Studies on the roles of livestock reservoirs in the epidemic of trypanosomosis and piroplasmosis

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トリパノソーマ症とピロプラズマ症の流行において家畜のレゼルボアが果たしている役割に関する研究

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Abbreviations

%	Percent
<	Less Than
>	Greater Than
±	Plus/Minus
°C	Degree Celsius
µl	Microliter
mm	Micrometer
BC48	48 kDa Merozoite Rhoptry Protein
CATT/*T. evansi*	Card Agglutination Test for Trypanosomes/*Trypanosoma evansi*
cELISA	Competitive ELISA
CFT	Compliment Fixation Test
DEAE	Diethylaminoethyl
DNA	Deoxyribonucleic Acid
dNTPs	Deoxyribonucleotide Triphosphate
EDTA	Ethylene Diamine Tetra Acetic acid
ELISA	Enzyme-Linked Immunosorbent Assay
EMA-2	Merozoite Antigen 2
IFAT	Indirect Fluorescent Antibody Test
ITS1	Internal Transcribed Spacer 1
kbp	Kilo Base Pair
kDNA	Kinetoplast DNA
kg	Kilogram
km^2	Kilometer Square
mg	Milligram
$MgCl_2$	Magnesium Chloride
min	Minute
ml	Milliliter

mM	Micromolar
OIE	World Organization for Animal Health
PARPs	Procyclic Acidic Repetitive Proteins
PCC	Philippine Carabao Center
PCR	Polymerase Chain Reaction
PCV	Packed Cell Volume
rDNA	Ribosomal DNA
RNA	Ribonucleic Acid
RoTat 1.2	Rode *Trypanozoon* Antigen Type 1.2
rpm	Round Per Minute
rTeGM6-4r-ELISA	Recombinant *T. evansi* GM6-based ELISA
sec	Second
TeCA-ELISA	*T. evansi* Crude Antigen-based ELISA
VAT	Variable Antigen Type
VSG	Variant Surface Glycoprotein

General introduction

1. Trypanosomosis and *Trypanosoma*

Definition and classification

Trypanosomosis (or trypanosomiasis) is the infection of an animal or a human with a trypanosome, which is a flagellate protozoan parasite of the genus *Trypanosoma* that lives in the blood of vertebrate animals and is transmitted by certain insects (Collins English Dictionary).

Classification of trypanosomes:

Phylum: Sarcomastigophora

Class: Zoomastigophora

Order: Kinetoplastida

Family: Trypanosomatidae

Genus: *Trypanosoma*

The genus *Trypanosoma* is divided into two groups: the salivarian group and stercorarian group. In the salivarian group, the parasites do not pass the stomach of the vector, and thus do not enter the intestine. They migrate from the stomach towards the salivary glands of the vector, where the infective stage of the parasite develops. They then infect the vertebrate host via the vector's saliva

when it bites the host to take its blood meal. In the stercorarian group, the parasites develop in the intestine of the vector and infect the vertebrate host via the feces (Hoare, 1966; Wéry and Paskoff, 1995; Nath Baral, 2010).

African trypanosomosis

Trypanosomosis is usually referred to as African trypanosomosis, but it is actually distributed worldwide. For example, *Trypanosoma evansi* causes diseases in different animal species in Central and South America, the Middle East, and Asia, and is considered a disease of economic importance in Southeast Asia (Wuyts *et al*., 1994).

African trypanosomosis is an infectious parasitic disease of domestic and wild animals, and humans caused by many species of protozoan parasites in the genus *Trypanosoma* in the family Trypanosomatidae, which live and multiply extracellularly in the blood stream and/or tissue fluids of their hosts (Molyneux *et al*., 1996). The main African pathogenic trypanosomes of animals belong to three subgenera in the salivarian group, which are mostly transmitted cyclically by tsetse flies (Hoare, 1972), as follows:

Subgenus *Nannomonas*:

Trypanosoma congolense, found in most domestic animals including cattle, horses, camels, sheep, goats, and dogs, and in many wild animals.

Subgenus *Duttonella*:

Trypanosoma vivax, found in horses and in domestic and wild ruminants.

Subgenus: *Trypanozoon*:

Trypanosoma brucei brucei, found in domestic and wild ruminants.

Trypanosoma evansi, found in several species of domestic animals, including camels, cattle, horses, water buffaloes, elephants, and dogs, and in many wild animals.

Trypanosoma equiperdum, found in equines.

Trypanosome infections of livestock are known as surra, nagana, and dourine, based on the causative *Trypanosoma* species. Surra is caused by *T. evansi*. Nagana is caused by *T. congolense*, *T. vivax*, and *T. b. brucei*. Other subspecies of *T. brucei*, namely *T. b. rhodesiense* and *T. b. gambiense*, are the causative parasites of human African trypanosomiasis, also known as sleeping sickness. Dourine is caused by *T. equiperdum* and transmitted during coitus (Nath Baral, 2010).

The geographical distribution of African trypanosomes is related to the geographical distribution of tsetse flies. However, there are several modes of trypanosome transmission other than tsetse flies, which lead to the geographical distribution of African trypanosomosis to be not exactly matching that of tsetse flies. The most important alternative mode of transmission is mechanical transmission by biting insects. Other modes of transmission include direct vertical, oral, sexual, and iatrogenic transmission (Gruvel, 1980; Nath Baral, 2010). *T. evansi*, due to it having lost particular

genetic materials, can no longer undertake its life cycle in tsetse flies, and thus mechanical transmission by biting insects is its principal way of infection (Hoare, 1972; Nath Baral, 2010). *T. vivax* is also mechanically transmitted by biting insects such as *Tabanus* and *Stomoxys* (Desquesnes and Dia, 2003, 2004; Baldacchino *et al*., 2013).

