Available online at www.asric.org
ASRIC Journal on Agricultural Sciences 1 (2022) 40-50

Molecular Detection of Blood parasites from Wild ruminants from Matabeleland Province, Zimbabwe

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Received 3 June 2022; revised 23 July 2022; accepted 17 August 2022

Abstract

A number of wild ruminant species have been recognized as reservoirs for blood parasites that also affect domesticated ruminants. This has often resulted in transmission of these parasites from wild animals to domestic animals with catastrophic results being observed. Detection and molecular characterisation of blood parasites from wild ruminants from Matabeleland province, Zimbabwe was carried out. A total of 32 blood samples from hunter killed wild ruminant species including African buffalo (*Syncerus caffer*), (n=8) waterbuck (*Kobus ellipsipyrymnus*) (n=2), eland (*Taurotragus oryx*) (n=1), black wildebeest (*Connochaetes gnou*)(n=5), impala (*Aepyceros melampus*) n=12, kudu(*Tragelaphus strepsiceros*)n=2,bushbuck(*Tragelaphus scriptus*)(n=1)were collected from wildlife parks located in Hwange, Nyamandlovu and Beitbridge. Blood parasites were detected by giemsa staining and total DNA was extracted followed by PCR to detect for *Theilleria spp,Babesia spp* and *Anaplasma spp*. Of the wild ruminants sampled 68% were infected with *Thieleria spp,*67% infected with *Babesia spp* and 59 % were infected with *Anaplasma spp*. Of the 32 samples convectional PCR was done to 17 samples using primers targeting 18SrRNA of *Theileria spp* of which 88% of the samples were positive for *Thieleria spp.* Touchdown PCR was also done to 16 of the samples using primer targeting 18SrRNA of *Babesia spp* and all samples were positive for *Babesia spp.* Results showed that all of the ruminants from Nyamandlovu and Hwange were positive for blood parasites compared to 65% from Beitbridge. Comparison of microscopic examination and PCR analysis showed higher sensitivity of detection in the PCR method as 7% of the samples that previously showed negative results on the microscope were positive in the PCR method. In total 67% of the samples were positive for microscopic analysis whilst a total of 74% was detected using the PCR analysis. Findings of this study suggest that wild ruminants are indeed reservoirs of *Anaplasma spp, Babesia spp and Theilleria spp*. and could play an important role in the epidemiology and spread of blood parasites and may represent a serious threat to the livestock industry.

Keywords: Wild Ruminants ; Blood Parasites; Molecular detection

1.0 INTRODUCTION

Wild ruminants have an important role in the epidemiology of blood parasites that affect domesticated ruminants. Zimbabwe is a country that over the years has had a lot of land use changes increasing the interaction between wild animals and domestic animals. Wildlife infection rates and wildlife parasites remain poorly documented in Zimbabwe despite the tourism industry being amongst the leading economic boosters. Neitz (1935) demonstrated the susceptibility of wild ruminants to anaplasmosis. Hobday (1975) demonstrated that blood parasites consisting of protozoa and bacteria were widespread in African wildlife species.

Transmission of blood parasites from wild ruminants to domesticated ruminants have been demonstrated in several studies (De Vos and Potgieter, 1994; Peter *et al*., 1998). A number of wild ruminant species have been recognized as reservoirs for blood parasites that also affect domesticated ruminants. African buffalo (*Syncerus caffer*) have been demonstrated to be the reservoir of tick borne pathogens such as heart water, corridor diseases and East Coast fever (Lawrence *et al*., 1994). Enormous populations of wild ruminants in wildlife parks can maintain large numbers of tick vectors associated with blood parasites. In Southern Africa, most domesticated ruminants can get in contact with wildlife species (Peter *et al*., 1998).

Interaction of wild ruminants and domesticated ruminants leads to a complex circulation dynamic of tick-borne pathogens between wild and domestic animals. Currently in Zimbabwe, the role of many wildlife species in the epidemiology of blood parasites of the genera *Babesia*, *Theileria* and *Anaplasma* in endemic areas are poorly defined. Information on the epidemiology of blood parasites in wild ruminants has applications to domesticated and wild animal health.

