5 min at 94ºC), followed by 35 cycles (each consisting of 30 s at 94ºC, 30s at 62ºC and 60s at 72ºC), and ending with an extension cycle (5min at 72ºC). Amplifications were performed on a Perkin-Elmer model 2400 thermal cycler.

For nested PCR reactions, 1 µl of the primary PCR products was used. A forward primer F79 (5'-GA(a/g)CA(t/c)GGnATnGA(t/c)CCnGTAA-3'), and a reverse primer R206 (5'-AC(a/t/g)GA(a/g)TCCATGGT(a/t/g)CCnGG(t/c)T-3') were used. The reactions were run

using the same profile as the primary PCR, with the exception of the number of cycles that were reduced to 30 as described by Caccio *et al*., (2000).

3.12 Data analysis

The collected data was analyzed using the DAG-STAT software programme for comparing diagnostic tests and determining the level of agreement between tests (Mckinnon, 2000).

CHAPTER FOUR
RESULTS

4.1 Samples collected

Of the 300 blood samples (EDTA tubes and slide smears) collected 120(40%) samples were found to be positive (*Theileria* only) by microscopy and the rest were negative. Of the 120 samples that tested positive by microscopy 80 samples were selected and subjected to the two tests (RLB and Beta-tubulin nested PCR). To determine the specificity of microscopy, of the 180 samples that tested negative microscopically 40 samples were selected at random and subjected to reverse line blot and beta-tubulin analysis.

4.2 Light-microscopy examination of thin blood smears

All the samples that tested positive by microscopy (120) showed intra-erythrocytic forms of *Theileria* piroplasms. No sample was found positive for *Babesia* piroplasms by this method.

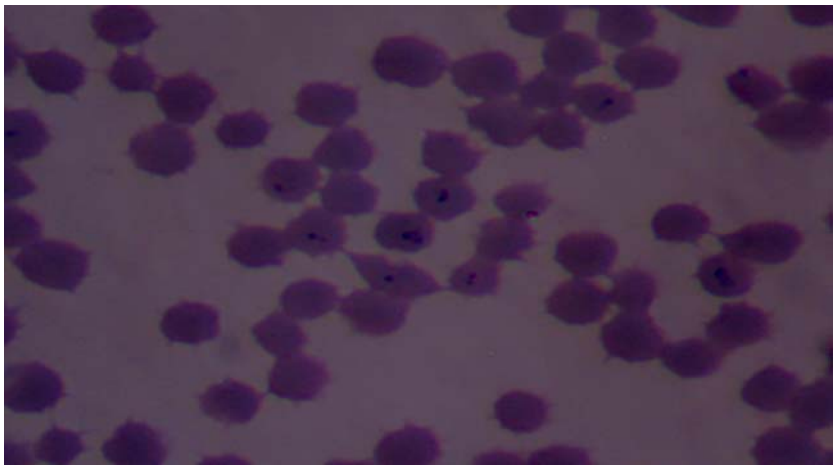

Figure 5: **Intra-erythrocytic forms of *Theileria* piroplasms at 100X objective magnification**

4.3 PCR amplification of the 18SrRNA gene

TheV4 region of the 18SrRNA gene of *Theileria* and *Babesia* was amplified and the results are as shown in Fig.6.