Special features of trypanosomes

i. Kinetoplast DNA

Trypanosomes are characterized by the existence of a structure called a kinetoplast, which corresponds to the agglutinated mitochondrial DNA (kDNA) of their uniquely large and elongated mitochondria (Borst *et al*., 1987). The kDNA network is composed of two types of DNA circles which have different sizes: minicircles of approximately 1 kbp and maxicircles of 22 kbp (Simpson, 1987; Simpson *et al*., 1987; Nath Baral, 2010). Maxicircle sequences are highly conserved, and code for ribosomal RNA and some mitochondrial proteins. Dozens of maxicircle sequences are present in each cell (Motyka *et al*., 2006). The number of minicircles in each cell is high (5,000–10,000 per trypanosome), and their sequences vary from one other (Sturm and Simpson, 1990; Nath Baral, 2010). The function of minicircles is unclear, and the absence of conserved sequences in them suggests that minicircles do not code for proteins (Ryan *et al*., 1988). However, minicircles code for small RNA sequences known as guide RNAs, which are necessary for decoding encrypted maxicircle transcripts (Lai *et al*., 2008). Different from other trypanosomes, the kDNA of *T. evansi*

completely lacks maxicircle DNA, and comprises minicircle DNA only (Borst *et al*., 1987; Nath Baral, 2010). The maxicircle DNA in *T. equiperdum* is partially conserved, so these two trypanosome species cannot develop in insects, as the maxicircles encode some mitochondrial genes that are necessary for the parasites to undergo stage conversion and proliferation in these vectors (Sánchez *et al*., 2015).

ii. Variant surface glycoprotein (VSG)

Inside the mammalian host, a monolayer of variant surface glycoprotein (VSG) covers the surface of the bloodstream form of trypanosomes. The VSG is a main antigen of the parasite whose antigenicity is continuously changed (antigenic variation) as an adaptive mechanism to avoid the host immune response (Nath Baral, 2010). This defines the variable antigen type (VAT) of each individual trypanosome. During the ascending phase of parasite number in bloodstream, the host adapted immune system recognizes parasites that are of the same antigenic type (homotype) and produces antibodies against this homotype, leading to the elimination of the parasites of the major VAT and the descending phase of parasitemia. At the same time, a parasite expressing one of the minor VATs or heterotypes can then multiply and become the new homotype, resulting in a new wave of parasitemia. Therefore, the expression of VSG is essential in the process of antigenic variation by the parasite aiming to exhaust the host immune system (Field *et al*., 2009).

Life cycle and transmission

Infection of the vertebrate host with tsetse-transmitted trypanosomosis starts when the tsetse fly vector injects the infective metacyclic stage of the parasite intradermally. The parasite rapidly transforms into the long, slender trypomastigote and divides by binary fission at the biting site (Barry and McCulloch, 2001). During the course of parasitemia in the mammalian host, the proliferative slender bloodstream form and the nonproliferative stumpy bloodstream form are developed during the ascending and at the peak of parasitemia, respectively (Matthews *et al*., 2004; Nath Baral, 2010). The stumpy form is preadapted for transition to the procyclic form that occupies and proliferates in the midgut of the tsetse fly when it takes the parasite in with the blood meal after biting an infected individual (Nath Baral, 2010). In the midgut of the infected tsetse fly, the stumpy bloodstream form very rapidly loses its surface coating of VSG and differentiates into the proliferative procyclic form, which expresses procyclic acidic repetitive proteins (PARPs, or procyclins) as their cell surface proteins (Ziegelbauer and Overath, 1990; Ziegelbauer *et al*., 1993; Gruszynski *et al*., 2003, 2006; Nath Baral, 2010). Two developmental stages in the fly are required for successful transmission of the parasite: establishment in the midgut, and then maturation in the mouth parts or salivary glands. The parasite migrates to the salivary glands or mouth part after proliferation in the tsetse midgut, and then the epimastigote form is generated and attaches to these glands using its flagellar membrane. After additional multiplication, infective metacyclic forms with metacyclic VSG as their cell surface protein are developed from epimastigote forms, and are then released into the lumen of salivary glands or

mouth part in preparation for being injected into a new vertebrate host (Matthews, 2005; Muhammad, 2009) (Figure 1).

Non-tsetse-transmitted trypanosomes, *T. evansi* and *T. vivax* in South America, are mechanically transmitted by blood-sucking insects, such as stable flies (*Stomoxys* spp.) and horseflies (*Tabanus* spp.) (Brun *et al*., 1998). However, tsetse flies can also perform as mechanical vectors. The biting insect passes the bloodstream form of the parasite from an infected animal to another one by engaging in frequent blood-sucking behavior. Since the trypanosomes die when the blood dries, the time between the two blood meals is crucial for effective transmission (Uilenberg and Boyt, 1998). All parasite developmental stages in the tsetse fly are not found in these mechanical vectors; for instance, the procyclic stage does not exist in them because the mechanically-transmitted trypanosomes cannot differentiate from bloodstream form to procyclic form as they lack the maxicircles in their kDNA (Borst *et al*., 1987). There are no tsetse flies outside of tsetse belt, except for small tsetse habitats in the southwestern Arabian Peninsula (Uilenberg and Boyt, 1998). Therefore, mechanical transmission has been confirmed to be the mode of transmission of *T. evansi* and *T. vivax* in South and Central America, as well as *T. evansi* in North Africa and Asia (Uilenberg and Boyt, 1998).

T. equiperdum, which is a principally tissue parasite that causes very low parasitemia in the blood of infected equids in cases of dourine, is transmitted directly from an infected to a healthy animal during coitus. The parasite is present in the mucous discharges of the penis and sheath of stallions and in the vaginal mucus of mares. Without any specific insect vector, the geographical

distribution of *T. equiperdum* and dourine is not limited to any particular region (Uilenberg and Boyt, 1998).

Iatrogenic transmission occurs when a needle or a surgical instrument that is contaminated with wet infected blood is used on more than one animal. This is common in cases of vaccination, treatment by injection, collection of blood samples from groups of animals in the field without changing or disinfecting needles, or when surgical procedures are performed on several animals at short intervals, such as dehorning or castration, without proper disinfection of the instruments (Uilenberg and Boyt, 1998).

Other means of transmission include the infection of carnivores with *T. evansi* and *T. b. brucei* through the mucosa of the mouth by ingesting the fresh meat or organs of infected animals that contain live trypanosomes. Congenital transmission of trypanosomes from an infected mother to her offspring happens either through the placenta during pregnancy, or during birth when bleeding occurs. Vampire bats are able to transmit *T. evansi* and *T. vivax* to healthy animals when they become infected by consuming blood from infected animals. The trypanosomes proliferate in the bats and then pass through their oral mucosa (Uilenberg and Boyt, 1998).