Wild ruminants are also susceptible to blood parasitic infections, mortality of wild animals due to tick-borne diseases has been reported on several occasions (Nijhof *et al.,* 2005). Most of these fatal cases mostly occur when wild animals are translocated to endemic areas. The ecology and epidemiology of blood parasites remain poorly understood in Zimbabwe investigations of the relations between blood parasites and wildlife are of interest to those involved in nature conservation and domestication of ruminants.

1.2 Problem Statement

Blood parasites have been a major challenge to livestock farming in Zimbabwe causing diseases and death with resultant decrease livestock productivity. The national herd decreased by 4.7% for the 2019/20 season. Anaplasmosis, Babesios and Heart water contributed to 50% of livestock death (DVTS.,2020). Studies on animal blood parasites have been mainly limited to domesticated animals, most of these studies lack adequate data on blood parasites circulating in wild ruminant species.

1.3 Justification

Blood parasites have been described in domestic and wild ruminants including cattle, African buffalo, antelopes, bushbucks and waterbucks; however, clinical signs are most noticeable in cattle (Kocan *et al.,* 2003). In many provinces, in Zimbabwe, livestock can be in contact with wildlife species, indicating complex circulation dynamics of blood parasites between wild and domestic animals. This implicates wildlife species as a source of *Anaplasma spp, Babesia spp* and *Theilleria spp* persistent infections. The distribution of blood parasites in ruminants in Zimbabwe have been significantly influenced by land utilization practices. The land reform programme in Zimbabwe has resulted in the utilisation farming areas close to wildlife parks (Williams.,2016). Livestock are susceptible to blood parasites, while wildlife susceptibility is poorly studied and understood. Studies done in countries in Southern Africa such as Botswana and South Africa has shown the prevalence of *Anaplasma spp,Babesia spp* and *Theilleria spp* wild ruminants (Khumalo *et al.*, 2016). However, in Zimbabwe there is limited information on the role played by wildlife species in the epidemiology of blood parasites such as *Anaplasma spp ,Babesia spp* and *Theilleria spp* in endemic areas; such information is important for both domestic and wild animal health. Livestock are susceptible to blood parasites, while wildlife susceptibility is poorly studied and understood. There is a need to identify the species of blood parasites from wild ruminants in different areas of Zimbabwe. The study has provided valuable preliminary data on the detection of blood parasites in wild ruminants. Understanding the epidemiology of blood parasites infections in wild ruminants will aid in the control of blood parasitic infections in domesticated ruminants particularly recent outbreaks recorded by Department of Veterinary Technical Services. Additionally, the data generated from this study provides a basic knowledge about species of *Babesia,Anaplasma* and *Theilleria* in wild ruminants. This should provide a basis for further studies on molecular epidemiology of the blood parasites in the current study areas. The detection of blood parasites is important, as some of these parasites cause high morbidity and mortality in wild and domesticated ruminants. Data on the prevalence and distribution of blood parasites can be used for future research on development to protect domesticated ruminants from blood parasites.

1.4 Aim

The aim of the study was to determine and understand the genetic diversity of some species of *Babesia, Anaplasma* and *Theileria* infecting wild ruminants from Matabeleland Province, Zimbabwe.

1.5 Objectives

1. To collect blood samples from wildebeest (*Connochaetes taurinus*), buffaloes (*Syncerus caffer*) , eland (*Taurotragus oryx*), impala (*Aepyceros melampus*), kudu (*Tragelaphus strepsiceros*), bushbuck (Tragelaphus scriptus), giraffe (*Giraffa camelopardalis*) and waterbuck (*Kobus defassa*).
2. To examine blood smears from blood samples for blood parasites.
3. To screen for specific target genes of *Babesia spp.,Theileria spp* and *Anaplasma spp*.