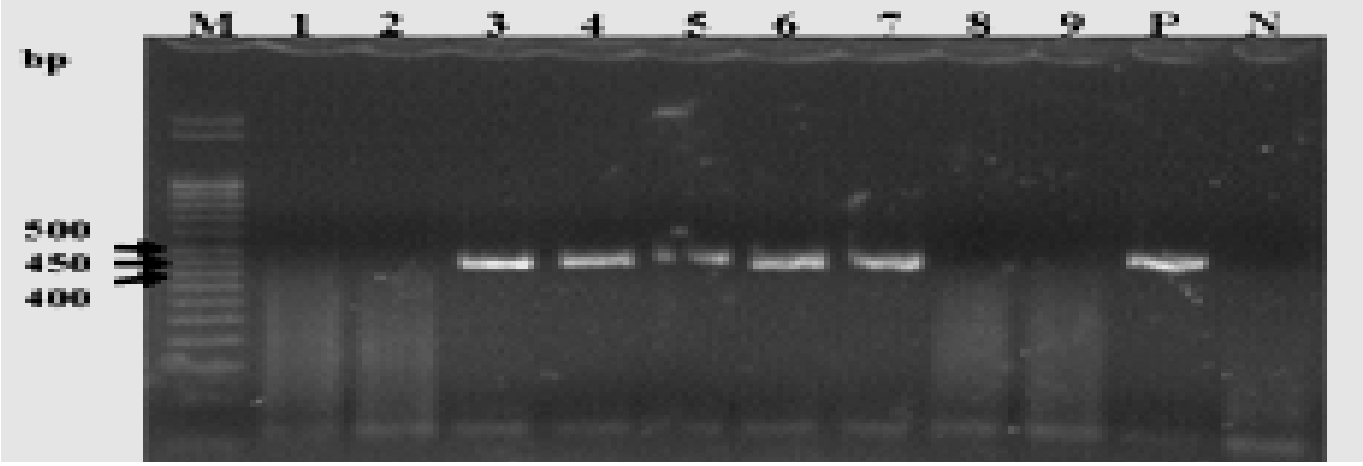

Figure 6; **PCR amplification of the 18SrRNA gene;** A 2% Agarose gel electrophoresis showing the representative results obtained with the primers targeting the V4 region of the 18SrRNA of *Theileria* and *Babesia* parasites. Lane M; 50bp Sigma Molecular weight DNA marker, Lane 1-9 are selected samples with 1,2,8, and 9 negative for *Theileria* and *Babesia*, while 3-7 are positive with 490-520bp band sizes, Lanes P and N are positive and negative controls respectively.

4.4 Binding of species-specific probes on to the Biodyne C membrane

Before analysis of DNA from blood for presence of *Theileria* and *Babesia* parasites, the RLB membrane was prepared by binding species-specific probes to the Biodyne C membrane. The species specific oligonucleotides that were bound on to the Biodyne C membrane used in this study were as follows; Theileria/Babesia catch-all, *Theileria annulata, Theileria parva, Theileria mutans, Theileria taurotragi, Theileria velifera, Theileria buffeli, Theileria spp(buffalo), Babesia bigemina*, and *Babesia bovis* respectively.

4.5 Reverse line blot analysis of blood samples testing positive by microscopy

Eighty samples testing positive by microscopy were subjected to RLB analysis and the results are as shown in Table 2. The RLB assay allowed the detection of individual species that simultaneously infected the cattle. Out of the 80 samples positive by microscopy, 57 (71.25%) samples were positive for the individual species while 23 (28.75%) samples were negative by the RLB assay. Of the 57 samples positive by RLB assay, 8 (14.04%) were infected by *T.mutans* only, 47 (82.46%) were simultaneously infected with *Theileria parva* and *Theileria*

spp (buffalo) but not with any other species, while 1 (1.75%) sample was simultaneously infected with *Theileria parva*, *Theileria spp (buffalo)* and *Babesia bovis.* Another sample (sample 31 in Fig 7) hybridized with the catch-all only but showed no species specific hybridization with the oligonucleotide probes used on the `membrane.

