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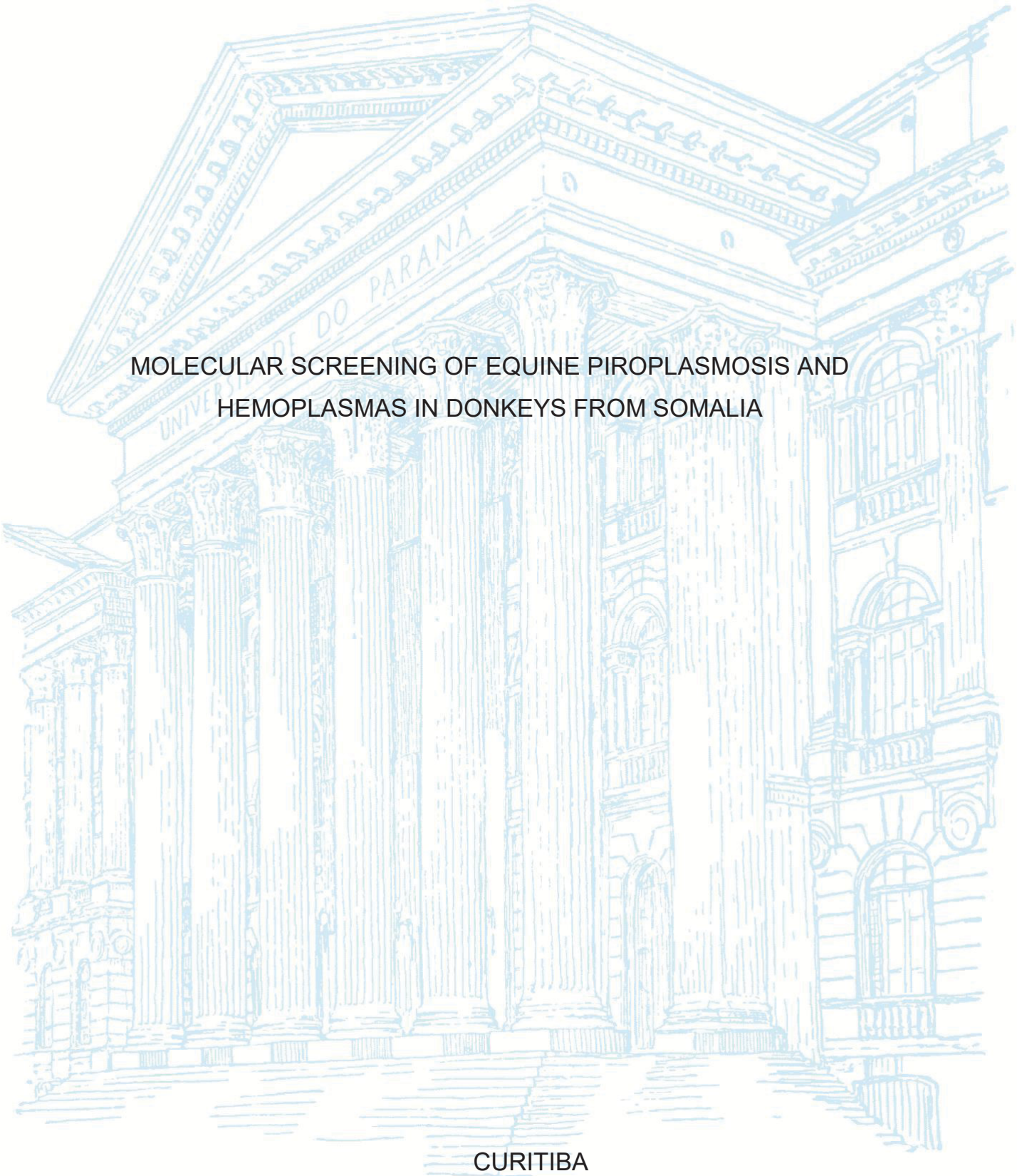

MOLECULAR SCREENING OF EQUINE PIROPLASMOSIS AND HEMOPLASMAS IN DONKEYS FROM SOMALIA

CURITIBA
2022

MOHAMED ABDULKADIR SHAIR

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Dissertação apresentada ao Programa de Pós-Graduação em Ciências Veterinárias, Setor de Ciências Agrárias, Universidade Federal do Paraná, como um requerimento parcial para obtenção do título de Mestre em Ciências Veterinárias

Orientador: Prof. Dr. Rafael Felipe da Costa Vieira
Co-orientador: Prof. Dr. Ahmed Abdulkadir Hassan-Kadle

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APPROVAL MINUTE

The Examining Board is designated by the Faculty of the Graduate Program of the Federal University of Paraná in CIÊNCIAS VETERINÁRIAS where invited to argue the THESIS of MASTER OF SCIENCES by **MOHAMED ABDULKADIR SHAIR** , entitled: **Molecular screening of equine piroplasmosis and hemoplasmas in donkeys from Somalia**, under the supervision of Dr. RAFAEL FELIPE DA COSTA VIEIRA, which and after assessment of the candidate and the work, the Examining Board decided for the APPROVAL in the present rite.

