UNIVERSIDADE FEDERAL DO PARANÁ

LARISSA DANTAS ROEDER FERRARI

MOLECULAR DETECTION OF HEMOPLASMAS IN CATTLE AND DROMEDARY CAMELS FROM THE FEDERAL REPUBLIC OF SOMALIA

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2022

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Tese apresentada ao Programa de Pós-Graduação em Ciências Veterinárias, Setor de Ciências Agrárias, Universidade Federal do Paraná, como requisito à obtenção do título de Doutor em Ciências Veterinárias.

Orientador: Prof. Dr. Rafael F. C. Vieira
Coorientadora: Dra. Thállitha S.W.J. Vieira

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DEDICATION

Dedico esta tese aos meus pais, **M**artin Roeder (*in memoriam*) e **M**aria da Penhas Dantas Roeder, assim como dedico ao meu marido **M**arcos Vinícius Ferrari.

Dos pais herdei a estabilidade necessária para alçar os mais altos voos; do marido ganhei as mais fortes asas.

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Aqoonla`aani waa iftiinla'aan (Somali proverb).

"The absence of knowledge is the absence of light".

RESUMO

Micoplasmas hemotrópicos (hemoplasmas) são bactérias pleomórficas gram-negativas que parasitam a superfície dos eritrócitos e podem causar anemia leve a severa em grande parte dos vertebrados, incluindo ruminantes. Hemoplasmas foram consistentemente descritos em bovinos e em diferentes espécies da Família Camelidae, mas dados sobre infecção em dromedários estão limitados a alguns relatos de caso. A população de bovinos na Somália é de aproximadamente 3.9 milhões de animais, e o rebanho de dromedários é o maior do mundo, com mais de 7 milhões de animais. Dados sobre a ocorrência de hemoplasmas na África Subsaariana são escassos, principalmente na Somália, onde nenhum estudo foi realizado até o momento. Desta maneira, este estudo objetivou verificar a ocorrência molecular de hemoplasmas em bovinos e dromedários da Somália. Um total de 74/131 (56.48%; 95% CI: 47.93-64.67%) bovinos foram positivos para *Mycoplasma* spp. na PCR em tempo real (qPCR) baseada no gene 16S rRNA. As amostras positivas para hemoplasma foram então submetidas a análises de PCR espécie-específica para *Mycoplasma wenyonii* e '*Candidatus* Mycoplasma haematobovis' baseadas no gene 16S rRNA. Um total de 34/74 (45.94%; 95% CI: 35.07-57.22%) bovinos estavam coinfectados com as duas espécies; 31/74 (41.89%; 95% CI: 31.32-53.26%) e 3/74 (4.05%; 95% CI: 01.39-11.25%) bovinos estavam somente infectados com *M. wenyonii* e '*Ca*. M. haematobovis', respectivamente. Seis de 74 (8,1%; 95% CI: 03.77-16.58%) amostras de bovinos foram negativas na PCR convencional espécie-específica, mas foram positivas na PCR semi-nested baseada no gene 16S rRNA de hemoplasmas. O sequenciamento do gene 16S rRNA de *Mycoplasma* sp. hemotrópico das amostras selecionadas confirmaram que os animais estavam infectados com *M. wenyonii* e '*Ca*. M. haematobovis'. As amostras de dromedários foram triadas por PCR em tempo real (qPCR) para hemoplasma e por PCR convencional focando o gene 16S rRNA deste grupo de bactéria. Todos as amostras de dromedários foram negativas em ambas as análises de PCR. Este é o primeiro estudo de detecção molecular de hemoplasmas em bovinos e triagem de hemoplasmas em dromedários na Somália.

Palavras-chave: Micoplasmas hemotrópicos, *Mycoplasma wenyonii*, '*Candidatus* Mycoplasma haematobovis', Bovinos, *Camelus dromedarius*, África Subasaariana

ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are small pleomorphic Gram-negative bacteria that parasitize the surface of red blood cells and may cause mild to severe anemia in a wide range of vertebrates, including ruminants. Hemoplasmas have been described in cattle and in different species from the Camelidae Family, but data on dromedary camels (*Camelus dromedarius*) are limited to few reports. Cattle population in Somalia is around 3.9 million heads, and the dromedary heard is the biggest in the world, with more than 7 million animals. Information on hemoplasmas in Sub-Saharan Africa are scarce, mainly in Somalia, where no studies have been performed to date. Accordingly, this study aimed to assess the molecular occurrence of hemoplasmas in cattle and dromedaries from Somalia. A total of 74/131 (56.48%; 95% CI: 47.93-64.67%) cattle were positive for hemotropic *Mycoplasma* spp. by real-time PCR (qPCR) based on the 16S rRNA gene. Hemoplasma-positive samples were later subjected to species-specific PCR assays for *Mycoplasma wenyonii* and '*Candidatus* Mycoplasma haematobovis' based on the 16S rRNA gene. A total of 34/74 (45.94%; 95% CI: 35.07-57.22%) animals tested positive for both species; 31/74 (41.89%; 95% CI: 31.32-53.26%) and three/74 (4.05%; 95% CI: 01.39-11.25%) cattle were solely infected by *M. wenyonii* and '*Ca*. M. haematobovis', respectively. Six out of 74 (8,1%; 95% CI: 03.77-16.58%) cattle were negative on species-specific conventional PCR assays but tested positive by a semi-nested PCR assay based on the 16S rRNA gene of hemoplasmas. Sequencing of the detected hemotropic *Mycoplasma* sp. 16S rRNA gene confirmed that animals were infected by *M. wenyonii* and '*Ca*. M. haematobovis'. Dromedary samples were screened using hemoplasma genus-specific real-time (qPCR) and conventional PCR assays targeting the 16S rRNA gene of this group of bacteria. All dromedaries tested negative for hemoplasma on both PCR assays. To the best of our knowledge, this is the first study on the molecular detection of hemoplasmas in cattle and hemoplasma screening in dromedaries from Somalia.

Keywords: Hemotropic mycoplasmas, *Mycoplasma wenyonii*, '*Candidatus* Mycoplasma haematobovis', Bovine, *Camelus dromedarius*, Sub-Saharan Africa

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LIST OF ABREVIATIONS

BLASTn	Nucleotide Basic Local Alignment Search Tool
CA	California
CI	Confidence Interval
cm	Centimeter
cPCR	Conventional Polymerase Chain Reaction
Ct	Cycle Threshold
DBS	Dried Blood Spot
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
Et al.	*et alia*
FTA	Flinders Technology Associates
GAPDH	Glyceraldehyde 3-Phosphate Dehydrogenase
GE	General Electric
HM	Hemoplasma
Km	Kilometer
LM	Light Microscopy
MAPA	Ministério da Agricultura, Pecuária e Abastecimento
mm	Millimeter
PBS	Phosphate-Buffered Saline
PCR	Polymerase Chain Reaction
PCV	Packed-Cell Volume
qPCR	Quantitative Polymerase Chain Reaction
RBC	Red Blood Cell
rRNA	Ribosomal Ribonucleic Acid
ssu rRNA	Small Subunit Ribosomal Ribonucleic Acid
SYBR	Synergy Brands, Inc.
UFPR	Universidade Federal do Paraná
UN	United Nations
UK	United Kingdom
µl	Microliter

µm	Micrometer

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1. INTRODUCTION

Hemotropic mycoplasmas (hemoplasmas) are small pleomorphic non-cultivable Gram-negative bacteria that parasitize red blood cells (RBCs) of a wide range of mammals. Hemoplasmas appear spherical, ring-shaped, or rod-shaped on blood smears and attached to the surface of the RBCs, causing cell deformity and damage with subsequent anemia (Messick, 2004). Originally known as *Haemobartonella* and *Eperythrozoon*, two genera of the order Rickettsiales, these species have been reclassified within the genus *Mycoplasma* (Neimark, 2001).