Epidemiology

The epidemiology and distribution of animal trypanosomosis depend on many factors. Tsetse-transmitted trypanosomosis depends mainly on the vector, *Glossina* spp., including the vector

type (savannah or riverine-forest species), its preferred hosts, the time of day that the flies are the most active, and the season (dry or rainy). Moreover, the susceptibility of the host species and breed is of great importance, as some breeds are susceptible, while others are trypanotolerant. The distribution of nagana, caused by *T. congolense*, *T. b. brucei*, and *T. vivax*, coincides with that of tsetse flies in Africa (Uilenberg and Boyt, 1998). It is found mainly in the tropical regions of Africa, over a range covering 10 million km^2 between 14° North and 20° South latitude in 37 African countries (Kuzoe, 1993; Molyneux *et al*., 1996) (Figure 2). However, the American strains of *T. vivax* are transmitted mechanically, and all attempts to transmit them experimentally by tsetse flies have failed (Uilenberg and Boyt, 1998).

The epidemiology of non-tsetse-transmitted trypanosomes is also affected by many factors, including host preference, daily behavioral patterns of the biting flies, whether flies come into contact with livestock during the hours that they are active, and the season, since biting flies are influenced by seasonal climatic differences. *T. evansi* is widespread outside of the tsetse belt in Africa, and also in Asia and South and Central America (Uilenberg and Boyt, 1998).

T. equiperdum infections are less restricted by climate. This species has been eradicated from North America and most of Europe, and its current distribution is poorly known. It is distributed in northern and southern Africa, some European countries, and parts of Asia (Uilenberg and Boyt, 1998).

Clinical symptoms

Important differences of clinical signs amongst infections caused by different trypanosomes in different hosts are observed. These differences vary in intensity from unapparent to strong according to the host species considered. In general, trypanosomosis is a wasting disease characterized by the progressive deterioration of animal condition accompanied by intermittent fever, anemia as a result of the hemolysis of red blood cells, decreasing of appetite, loss of weight, losses in production, weakness, nervous signs, collapse, and death (Gardiner and Mahmoud, 1990; Uilenberg and Boyt, 1998; Bhatia *et al*., 2006; Jaiswal *et al*., 2015).

Cattle and buffaloes are usually asymptomatic carriers, with occasional acute or peracute infections. The disease is chronic with mild clinical signs in cattle and buffaloes in Latin America and Africa, while it is acute in Asia with anemia, recurrent fever, loss of weight, and losses in production (Payne *et al*., 1993; Jaiswal *et al*., 2015). In chronic cases, signs of dullness, progressive emaciation, lacrimation, intermittent fever, anemia, and edema in the lower parts of the body have been reported (Muraleedharan and Srinivas, 1985; Rajguru *et al.*, 2000; Jaiswal *et al*., 2015).

In camels, both acute and chronic forms of the disease occur in all age groups, especially young animals. Signs of acute cases are high fever, weakness, anemia, emaciation, and death. In chronic cases, which are more frequent, the course of the disease continues for three years, including the clinical signs of recurrent fever, progressive weakness, dullness, loss of appetite, weight loss, edema in the lower parts of the body, petechial hemorrhages on the mucous membranes and anemia (Gutierrez *et al*., 2005; Jaiswal *et al*., 2015).

Among equines, horses are highly susceptible to trypanosome infections, but the mule, donkey, and ass are less susceptible. Equines show classical clinical signs, such as recurrent fever, weakness, severe weight loss, anemia, general or local cutaneous discharges, abortion, petechial hemorrhages on the mucosa of vagina and vulva and in the frontal chamber of the eye, nervous signs, movement disturbances, and edema of the ventral parts of the body, particularly the lower abdomen, legs, testicles, and reproductive organs. Signs of chronic disease are anemia, loss of weight, and jaundice, with urine of dark yellow color (Jani and Jani, 1993; Varshney and Gupta, 1996; Laha *et al*., 2004; Jaiswal *et al*., 2015).

Trypanosomosis in sheep and goats is usually mild or asymptomatic. However, unilateral superficial ulceration of the cornea and retinochoroiditis, while there is no obvious loss of vision, were detected in experimentally infected goats (Morales *et al*., 2006; Jaiswal *et al*., 2015). In pigs, clinical signs are very mild, including fever, loss of appetite, emaciation, and abortion (Arunasalam *et al*., 1995; Jaiswal *et al*., 2015).

Dogs are highly susceptible to trypanosomosis, with clinical signs including recurrent fever, weakness, emaciation, edema of the head, neck, abdomen, and legs, anemia, lymphadenopathy, muscular spasms, paralysis of the hindquarters, tachycardia, and myocarditis (Varshney *et al*., 1998; Jaiswal *et al*., 2015). Cats show clinical signs which are, in general, similar to the clinical signs of dogs (Thirunavukkarasu *et al*., 2000; Jaiswal *et al*., 2015).

Diagnosis

The diagnosis of trypanosomosis is based on the detection of the parasite (antigen) or antibodies against it. Many techniques can be used for the direct detection of the parasite, including such parasitological techniques as examining wet blood films, fresh lymph preparations, and Giemsa-stained blood smears. These techniques are simple and inexpensive, but their sensitivity is limited. The microhaematocrit centrifugation technique is used for the detection of the parasite in the buffy coat fraction of blood samples, and to determine the packed cell volume (PCV) value that indicates the presence and degree of anemia. This method has higher sensitivity than microscopy-based techniques, but it does not identify the species of trypanosomes present, although it may give some indication of the motility of the parasite (Uilenberg and Boyt, 1998). Mini anion-exchange chromatography with diethylaminoethyl cellulose (DEAE-C) and inoculation of laboratory mice are also used for the detection of trypanosomes (Brun *et al*., 1998). Anti-trypanosome antibodies can be detected by employing serological diagnostic methods, including the trypanolytic test, card agglutination test (CATT), indirect fluorescent antibody test (IFAT), and enzyme-linked immunosorbent assay (ELISA) (Brun *et al*., 1998). Moreover, highly sensitive molecular methods have been developed to detect these parasites using the polymerase chain reaction (PCR)-based amplification of trypanosomal DNA (Brun *et al*., 1998).