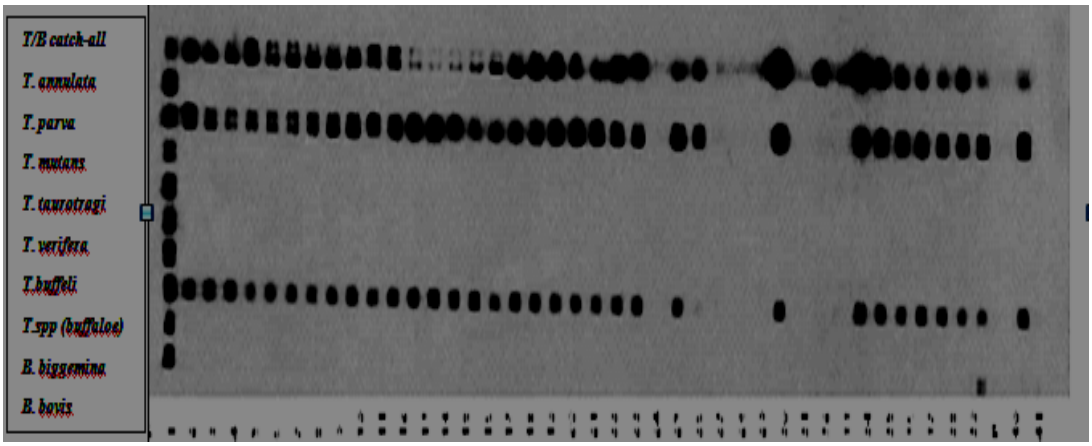

Figure 7; **Reverse line blot of 40 samples positive by microscopy.** Species-specific oligonucleotide probes were applied to the horizontal rows of the RLB and are shown to the left of the blot (T/B catch-all, *Theileria/Babesia* catch-all; *Theileria annulata, Theileria parva, Theileria mutans, Theileria taurotragi, Theileria velifera, Theileria buffeli, Theileria spp(buffalo), Babesia bigemina*, and *Babesia bovis* respectively). slot 1 is the negative control (buffer) ,slot 2 is the membrane positive contol(Isogen) and Slot 26 is the positive control (*Theileria parva*). The remaining slots are test samples.

Table 2: Number of bovine *Theileria* and *Babesia* species detected by RLB using a locally prepared membrane (n=80)

Species	Number detected
Babesia bovis	1
Babesia bigemina	0
Theileria buffeli	0
Theileria Velifera	0
Theileria taurotragi	0
Theileria mutans	8
Theileria parva	48
Theileria annulata	0
Theileria spp(buffalo)	48

4.6 Analysis of selected blood samples using a commercial membrane

The commercial membrane contained more species specific oligonucleotides than the locally prepared membrane as shown in Table 3 and results are shown in Fig.8.

Out of the 40 samples testing negative by microscopy, 17 (42.5%) samples were found positive while 23 (57.5%) samples were negative by RLB assay. 11 (64.7%) of the positive samples had mixed infection, while 6 samples (35.3%) had single infections, as shown in figure 8.

All the samples with mixed infection were positive for *Theileria parva* and any other two of the *Theileria* species (table 3). No sample showed mixed infection of *Theileria parva* and any *Babesia* species. Out of the 6 samples showing single infection, 5 samples were positive for *Theileria parva* while 1 sample was positive for *B.bovis.*