Hemoplasmas have been described infecting domestic dogs (*Canis lupus familiaris*) (Vieira et al., 2015), cats (Marcondes et al., 2018), humans (dos Santos et al., 2008; Alcorn et al., 2021; Hattori et al., 2020), wild animals (André et al., 2011; Gonçalves et al., 2015; Massini et al., 2019; Vieira et al., 2009; Willi et al., 2007), cattle (*Bos taurus*) (Adler e Ellenbogen, 1934; Tagawa et al., 2008), buffaloes (*Bubalus bubalis and Syncerus caffer*) (Su et al., 2010; Santos et al., 2018; Gonçalves et al., 2018), small ruminants (Johnson et al., 2016; Machado et al., 2017; Mongruel et al., 2020) and animals from the Camelidae Family, such as llamas (Mclaughlin et al., 1990; Tornquist et al., 2010) and alpacas (Tornquist et al., 2010; Guimarães et al., 2012).

Historically, hemoplasma diagnosis has been made on the identification of organisms on stained blood smears. Although, this technique has low sensitivity mainly because bacteria almost disappear from peripherical blood during chronic infection (Messick, 2004). Consequently, molecular methods such as conventional polymerase chain reaction (PCR) and Real-Time PCR (qPCR) are considered the gold standard method for the diagnosis of hemoplasma infections due to their high sensitivity and sensibility (Hoelzle, 2007).

Two hemoplasma species have been reported infecting cattle: *Mycoplasma wenyonii* and '*Candidatus* Mycoplasma haematobovis'. In chronically infected animals, clinical signs include fever, decreased milk productivity, lymphadenopathy, anorexia, and depression (Messick, 2004; Tagawa et al., 2013; Smith et al., 1990). The main clinical signs during chronic hemoplasmosis are lower milk yield and lower calf birth weight (Tagawa et al., 2013). Less commonly, it can be cause lameness and infertility (Hoelzle, 2011). Abortion and delayed estrus have also been reported during acute infection (Quilan, 1985; Sutton et al., 1977; Smith et al., 1990). Latent hemoplasma infections are frequently reported, producing low-grade parasitemia (Hoelzle, 2011).

Hemoplasmas have been reported infecting different species from the Camelidae Family, however, data on hemotropic *Mycoplasma* sp. infection on dromedaries are limited to a few reports in animals from Iran (Nazifi et al., 2009; Sharifiyazdi et al., 2018; Esmaeilnejad et al., 2019).

The Federal Republic of Somalia is an agricultural country located in the easternmost part of Africa, on the Horn of Africa, and has approximately 3.9 million cattle and 7.1 million dromedaries, being considered the country with one of the biggest dromedary heard in the world. Livestock remains the main activity of the country's economy, which mainly exports meat, meat preparations, and live animals, which are the most exported to Middle East countries, including Saudi Arabia, Yemen, Oman, Qatar, and United Arab Emirates (FAO, 2018).

One of the major risks to the exporting market and public health is transboundary zoonotic diseases due to the constant mobility of a high number of animals, which facilitates the dissemination of many pathogens. Also, animals from different groups circulating in common areas, such as watering points, markets, grazing spaces, and along trading routes are very common in Somalia and maintain a high disease transmission index (FAO, 2018). Besides transboundary infectious diseases, zoonotic and vector-borne diseases are also the main concern for Somalia´s economic and public health. Thus, there is a need to generate information about the epidemiological situation and molecular characterization of hemotropic *Mycoplasma* spp. in cattle and dromedaries from Somalia.

2. LITERATURE REVIEW

2.1 HEMOPLASMAS

Hemoplasmas, formerly known as *Haemobartonella* and *Eperythrozoon* spp., order Rickettsiales, were reclassified as species of the genus *Mycoplasma*. They are small pleomorphic bacteria that lack a cell wall and parasitize the surface of red blood cells (RBCs) of a wide range of mammals. These bacteria attach (Fig. 1) but do not penetrate into the RBC structure (Messick, 2004) and may be visualized as spherical, ring-shaped, or rod-shaped on stained blood smears (Neimark, 2001), with size ranging from 0.3 to 3 μm in diameter (Messick, 2004).

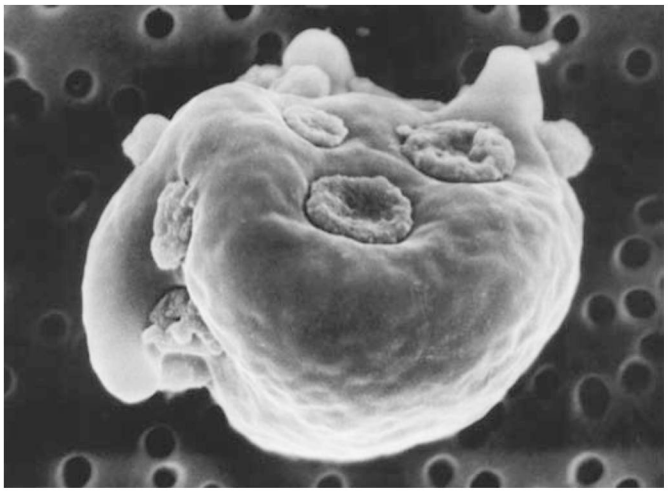

Figure 1. Scanning electron micrograph of several hemoplasma parasites (*Mycoplasma suis*) within shallow depressions on the surface of a red blood cell (RBC). 1 cm = 1 lm. (MESSICK, 2004)

Hemoplasmas have not been cultivated *in vitro* and only remain viable through transmission between hosts (Neimark, 2001; Messick, 2004). Phylogenetically, the hemotropic mycoplasmas cluster can be sub-divided into two well-supported groups: *haemosuis* and *haemofelis* (Volokhov et al., 2012), based on nucleotide sequence data for the 16S rRNA gene among species of the family *Mycoplasmataceae*.

Many of the hemoplasma species still have the *Candidatus* taxonomic status (Oren et al., 2020; Volokhov et al., 2012), including '*Ca*. M. haematobovis'. This status should be used to describe prokaryotic organisms for which the nucleic acid sequence is available, but which still lack characteristics required for the description of a new species according to the

International Code of Nomenclature of Prokaryotes (Murrary and Stackebrandt, 1995). When an organism with *Candidatus* status is later isolated and the pure culture sufficiently described, it must be classified and named according to the rules of the Code, and the former *Candidatus* organism's name is deleted from the *Candidatus* list (Oren et al., 2020).

Hemoplasmas are known for infecting several mammal species. They have been detected in domestic animals, such as *M. haemofelis* in cats (Jensen et al., 2001) and *M. haemocanis* (Kikuth, 1928) and *M. haematoparvum* (Sykes et al., 2004) in dogs and wild canivores (André et al, 2011). Hemoplasmas have also been detected in bats (Ikeda et al., 2017; Collere et al., 2022), non-human primates (Cubilla et al., 2017), rodents (Vieira et al., 2021), opossums (Massini et al., 2019), ring-tailed coatis (Collere et al., 2021) and procyonids (de Sousa et al., 2017).

Six species have been described to infect ruminants: *M. wenyonii*, '*Ca*. M. haematobovis', *Mycoplasma ovis*, '*Candidatus* Mycoplasma haemolamae', '*Candidatus* Mycoplasma erythrocervae' and '*Candidatus* Mycoplasma haemocervae'. In cattle, two species were described: *M. wenyonii* and '*Ca*. M. haematobovis' (Tagawa et al., 2008). '*Ca*. M. haemolamae' can be found in species from the Camelidae Family, such as llamas (*Lama glama*) and alpacas (*Vicugna pacos*) (Tornquist et al., 2010) from Chile and Peru, and there are few reports in one-humped camels (*Camelus dromedarius*) from Iran (Nazifi et al., 2009; Sharifiyazdi et al., 2018; Esmaeilnejad et al., 2019). Sheep and goats are commonly affected by *M. ovis* (Machado et al., 2017; Mongruel et al., 2020), and buffaloes can be infected by the same hemoplasmas as cattle (Santos et al., 2018; Gonçalves et al., 2018). '*Ca*. M. erythrocervae' and '*Ca*. M. haemocervae' were first described infecting sika deer (*Cervus nippon*) (Watanabe et al., 2010).