T. evansi type A is characterized by the existence of the gene for the Rode *Trypanozoon* antigen type 1.2 (RoTat 1.2) VSG, which is expressed early during infections and allows anti-RoTat 1.2 VSG antibodies to be detected in animals infected with *T. evansi* type A. In contrast, *T. evansi*

type B lacks this gene and, consequently, infections with this type of the parasite cannot be detected with serological and molecular tests based on RoTat 1.2 VSG, such as the CATT/*T. evansi* and RoTat 1.2 PCR approaches (Birhanu *et al*., 2016).

The internal transcribed spacer (ITS) sequence of ribosomal DNA (rDNA) is a preferred target for common PCR tests because of highly conserved primer region with different amplicon sizes among trypanosome species. It is used to detect several different species of *Trypanosoma* (Njiru *et al*., 2005).

Treatment and control

Chemotherapy and chemoprophylaxis are the main methods used in the control of animal trypanosomosis. The selection of the drug, dosage, and way of application to be used for treatment depend on the animal species and the management applied in a certain area, along with the chemosensitivity of the trypanosome strain considered (Brun *et al*., 1998). Diminazene aceturate and isometamidium chloride are widely applied for the treatment of animal trypanosomosis (Holmes *et al*., 2004) in addition to suramin, which is widely applied for the treatment of *T. evansi* infections. However, the increasing in the number of reports of resistance to these drugs is worrying (Geerts *et al*., 2001; Delespaux and de Koning, 2007; Giordani *et al*., 2016). Most trypanocidal drugs have therapeutic rather than prophylactic activity, so the decision to use a drug for treatment or prevention depends on drug availability, cost, the risk of infection, and logistics (Giordani *et al*., 2016).

Generally, drugs should be used for the treatment of animals with confirmed infections and/or with a clinical disease attributable to trypanosomes in areas of low prevalence. On the other hand, in areas of high prevalence, application of drugs to the whole herd for prevention is more cost-effective, reduces morbidity and mortality, and helps to avoid the effects of infection on productivity (Gu *et al*., 1999; Giordani *et al*., 2016). Currently, trypaniocidal drugs are not used in combination for treatment of animal trypanosomosis. Instead, the alternating use of different drugs with low risks of cross-resistance, particularly diminazene and isometamidium, is recommended, where possible, in a case of relapse. In this case, the relapsed animal should be treated with a different type of drug from the one previously used, so as not to reinforce drug resistance (Giordani *et al*., 2016). However, this approach is not always practicable due to the chemical relationships between several trypanocidal drugs. Thus, to maintain the efficiency of currently available compounds, it is important that therapeutic and prophylactic dosage courses are rational and based on the drug-susceptibility of trypanosomes (Giordani *et al*., 2016).

The control of trypanosomosis cannot be well thought-out separately from the control of its vectors. Insecticides and traps, in addition to the sterile male technique, are the most commonly used methods of vector control that aim to reduce the fly population densities and, consequently, trypanosomosis incidence to levels at which cost-effective animal production are possible (Uilenberg and Boyt, 1998).

Currently, there is no vaccine against trypanosomosis. Trypanosomes have an unlimited ability to change their surface antigens, and do so frequently. This means that the antibodies produced against

some types of antigens will no longer be effective when new antigens appear, and the immune system in infected host has to start producing new antibodies for each new infection. Moreover, antigens differ among different trypanosome species, subspecies and strains of the same species. The fact that trypanosomosis can occur as a mixed infection of two or more different species should also be considered. However, with the rapid developments in molecular biology in recent years, the prospects for the use of vaccines against trypanosomes may change in the future (Uilenberg and Boyt, 1998).

2. Equine piroplasmosis

Definition and classification

Equine piroplasmosis, usually known as biliary fever, is an infectious acute, subacute, or chronic vector-borne disease. It is caused by *Theileria equi* and *Babesia caballi*, and transmitted by ticks. It affects all equids, including horses, donkeys, mules, and zebras (Schein, 1988; Friedhoff *et al*., 1990; De Waal, 1992).

Classification of piroplasms:

Phylum: Apicomplexa

Class: Aconoidacida

Order: Piroplasmida

Families: Theileriidae, Babisiidae

Genera: *Theileria, Babesia*

Life cycle and transmission

The life cycle of both piroplasms involves different stages occurring in the host and vector organisms. Infectious sporozoites (the asexual transmission stage) are transmitted through the saliva of the tick (vector) to the host. In the equine host, *B. caballi* sporozoites directly enter erythrocytes. In the erythrocytes, sporozoites multiply and differentiate into trophozoites and then develop into merozoites (the asexual blood stage). After erythrocytes rupture, the merozoites released and enter other new erythrocytes (Figure 3). *Th. equi* is different from *B. caballi* in that its sporozoites first enter peripheral blood mononuclear cells, where they develop first into large schizonts, then the mature merozoites are released and enter erythrocytes after approximately 9 days (Figure 4). For both parasites, asexual proliferation in the equine host increases the numbers of merozoites and infected erythrocytes. Some of those merozoites differentiate into gametocytes within the peripheral blood of the equine host. When the ticks ingest the merozoites (and/or gametocytes) with the blood meal, the parasites undergo sexual reproduction within the tick midgut. In this stage, the gametocytes develop into gametes, and zygotes are generated by the combination of male and female gametes. The zygotes development differs according to the parasite and the tick species. After 6–24 days of infection, the result of the development of the parasite is the presence of sporozoites in the salivary glands of the tick (Wise *et al*., 2013).

Iatrogenic transmission occurs through the use of infected blood-contaminated medical equipment, such as needle sharing between positive and negative animals. This type of transmission can also be caused by using infected animals as blood donors for non-infected animals. Transmission from infected mares to their fetuses across normal placentas has also been reported. (Wise *et al*., 2013).