The granting of the title of master of sciences is contingent upon the fulfillment of all the requirements indicated by the Examining Board and terms determined in the regulation of the Graduate Program.

CURITIBA, August 12th, 2022.

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DEDICATION

I dedicate this work with great appreciation to my wife,

Ramla Mohamed.

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“Learn from yesterday, live for today, hope for tomorrow. The important thing is not to stop questioning” **(Albert Einstein)**

RESUMO

A piroplasmose equina (PE) é uma doença infecciosa transmitida por carrapatos de equídeos (cavalo, burro, mula e zebra), causada por *Babesia caballi* e *Theileria equi*. A PE é uma doença de notificação compulsória que possui ampla distribuição na maioria dos países tropicais e subtropicais do mundo. Os micoplasmas hemotrópicos (hemoplasmas) são os agentes causadores da anemia hemolítica infecciosa em mamíferos em todo o mundo. No entanto, poucos relatos em cavalos foram realizados até o momento. Faltam dados epidemiológicos e caracterização molecular da infecção por PE e hemoplasma na Somália, uma vez que os burros são usados para várias atividades, incluindo transportar mercadorias nas costas ou puxar carrinhos carregados com mercadorias como lenha, alimentos, água e materiais de construção. Considerando a importância socioeconômica e a falta de dados sobre esses patógenos em jumentos na Somália, este estudo teve como objetivo rastrear burros para infecção por PE e hemoplasma. Um total de 30 burros machos foram avaliados, amostrados de sangue e inspecionados quanto à presença de ectoparasitos. O DNA foi extraído do sangue total e as amostras foram ainda rastreadas usando ensaios de PCR em tempo real específicos da espécie visando o gene gene endógeno de mamífero gliceraldeído-3-fosfato desidrogenase (gapdh), ema-1 de *T. equi* e o gene 18S rRNA de *B. caballi,* e ensaio qPCR específico de gênero visando o 16S rRNA gene dos hemoplasmas. Os jumentos não estavam infestados por carrapatos no momento da amostragem. O gene gapdh foi consistentemente amplificado a partir de todas as amostras de jumentos. No geral, a prevalência de 22/30 (73,3%, IC 95%: 55,6 – 86,8%) foi qPCR-positiva para T. equi e B. caballi. Enquanto uma única infecção por T.equi foi observada em 3/30 (10%, IC 95%: 2,7 – 24,9%) dos jumentos. Todas as 30 amostras de jumento testaram negativo para hemoplasma por qPCR. Mais estudos avaliando um número maior de burros são necessários para estabelecer a presença de hemoplasmas na Somália. Até onde sabemos, este é o primeiro estudo sobre a investigação molecular de PE e hemoplasmas em jumentos da Somália.

Palavras-chave: *Theileria equi, Babesia caballi,* Hemotropic *Mycoplasma* spp., burro doméstico, micoplasmas hemotrópicos, África Subsaariana.

ABSTRACT

Equine piroplasmosis (EP) is an infectious tick-borne disease of equids (horse, donkey, mule, and zebra) caused by *Babesia caballi* and *Theileria equi*. EP is a notifiable disease that has a wide distribution in most tropical and subtropical countries worldwide. Hemotropic mycoplasmas (hemoplasmas) are the causative agents of hemolytic infectious anemia in mammals worldwide. However, few reports on horses have been performed to date. Epidemiological data and molecular characterization of EP and hemoplasma infection in Somalia is missing since donkeys are used for various activities including to carry goods on their backs or pulling carts loaded with goods like firewood, foods, water, and construction materials. Considering the social-economic importance and the lack of data on these pathogens in donkeys in Somalia, this study aimed to screen donkeys for EP and hemoplasma infection. A total of 30 male donkeys were evaluated, blood sampled, and inspected for ectoparasites. DNA was extracted from whole blood, and samples further screened using by species-specific real-time PCR assays targeting the mammal endogenous gene glyceraldehyde-3-phosphate dehydrogenase (gapdh), *ema-1* gene of *T. equi* and the 18S rRNA gene of *B. caballi*, and genus-specific qPCR assay targeting the 16S rRNA gene of hemoplasmas. Donkeys were not infested by ticks at the time of sampling. The *gapdh* gene was consistently amplified from all donkeys' samples. Overall, prevalence of 22/30 (73.3%, 95% CI: 55.6 – 86.8%) were qPCR-positive for *T. equi* and *B. caballi*. While a single infection for *T.equi* was observed in 3/30 (10%, 95% CI: 2.7 – 24.9%) of donkeys. All 30 donkey samples tested negative for hemoplasma by qPCR. Further studies evaluating a higher number of donkeys are needed to establish the presence of hemoplasmas in Somalia. To the best of our knowledge, this is the first study on the molecular investigation of EP and hemoplasmas in donkeys from Somalia.

Keywords: *Theileria equi*, *Babesia caballi*, Hemotropic *Mycoplasma* spp., domestic ass, hemotropic mycoplasmas, Sub-Saharan Africa.