Mycoplasma wenyonii was first described in 1934 by Adler e Ellenbogen, and since then many studies have been conducted throughout the world. The first description of its whole genome sequence was made by dos Santos et al. (2012).

'*Ca*. M. haematobovis' was first described in Japan (Tagawa et al., 2008) and since then has been detected in cattle and buffalo in China (Su et al., 2010), Malaysia (Agina et al., 2021), Brazil (Girotto et al., 2012; de Mello et al., 2019), Philippines (Galon et al., 2020), Germany (Hoelzle et al., 2011), Mexico (Martínez-Ocampo et al., 2016), England (Ayling et al., 2012), Hungary (Hornok et al., 2011), New Zealand (Mcfadden et al., 2016), Cuba (Díaz-Sánchez et al., 2019), Nigeria (Happi et al., 2020), Wales (Deeney et al., 2021), Uganda (Byamukama et al., 2020), Mozambique (Gonçalves et al., 2016), and Switzerland (Meli et al., 2010).

2.2 PATHOGENICITY AND CLINICAL SIGNS IN CATTLE AND DROMEDARIES

Although many cases of hemoplasma infections are asymptomatic or cause latent infection (Tagawa et al., 2013), there are clinical signs associated with the bacteria. In cattle, when latent, typically infections with this organism produce low-grade bacteremia and mild anemia (Hoelzle et al., 2011), but occasionally infected animals can present acute signs such as swollen limbs, scrotal edema, fever, poor coat condition, decreased milk production, weight loss, infertility and death (Neimark et al., 2001). The most common clinical signs caused by *M. wenyonii* chronic infection in cattle are anemia, swelling of the teats and the distal portions of the hind limbs, transient fever, lymphadenopathy, weight loss, rough haircoat, and decreased milk production (Smith et al., 1990; Messick, 2004). Although both species can cause clinical signs in bovids, it was noted that cattle infected with '*Ca*. M. haematobovis' develop more severe clinical signs compared to animals infected with *M. wenyonii* (Tagawa et al., 2013).

In Brazil, a previous study did not find association between the packed cell volume (PCV) of clinically healthy cows with and without hemoplasma infection (Girotto et al. 2012). Also, although a high prevalence (61%) of '*Ca*. M. haematobovis' has been found in that study, infected cattle did not appear to be clinically affected (Girotto et al., 2012). Similar data were found by Meli et al. (2010), where the majority of infected animals were also healthy.

Also, a previous study in New Zealand has found that bovine hemoplasmas may be associated with anemia but finding these agents in blood could also be an incidental finding (Mcfadden et al., 2016). It was not possible, in that study, to demonstrate an association between the presence of hemoplasmas and anemia. In Nigeria, the relation between anemia and infected cattle was not statistically significant, even when animals were co-infected with *Trypanosoma* spp. and hemotropic *Mycoplasma* spp., or co-infection with *Trypanosoma* spp., microfilaria and hemotropic *Mycoplasma* spp. (Happi et al., 2020). Anemia, decreased milk production, infertility, and lameness were the clinical signs that animals presented during an outbreak of infection with hemoplasmas in cattle on three farms in Northern Germany (Hoelzle et al., 2011). In France, a previous study has found a high rate (25,6%) of *M. wenyonii* infection in animals selected on basis of clinical suspicion compared to those randomly selected (4,9%) in the same area. The significant clinical signs and hematological

findings were anemia without hemoglobinuria, edema of the limbs and/or the udder, and transient milk drop (Nouvel et al., 2019).

Data on hemoplasma infection in dromedaries and possible associated clinical signs are still lacking. There is one case-report describing co-infection by '*Ca*. M. haemolamae' and *Trypanosoma evansi* in a one-humped camel (*Camelus dromedarius*) from Iran (Esmaeilnejad et al., 2019). The infected animal was presenting anorexia, weakness, depression, and anemia. Another study found hemotropic *Mycoplasma* spp. in dromedaries in central Iran (Nazifi et al., 2009) by the direct observation of stained blood smears. In southern Iran, two/100 (2.0%) dromedaries were positive for hemoplasmas by molecular diagnosis (Sharifiyazdi et al., 2018). Hematological values of both infected animals were normal, except for PCV (21.1 and 24.45%) and red blood cell count (5.27 and 5.21 10^9/L). In wildlife, hemoplasma's pathogenicity is poorly known, but appears to be subclinical (Millán et al., 2021).

Regarding age susceptibility, a study in Japan (Tagawa et al., 2012) has found that cattle from one to three years old seems to be more susceptible to hemoplasma infection than other age groups. On the other hand, a recent study has found that cows one to four years old had the lowest *M. wenyonii* infection rate and the highest co-infection rate (*M. wenyonii* and '*Ca*. M. haematobovis') (Tatsukawa et al., 2020).

2.3 TRANSMISSION

The knowledge concerning the epidemiology, transmission routes, and other aspects of hemoplasmas (and their associated diseases) is still lacking. Transmission routes may include arthropod vectors such as fleas, hard ticks, and mosquitoes (Hornok et al., 2011; Gonçalves et al., 2015; Vieira et al., 2021).

Hornok et al. (2011) made the first report of transplacental infection of *M. wenyonii* and '*Ca*. M. haematobovis' in cattle in northern Hungary. It was suggested that the neonatal PCR-positivity of calves to bovine hemoplasmas (10.5%) might have been a consequence of transplacental infection of the fetus, since other post-parturient routes, including colostral, galactogenic, and vector-borne infection were rule out. Additionally, the study has found the presence of '*Ca*. M. haematobovis' and *M. wenyonii* in blood-sucking dipterans (*Haematobia irritans, Stomoxys calcitrans, Tabanus bromius* and *T. bovinus*) and a mechanical transmission has been suggested. A previous study conducted in Germany also supports

the vertical transmission of hemoplasma in cattle (Niethammer et al., 2018). They found two immediately *postpartum* positive calves (2/25) for '*Ca*. M. haematobovis', one was born from a co-infected mother (*M. wenyonii* and '*Ca*. M. haematobovis') while the other calf came from a PCR-negative cow, a fact that could be explained by a low bacterial load during dam sampling. Besides possible proof of vertical transmission of hemoplasma in cattle, authors suggest that is a minor pathway of infection due to the low frequency found.

In a recent study conducted in Japan (Tatsukawa et al., 2020), a very high rate of hemoplasma infection in cattle (366/400, 91.5%) has been found. From this total, 161 animals (40.3%) were infected only with *M. wenyonii*, 38 animals (9.5%) with '*Ca*. M. haematobovis' and 167 animals (41.8%) showed co-infection. It was suggested that not only the ticks but also other arthropod vectors may contribute to hemoplasma transmission since they have found a higher infection rate with '*Ca*. M. haematobovis' in the housed cows compared to the grazing animals.

In fact, transmission by blood-sucking arthropods has been suggested as the most common route for hemoplasma infection (Hofmann-Lehmann et al., 2004). Galon et al. (2020) did not relate tick infestation with hemoplasma infection in cattle and buffaloes. Considering other species, previous studies have suggested that hemoplasma transmission could occur through aggressive interactions between cats (Museux et al., 2009) and wild rodents (Cohen et al., 2018).