Epidemiology

Equine piroplasmosis has a worldwide distribution within various equine populations occurring where disease vectors exist (Thompson, 1969). According to the information from the World Organization for Animal Health (OIE) about the geographic distribution of infected equines, the disease is endemic to Africa, the Middle East, Asia, Caribbean islands, Central and South America, and Southern Europe. The disease was identified in all regions within South America except the southernmost areas of Chile and Argentina. Equine piroplasmosis is widespread in African countries, and the highest prevalence was reported in South Africa. *Th. equi* is more prevalent than *B. caballi*, but because not all affected countries report all confirmed cases to the OIE, it is currently difficult to accurately understand the global distributions of these parasites (Wise *et al*., 2013).

Ixodid ticks (hard ticks) exist in tropical, subtropical, and some temperate climates (Sonenshine and Roe, 2013). Many ixodid tick species have been identified to be vectors that transmit

piroplasmosis. *B caballi* is transmitted by 15 different tick species (7 *Dermacentor* spp., 6 *Hyalomma* spp., and 2 *Rhiphicephalus* spp.), and *Th. equi* is transmitted by 14 species (4 *Dermacentor* spp., 4 *Hyalomma* spp., 5 *Rhiphicephalus* (*Boophilus*) spp., and *Amblyomma cajennense*). *B. caballi* is transmitted transstadially and transovarially by its vectors (De Waal, 1990; Wise *et al*., 2013). Transstadial transmission means that the tick is infected with the parasite in one life stage, and has the ability to transmit the infection during the following life stages. Transovarial transmission occurs when the female obtains parasites that then enter the ovaries and are consequently transmitted to the offspring, so that parasites are maintained across tick generations (Wise *et al.*, 2014). *Th. equi* generally undergoes transstadial and intrastadial transmission. Intrastadial transmission occurs when infection and transmission of the parasite happens within one life stage of the vector (with no stage transition before transmission). In this case, the parasite is preserved within the tick during its development (Ueti *et al*., 2008).

Clinical symptoms

After transmission, clinical signs progress within 12–19 days for *Th. equi* infection and 10–30 days for *B. caballi* infection, depending on such factors as the parasite infective dose and host immunity (De Waal, 1992). In endemic areas, the mortality rate of infected animals has been estimated to be 5%–10%, depending on the parasite species, infective dose, general health condition

of the infected animal, and application of drugs for treatment. Usually, the clinical disease results from the infection with *Th. equi* is more severe than that of *B. caballi* (Maurer, 1962).

Clinical signs and severity of the disease can vary significantly from one region to another. In most cases, the animals become mostly asymptomatic carriers, with a persistent infection that lasts for life with *Th. equi*, and possibly *B. caballi*, although some studies have indicated that the *B. caballi*- infected animal can go through self-clearance of the parasite without treatment (Wise *et al*., 2013). These persistent subclinical infections are assumed to be part of the parasite's immune evasion strategy. Different theories regarding the position of the parasite restoration in asymptomatic animals have been reported. The mechanism(s) of persistent infection is still remaining unknown. Infections in asymptomatic pregnant females can end with abortion or neonatal infection. Because these carriers may act as reservoirs for the disease via different modes of transmission, they are considered the biggest challenge to non-endemic countries that trying to avoid apparently healthy carriers from entering their borders (Wise *et al*., 2013). Introduction of non-infected animals into an endemic area can result in the rapid onset of severe, peracute disease, in which collapse and sudden death can occur (Basset *et al*., 1931).

Acute infections start with the development of nonspecific signs, including high fever, fatigue, loss of appetite, weight loss, and peripheral edema (Maurer, 1962). Then, followed by signs of hemolytic anemia, including yellow or pale mucous membranes, weakness, tachypnea, tachycardia, and pigmented urine (either hemoglobinuria or bilirubinuria) (Ambawat *et al*., 1999;

Zobba *et al*., 2008; Wise *et al*., 2013). Some infected animals show gastrointestinal complications signs, such as colic, impactions and diarrhea (Wise *et al*., 2013).

Chronic infections cause only nonspecific clinical signs such as fatigue, loss of appetite, loss of weight, and poor performance. Due to the increasing in the rate of extravascular hemolysis that takes place within the spleen in less severely affected animals, the spleen is enlarged, as detected by rectal palpation, and mild anemia also occurs (Allen *et al*., 1975; De Waal,1992; Friedhoff and Soulé, 1996; Wise *et al*., 2013).

Diagnosis

Different diagnostic methods can be used alone or in combination to diagnose infections with piroplasms. A Giemsa-stained thin blood smear, can be examined using a light microscope to identify the organisms within the erythrocytes during the acute phase of infection. The blood smears must be carefully examined because the parasitemia remains so low, even during severe infections, and it is not uncommon to get false-negative results (Friedhoff and Soulé, 1996; OIE, 2008; Wise *et al*., 2013). It is easy to distinguish *Th. equi* and *B. caballi* from each other. Inside erythrocytes, *B. caballi* looks like 2 large pyriform (pear-shaped) merozoites with approximately 2–5 μm in length, while *Th. equi* merozoites appear as polymorphic, small piroplasms, sometimes in a separate Maltese cross-formation. They are smaller than *B. caballi* and measure 2–3 μm in length. The percentage of infected erythrocytes during clinical infections caused by *B. caballi*, is less than 1%, and may be less

than 0.1%, while during clinical diseases caused by *Th. equi*, the percentage of infected erythrocytes is usually between 1 and 5%, but it can exceed 20% in severe cases. In chronic cases or unapparent infections, the numbers of parasites are usually too low for reliable detection in the blood smears (Wise *et al*., 2013).

Many serological tests have been developed to increase the sensitivity of piroplasmosis diagnosis, especially in carrier animals without any clinical signs. Such tests include IFAT, CFT, western blotting, and competitive ELISA (cELISA). Highly sensitive PCR tests, including real-time PCR, nested PCR, and nested PCR with hybridization, are increasingly used for the detection of piroplasms by amplifying and detecting specific fractions of their DNA (Wise *et al*., 2013).