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LIST OF ABBREVIATIONS

ARTC	- Abrar Research and Training Centre
AU	- Abrar University
cPCR	- Conventional Polymerase Chain Reaction
Ct	- Cycle threshold
EDTA	- Ethylenediamine tetraacetic acid
DNA	- Deoxyribonucleic acid
Et al.	- et alia
GAPDH	- Glyceraldehyde 3-Phosphate Dehydrogenase
GE	- General Electric
qPCR	- Quantitative Polymerase Chain Reaction
RBC	- Red Blood Cell
rRNA	- Ribosomal Ribonucleic Acid
FTA	- Flinders Technology Associates
CA	- California
DBS	- Dried Blood Spot
BLASTn	- Nucleotide Basic Alignment Search Tool
Ssu rRNS	- Small Subunit Ribonucleic Acid
SYBP	- Synergy Brands, Inc
UFPR	- Universidade Federal do Parana
UN	- United Nation
µl	- Microliter
UK	- United Kingdom
EMA-1	- equi merozoite antigen 1

TABLE OF CONTENT

1 GENERAL INTRODUCTION

1.1 BACKGROUND

Livestock in Somalia are the main source of individual and national wealth (FAO, 2004). Moreover, the sector contributes about 40 percent in the Gross Domestic Products (GDP) of the country and more than 50% of export earnings (CIA, 2020). More than half of the population living in rural areas depends directly or indirectly on livestock production, and the sector is the largest contributor to Somalia's GDP, livelihoods, and economic growth. Yet its performance is undermined by several factors, including poor animal nutrition, cross-boundary diseases, eroded genetic resources, and a lack of natural resource management and institutional weaknesses (FOA, 2019).

Equine piroplasmosis (EP) is an infectious tick-borne disease of equids (horse, donkey, mule, and zebra) caused by *Babesia caballi* and *Theileria equi* (Wise et al., 2019; Qablan et al., 2013). EP is a notifiable disease that has a wide distribution in most tropical and subtropical countries around the world (OIE 2019; Onyiche et al., 2019; Friedhoff et al., 1990). The tick genera involved in the transmission of EP include *Amblyomma, Hyalomma, Rhipicephalus, Haemaphysalis, Dermacentor,* and *Ixodes* (Tirosh-Levy et al., 2020; Oguntomole et al., 2018; Scoles and Ueti 2015). In Somalia, four these genera *Rhipicephalus, Hyalomma, Amblyomma,* and *Haemaphysalis* have been reported (Hassan et al., 2013; Kaiser & Hoogstraal, 1968; Pegram, 1976; Iori et. al., 1996; Walker et. al., 2003, Isse et. al., 2017). In donkeys, EP leads to major economic importance since the affected animals demonstrate loss of appetite and a significant reduction in strength, draughts power, and these diseases may threaten the animal’s survival (Ahmadi et al., 2020).

Hemotropic mycoplasmas (hemoplasmas) are obligate erythrocyte bacteria that infect a wide variety of vertebrates worldwide (Willi et al., 2006; Messick, 2004; Millán et

al., 2020). Hemoplasmas are the causative agents of acute or chronic infectious anemias in mammalian species, including humans (Messick, 2004).

To date, there are no studies regarding the occurrence of EP and hemoplasmas in donkeys in Somalia. The economic relevance of donkeys in Somalia is due to the various activities performed mainly by family farmers used to carry goods on their backs or pull carts loaded with goods like firewood, foods, water, and construction materials. Considering the social-economic importance and the lack of information regarding the epidemiology of these pathogens in donkeys this study aimed to screen a population of this animal species for EP and hemotropic *Mycoplasma* spp.

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2 LITERATURE REVIEW

2.1 Equine Piroplasmosis

2.1.2 Etiology

Equine piroplasmosis (EP) is an intraerythrocytic parasitic disease of equids caused by *Babesia caballi* and *Theileria equi* (Wise et al., 2013). Previously known as *Babesia equi,* the species was reclassified as *T. equi* in 1998 due to the extra-erythrocytic life stage in lymphocytes and the lack of transovarial transmission (Mehlhorn and Schein 1998; Allsopp et al., 1994). A few years later, it was discovered of these parasites *Babesia* and *Theileria* could infect the erythrocytes of equids (Wise et al., 2013).

2.1.3 Epidemiology

Ixodid ticks are the biological vectors of EP, with infected animals acting as carriers of its etiological agents for a long period of time (Sumbria et al., 2014). Previous studies on EP were reported in many parts of the world, including Africa (Idoko et al., 2020; Dahmana et al., 2019), based on microscopic examination of stained blood smears and serological and molecular methods (Knowles et al., 1992; Davitkov et al., 2017; Sunday et al. 2020; Camino et al., 2021; Coultous et al., 2020; Oladosu and Olufemi 1992).

In donkeys, EP leads to major economic importance due to the affected animals manifest loss of appetite and a significant reduction in strength, draughts power and these diseases may threaten the animal's survival (Ahmadi et al., 2020). Donkeys usually show the chronic EP and most of the time they lack any specific signs (Ahmadi et al., 2020).