Gonçalves et al. (2020) did not find molecular evidence of hemoplasma cross-species transmission among wild and synanthropic rodents, capybaras, and opossums. It was the first molecular evidence of hemoplasmas in ticks and lice from Brazil (Gonçalves et al., 2020). Although *M. haemocanis* has been successfully experimentally transmitted to a dog by *Rhipicephalus sanguineus* sensu lato ticks (Seneviratna, Weerasinghe, & Ariyadasa, 1973), and porcine hemoplasmas DNA has been recently detected in ticks collected from wild boars in southeastern Brazil (SANTANA et al., 2022), the role of ticks and other arthropod vectors in the transmission of hemoplasma is still unknown. Moreover, the hemoplasma cross-species transmission between humans, dogs, and horses was not observed, despite overexposure to agents and vectors (Vieira et al., 2015).

2.4 DIAGNOSIS

Historically, the diagnosis of hemoplasma infection has been made by the direct observation of bacteria on Romanowsky-stained blood smears by light microscopy (LM) and

by molecular methods. PCR is a highly sensitive and specific method considered the gold standard for the diagnosis of hemoplasmas (Hoelzle et al., 2007). Also, direct examination of peripheral blood smear could misidentify the hemoplasmas due to the presence of Howell-Jolly bodies and other confounding artifacts (Nishizawa et al., 2010). Another disadvantage of direct examination is that the species or genotype of the organism cannot be morphologically determined (Happi et al., 2020).

In Nigeria, Happi et al. (2020) made a comparative evaluation of light microscopy and PCR for the diagnosis of hemoplasmas in cattle. It was found that 17% (17/100) were positive for hemotropic *Mycoplasma* spp. by light microscopy and 64% (64/100) by a PCR assay based on 16S rRNA gene of hemoplasmas. The study was the first one to describe '*Ca*. M. haematobovis' in cattle (*Bos taurus*) in Africa (Happi et al., 2020), after Gonçalves et al. (2018) that described '*Ca*. M. haematobovis' and *M. wenyonii* in African buffalo (*Syncerus caffer*).

Due to the lack of cell culture isolation of hemoplasmas, PCR techniques are especially important since they are the only way to provide a sensitive and specific diagnosis of hemoplasma infections (Ade et al., 2018; Hoelzle, 2007). However, the combination of both PCR and LM methods gives a more sensitive and accurate diagnosis and could be a wise choice during research investigations (Happi et al., 2020).

2.5 THE FEDERAL REPUBLIC OF SOMALIA

Somalia, officially The Federal Republic of Somalia (Somali: Jamhuuriyadda Federaalka Soomaaliya) is a country located in the Horn of Africa, bordered by Ethiopia to the west, Djibouti to the northwest, Kenya to the southwest, and Indian Ocean to the east (Fig. 2). Geographically, Somalia has the longest coastline in mainland Africa, with 3,333 Km in extension, and considering all African countries, is the second only behind the insular nation of Madagascar. Somalia's terrain consists mainly of plateaus, plains, and highlands with landscapes of thornbush savanna and semidesert (UNIDO, 2021). Apart from a mountainous coastal zone in the north and several pronounced river valleys, most of the country is extremely flat, with few natural barriers to restrict the mobility of the nomads and their livestock (Britanica, 2022). The climate in Somalia is semi-arid to arid, mainly dry, and hot, with sporadic rainfall. The rainy season is from April to June, and the dry season is from December to March. One important point about Somalia´s climate is the absence of any large seasonal temperature change during the year, with an annual average temperature of

27 degrees Celsius in Mogadishu and without much variation between the warmest and the coldest months.

Figure 2. Somalia map. Source: UN Cartographic Section

The country has an estimated population of around 15 million people (UNDESA, 2019). Despite rapid urbanization, about 49% of the population still lives in rural and nomadic areas, of which slightly more than half are classified as nomads, slightly less than half derive their livelihood from crop cultivation, and a much smaller share from fishing (FAO, 2018).

Regarding livestock population, Somalia has approximately 28.7 million goats, 13.6 million sheep, 7.1 million dromedaries, and 3.9 million cattle. The dromedary population has a uniform presence across the country, while the cattle population is mostly concentrated in the river valleys: Juba River Valley, Inter-riverine Regions, and the Shabelle River Valley (UNIDO, 2021).

Although many types of livestock production systems can be found in Somalia, the three main production systems are nomadic and transhumant pastoralism, agropastoralism, and urban and peri-urban rearing systems. Nomadic pastoralism, which is the predominant production system, is characterized by the high mobility of people with their animals (Fig. 3) in search of grazing and water, with no or little agriculture (FAO, 2018). Although the country's vast landmass with large areas for grazing and fodder production of livestock, animals are generally fed with natural pasture and with maize and sorghum straw. There is no system in place to grow fodder crops and process them for long-term storage (Haji and Shirdon, 2020).

Even in difficult times, such as the Somali civil war in 1990, the livestock sector remained partially functional, mainly due to the demand for live animals from Saudi Arabia during the annual Hajj pilgrimage[1]. Exports of small ruminants (sheep and goats) to Saudi Arabia during this period represent almost 70% of Somalia's total exports of these animals (Haji and Shirdon, 2020).

Agriculture and livestock farming are the backbone of the Somali economy, contributing over 90% of the country's total exports (Table 1) and employing over 80% of its population (FAO, 2018), but the main productivity limitations include unpredictable and extreme weather patterns, underdeveloped and fragmented markets, poor value addition and lack of access to quality inputs such as seeds, fertilizers, and animal vaccines (Haji and Shirdon, 2020).

Livestock and crops remain, until these days, the main activities of the country's economy. Approximately ¾ of all exports by value are livestock related, such as meat, meat preparations, and live animals, with most of the live animals being exported to Middle East countries, including Saudi Arabia, Yemen, Oman, Qatar, and the United Arab Emirates. Considering value, livestock represents almost 61% of the gross domestic product (GDP) (FAO, 2018).

[1] Hajj is a pilgrimage made to the Kaaba, the "House of God", in the sacred city of Mecca in Saudi Arabia.

Normally, smaller animals like goats and sheep are reared and sold as the whole animal, while the primary reason for rearing cattle and dromedary is selling milk. Dromedaries being sturdy animals can survive and produce milk even in adverse conditions when there is a shortage of fodder, and in Somalia, dromedary milk has been culturally preferred milk compared to the other livestock (UNIDO, 2021).

Product Description	1981-85	1986-90	1991-95	1996-2000	2001-05	2006-10	2011-14	2015
Live animals chiefly for food	88.4	55.7	66.6	72.9	76.7	126.3	377.1	493.9
Animals, live, zoo animals, dogs, cats	0.0	0.0	4.2	6.5	0.6	5.2	42.8	38.7
Oil seeds and oleaginous fruit	0.0	0.7	0.1	1.0	2.1	6.2	40.0	34.3
Cork and wood	0.1	0.0	1.1	6.9	16.1	31.0	25.0	0.0
Hides, skins and furskins, raw	3.9	5.3	2.4	2.3	6.6	8.7	8.6	3.8
Crude animal and vegetable material	4.8	7.1	3.8	3.0	3.3	5.2	6.0	20.7
Vegetables and fruit	14.7	29.5	5.0	7.2	0.6	4.5	5.9	4.9
Of which: Bananas, fresh or dried	*13.8*	*28.1*	*4.3*	*6.6*	*0.0*	*0.0*	*0.0*	*0.6*
Other citrus fruit, fresh or dried	*0.1*	*0.1*	*0.1*	*0.0*	*0.1*	*0.6*	*4.5*	*4.0*
Other vegetables & fruits	*0.5*	*0.8*	*0.6*	*0.5*	*0.5*	*4.0*	*1.3*	*0.3*
Cereals and cereal preparations	0.0	0.0	0.0	1.0	4.7	0.2	4.8	0.1
Meat and meat preparations	0.1	0.0	0.1	1.7	7.5	13.2	4.0	9.6
Fish, crustaceans, mollucs, preparation	2.1	9.0	10.1	8.1	18.3	7.6	3.6	27.8
Dairy products (all)	0.0	0.0	0.1	0.3	1.2	0.4	0.2	0.0
Fixed vegetable oils and fats	0.2	0.1	0.0	1.3	1.6	0.1	0.0	0.1
Sugar,sugar preparations and honey	0.7	1.5	0.2	0.1	1.9	0.3	0.0	0.0
Animal-vegetable oils-fats, processed	0.0	-	0.0	0.0	0.0	0.0	0.0	0.0
Animal oils and fats	0.0	-	0.0	0.0	0.1	0.0	-	0.0
Total (Top 15 agric exports)				115.0	109.0	93.7	112.3	141.1
Total Agric Exports)	115.0	109.0	93.7	112.3	141.1	208.9	518.1	634.0
Total Exports	119.6	114.3	102.5	119.9	169.5	282.5	559.1	688.5

Tabel 1. Somalia´s top 15 agricultural exports, 1981-2015 (millions of dollars, annual averages). Source: Somalia Country Economic Memorandum.