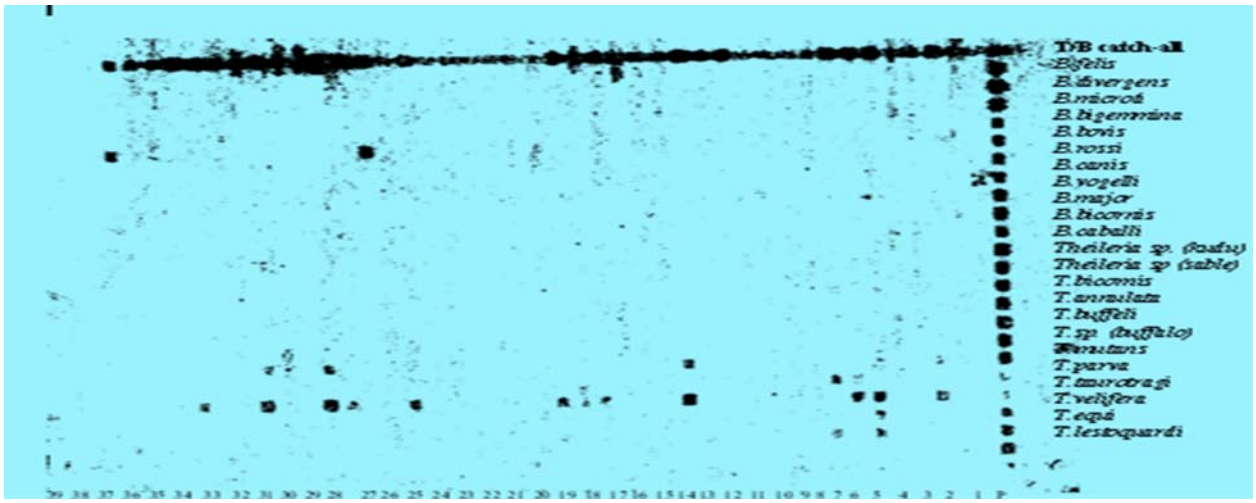

Fig.8 Reverse line blot of 40 samples negative by microscopy. The Species-specific oligonucleotide probes on the commercial membrane were applied in search a way that T/B (*Theileria/Babesia*) catch-all was the first (top) and *T.lestoquardi* the last (bottom). The positive control used was *B.bovis* (slot 37).

Table 3: **Number of *Theileria* and *Babesia* species detected by RLB (commercial membrane) on samples negative by microscopy. (n=40)**

Oligonucleotide Species on the biodyme C membrane	**Number of species detected (%)**
Babesia divergens	0
Babesia bigemina	0
Babesia bovis	1
Babesia major	0
Theileria sp.(kudu)	1
Theileri sp.(sable)	0
Theileria annulata	0
Theileria buffeli	2
Theileria sp.(buffalo)	4
Theileria mutans	6
Theileria parva	15
Theileria taurotragi	6
Theileria velifera	3
Theileria lestoquardi	0
Total number of species detected	38

4.7 Beta-tubulin targeted nested PCR results

Eighty samples positive by microscopy were subjected to beta-tubulin nested PCR analysis and the results are presented in Table 4. Fifty samples (62.5%) tested positive while 30 samples (37.5%) tested negative with beta-tubulin nested PCR analysis. The gel (Fig.9) shows some representative samples tested with the beta-tubulin nested PCR analysis. Two to three bands (300-400bp) were identified in some samples suggesting mixed infection in some samples though it was not possible to know the specific species in question. Fourty samples testing negative by microscopy were subjected to beta-tubulin nested PCR analysis. Eleven samples (27.5%) were found to be positive while 29 samples (72.5%) were found to be negative. The beta-tubulin primers did not amplify *Trypanosoma* tubulin (Fig. 9).

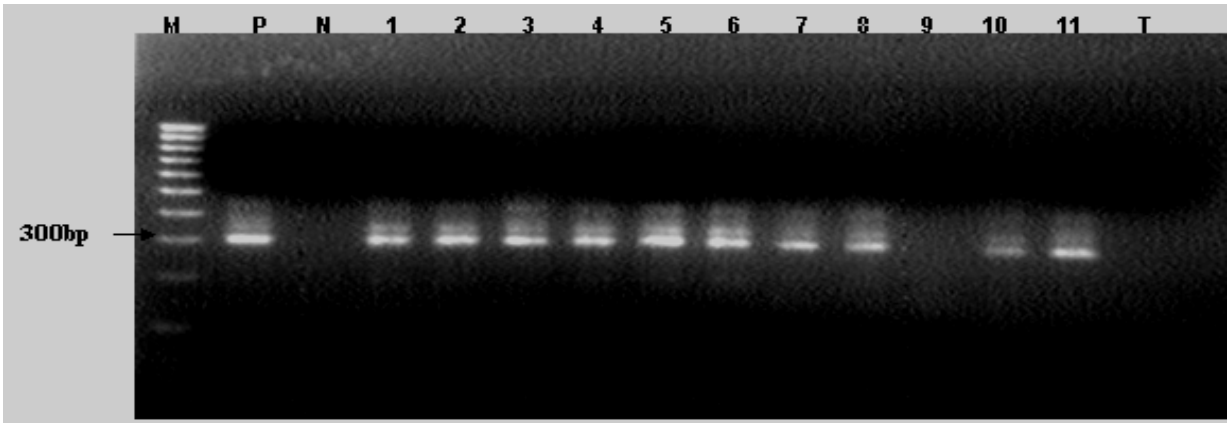

Fig 9; **PCR amplification of the beta-tubulin gene.** A 2% Agarose gel electrophoresis showing the representative results obtained with the primers targeting beta-tubulin of *Theileria* and *Babesia* parasites. Lane M; 100bp Sigma Molecular weight DNA marker, Lane P was Positive control, Lane N was Negative control, , Lanes 1-11 were Test samples and Lane T was *Trypanosoma* DNA.