The tick genera involved in the transmission of EP include *Dermacentor, Hyalomma, Haemaphysalis, Ixodes, Rhipicephalus* and *Amblyomma* (Scoles et al., 2015; Tirosh-Levy et al., 2020). Transstadial transmission has been reported for both EP agents in several tick species; however, transovarian transmission has been only reported for *B. caballi* (Scoles et al., 2015). Transplacental transmission in the equine host has been documented for both *B. caballi* and *T. equi* (Allsopp et al., 2007; De Waal 1992). Recently, transplacental transmission was reported in mules particularly associated with *T. equi* (Françoso et al., 2018). *Theileria equi* has been associated as a major cause of abortion (Phipps and Otter 2004; De Waal 1992; Penzhorn et al., 1999), although the role of this parasite as a cause of abortion is still unknown (Tirosh-Levy et al., 2020).

Asymptomatic carriers of piroplasms from endemic areas are of remarkable epidemiological relevance as they serve as reservoirs for ticks and increase the risk for iatrogenic transmission (Tirosh-Levy et al., 2020). Additionally, the transmission of EP can occur iatrogenically through infected blood via blood transfusions and sharing contaminated needles or surgical instruments (Onyiche et al., 2019; Short et al., 2012; Gerstenberg et al., 1998). There is no proof that an infection can transmit during routine reproductive operations. The transmission of both parasites *in utero* has been reported, though the specific mechanism by which this occurs is still unknown (Allsopp et al., 2007).

2.1.4 Pathogenesis and Clinical Findings of EP

Clinical signs of EP varies with some infected animals asymptomatic (Zobba et al., 2008). In general, infection with *T. equi* is more severe than *B. caballi* (Maurer 1962). Manifestation of clinical signs can take various forms, such as acute, subacute, or chronic (De Waal 1992).

Acute EP infections may induce high fever, hemolytic anemia, inappetence, weight loss, hemoglobinuria, jaundice, malaise, lethargy, anorexia, and even death (Wise et al., 2013; de Waal et al., 2004), whereas chronic infections, more common in endemic regions, are usually asymptomatic with animals exhibiting nonspecific signs such as weight loss, as well as poor performance condition (Onyiche et al., 2019; Donnellan et al., 2009). Thrombocytopenia has also been described (Wise et al., 2013).

Donkeys show usually the chronic form of EP rather than horses (Sumbria et al., 2014). In addition, abortion and neonatal death have been reported following intrauterine infections (Wise et al., 2013). While the first clinical manifestations, horses with EP often remain persistent subclinical (unapparent) carriers for long periods of time without treatment (Rothschild, 2013; Wise et al., 2013).

2.1.5 Diagnosis of EP

The microscopical detection of EP agents on stained blood smears has been historically used (Rothschild, 2013; Camino et al., 2021), although presents low sensitivity during low parasitemia (Wise et al., 2013). Several serological assays have been reported to increase diagnostic sensitivity in equids chronically infected with *B. caballi* and *T. equi*. Some of these diagnostic assays include the complement fixation test (CFT), enzyme-linked immunosorbent assay (ELISA), the immunochromatographic test (ICT), Western blot, and indirect immunofluorescence assay (Camino et al., 2021). Each technique has advantage or a disadvantage depending on specificity, and sensitivity (Onyiche et al., 2019).

Detection of EP agents using olymerase Chain Reaction (PCR) shows high sensitivity (Motloang et al., 2008). PCR is the diagnosis of choice for equids in chronic EP infection by both *B. caballi* and *T. equi* (Bhoora et al., 2010; Onyiche et al., 2020). Variationsin PCR, including conventional PCR, Nested-PCR, real-time PCR, and

reverse line blot hybridization, has been employed in various epidemiological investigations of EP (Ros-García et al., 2013).

2.2 Hemotropic mycoplasmas

2.2.1 History

Hemotropic mycoplasmas, also known as hemoplasmas, are small, pleomorphic bacteria that parasitize red blood cells (RBCs) of a wide range of mammals (Messick, 2004). Blood parasites in mice (*Epreythrozoon coccoides*) and dogs (*Haemobartonella canis*) were first observed in Germany in 1928, (Schilling 1928), Adler & Ellenbogen (1934), reported finding similar parasites in anemic cattle in Palestine. Also, in the early 1930s, *Eperythrozoon* spp. infection in pigs, characterized by icterus and anemia, was first recognized in the United States (Kreier & Ristic 1984; Mazaheri et al., 2014).

Hemotropic mycoplasmas were previously classified into two genera (*Haemobartonella* and *Eperythrozoon*) in the Anaplasmataceae family. They were reclassified into the genus *Mycoplasma* based on phenotypic and genotypic information (Neimark et al., 2001; Rikihisa et al., 1997). The first *haemobartonella*-like infection in horses based on microscopic findings was reported in Nigeria (Gretillat, 1978). Later, hemoplasmas, closely related to '*Candidatus* Mycoplasma haematobovis' (formerly '*Candidatus* Mycoplasma haemobos'), were detected infecting two horses from Germany (Dieckmann et al., 2010). A recent study of hemotropic Mycoplasma ovis-like species in horses was reported in Iran (Kalantari et al., 2019).