The area where today is Somalia is among the most probable location of the ancient kingdom Land of Punt, a trading partner of Egypt that was known because of his export products, such as incense, ebony, short-horned cattle, and wild animals (Tyldesley, 1998), showing how intrinsic to Somalia roots and Somalian people is the trading market of animals.

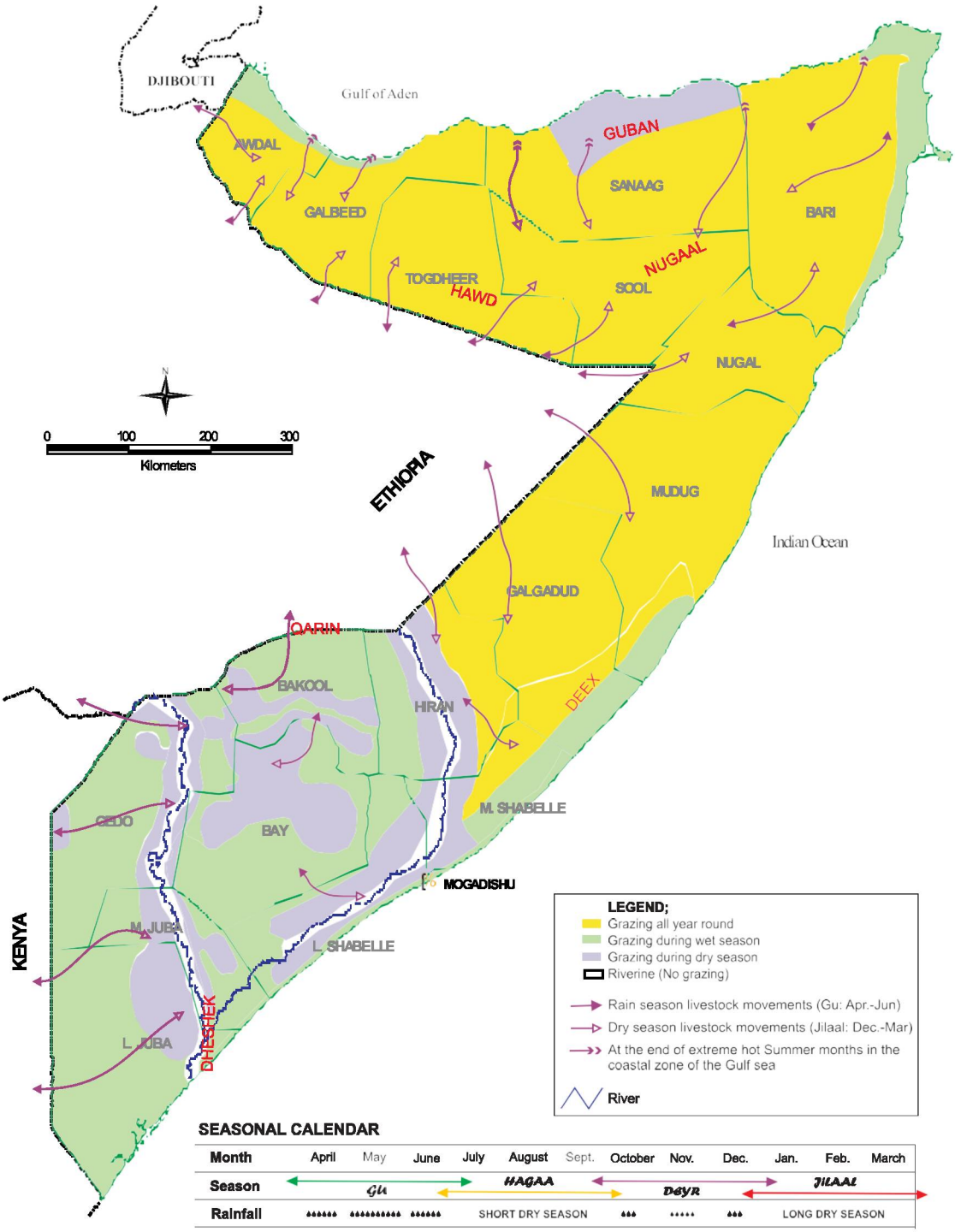

Figure 3. Migration and Seasonal Pattern of Livestock Movement in Somalia.
Source: FSNAU
(http://www.google.it/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=0ahUKEwi4zrfhrt_QAhVIXhQKHXDfCZUQFggoMAA&url=http%3A%2F%2Fwww.fsnau.org%2Fdownloads%2FLivestock%2520Seasonal%2520Migration.pdf&usg=AFQjCNGR_f_3LRwMU3HSje7fYTScGGW62A).

3. HYPOTHESIS

Hemotropic *Mycoplasma* spp. infects cattle and dromedaries from Somalia.

4. OBJECTIVES

4.1. General objective

To perform the molecular detection and characterization of hemoplasmas in cattle and dromedaries from Somalia.

4.2. Specific objectives

- To investigate the occurrence of hemoplasmas DNA in cattle and dromedary blood samples;
- To sequence 16S rRNA of hemotropic *Mycoplasma* species infecting cattle and dromedaries and compare the sequences with other hemoplasmas deposited in GenBank database;

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6. CHAPTER I: Hemoplasmas in cattle from Somalia

ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are small gram-negative bacteria that parasitize red blood cells and can cause mild to severe anemia in a wide range of vertebrates, including ruminants. The cattle population in Somalia is around 3.9 million heads, with animals more concentrated around the river areas, mainly in the Juba River and Shabelle River Valleys. Information on hemoplasmas in Sub-Saharan Africa is scarce, mainly in Somalia, where no studies have been performed to date. Accordingly, this study aimed to assess the molecular occurrence of hemoplasmas in cattle from Somalia. Thirty out of 131 (22.90%; 95% CI: 16.54-30.81%) cattle were infested by ticks: *Rhipicephalus pulchellus* (68.18%), *Amblyomma gemma* (18.18%), *Amblyomma lepidum* (9.09%), *Hyalomma marginatum* (1.51%), *Hyalmomma rufipes* (1.51%), and *Rhipicephalus pravus* (1.51%). A total of 74/131 (56.48%; 95% CI: 47.93-64.67%) cattle were positive for hemotropic *Mycoplasma* spp. by real-time PCR (qPCR) based on the 16S rRNA gene. Hemoplasma-positive samples were later subjected to species-specific PCR assays for *Mycoplasma wenyonii* and '*Candidatus* Mycoplasma haematobovis' based on the 16S rRNA gene. A total of 34/74 (45.94%; 95% CI: 35.07-57.22%) animals were coinfected by both species; 31/74 (41.89%; 95% CI: 31.32-53.26%) and 3/74 (4.05%; 95% CI: 01.39-11.25%) cattle were solely positive to *M. wenyonii* and '*Ca*. M. haematobovis', respectively. Six out of 74 (8.1%; 95% CI: 03.77-16.58%) cattle were negative on species-specific conventional PCR assays but tested positive by a semi-nested PCR assay based on the 16S rRNA gene of hemoplasmas. Sequencing of the detected hemotropic *Mycoplasma* sp. 16S rRNA gene confirmed that animals were infected by *M. wenyonii* and '*Ca*. M. haematobovis'. To the best of our knowledge, this is the first study on the detection of hemoplasmas in cattle from Somalia.