2.0 MATERIALS AND METHODS

2.1 Sample Collection

A total of 32 blood samples from hunter killed wild ruminant species including African buffalo (*Syncerus caffer*), (n=8) waterbuck (*Kobus ellipsipyrymnus*) (n=2), eland (*Taurotragus oryx*)

(n=1), black wildebeest (*Connochaetes gnou*)(n=5), impala (*Aepyceros melampus*) n=12, kudu (*Tragelaphus strepsiceros*) n=2,bushbuck(*Tragelaphus scriptus*)(n=1),giraffe(*Giraffa camelopardalis*) (n=1) were obtained from wildlife parks located in Hwange, Nyamandlovhu and Beitbridge. Blood samples were collected into 10ml heparin tubes.

2.2 Laboratory Techniques

The 32 blood samples collected were analysed in the Microbiology and Molecular Biology laboratories of the Department of Applied Biology and Biochemistry in the Faculty of Applied Sciences at National University of Science and Technology as described below**.**

2.2.1 Giemsa Staining

A thin smear of each of the blood samples was prepared on a glass slide, air-dried, and fixed with 100% methanol and stained with Giemsa for 45 minutes.

2.2.2 DNA Extraction

DNA was extracted from anticoagulant-treated blood from all the 32 blood samples using the QIAamp® DNA mini kit (QIAGEN, Southern Cross Biotechnologies). The manufacturer's extraction protocol was followed. Twenty microliters of proteinase K was added into a 2 ml micro centrifuge tube. To this 100 µl anticoagulant-treated blood was added, then volume was adjusted to 220 µl with PBS. Two hundred microliters of lysis buffer were added and mixed thoroughly by vortexing. The blood samples were incubated at 56°C for 10 min followed by addition of 200 µl ethanol (96%), this was then mixed thoroughly by vortexing. The mixture was pipetted into a DNeasy Mini spin column placed in a 2 ml collection tube. This was centrifuged at ≥6000 x g (8000 rpm) for 1 min. The flow-through and collection tube were discarded. The spin column was placed in a new 2 ml collection tube. 500 µl Buffer AW1 were added and centrifuged for 1 min at ≥6000 x g. The flow-through and collection tube were discarded. The spin column in was placed into new 2 ml collection tube, followed by addition of 500 µl Buffer AW2 and centrifuged for 3 min at 20,000 x g (14,000 rpm). The flow-through and collection tube were discarded. The spin column was transferred into to a new 2 ml microcentrifuge tube. DNA was eluted by adding 50 µl of elution buffer to the centre of the spin column membrane. This was incubated for 1 min at room temperature followed by centrifugation for 1 min at ≥6000 x g. The extracted DNA was stored at 4°C.

2.2.3 Polymerase Chain Reaction (PCR).

PCR was done for all the 32 DNA samples using *Theileria* genus-specific forward primer and reverse primer. Primers used amplified the V4 hypervariable region of the parasite 18S rRNA gene as previously

described by Nijhof *et al.,* 2005. The PCR reaction mixture consisted of 5µ of Taq 2x Master mix (containing Taq DNA polymerase, dNTPs, $MgCl_2$, KCl and

stabilisers),0.2µ of Forward Primer,0.2µ of Reverse Primer,0.8µ of MgCl,2µ of DNA template and 1.8µ of nuclease free water to a total volume of 10µ.A positive and negative controls (master mix without DNA template) were included to monitor false positive and false negative results. The amplification included a 3 min denaturation step at 95°C followed by 35 repeats of 30 s at 95°C, 45 s at 55°C, and 2 min at 68°C and a final extension at 68°C for 10 min.

2.2. Gel Electrophoresis

Gel electrophoresis was done for all the 32 DNA sample amplicons including 1kb molecular ladder on first lanes on 1% agarose, all the amplicons were stained with a dye. The gel was run at 100V,300MA for 45 minutes and viewed under UV light. Selected positive amplicons were purified and were sent for sequencing at Inqaba Biotech

3.0 RESULTS

3.1 Giemsa Staining

Identification of parasites was based on the presence of intra- erythrocytic bodies in blood smears when they were viewed under a light microscope. *Anaplasma marginale* was identified by the appearance of dense intra-erythrocytic rounded bodies located on the edges of red blood cells (Fadly.,2012). *Babesia spp.* were identified by the presence of pairs of merozoites in blood smears (Mweemba *et al*.,2012). *Theileria sp* were identified by presence of schizonts in blood smears (Fadly, 2012).