Table 2: **Comparison of Reverse Line Blot and the beta-tubulin targeted nested PCR using microscopy as the reference test.**

		MICROSCOPY	
TEST		*POSITIVE (80)*	*NEGATIVE (40)*
RLB	POSITIVE (74)	57	17
	NEGATIVE (46)	23	23
ß-tubulin nested PCR	POSITIVE (61)	50	11
	NEGATIVE (59)	30	29

Using microscopy as gold standard, the sensitivity of RLB technique for the detection of *Theileria* and *Babesia spp* was 71.25% (95% CI: 60.05%-80.82%) and the specificity was 57.50% (95% CI:40.89%-72.960%) while the sensitivity of the beta-tubulin targeted nested PCR was 62.5% (95% CI: 50.96%-73.08%) and the specificity was 72.5% (95% CI:56.11%-85.40%). The Positive Predictive Value using RLB was 77.03 % (95% CI: 65.79%-86.01%) and the Negative Predictive Value was 50 % (95% CI: 34.90%-65.10%) while the Positive Predictive Value using beta-tubulin targeted nested PCR was 81.97 % (95% CI: 70.02%-90.64%) and the Negative Predictive Value was 49.15% (95% CI: 35.89%-62.50%).

The Kappa statistic between microscopy and RLB was 0.2771 (95% CI: 0.1008-0.4534). This indicated a fair agreement between the two techniques (Microscopy and RLB). The Kappa

statistic between Microscopy and beta-tubulin targeted nested PCR was 0.3128 (95% CI: 0.1514-0.4743). This Kappa value indicated fair agreement between the two techniques (Microscopy and beta-tubulin targeted nested PCR).

Table 5: **Comparison of Reverse Line Blot and the beta-tubulin targeted nested PCR**

		RLB	
		POSITIVE (74)	*NEGATIVE (46)*
ß-tubulin-PCR	POSITIVE (62)	62	00
	NEGATIVE (58)	12	46

Sixty two samples positive by RLB were found to be positive by beta-tubulin targeted nested PCR and 12 samples positive by RLB were found to be negative by beta-tubulin targeted nested PCR, while no sample found to be negative by RLB was detected as positive by beta-tubulin targeted nested PCR. The 46 samples found negative by RLB were also negative by beta-tubulin targeted nested PCR as shown in Table 5. The Kappa statistic for level of agreement in detection of Tick-borne piroplasms between RLB assay and beta-tubulin targeted nested PCR was 0.7984 (95% CI: 0.6925-0.9044). This Kappa indicated substantial agreement between the two tests.

CHAPTER FIVE
DISCUSSION

The aim of this study was to compare Reverse Line Blot and beta-tubulin targeted nested PCR in the detection of tick-borne piroplasms of apparently normal cattle so that one could be adopted for routine use in clinical diagnosis and epidemiological studies. Microscopic examination of Giemsa-stained blood smears was used as a reference test in this study. Altay *et al*, (2008) used microscopy as gold standard in a similar study in Turkey.

Cattle with subclinical Theileriosis and Babesiosis become chronic carriers of the piroplasm and, hence, sources of infection for tick vectors. Therefore, latent infections are important in the epidemiology of the diseases. The diagnoses of piroplasm infections are based on clinical findings and microscopic examination of Giemsa-stained blood smears. However, this method is not sensitive enough *Babesia* or sufficiently specific to detect chronic carriers, particularly when mixed infections occur. Molecular techniques enable sensitive and specific detection of the parasites (Altay *et al*., 2008).