Keywords: Hemotropic mycoplasmas, *Mycoplasma wenyonii*, '*Candidatus* Mycoplasma haematobovis', bovine, Sub-Saharan Africa

6.1 Introduction

Hemoplasmas (hemotropic mycoplasmas) are small pleomorphic Gram-negative uncultivable bacteria that parasitize the surface of red blood cells (RBCs) of a wide range of mammals (Messick, 2004). Two hemoplasma species have been reported infecting cattle to date: *Mycoplasma wenyonii* and '*Candidatus* Mycoplasma haematobovis' (formerly '*Candidatus* Mycoplasma haemobos') (Oren et al., 2020).

Mycoplasma wenyonii, formerly known as *Eperythrozoon wenyonii* (Neimark e Kocan, 1997), was first described in blood smears of bovines from Jerusalem (Adler e Ellenbogen, 1934). The first molecular detection of '*Ca.* M. haematobovis' in cattle occurred 74 years later in Japan (Tagawa et al., 2008). Since then, *M. wenyonii* and '*Ca.* M. haematobovis' infection have been described in cattle worldwide (Su et al., 2010; Meli et al., 2010; Hoelzle et al., 2011; Hornok et al., 2011; Ayling et al., 2012; Girotto et al., 2012; Mcfadden et al., 2016; Oren et al., 2020). In Africa, *M. wenyonii* and '*Ca.* M. haematobovis' were reported in wild buffaloes (*Syncerus caffer*) from Mozambique (Gonçalves et al., 2016), and cattle from Uganda (Byamukama et al., 2020) and Nigeria (Happi et al., 2019), with hemotropic *Mycoplasma* spp. prevalence ranging from 15.4% to 64%.

In cattle, *M. wenyonii* and '*Ca.* M. haematobovis' chronic infection may cause anemia, fever, decreased milk productivity, lymphadenopathy, anorexia, and depression (Messick, 2004; Tagawa et al., 2013; Smith et al., 1990). Abortion and delayed estrus have also been reported during acute infection (Quinlan, 1985; Sutton et al., 1977; Smith et al., 1990). The main clinical signs during chronic hemoplasmosis are lower milk yield and calf birth weight (Tagawa et al., 2013). Although acute infections are rare in cattle (Smith et al., 1990),

economic losses have been associated with chronic hemoplasma infection (Tagawa et al., 2013).

Livestock remains the main activity of Somalia`s economy, which exports meat, meat preparations, and live animals, mostly to Middle East countries, including Saudi Arabia, Yemen, Oman, Qatar, and United Arab Emirates (FAO, 2018). The cattle population in Somalia is approximately 3.9 million heads, and they are more concentrated around the river areas, mainly Juba and Shabelle River Valleys, in southern Somalia (FAO, 2018). The Lower Shabelle region is an agricultural area, where agropastoralism is very common due to higher land productivity and rainfall (FAO, 2018; UNIDO, 2021). The major risks to the exporting market and public health are transboundary infectious diseases, due to the constant mobility of a high number of animals, which facilitates the dissemination of pathogens (Mohamed et al., 2014). Thus, considering the importance of Somalia`s livestock to the economy and the lack of data on hemoplasma infection in cattle, the present study aimed to investigate the molecular occurrence of hemotropic *Mycoplasma* spp. and associated tick species in cattle from Lower Shabelle Region, Somalia.

6.2 Materials and methods

Study area and sampling

This study was carried out between November 2019 to January 2020, which is the dry season in Somalia, on Wanle Weyn (2°08´17.16"N 45°07´16.32"E), Awdhegle (1.9805°N 44.8330°E) and Afgoye (2°08′47.67″N 45°07′ 08.11″E) districts, Lower Shabelle Region. Districts were selected due to cattle population and accessibility. This region presents a Mid-Latitude Steppe and Desert Climate (Köppen: Bsh) and is characterized by higher land productivity and higher rainfall compared to the rest of Somalia (FAO, 2018). Herds studied

herein were selected based on the accessibility and owner's willingness to cooperate with this study.

A non-probabilistic convenience sampling was performed. A total of 131 cattle EDTA-blood samples were collected by jugular venipuncture (122 females and 9 males). Animals were physically restrained, and blood samples (5 mL) were collected by venipuncture of the jugular vein using vacuum tubes containing EDTA (BD Vacutainer®, Franklin Lakes, NJ, USA) for PCR analysis and kept at −20 °C until testing.

Animals were submitted to a visual inspection for the presence of ticks, beginning at the head and neck and proceeding systematically down the mane until the tail was reached. Thereafter, the tail, perianal region, foreleg, axillary region, ventral region, hindlegs, inguinal region, and external ears were also examined (Duell et al., 2013). Ticks infesting cattle were removed using a commercial hook (O'TOM®/Tick Twister®, Lavancia, FRA), and kept in absolute ethanol labeled tubes for identification according to morphological taxonomic keys (Walker et al., 2000, 2003; Apanaskevich and Horak, 2009).

DNA extraction and PCR assays

DNA was extracted from 200 µL of whole blood using a commercial kit (Illustra® Blood Genomic Prep Mini Spin Kit, GE Healthcare, Little Chalfont, UK), according to the manufacturer's instructions. For molecular characterization, DNA was extracted from ticks' right fourth leg using a commercial kit (MagaZorb® DNA Mini-Prep Kit, Promega, Madison, Wisconsin), according to the manufacturer's instructions. Ultrapure water was used in parallel as a negative control to monitor cross-contamination.

DNA samples extracted from cattle blood samples were submitted to a conventional PCR for the mammal endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) (Birkenheurer et al., 2003), while tick DNA samples were subjected to a PCR

targeting the mitochondrial 16S rRNA gene of ticks (Fukunaga et al., 2001) to monitor the DNA extraction. Thereafter, cattle DNA samples were screened using a universal SYBR green real-time PCR (qPCR) assay targeting the 16S rRNA gene of hemoplasmas, as previously described (Willi et al., 2009). Standard curve was calibrated using serial dilutions of gBlock™ (Integrated DNA Technologies, Coralville, IA, USA). All parameters were analyzed according to the standards established by MIQE (Minimum Information for Publication of Quantitative real-time PCR Experiments) (Bustin et al., 2009). Samples with a median Cycle quantification (Cq) value < 32 were considered positive (Vieira et al., 2015). All positive samples for hemotropic *Mycoplasma* spp. by the qPCR assay were subjected to species-specific conventional PCR assays for 16S rRNA of *M. wenyonii* and '*Ca*. M. haematobovis', as previously described (Nishizawa et al., 2010). Moreover, all qPCR hemoplasma-positive samples, which tested negative on species-specific PCR for *M. wenyonii* and '*Ca*. M. haematobovis', were submitted to semi-nested PCR assay based on 16S rRNA gene of hemoplasmas, as previously described (Di Cataldo et al., 2020). *M. wenyonii* and '*Ca*. M. haematobovis' (dos Santos et al., 2018) were used as positive controls, and ultrapure nuclease-free water was used as the negative control in all analyses.

Sequencing

Amplicons obtained from seven hemoplasma-positive samples and eight ticks were sequenced in both directions by Sanger method (Sanger et al, 1977). Partial nucleotide sequences of the 16S rRNA gene of hemoplasmas were deposited in GenBank® database (accession nos. ON614702, ON614703, ON614704, ON614705, ON614706, ON614707, ON614708, respectively). Partial nucleotide sequences of the mitochondrial 16S rRNA gene of ticks were deposited in GenBank® database (accession nos. ON532090, ON532091, ON532092, ON532093, ON532094, ON532095, ON532096, ON532097).