Treatment and control

In endemic areas, piroplasmosis treatments are only used to decrease the occurrence or severity of clinical signs and reduce fatalities. The goal of such treatments is not the clearance of the organisms in these regions, as life-long immunity is supposed to interact with the chronic, asymptomatic infections. However, in non-endemic areas trying to stay free of piroplasmosis, treatment of infected animals with the purpose of achieving clearance is required. Treatment of *Th. equi* infections is more difficult than that of *B. caballi* infections (Wise *et al*., 2013). Several drugs have been used with success to relieve the clinical signs of piroplasmosis, among which imidocarb dipropionate is considered to be the most effective. This drug is administered

intramuscularly. Imidocarb dihydrochloride, the alternate form of imidocarb dipropionate, causes more severe damage of muscles at the site of injection (Frerichs *et al*., 1973; Meyer *et al*., 2005). Diminazene aceturate and diminazene diaceturate have also been successfully used against both parasites (Wise *et al*., 2013), but both of them have been reported to cause significant damage of the muscles at the site of injection. Diminazene aceturate is more effective than diaminazene diaceturate, and their efficacy increases with the second dose (Rashid *et al*., 2008). The intravenous administration of the antibiotic oxytetracycline at a dose of 5–6 mg/kg once daily for 7 days, was found to have efficacy against *Th. equi*, but it is not effective against *B. caballi* (Zobba *et al*., 2008; Wise *et al*., 2013). In addition to treatment with antiprotozoal drugs, animals with acute infections require supportive treatments, including intravenous administration of fluids, pain killers, nonsteroidal anti-inflammatory drugs, and blood transfusions. Sufficient hydration is crucial upon the start of and throughout the treatment with imidocarb (Wise *et al*., 2013).

Prevention of infection in endemic countries is almost impossible. In non-endemic countries, the most important means of prevention is the ruling of animals' movement. Animals must test serologically negative for these parasites at the importing country using CFT, IFAT or cELISA. The entrance of positive animals to another country is generally denied. All imported equids from endemic regions must go through firm quarantine, and must be carefully inspected for ticks. To confirm that ticks will not be introduced along with the animals to another country, treatment with acaricides is applied before the movement of animals from an endemic country. This controlling system, enacted by the OIE, has been successful, since only sporadic cases continue to arise in

non-endemic countries, and these are infrequently caused by tick transmission; rather, these cases are usually caused by the use of equipment that are contaminated with blood, and through practices containing the sharing of needles, or following blood transfusions from untested animals (Taylor *et al*., 1969; Mahoney *et al*., 1977; Short *et al*., 2012; Wise *et al*., 2013). There are no available vaccines to induce immunity against *Th. equi* and *B. caballi* infections.

The vaccination and treatment strategies applied in a region depend on the status of infection therein (endemic or non-endemic). In an endemic region, it would not be appropriate to use a transmission-blocking vaccine, while a vaccine that prevents transmission of parasites or clinical disease and death caused by them is needed for non-infected animals that are moved into endemic countries (Wise *et al*., 2013). Chemotherapeutics, which help in the control of acute parasitemia and the associated clinical signs without eliminating the infection, are important in endemic countries. In non-endemic countries, the goal is to keep the infection-free status of animals; thus, when infected animals are identified in such countries, a chemotherapeutic that can efficiently eliminate persistent infections is required. To keep on infection-free status, the essential methods include perfect screening diagnostic tests. Moreover, deep knowledge of vector tick populations, biology and their ability to transmit *Th. equi* and/or *B. caballi* is required. Increasing globalization of the equine industry, in addition to continuous changes in climates, provide great challenges for the control of persistent infections like those caused by *Th. equi* and *B. caballi*. Similar to all vector-borne diseases, protective methods must be followed to improve disease control over the surveillance of equine populations and obtaining detailed information of vector ability and environments. Detailed

knowledge about the life cycles, transmission, immune responses, proper diagnosis, and treatment of these parasites is necessary to control the important diseases caused by them, while also supporting the development of the movement of equines internationally for trade purposes (Wise *et al*., 2013).

3. Aim and objectives

Aim

The aim of this study was to detect and characterize trypanosomes and piroplasms in different animal species by performing serological tests and using molecular techniques to update the

epidemiological data of trypanosomosis and piroplasmosis in Sudan and the Philippines, while taking into consideration the role of apparently healthy and locally important animals as reservoirs in the epidemiology of these diseases.

Specific objectives

1. To update the prevalence of equine trypanosomosis and equine piroplasmosis in donkeys in Sudan by utilizing serological and molecular detection techniques.
2. To perform a PCR-based detection and identification of trypanosomes in the Visayas region of the Philippines, to assess the prevalence of trypanosome-caused diseases in different animal species in this area; and to assess the role of goats as reservoirs of trypanosomosis in the epidemiology of this disease in the Philippines.