Using microscopy out of the 300 samples examined, 120 (40%) were positive with intra-erythrocytic forms of *Theileria* parasites and no *Babesia* parasites were detected. This may be due to the higher prevalence of *Theileria* species than *Babesia* species in areas around Lake Mburo and Uganda. This is in agreement with Muhanguzi, (2009), who reported a higher prevalence of *Theileria* species (19.8%) than species (0.6%) in Kashari County which also borders L. Mburo National Park using RLB. It was not possible to discern the *Theileria* species using microscopy or even detect the presence of mixed infections. This is because parasite species identification using conventional methods is difficult, particularly when mixed infections occur (Altay *et al*., 2008). RLB permitted simultaneous detection of multiple

infections in single animals. A substantial proportion of the cattle showed mixed infection (84.21%) with the majority (82.46%) infected with two species only; that is, *Theileria parva* and *Theileria spp(buffalo)* while eight cattle(14.04%) were infected with *T.mutans* alone while one animal had mixed infection with three species that is, *Theileria parva*, *Theileria spp(buffalo)* and *Babesia bovis.* However, *Theileria spp (buffalo)* and *Babesia bovis* presented with weak signals. *Theileria parva* and *B.bovis* are the most pathogenic species of cattle in their genera.

The most serious tick-borne disease in eastern and central Africa is East Coast Fever (ECF), caused by the intracellular protozoan parasite *Theileria parva* (Muraguri *et al*., 1999 and Oura *et al*., 2004). In this study the average prevalence of *Theileria parva* was found to be 60 %(48 cattle out of 80). This agrees with the average prevalence of *Theileria parva* in South Western Uganda of 54% reported by Oura *et al*., (2004). In this study the prevalence of *Theileria mutans* was found to 14.04 %. This study agrees with the findings of Muhanguzi (2009) who reported an 18.4% prevalence of *Theileria mutans* species in a County (Kashari) neighboring Lake Mburo National Park. *T.mutans* is a parasite of cattle and buffaloes and is widespread in sub-Saharan Africa, where the distribution follows that of its tick vectors (Walker and Olwage, 1987). *T.mutans* is generally regarded as only mildly pathogenic to cattle, although there are records of *T.mutans* being pathogenic and even fatal in East Africa, and anaemia can become severe in cases of pathogenic strains (Brown *et al.*, 1990). In this study the average prevalence of *Theileria spp (buffalo)* was 60% and was found as a mixed infection with *T.parva*. This could be because of the interactions between cattle and wild animals especially buffaloes since *Theileria spp(buffalo)* and *T.mutans* are piroplasms of buffaloes and other wild ruminants. The role of *T.mutans* and *Theileria spp(buffalo)* in the development of disease in cattle needs to be

further investigated. One sample out of 80 hybridized with the catch-all but did not hybridize with any of the species specific oligonucleotide probes used on the locally activated membrane. This could be because of the few number of oligonucleotide probes used in this part of the study. Therefore it was not necessary to sequence its PCR product to determine the species. This was probably not a novel species. Use of many species specific probes on the membrane in future would perhaps solve such problems (Gubbels *et al*., 1999).

Out of the 40 samples testing negative by microscopy, RLB was able to detect 17 (42.5%) as positive while 23 were negative. This can be explained by the fact that microscopy technique is usually adequate for detection of acute infections, but not for detection of carrier animals, where parasitaemias may be low (Friedhoff and Bose, 1994) since the study used only apparently normal animals and not clinical cases. The Kappa statistic between Microscopy and RLB technique was 0.2771(95% CI: 0.1008-0.4534). This indicated a fair agreement between Microscopy and RLB technique.

Out of the 80 samples testing positive by microscopy, 50 (62.5%) tested positive while 30 (37.5%) tested negative with beta-tubulin nested PCR analysis. Out of the 40 samples testing negative by microscopy 11(27.5%) were positive while 29 (72.5%) were negative by this technique. The beta-tubulin nested PCR was able to determine the genus but it was not possible to determine the species. This is because the sizes of PCR amplicons of most species have not been established. The previous study by Caccio *et al*., (2000) used known species of *Theileria* and *Babesia* and the sizes of their PCR products were determined. In this part of the study only *Theileria* parasites were detected and no *Babesia* specie was identified using this technique. The beta-tubulin primers did not amplify *Trypanosoma* tubulin DNA. This showed that the

primers were specific to *Theileria* and *Babesia* tubulin and probably not to any other parasite tubulin.