Babesia spp and Theileria spp were detected in all blood smears from Hwange National Park while 87.5% had Anaplasma spp infection. All the buffaloes had mixed infection of the blood parasites. Blood smear results from Beitbridge showed that 75% of Wildebeest had *Babesia spp, Theileria spp and Anaplasma spp* infection. All wildebeest which were positive for blood parasites had mixed infections. Blood smears from waterbuck were negative for blood parasites. Eland and bushbuck were positive for *Babesia spp, Theileria spp* and *Anaplasma spp* infection and they all had mixed infections. Blood smear results from all the impalas from Beitbridge showed that they had no blood parasites as shown on Table 3.1.

Blood smear results from Nyamandlovu showed that wildebeest, kudu and giraffe all had *Babesia spp, Theileria spp* and *Anaplasma spp* infection and they all had mixed infections.80% of the Impalas had *Babesia spp, Theileria spp* and *Anaplasma spp* and mixed infection.

Table 3:1 Prevalence of blood parasites detected from blood smears from wild ruminants

Animal species							
Parasite	Buffaloes	Wildebeest	Waterbuck	Eland	Impala	Bushbuck	Giraffe
Theileria spp	100%	87.5%	0%	100%	90%	100%	Positive
Babesia spp	100%	87.5%	0%	100%	80%	100%	Positive
Anaplasma spp	87.5%	87.5%	0%	100%	40%	100%	Positive

Figure 3.1 shows presence of parasites in giemsa stained blood. Most samples showed presence of piroplasms that are typical of Theilleria infection.

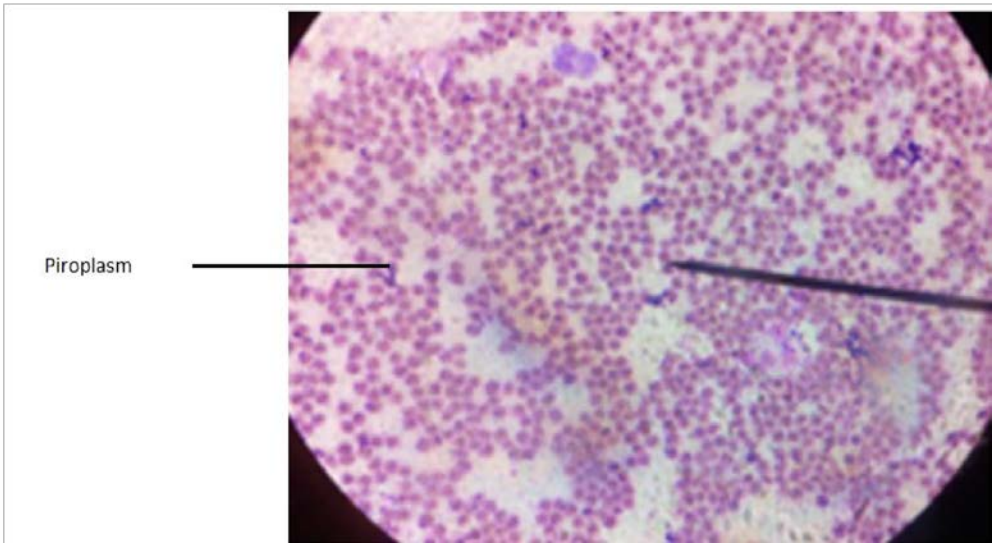

Figure 3.1: Giemsa stained blood smears of infected Buffalo from Hwange revealed the presence of intra-erythrocytic forms morphologically compatible with *Theileria* piroplasms. (Magnification x 100)

3.2. Molecular Assays

On doing a PCR reaction on 17 representative samples, there was amplification on 17 out of the 17 samples as shown on Figure 3.2. Gel electrophoresis of PCR products obtained after using *Theileria* genus-specific primers obtained for all representative blood samples collected from wild ruminants demonstrated the presence of *Theileria* species in the animals. The PCR products were 1700bp as shown and were clear distinct bands showing success in
amplification. A representative gel picture result is shown on Figure 3.2.