Hemoplasmas have been described infecting different mammalian hosts such as cattle (Hoelzle et al., 2011), sheep (Adejinmi et al., 2004), cats (Messick, 2004), non-human primates (Peters et al., 1974; Peters et al., 1973), wild animals (Vieira et al., 2015a; Vieira et al., 2015b), and human beings (Maggi et al., 2013). In Somalia, there are no studies regarding the occurrence of hemoplasmas in donkeys.

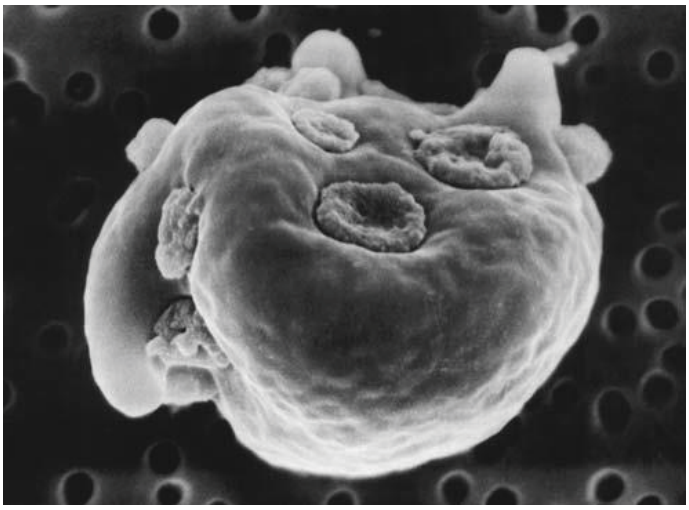

Figure 1 Scanning electron micrograph of several hemoplasma parasites (*Mycoplasma haemosuis*) within shallow depressions on the surface of an erythrocyte. 1 cm = 1 lm. (Messick, 2004).

2.2.2 Transmission

Hemoplasmas are not freely available in nature but are able to survive as parasites in their host (Dawood et al., 2022). The main forms of transmission blood-sucking arthropods like fleas and ticks have also been suggested as the major vectors (Hornok et al., 2011). The brow dog tick, *Rhipicephalus sangunieus* may play a role in the transmission of hemoplasmas canine (Seneviratna et al., 1973), also study from Brazil detect hemoplasma in ticks (Vieira et al., 2021).

Transmission of hemoplasmas can also occur via infected blood, as through blood transfusion, or the use of contaminated needles, and aggressive interactions (Barker & Tasker 2013). Vertical transmission from mother to the offspring has been reported in cats (Barker & Tasker 2013) and cattle (Girotto-Soares et al., 2016). Horizontal transmission, possibly associated with fighting is suspected in cats (Barker & Tasker 2013). Mites may play a role in the mechanical transmission of hemoplasmas, as reported (Willy et al., 2010). The role of ticks and fleas with other arthropod vectors in the transmission of hemoplasmas is still unknown. Moreover, the hemoplasma cross-species transmission between humans, dogs and horses was not observed (Vieira et

al., 2015). Thus, considering that there is no consensus on transmission routes of hemoplasma and their potential arthropod vectors, further investigation is needed.

2.2.3 Pathogenicity and clinical signs

Clinical signs are not specific but generally include anemia, pallor mucosa, lethargy, anorexia, weight loss, and depression. Constant fever, especially in the acute stage of the disease, can often be seen (Messick, 2004). Splenomegaly and lymphadenopathy may occur due to extramedullary hematopoiesis (Mazaheri et al., 2014). Sometimes, jaundice caused by hemolysis is seen (Hoelzle et al., 2011). Globally, few reports have been published on the presence of the bacteria in horses' blood. Infected horses show clinical signs such as decreased stamina, weight loss, anemia, fever, lymphadenitis, and impaired blood flow (Dieckmann et al., 2012). To date, no studies on hemoplasmas have been performed in donkeys.

The anemia is reversible, combined with reticulocytosis, anisocytosis, macrocytosis, and polychromasia. Hematocrit may decrease to below 20%, depending on the severity of the infection (Foley et al., 1998; VanSteenhouse et al., 1993). *Mycoplasma haemofelis* is a common cause of normoblast presence in the blood (Hammer and Wellman, 1999). However, the anemia may be irreversible sometimes (de Gopegui et al., 1995). Infections with hemoplasmas can induce acute haemolysis, associated with anorexia, lethargy, dehydration, weight loss and sudden death of infected animals (Willi et al., 2010).

2.2.4 Diagnosis of hemoplasmas

Detection of hemoplasmas attached to erythrocytes through light microscopy of blood smears stained with Romanowsky-type stains have been historically used as diagnostic method (Messick, 2004). Microscopic observation demonstrates bacteria in single, pairs or chains on the surface of erythrocytes but may also be seen free in the plasma (Bobade and Nash 1987; Messick, 2004).

If highly concentrated EDTA is used, bacteria may be detached from the erythrocytes. Therefore, it is better to prepare smears immediately after blood sampling or use other anticoagulants such as EDTA (Alleman et al., 1999). PCR is a highly sensitive diagnostic method, amplifying certain fragments of the DNA to identify the microorganisms (Hoelzle et al., 2007; Messick, 2004).