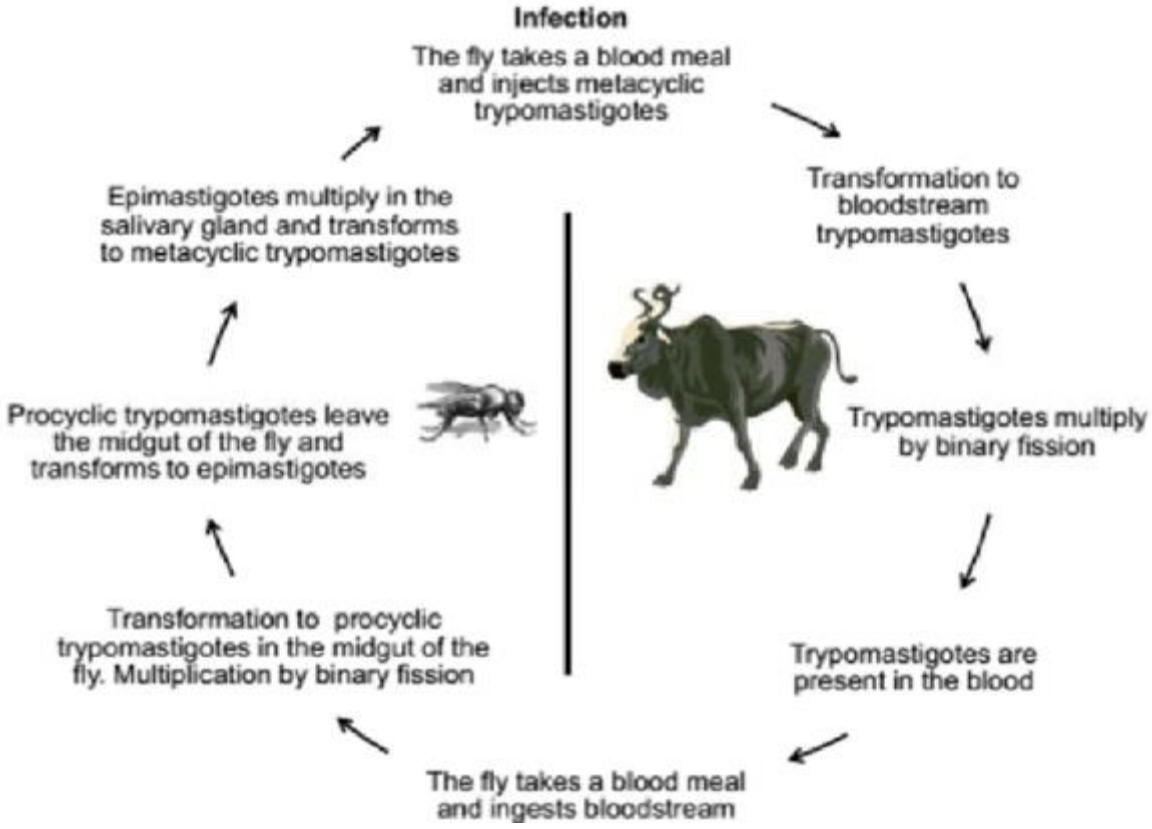

Figure 1: Life cycle of tsetse-transmitted *Trypanosoma* spp. (Dagnachew and Bezie, 2015)

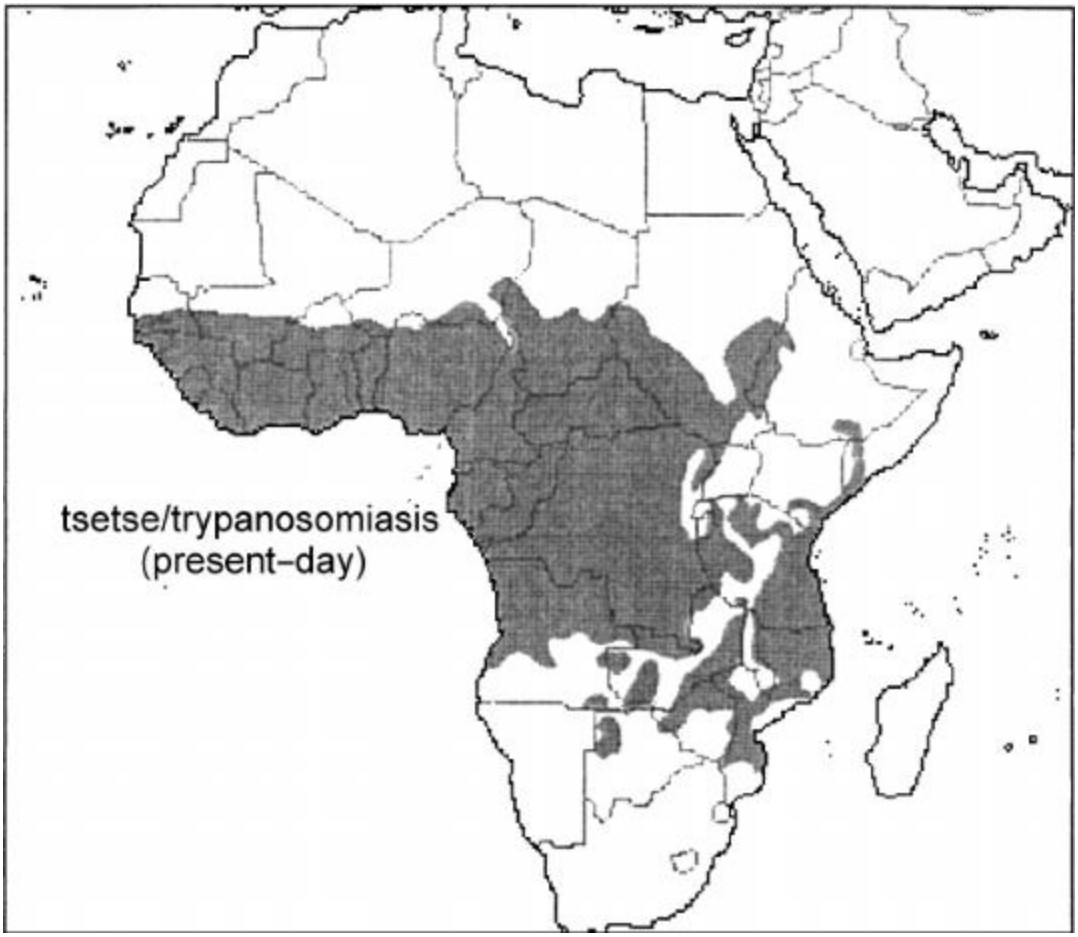

Figure 2: The distribution of tsetse flies in Africa over a range comprising 10 million km^2 between 14° North and 20° South latitude in 37 African countries (Gifford-Gonzalez, 2000)