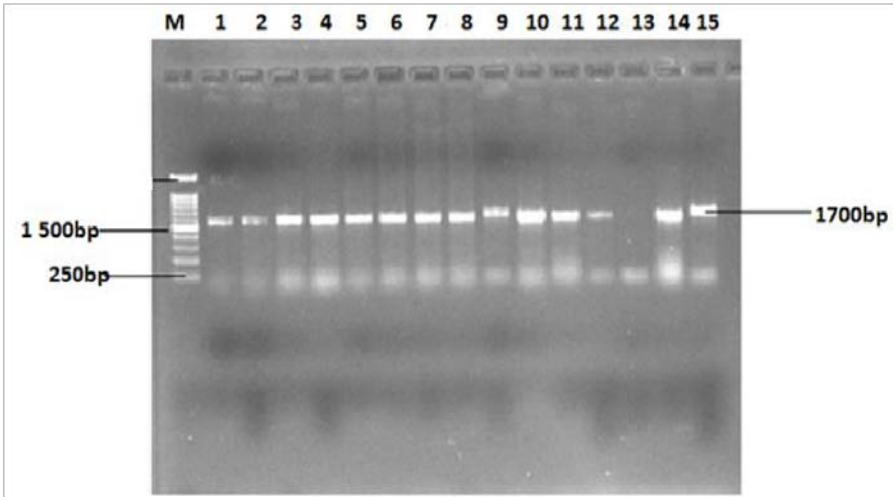

Figure 3.2: Agarose gel electrophoresis of conventional PCR products using *Theileria* 18SrRNA specific primers M 1500bp ladder DNA marker.

Analysis of PCR products after agarose gel electrophoresis of the DNA extracted from blood samples showed that the following animal species had *Theileria spp* infection, 100% of the buffaloes ,66% of the impalas ,50% of the waterbuck, giraffe,90% of the wildebeest,100% of the bushbuck,100% of the eland and 100% of the kudu. All microscopically positive samples were confirmed by PCR. Waterbucks were detected by PCR and they had tested negative on microscopic examination. No *Theileria* piroplasms were seen on blood smears of samples that were negative in PCR. The results are shown on Table 3.2

Table 3.2: Positive samples after agarose gel electrophoresis of conventional PCR products using *Theileria* 18SrRNA specific primers.

Species	**Eland**	**Buffalo**	**Impala**	**Water Buck**	**Giraffe**	**Wildebeest**	**Bush Buck**	**Kudu**
Percentage Positive samples	100%	100%	66%	50%	100%	90%	100%	100%

Comparison of microscopic examination and PCR analysis showed that in total 67% of the samples were both positive for microscopic and PCR analysis whilst 7% of the samples which had been negative for microscopic examination were positive for PCR analysis as shown on Table 3.3.

Table 3.3: Comparison of microscopic examination and PCR analysis results in *Theileria* infection diagnosis in wild ruminants.

PCR analysis				
Microscopic Examination		Positive	Negative	Total
	Positive	67%	0	67%
	Negative	7%	28%	33%

3.2.1. Touch Down PCR for Detection of *Babesia*

A total of 16 samples from the 32 samples run for the touchdown PCR came out positive for *Babesia* with an approximately 400bp product being produced. The product showed distinct bands that are typical of *Babesia* infection as shown on figure 3.4.