Statistical analysis

The Chi-square test was used to determine if an individual variable (presence of ticks) was associated with hemoplasma infection. *Odds ratio* (OR), 95% confidence interval, and p-value were calculated. Results were considered significantly different when $p < 0.05$. Data was compiled and analyzed by Epi InfoTM Software (version 7.1.5, CDC).

6.3 Results

A total of 66 (37 males, 26 females, and three nymphs) ticks were collected from 30/131 (22.90%; 95% CI: 16.54-30.81%) cattle with a mean of 2.2 ticks per animal. Adult ticks were identified as *Rhipicephalus pulchellus* (43/66; 65.15%, 19M, 24F), *Amblyomma gemma* (12/66; 18.18%, 12M), *Amblyomma lepidum* (6/66; 9.09%, 5M and 1F), *Hyalomma marginatum* (1/66; 1.51%, 1F) and *Hyalmomma rufipes* (1/66; 1.51%, 1M). Two nymphs were identified as *R. pulchellus* and one *Rhipicephalus pravus* (GenBank® accession no. ON532094) tick.

The *gapdh* was consistently amplified in all 131 samples cattle samples. A total of 74/131 (56.48%; 95% CI: 47.93-64.67%) animals tested positive for hemotropic *Mycoplasma* spp. by qPCR. The efficiency of the qPCR for hemoplasma was 97.976%, the r^2 0.998, the slope -3,371, and the y-intercept 34.024. The melt curve mean of gBlock was 77.398 and the melting temperature of positive samples varied from 73.587 to 79.943.

Using species-specific PCR assays, 34/74 (45.94%; 95% CI: 35.07-57.22%) cattle tested positive for both species, *M. wenyonii* and '*Ca*. M. haematobovis', being 31/74 (41.89%; 95% CI: 31.32-53.26%) positive for *M. wenyonii*, and 3/74 (4.05%; 95% CI: 01.39-11.25%) positive solely for '*Ca*. M. haematobovis'. Six out of 74 (8.1%; 95% CI: 03.77-16.58%) hemoplasma-negative cattle by the species-specific PCR assays tested

hemoplasma-positive by the semi-nested PCR assay. Considering all 74 animals that tested positive for hemoplasmas, 17 were infested by ticks. Association between positivity to hemoplasmas and the presence of ticks was not found (OR = 0.836; 95% CI: 0.372 – 1.888%; P = 0.331).

BLASTn analysis of three *M. wenyonii* 16S rRNA gene sequences detected herein showed 99.88-100% identity with multiple sequences of *M. wenyonii* previously detected in cattle from Brazil, China, and Cuba (GenBank® accession nos. MT341494, EF221880, MG948624). Two '*Ca.* M. haematobovis' sequences (461bp) showed 99.88-100% identity with multiple '*Ca.* M. haematobovis' sequences previously detected in animals from Brazil and Cuba (GenBank® accession nos. MT341495 and MG948631). Additionally, two of the hemoplasma-positive samples by the semi-nested PCR assay (1,107bp) showed 99.49-99.62% identity with multiple sequences of *M. wenyonii* previously detected in cattle from Japan and Cuba (GenBank® accession nos. EU367963 and MG948627).

6.4 Discussion

Herein, 56.48% cattle tested positive for hemotropic *Mycoplasma* spp., lower than the prevalence reported in Nigeria (64%) (Happi et al., 2019), Phillippines (80%) (Ybañez et al., 2019), Cuba (63.4%) (Díaz-Sánchez et al., 2019), Germany (62.27%) (Ade et al., 2018), and Japan (91.5%) (Tatsukawa et al., 2021), but higher than those found in Malaysia (40.98%) (Agina et al., 2021), Philippines (8.33%) (Galon et al., 2020), and Uganda (32.2%) (Byamukama et al., 2020). To the best of the author's knowledge, this study provides the first molecular evidence of hemoplasmas in cattle from Somalia.

In the African continent, a previous study has described '*Ca.* M. haematobovis' in cattle from Nigeria (Happi et al., 2019), and '*Ca.* M. haematobovis' and *M. wenyonii* in animals from Uganda (Byamukama et al., 2020). In African buffaloes (*Syncerus caffer*) from

Mozambique, 14.45% (14/97) and 4.1% (4/97) animals tested positive for *M. wenyonii* and '*Ca*. M. haematobovis', respectively, when used species-specific PCR assays (Gonçalves et. al, 2018). Herein, 45.94% of the animals were co-infected by both agents, while 41.89% and 4.05% were solely positivity for *M. wenyonii* and '*Ca*. M. haematobovis', respectively. In cattle from Cuba, the overall occurrence of *M. wenyonii* (66.2%) was also higher than '*Ca*. M. haematobovis' (41.2%) (Díaz-Sánchez et al., 2019). It is important to state that screening animals presenting clinical signs and/or hematological abnormalities such as anemia, edema of the limbs and/or the udder, and transient milk drop may increase the odds to detect *M. wenyonii* (Nouvel et al., 2019). Although most hemoplasma infections remain subclinical, co-infections with other pathogens, such as *Anaplasma marginale*, *Anaplasma phagocytophilum*, *Babesia* spp., and *Theileria* spp. may increase hemoplasmas' pathogenicity (Hofmann-Lehmann et al., 2004). Chronic hemoplasma infection may cause important economic losses (Tagawa et al., 2013), with animals remaining carriers. Unfortunately, it was not possible to clinically evaluated cattle in the present study.

Although transmission of hemoplasmas through aggressive interactions has been reported for cats (Museux et al., 2009) and wild rodents (*Gerbillus andersoni*) (Cohen et al., 2018), studies on the transmission of bovine hemoplasmas are scarce. The transplacental transmission of hemoplasmas in cattle has been reported (Girotto et al., 2012; Hornok et al., 2011), while the mechanical transmission through the horn (*Haematobia irritans*) and horse flies (*Tabanus bovinus*, *T. bromius*) has been suggested (Hornok et al., 2011). Moreover, although *Mycoplasma haemocanis* has been experimentally transmitted by *Rhipicephalus sanguineus* sensu lato ticks in dogs (Seneviratna, Weerasinghe and Ariyadasa, 1973), and '*Ca.* M. haematohydrochoerus' DNA has been detected in *Amblyomma sculptum* (Gonçalves et al., 2020) and salivary glands of *Amblyomma dubitatum* ticks (Vieira et al., 2021) infesting hemoplasma-infected capybaras (*Hydrochoerus hydrochaeris*) from Brazil,

the transmission of hemoplasmas through hard ticks still needs further investigation. Somalia has suitable ecological conditions for arthropod vector breeding, spread, and zoonotic vector-borne disease transmission (Hassan-Kadle et al., 2019; 2020; 2021). Herein, most animals were infested by *R. pulchellus* (65.15%) and *A. gemma* (18.18%), with 22.97% (17/74) hemoplasma-positive animals infested by ticks. However, an association between positivity to hemoplasmas and the presence of ticks was not found ($p = 0.331$). Further studies should be performed to evaluate risk factors for hemoplasma infection in animals from Somalia.

In conclusion, this study provides the first molecular evidence of bovine hemoplasmas infection in cattle from Somalia. Our data indicate a high hemoplasma prevalence among cattle in the studied region, with high level of co-infection by *M. wenyonii* and '*Ca.* M. haematobovis'. Further studies are needed to evaluate the epidemiology, clinical and economic impact of hemoplasmas on the livestock industry in Somalia.