Studies have shown that diagnostic rates of cytopathology and PCR are nearly 37.5 and 100%, respectively. *M. haemofelis* can be detected by PCR after eight days of infection until no antibiotics are used. PCR shows reliable results after the completion of three to 35 days of antibiotic therapy (Berent et al., 1998; Foley et al., 1998). Usually, PCR may remain positive for a long time in asymptomatic animals. Positive results do not always reflect the occurrence of clinical signs but can show previous infections (Tasker and Lappin, 2002).

2.2.5 Donkeys' importance to Somali community

Donkey population has declined in most developed countries in America and Europe. In Africa, donkeys are very important in the rural areas and for transport in the urban areas (Starkey, 1995). The donkey population in the African continent has increased from 15.6 million donkeys in 2004 to 19.3 million donkeys in 2013, and only in Sub-Saharan Africa, the population increased from 8,9 million in 1997 to 20 million donkeys in 2018 (Norris et al., 2021; FAOSTAT, 2013).

Donkeys are mainly owned by small-scale farmers and are used to carry goods on their backs or pull carts loaded with goods such as firewood, animal feed, grains, water, and building material. Compared to motor vehicles, animals are slower and do not have the same carrying capacity, but the animals have other advantages (Wold et al., 2004). Farmers that can afford cart or pack animals get higher prices for their crops when transporting it by themselves to markets because they avoid paying margins to

traders (Wold et al., 2004). Donkeys are also used in agricultural operations as ploughing (Pearson et al., 2001).

However, transboundary infectious diseases, zoonotic and vector-borne diseases are also among the main concern for Somalia´s economic and public health. Therefore, there is a need to generate information on equine piroplasmosis and hemotropic *Mycoplasma* spp. Infection in donkeys from Somalia.

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3 RATIONALE AND OBJECTIVES

3.1 RATIONALE

There are no studies regarding the occurrence of EP agents and hemoplasmas in donkeys in Somalia. The economic relevance of donkeys in Somalia is due to the various activities performed mainly by family farmers used to carry goods on their backs or pull carts loaded with goods like firewood, foods, water, and construction materials.

Considering the social-economic importance and the lack of information regarding the epidemiology of these pathogens in donkeys,

3.2 HYPOTHESIS

Equine piroplasms and hemotropic *Mycoplasma* spp. infect donkeys from Somalia.

3.3 OBJECTIVES

3.3.1 General Objective

- To screen donkeys for EP agents and hemoplasma species and to evaluate factors associated with infection in Somalia.

3.3.2 Specific objectives

- To collect and identify tick species parasitizing donkeys;
- To screen donkey blood samples for *B. caballi* and *T. equi*, and hemoplasmas using molecular assays;
- To molecular characterize EP agents and hemoplasmas detected in donkeys and compare with those deposited in the Genbank® database;
- To evaluate factors associated with infections by EP and hemoplasmas in donkeys from Somalia.

4 MANUSCRIPT: MOLECULAR SCREENING OF EQUINE PIROPLASMA AND HEMOPLAMAS IN DONKEYS FROM SOMALIA

4.1 ABSTRACT

Equine piroplasmosis (EP) is an infectious tick-borne disease of equids (horse, donkey, mule, and zebra), caused by *Babesia caballi* and *Theileria equi*. EP is a notifiable disease that has a wide distribution in most tropical and subtropical countries around the world. Hemotropic mycoplasmas (hemoplasmas) are the causative agents of hemolytic infectious anemia in mammals worldwide. However, few reports on horses have been performed to date. Epidemiological data and molecular characterization of EP and hemoplasma infection in Somalia are missing since donkeys are used for various activities, including carrying goods on their backs or pulling carts loaded with goods like firewood, foods, water, and construction materials. Considering the social-economic importance and the lack of data on these pathogens in donkeys in Somalia, this study aimed to screen donkeys for EP and hemoplasma infection. A total of 30 males donkeys were evaluated, blood sampled, and inspected for ectoparasites. DNA was extracted from whole blood, and samples further screened using by species-specific real-time PCR assays targeting *ema-1* gene of *T. equi* and the 18S rRNA gene of *B. caballi*, and genus-specific qPCR assay targeting the 16S rRNA gene of hemoplasmas. Donkeys were not infested by ticks at the time of sampling. The mammal endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was consistently amplified from all donkeys' samples. Overall, prevalence of 22/30 (73.3%, 95% CI: 55.6 – 86.8%) were qPCR-positive for *T. equi* and *B. caballi*. While a single infection for *T.equi* was observed in 3/30 (10%, 95% CI: 2.7 – 24.9%) of donkeys. All 30 donkey samples tested negative for hemoplasma by qPCR. To the best of our knowledge, this is the first study on the molecular investigation of EP and hemoplasmas in donkeys from Somalia.

Keywords: *Theileria equi*, *Babesia caballi*, Hemotropic *Mycoplasma* spp., domestic ass, hemotropic mycoplasmas, Sub-Saharan Africa.