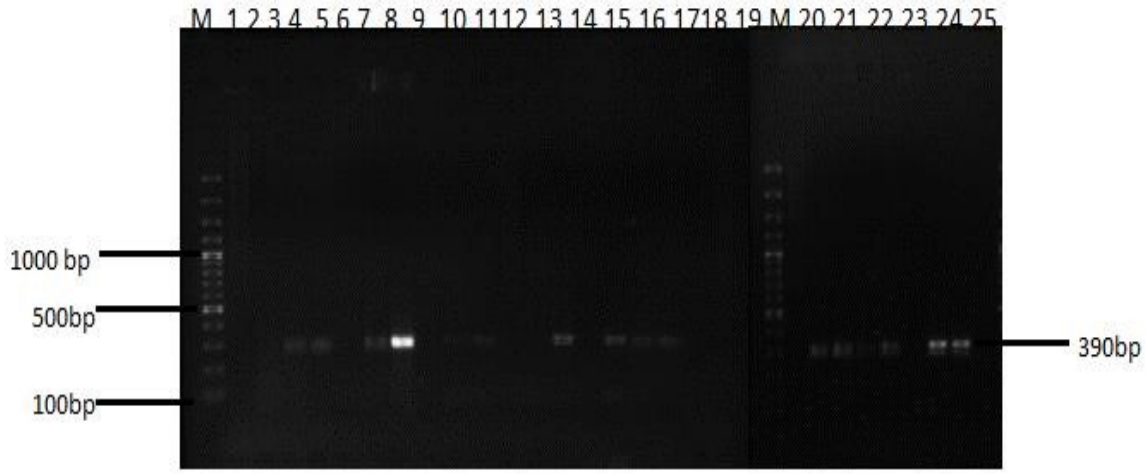

Figure 3:3 Agarose gel electrophoresis of conventional PCR products using *Babesia spp* 18SrRNA specific primers
M 1500bp ladder DNA marker

4.0 DISCUSSION

In this study, the occurrence of blood parasites (*Theileria, Babesia* and *Anaplasma*) from wild ruminants from wildlife parks located in Hwange, Beitbridge and Nyamandlovhu was determined by giemsa staining and convectional PCR. Giemsa staining results demonstrated the presence of *Theileria* and *Babesia* parasites in all the buffaloes sampled from Hwange National Park. Microscopy results also indicated that 87.5% of the buffaloes had *Anaplasma* infections. All the buffaloes had mixed infections. This study has confirmed the findings of previous studies that buffaloes are commonly infected with *Theileria spp* (Pienaar, *et al.*, 2011; Chaisi, *et al*., 2011). Our study was in agreement with other studies done in Southern Africa which have indicated that the African buffalo is the natural reservoir host of both pathogenic and non-pathogenic *Theileria* species (Pienaar, *et al*., 2011; Chaisi, *et al*., 2011; Young, *et al.*,

1977). *Theileria sp*. has previously been identified from healthy African buffaloes in South Africa (Nijhof, *et al*., 2005).

Babesia spp were detected in all the buffaloes from Hwange National Park. Previous studies done have indicated that the African buffalo can also be infected with *Babesia spp*. In a study done by Chaisi *et al.* (2011) in South Africa, 21% of Hluhluwe-iMfolozi Park buffalo samples hybridized with the *Theileria/Babesia* genus specific when they used reverse blot hybridisation method.

Anaplasma spp were also detected in 87.5% of blood samples from buffaloes from Hwange National Park. *Anaplasma spp* infection has previously been described in buffaloes which can act as reservoirs of the parasite (Kocan, *et al.,* 2003). Brocklesby and Vidler (1966) observed Anaplasma bodies similar to *A. centrale* in buffalo erythrocyte.

In a study done by Oura *et al*. (2011) in South Africa by using reverse line blot assay they showed that ten buffaloes from Murchison Falls National Park, 14 buffaloes from Kidepo Valley National Park and seventeen from Queen Elizabeth National Park were carriers of *A. centrale*.

Giemsa staining results of blood smears from Beitbridge showed that 75% of wildebeest had *Babesia spp, Theileria spp* and *Anaplasma spp* infection. *Babesia spp* and *Theileria spp* have been previously described in wildebeest. In a study done by Camma *et al* (2012) they showed that, six out of ten wildebeest were positive for *Theileria/Babesia* genus-specific probes when they used real time PCR to detect blood parasites from wildebeest from Etosha National Park in Namibia. Seventy-five percent of wildebeest were also positive for *Anaplasma spp.* In a study done by Neitz and Du toit (1935) it was found that a black-wildebeest was susceptible to *A. marginale* but no clinical symptoms were noticed.