In this study, RLB was found to be more sensitive compared to beta-tubulin targeted nested PCR though the latter was more specific than the former. Previous studies by Oura *et al*., (2004) Gubbels *et al*., (1999); Schnittger *et al.*, (2004) have emphasized on the high sensitivity of the RLB assay. However, the two tests showed a substantial agreement since 62 samples positive by RLB were positive by beta-tubulin targeted nested PCR, 12 samples positive by RLB were negative by beta-tubulin targeted nested PCR, while no sample negative by RLB was detected as positive by beta-tubulin targeted nested PCR. The 46 samples negative by RLB were also negative by beta-tubulin targeted nested PCR. The level of agreement of the two tests in detection of Tick-borne piroplasms was determined by the Kappa statistic of 0.7984 (95% CI: 0.6925-0.9044).

Microscopy technique under-diagnosed both mixed infections and single *Theileria* and *Babesia* infections since RLB and beta-tubulin nested PCR were able to detect positive samples from the negative samples by microscopy. Although, rapid and cost effective, microscopy remains the most appropriate method for clinical diagnosis in a field setting, it lacks the sensitivity and specificity to be considered as a gold standard. Conversely, PCR-based molecular techniques allow sensitive detection of specific piroplasms. The RLB assay is a powerful tool and practical assay, since it is able to detect extremely low parasitaemia rates and simultaneously identify *Theileria* and *Babesia* species (Gubbels *et al*., 1999; Schnittger *et al*., 2004). It is useful in large-scale epidemiological screening to detect carriers of various infectious and non-infectious haemoparasites. The beta-tubulin targeted nested PCR though

less laborious than the RLB can be used for diagnosis of clinical cases but not field epidemiological studies in which establishment of the species is required.

This study faced the same problem faced by others when comparing *Theileria* and *Babesia* detection assays; the traditional gold standard clearly lacks the sensitivity and specificity of the newer assays. Although microscopy remains the most appropriate method for clinical diagnosis in a field setting, its use as a gold standard in apparently normal cattle may result in apparent false positive/negative results by superior techniques as seen in this study. Future studies should consider using more than one established molecular methods as a new gold standard.

CONCLUSIONS

- In this study, RLB was found to be more sensitive than beta-tubulin targeted nested PCR in detection of Tick-borne piroplasms.
- Beta-tubulin targeted nested PCR was found to be more specific than RLB.
- *Theileria* piroplasms were the most prevalent parasites found in blood with *T.parva* and *Theileria spp(buffalo)* being the most prevalent species.
- The biggest advantage RLB had over beta-tubulin targeted nested PCR was the higher sensitivity and the ability to detect species of *Theileria* and *Babesia* since the later detected only the genus.
- Our results suggest that RLB is still the best molecular technique for the detection of *Theileria* and *Babesia* parasites in apparently normal cattle and in epidemiological studies.
- The results further suggest that the beta-tubulin nested PCR method can be used in the diagnosis of Theileriosis and Babesiosis and for detection of the carrier status of cattle as a rapid, specific, convenient, reproducible and simple method to support other diagnostic tools especially microscopy since RLB is more laborious.

RECOMMENDATIONS

- More research needs to be done on the beta-tubulin targeted nested PCR using known species of *Theileria* and *Babesia* such that the specific sizes of amplicons can be established.
- Furthermore, restriction digests need to be done on all species of *Theileria* and *Babesia* to establish a restriction map of each of the species. This would make the beta-tubulin targeted nested PCR suitable for confirmatory diagnosis in the laboratory since it is less laborious than RLB technique though its sensitivity remains questioned.
- Future studies on comparison of diagnostic tests should accommodate a larger sample size covering a wider area and should use an established molecular technique as the gold standard.