4.2 INTRODUCTION

Livestock in Somalia are the main source of individual and national wealth (FAO, 2004). Moreover, the sector contributes about 40 percent of the Gross Domestic Products (GDP) of the country and more than 50% of export earnings (CIA, 2020). More than half of the population living in rural areas depends directly or indirectly on livestock production, and the sector is the largest contributor to Somalia's GDP, livelihoods, and economic growth. Yet its performance is undermined by a number of factors, including poor animal nutrition, cross-boundary diseases, eroded genetic resources and a lack of natural resource management and institutional weaknesses (FOA, 2019).

Equine piroplasmosis (EP) is an infectious tick-borne disease of equids (horse, donkey, mule, and zebra), caused by *Babesia caballi* and *Theileria equi* (Wise et al., 2019; Qablan et al., 2013). EP is a notifiable disease that has a wide distribution in most tropical and subtropical countries around the world (OIE 2019; Onyiche et al., 2019; Friedhoff et al., 1990). The tick genera involved in the transmission of EP include *Amblyomma, Hyalomma, Rhipicephalus, Haemaphysalis, Dermacentor,* and *Ixodes* (Tirosh-Levy et al., 2020; Oguntomole et al., 2018; Scoles and Ueti 2015). Four of these genera have been reported in Somalia (Hassan et al., 2013; Kaiser & Hoogstraal, 1968; Pegram, 1976; Iori et. al., 1996; Walker et. al., 2003, Isse et. al., 2017). In donkeys, EP leads to major economic importance since the affected animals demonstrate a loss of appetite and a significant reduction in strength, draughts power and these diseases may threaten the animal’s survival (Ahmadi et al., 2020).

Hemotropic mycoplasmas (hemoplasmas) are obligate erythrocyte bacteria that infect a wide variety of vertebrates worldwide (Willi et al., 2006; Messick, 2004; Millán et

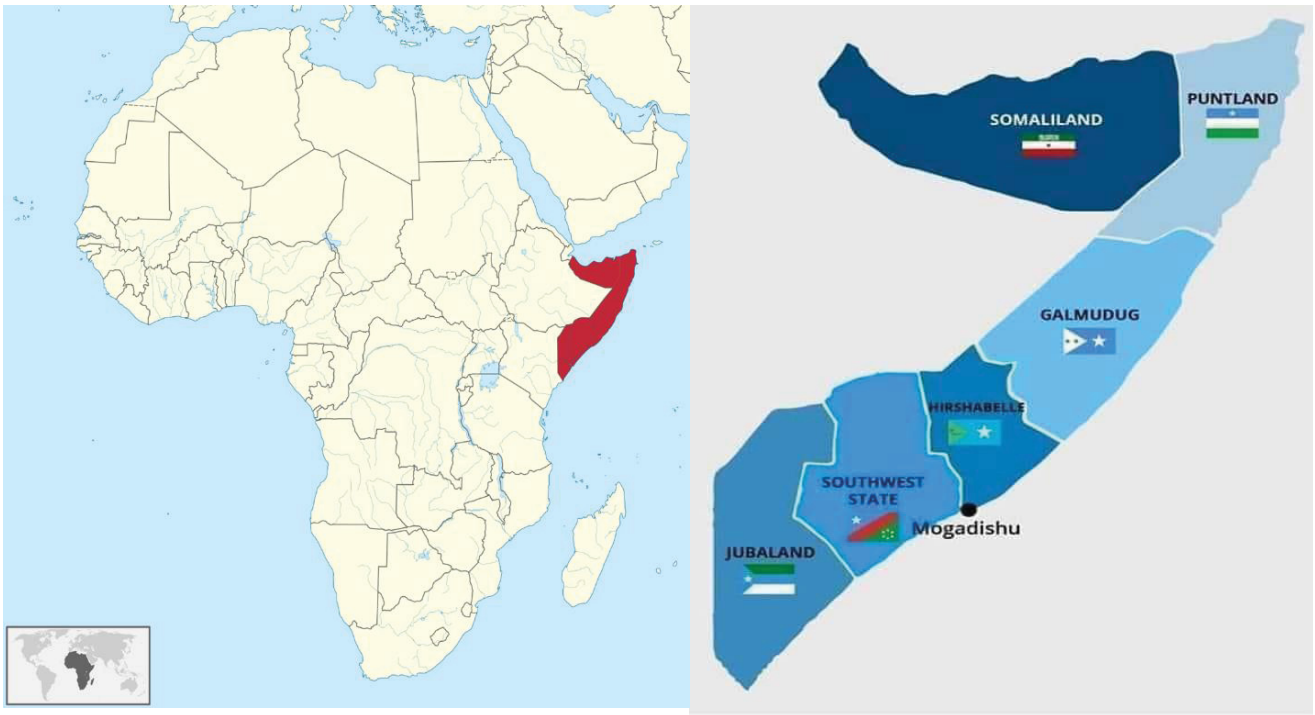
al., 2020). Hemoplasmas are the causative agents of acute or chronic infectious anemias in mammalian species, including humans (Messick, 2004).

To date, there are no studies regarding the occurrence of EP and hemoplasmas in donkeys in Somalia. The economic relevance of donkeys in Somalia is due to the various activities performed mainly by family farmers used to carry goods on their backs or pull carts loaded with goods like firewood, foods, water, and construction materials. Considering the social-economic importance and the lack of information regarding the epidemiology of these pathogens in donkeys this study aimed to screen a population of this animal species for EP and hemotropic *Mycoplasma* spp.