Water bucks which were negative for microscopic analysis were positive for PCR,50% of the waterbuck were positive for *Theilleria spp*. Several authors have also suggested that waterbuck are not easily infected with blood parasites. In a study done by Grootenhuis (1995) exposed batches of adult *R. zambeziensis* ticks, highly infected from buffalo with *T. parva* parasites and which were shown to be highly infective to cattle, were also fed on four waterbucks, none of which became infected. Waterbucks have also been previously described to be carriers of blood parasites (Stagg *et al*.,2009). Nevertheless, giemsa staining may fail to detect blood parasites in carrier animals. Stagg *et al* (2009) successfully infected waterbucks with *Theileria parva* sporozoites derived from ticks infected by feeding on African buffalo (*Syncerus caffer*). All waterbuck underwent mild infections with the development of sporadic schizont and piroplasm parasitosis when inoculated with sporozoite doses lethal to cattle.

All the eland and bushbuck in this study were positive for *Babesia spp, Theileria spp* and

Anaplasma spp infection and they all had mixed infections. The results were in agreement with Carmichael and Hobday (1975) who carried out a similar study in Botswana in their study they used giemsa staining to detect blood parasites from several Bovidae and their results showed that *Babesia spp* occurred in erthryocytes of eland and bushbuck.

Blood smear results from all the impalas from Beitbridge showed that they had no blood parasites. Impalas have also shown to be carriers of blood parasites and normally blood carrier animal are not detectable by the use of giemsa staining. In study done by Oura *et al*. (2011) in Uganda they showed in their study that majority of impala sampled in their study were carriers of *A. centrale*, and all were carriers of an unidentified *Babesia/Theileria* species.

Giemsa staining results from Nyamandlovhu showed that wildebeests, kudus and giraffe all had *Babesia spp, Theileria spp* and *Anaplasma spp* infection and they all had mixed infections. Eighty percent of the impalas had *Babesia spp, Theileria spp* and *Anaplasma spp*. Compared to impalas from Beitbridge, impalas from Nyamandlovhu were all positive for both of the blood parasites under study. All kudus and a giraffe were also positive for *Babesia spp ,Theileria spp* and *Anaplasma spp.* The results were also in agreement with Carmichael and Hobday (1975) who carried out a study in Botswana who stained blood smears from several Bovidae and they showed that kudus,impalas and girrafes were positive for blood parasites.

Samples which were positive for PCR but negative for microscopy were from waterbucks. Theileria *spp* cannot be reliably detected and differentiated according to their piroplasms structure by microscopy, especially in subclinical infections (Razmi *et al.*,2003). Razmi *et al*. (2003) suggested that to identify the role of each wild ruminant species in the epidemiology of theileriosis, sensitive and specific diagnostic tests, such as polymerase chain reaction (PCR), are required to be used.

All the buffalo samples from Hwange were positive for PCR gel electrophoresis analysis though they appeared to be healthy. This is the first report on Theileria *spp* in buffaloes reported from Hwange. Eygelaar *et al.,* (2015) carried out a similar study in Zimbabwe Gonarezhou National Park. Quantitative real-time PCR (qPCR) detected 81% samples from buffaloes infected with *T. parva.*

All wild ruminants which tested positive appeared to be in good health though they tested positive for the blood parasites.

5.0 CONCLUSION

In conclusion, findings of this study suggest that wild ruminants are reservoirs of *Anaplasma spp*, *Babesia spp* and *Theilleria spp*. infection and could play an important role in the epidemiology and spread of blood parasites and may represent a serious threat to the livestock industry. It can also be concluded that the African buffalo compared to other ruminants can act as reservoir of several blood parasites. Findings of the study also suggest that wild ruminants act as carriers of blood parasites since all the animals sampled in this study appeared to be healthy but results showed that most of them were positive for blood parasites. The results also show that the PCR method is more sensitive when it comes to detection for blood parasites.

Development and use of molecular techniques make it easier and faster to detect for parasites in a non invasive manner. It becomes imperative to further study the phylogeny of these blood parasites.

6.0 RECOMMENDATIONS

There is need for further epidemiological surveys of blood parasites in wild ruminants using more specific and sensitive diagnostic tools or assays.

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