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Appendix1

Diagnostic & Agreement Statistics

Criterion		Test: Positive	Test: Negative	
	Positive	62	0	62
	Negative	12	46	58
		74	46	120

95% Confidence Intervals — Clear | Calculate | % | 0.0

Index	*Symbol*	*Estimate*	*se*	*Lower 95% CI*	*Upper 95% CI*
Sensitivity	SE	1.0000	0.0000	0.9422	#NUM!
Sensitivity of a random test	SE_RAN	0.6167			
Quality index of sensitivity	κ(1,0)	1.0000			
Specificity	SP	0.7931	0.0532	0.6665	0.8883
Specificity of a random test	SP_RAN	0.3833			
Quality index of specificity	κ(0,0)	0.6645			
Efficiency (Correct classification rate)	EFF	0.9000	0.0274	0.8318	0.9473
Efficiency of a random test	EFF_RAN	0.5039			
Quality index	κ(.5,0)	0.7984	0.0541	0.6925	0.9044
Youden's index	J	0.7931	0.0532	0.6889	0.8974
Predictive value of positive test	PVP	0.8378	0.0428	0.7339	0.9133
Pred. value of a positive random test	PVP_RAN	0.5167			
Predictive value of negative test	PVN	1.0000	0.0000	0.9229	#NUM!
Pred. value of a negative random test	PVN_RAN	0.4833			
False positive rate	FP	0.2069	0.0532	0.1117	0.3335
False negative rate	FN	0.0000	0.0000	#NUM!	0.0578
Misclassification rate	1-EFF	0.1000	0.0274	0.0527	0.0336
Prevalence	P	0.5167	0.0456	0.4237	0.6088
Test level	Q	0.6167	0.0444	0.5235	0.7039
Likelihood ratio of positive test	LR+	4.8333	1.2932	2.9202	7.9998
Likelihood ratio of negative test	LR-	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Odds ratio	OR	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Odds ratio (Haldane's estimator)	OR'	465.0000	4.2852	26.8420	8055.4852

Warning: Empty cell(s) in table will result in uncalculatable values for some statistics.

Kappa and Related Indices					
Cohen's Kappa	κ	0.7984	0.0541	0.6925	0.9044
		This kappa indicates substantial agreement.			
		Test of Ho: Kappa=0: z=8.93, p =0.0000			
		t.t.t.			
Observed Agreement	PO	0.9000	0.0274	0.8318	0.9473
Chance Agreement	PE	0.5039			
Positive Agreement	PA	0.9118	0.0254	0.8620	0.9615
Negative Agreement	NA	0.8846	0.0331	0.8198	0.9495
Byrt's Bias Index	BI	-0.1000			
Byrt's Prevalence Asymmetry Index	PI	-0.1333			
Bias Adjusted Kappa	BAK	0.7964			
Prevalence & Bias Adjusted Kappa	PABAK	0.8000			
Alternative Indices of Association					
Dice's Index	p(s)	0.9118	0.0254	0.8620	0.9615
Yule's Q (Gamma)	γ	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Phi	ϕ	0.8152	0.0459	0.7251	0.9052
Scott's agreement index	π	0.7964			
Tetrachoric Correlation	r(t)	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
Goodman & Kruskal's tau (Crit. dep.)	τ(asy)	0.6645	0.0749	0.5177	0.8113
Lambda(Symmetric)	λ(sym)	0.7692	0.0662	0.6395	0.8989
Lambda(Criterion dep.)	λ(asy)	0.7931	0.0532	0.6889	0.8974
Uncertainty Coefficient (Symmetric)	U(sym)	#NUM!	#NUM!	#NUM!	#NUM!
Uncertainty Coeff. (Criterion dep.)	U(asy)	#NUM!	#NUM!	#NUM!	#NUM!

Test	*Chi Square*	*P*
Pearson Chi Square	79.74	0.0000
with Yate's correction	76.42	0.0000
Likelihood Ratio Chi Square	#NUM!	#NUM!

Minimum Expected Frequency	22.23	
Cells with Expected Frequency < 5	0	of 4 (0%)
Cells with Expected Frequency < 1	0	of 4 (0%)

Test	*Chi Square*	*P*
McNemar's Test	12.00	0.0005
with Yate's correction	10.08	0.0015