4.3 MATERIALS AND METHODS

4.3.1 Study area

Somalia is a country located in the Horn of Africa which the capital city is Mogadishu. Officially the country consists of six federal member states, namely Galmudug, Hirshabelle, Jubaland, South west, Puntland, Somaliland and the municipality of Benadir. It is bordered by Ethiopia to the west, Djibouti to the northwest, the Gulf of Aden to the north, the Somali Sea and Guardafui Channel to the east, and Kenya to the southwest. **Figure 2:** Somalia Maps with a Federal member states



With a land area of 637,657 square kilometers. It has the longest coastline of mainland in Africa length 3,333 kilometers. The country lies between Latitude 5.1521° N, Longitude 46.1996° E. The census population is 15.01 million (World Bank 2018).

Benadir region is one of the eighteen regions of Federal Republic of Somalia (World Bank 2018). It is bordered by northwest to the Middle Shabelle and Lower Shabelle and southeast by Indian Ocean. The region lies between latitude 2.1187° N, and longitude 45.3369° E. It has the population estimated to be about 2.1million people (Abdirisak et al., 2019). These region is located the largest market in the nation and is where most donkeys are found.

Figure 3: Benadir region Somalia Map showing study area in red ring

4.3.2 Sampling

Blood samples (up to 5 mL) from the 30 donkeys' males were collected aseptically from the jugular vein using sterile syringe into tubes containing EDTA (BD Vacutainer, Franklin Lakes, NJ, EUA). All collected blood samples were transported to the Abrar Research and Training Centre laboratory in Abrar University Mogadishu, Somalia (ARTC) for PCR analysis and kept at −20 °C until transported to the Vector borne laboratory in UFPR in Curitiba Brazil.

The whole body surfaces of the donkey's particular predilection sites of ticks were carefully examined. A non-probabilistic convenience sampling was performed.

Animals were selected purposively based on the accessibility and owner's willingness to cooperate for this study.

4.3.3 DNA extraction

DNA was extracted from 200 µL of whole blood using a commercial kit (MagMax™ Core Nucleic Acid Purification Kit, Applied Biosystems, MA, US), according to the manufacturer's instructions. Negative control purifications using nuclease-free water were performed in parallel to monitor cross-contamination in each batch of 30 samples.

4.3.4 Real-time PCR assays (qPCR)

All donkeys' DNA samples were tested for the presence of the mammal endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) to monitor DNA extraction. Samples were initially screened by species-specific real-time PCR assays targeting the *ema-1* gene of *T. equi* and the 18S rRNA gene of *B. caballi* (Lobanov et al., 2018). Thereafter, samples were screened using genus-specific qPCR assay targeting the 16S rRNA gene of hemoplasmas (Willi et al., 2009). Horse DNA known to be infected by *B. caballi* or *T. equi* were used as positive controls (Valente et al., 2019). For hemoplasmas, gBlock™ (Integrated DNA Technologies, Coralville, IA, USA) containing *Mycoplasma haemofelis* sequence was used as positive control. Nuclease-free water was used as negative control in all qPCR reactions.

4.4 RESULTS and DISCUSSION

Donkeys were not infested by ticks at the time of sampling. The mammal endogenous gene *gapdh* was consistently amplified from all donkeys' samples Overall, prevalence of 22/30 (73.3%, 95% CI: 55.6 – 86.8%) were qPCR-positive for *T. equi* and *B. caballi*. While a single infection for *T.equi* was observed in 3/30 (10%, 95% CI: 2.7 –

24.9%) of donkeys. However, all donkey samples tested negative for hemoplasmas by qPCR.

Previous studies have found that anemia, weight loss, anorexia, and tick infestation were associated to hemoplasma infection in equids (Mazaher et al., 2014; Dieckmann et al., 2010; Vieira et al., 2015). Hemoplasma infection was reported in horses in Nigeria (Happi and Oluniyi, 2020), Germany (Dieckmann et al, 2010), and Iran (Mazaheri et al., 2014).

This study provided a comprehensive overview of EP and hemoplasmas in Somalia. The overall prevalence of *B. caballi* and *T. equi* were 73.3% for qPCR assay. While a single infection 10% of *T. equi* in donkeys in this study was high 83.3%. Prevalence found in this study was higher than previous studies performed in equine from Africa which have shown prevalence rates ranging from 1.6% to 50% of piroplasma infection, and lower then prevalence rates 86.4% of EP infection by microscopical and molecular characterization (Onyiche et al., 2020; Gizachew et al., 2013; Hawkins et al., 2015; Oduori et al., 2015; Zobba et al., 2008). This difference may be due to the various in agroecological system of the area, donkeys' management and endemicity of the tick.

In conclusion, this is the first study on EP and hemoplasma infections in donkeys from Somalia. Our data shows a high prevalence of EP, than hemoplasma infection in donkeys in the studied region. Further studies are needed to evaluate the epidemiology, clinical and economic impact of equine piroplasmosis on the donkeys in Somalia.

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