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7. CHAPTER II: Molecular detection of hemoplasmas in camels (*Camelus dromedarius*) from Mogadishu, Somalia

ABSTRACT

Hemotropic mycoplasmas (hemoplasmas) are small pleomorphic bacteria that parasitize the surface of red blood cells of several mammals and may cause mild to severe anemia. Hemoplasmas have been described in different species from the Camelidae Family, such as llamas and alpacas, but data on dromedaries are limited to a few reports. Somalia possesses one of the biggest dromedaries heard in the world, with more than seven million heads, and to date, studies on hemoplasmas and tick-borne pathogens are lacking. Accordingly, the aim of this study was to screen dromedaries for hemotropic Mycoplasma sp. by PCR-based assays. A total of 104 dromedary FTA-blood samples were collected from different areas of Mogadishu, Somalia. After DNA extraction, samples were screened using hemoplasma genus-specific real-time (qPCR) and conventional PCR assays targeting the 16S rRNA gene of this group of bacteria. The endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was consistently amplified from all dromedary samples. All animals tested negative for hemoplasma on both PCR assays. To the best of our knowledge, this is the first study on the molecular screening of hemoplasmas in dromedaries in Somalia.

Keywords: Hemotropic mycoplasmas, *Camelus dromedarius*, Sub-Saharan Africa

7.1 INTRODUCTION

Hemotropic mycoplasmas, or hemoplasmas, are small uncultivable gram-negative bacteria that can be found attached to erythrocytes in a variety of mammals worldwide (Messick, 2004). In camelids, '*Ca*. M. haemolamae' is considered an endemic pathogen of llamas (*Lama glama*) and alpacas (*Vicugna pacos*), and it has been found infecting camelids in different countries, such as Canada (Almy et al., 2006), United States (Lascola et al., 2009; Pentecost et al., 2012), Peru (Tornquist et al., 2010), Chile (Tornquist et al., 2010; Ramos et al., 2021), Switzerland (Meli et al., 2010; Kaufmann et al., 2011) Germany (Kaufmann et al., 2011), England (Crosse et al., 2012), New Zealand (Van andel et al., 2013; Dittmer et al., 2018) and Austria (Franz et al., 2016). Although is known that hemoplasmas can infect members of the Camelidae family (Mclaughlin et al., 1990; Tornquist et al., 2010; Guimaraes et al., 2012), data on camels (*Camelus dromedarius*) are still scarce (Nazifi et al., 2009; Sharifiyazdi et al., 2018; Esmaeilnejad et al., 2019).

The first report of hemoplasmas infecting dromedaries was based on direct bacteria visualization by light microscopic of blood smears in Iran (Nazifi et al., 2009). Later, the first molecular hemoplasma detection based on 16S rRNA gene was described infecting two Iranian dromedaries as *Mycoplasma suis* and hemotropic *Mycoplasma* sp. (Sharifiyazdi et al., 2018). Another study from Iran reported co-infection with '*Ca*. M. haemolamae' and *Trypanosoma evansi* in an anemic dromedary (Esmaeilnejad et al., 2019).

'*Ca*. M. haemolamae' infection may cause severe hemolytic anemia, fever, jaundice, hemoglobinuria, and weight loss in camelids (Mclaughlin et al., 1990), and both chronic and acute infections have been observed in the field (Mclaughlin et al., 1990; Meli et al., 2010). Even if most the infected camelids have the asymptomatic chronic form of the infection, stresses such as parturition and gestation may contribute to the development of severe, life-threatening anemia (Meli et al., 2010).

Livestock is the traditional repository of household wealth in Somalia, the largest export, and an important source of livelihood for a large part of the population. The dromedary population in Somalia, around 7.1 million head, is the world's biggest dromedary population. The main production systems are nomadic pastoralism and agro-pastoralism, but large livestock-rearing operations with dairy animals (mostly dromedaries) have been on the rise in settled mixed farming (FAO,2018).

Considering the importance of one-humped camel livestock in Somalia and the lack of information about hemoplasma infection in this animal specie, this study aimed to screen dromedaries for *Mycoplasma* sp. infection using molecular techniques.

7.2 MATERIALS AND METHODS

7.2.1 Sampling

A total of 104 dromedary blood samples from Mogadishu, Somalia, were collected from December 2018 to April 2019, which corresponds to the dry season in Somalia. All the blood samples were collected by jugular venipuncture (n= 104; 101 females and 3 males) and stored in EDTA microtubes. Approximately 125µl of each dromedary blood sample was applied on filter paper (FTA® card, Whatman®, GE Healthcare, Buckinghamshire, United Kingdom) and was delivered to the Vector Borne Diseases Laboratory (UFPR, Brazil), accordingly to the Ministério da Agricultura, Pecuária e Abastecimento (MAPA) recommendations.

7.2.2 DNA extraction

DNA was extracted from dried blood spots (DBS) using a commercial kit (IndiMag® Pathogen Kit, Qiagen for Indical Bioscience, Leipzig, Germany) according to the manufacturer's instructions. A total of 3 discs were removed from each FTA® card using a 3.00mm Punch (Harris Uni-Core™, Qiagen, Hilden, Germany) and placed in a clean RNase/DNase-free 1.5 ml microcentrifuge tube. Pre-treatment was performed by adding 200µl PBS to the macerated FTA discs using an 18-gauge needle, followed by heating for 10 minutes at 96°C. FTA discs without blood and RNase/DNase-free water was used in parallel as a negative control to monitor cross-contamination. DNA samples were stored at –20°C prior to PCR assays.

7.2.3 Polymerase Chain Reactions (PCR)

Conventional PCR (cPCR) for the mammalian endogenous gene glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) was performed in all samples to ensure successful DNA extraction (Birkenheurer et al., 2003).

All samples were initially screened using a pan-hemoplasma SYBR green real-time PCR (qPCR) assay, as previously described (Willi et al., 2009). Thereafter, all samples were subjected to conventional PCR assay targeting a fragment (~950 bp) of the 16S rRNA gene for hemoplasmas (Hoelzle et al., 2011).

7.3 RESULTS AND DISCUSSION

Although housekeeping gene was successfully amplified, all 104 DNA dromedary samples tested negative by qPCR and conventional PCR assays targeting 16S rRNA gene for hemoplasmas. qPCR and conventional PCR assays were used in this study due to the probable inability of the primers used in the qPCR assay to amplify DNA from genetically diverse hemoplasmas, especially when screening biological samples from wild animals (de Oliveira et al., 2022).

A previous study showed no evidence of hemoplasmas infection on blood smears visualization and PCR assays in 55 clinically health one-humped camels from United Arab Emirates (Wernery et al., 2014).

To the best of author's knowledge, molecular detection of hemoplasma infection in dromedaries was only previously described in Iran (Sharifiyazdi et al., 2018; Esmaeilnejad et al., 2019). A study has detected two Iranian dromedaries infected by *M. suis* and hemotropic *Mycoplasma* sp. belonging to "haemofelis-group" (Sharifiyazdi et al., 2018). A case report of '*Ca*. Mycoplasma haemolamae' (CMhl) coinfection with *Trypanosoma evansi* was described in a one-humped camel presenting anorexia, weakness, depression, and pale mucosa, using species-specific PCR targeting the hemoplasmas ssu rRNA gene (Esmaeilnejad et al., 2019). Hemotropic mycoplasma infection in healthy dromedaries appears to be rare, while the significance of infection in apparently sick dromedaries requires further investigation.

Although hemoplasma dromedary infection appears to be lower than infection in other ruminant species, further studies are necessary to establish if the hemoplasmas species infecting dromedary camels are just a finding and the role of hemoplasmas in the pathogenesis of the diseases.

8. GENERAL CONCLUSIONS

The present study has shown that hemoplasmosis is prevalent in cattle from Lower Shabelle, but isn´t in dromedaries from Mogadishu city, Somalia. This is the first study on the molecular detection of *Mycoplasma* spp. in cattle in Somalia, with the identification of *Mycoplasma wenyonii* and '*Candidatus* Mycoplasma haematobovis'. Coinfections in cattle with these two *Mycoplasma* species were also reported in the present study. Further epidemiological studies are needed to investigate hemoplasma infection in cattle and to better explore the possibility of hemoplasma infection in dromedaries in Somalia.

Also, epidemiological data, such as animal trade, potential vectors, other pathogen co-infections, public health impact, and risks of chronic infection should be investigated in cattle from Somalia to understand disease progression at the population level.

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