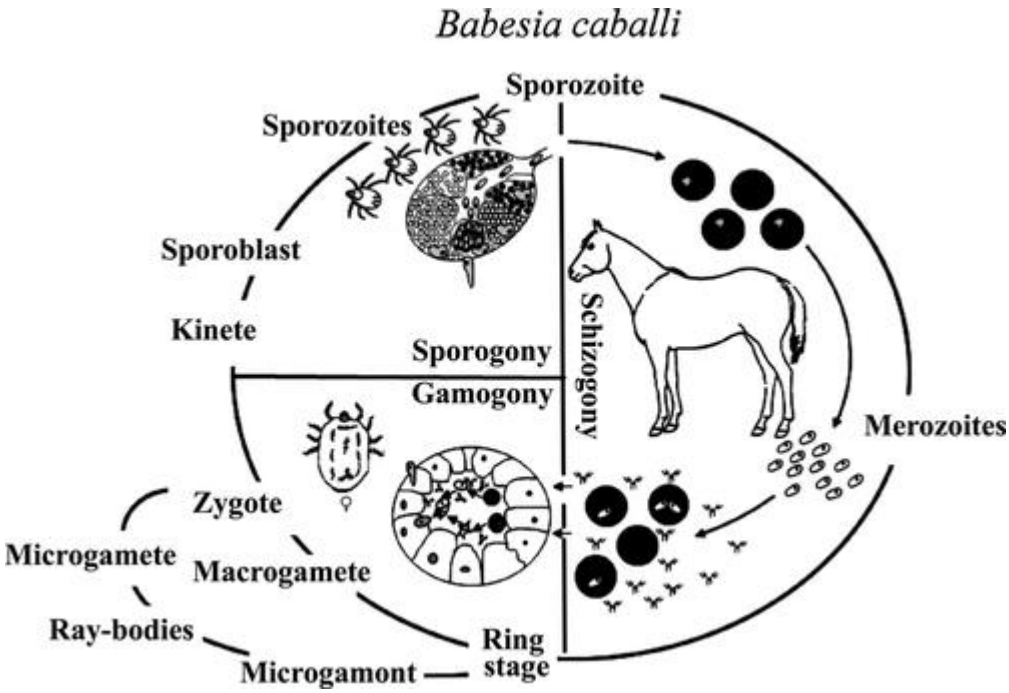

Figure 3: Life cycle of *Babesia caballi (*Wise *et al*., 2013)

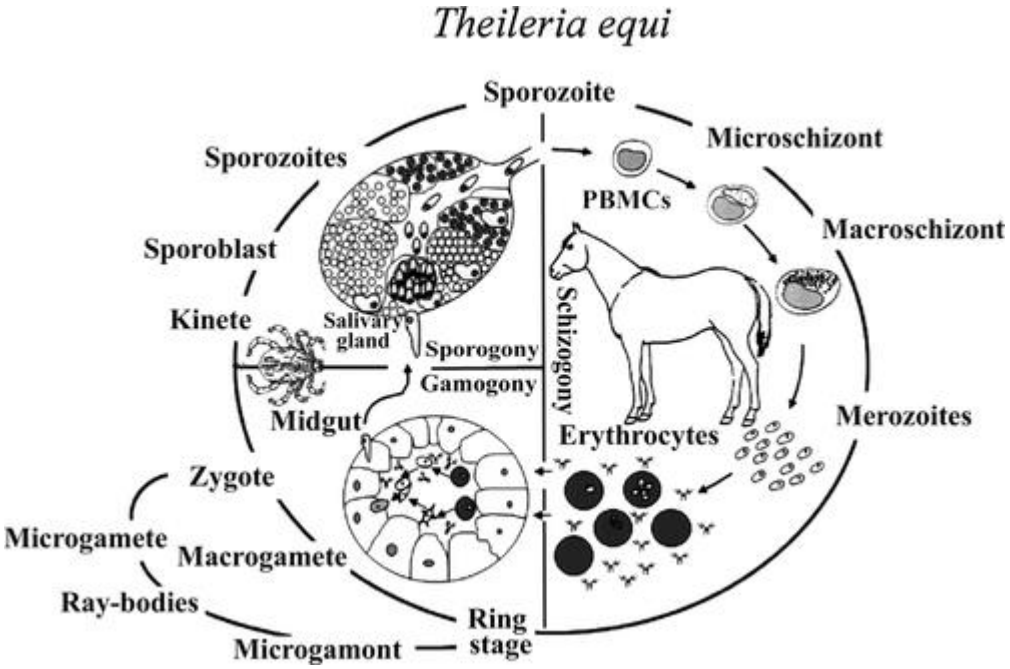

Figure 4. Life cycle of *Theileria equi (*Wise *et al*., 2013)

Chapter 1

Serological and molecular detection of selected hemoprotozoan parasitic infections in donkeys in Sudan

Introduction

The donkey (*Equus asinus*) is believed to have originated from Africa, where it was domesticated from the African wild ass (*Equus africanus*) in Nubia (Northern Sudan) by around 4000 BC (Clutton-Brock, 1999). The widespread use of donkeys in rural and urban areas of Africa for transportation and farm work indicates that these animals have an important role in the economies of many developing countries, including Sudan (Fielding, 1988). Sudan is home to approximately 7.51 million donkeys, meaning it has the fifth largest donkey population in Africa (OIE, 2018; FAO, 2014; MARF, 2009). These donkeys have a significant presence in agriculture and transport operations in urban areas and on the margins of cities, where poor people use donkeys as means of transportation and a source of daily income (Wilson, 2017). Donkeys have often been described as sturdy animals, so there is a perception that they do not require much care when they get sick, and their health problems are neglected in most parts of Sudan despite their huge contribution to the quality of human life (Sowar, 2006). However, donkeys are susceptible to a wide variety of diseases, for which they are usually asymptomatic carriers (Angara *et al.*, 2011).

Trypanosomosis and equine piroplasmosis are two of the most important hemoprotozoan parasitic diseases in equines. Donkeys seem to have the greatest resistance to tsetse-transmitted trypanosomosis among equids, but the disease becomes a clinical problem in them when accompanied by precipitating factors, such as the stress of work (Wilson, 2017). In early 1915, trypanosomes were found to cause trypanosomosis in a group of equines in Sudan, resulting in 100% mortality, owing to their use as transport animals in tsetse-infested areas (Webb, 1915). The parasite causing this outbreak was identical to *Trypanosoma brucei*. Thereafter, in the 1930s, *T. congolense* was identified as the causative agent of the disease in horses from tsetse-infested areas in Sudan (Bennett, 1936). Infection with mechanically-transmitted *T. evansi* was first diagnosed in horses in 1952 (El Karib, 1961). Dourine, a type of sexually transmitted trypanosomosis caused by *T. equiperdum*, was identified in a donkey mare in Nyala, Western Sudan, in 1961 (Uilenberg, 1961), although World Organization for Animal Health (OIE) data indicate that this disease has never been recorded in Sudan (OIE, 2018). This disease has not been reported again since this first report. Recently, *T. brucei* subspecies, *T. vivax*, *T. simiae*, and *T. congolense* have been reported in equines in Sudan (Salim *et al*., 2014). Although trypanosomosis is usually reported in veterinary clinics in Sudan, its epidemiology is still unclear, particularly in donkeys. Importantly, trypanosomosis can contribute to reductions in the strength and survival of donkeys (Svendsen *et al*., 1997). Moreover, one report described a significant association between trypanosome infection and mean body condition score in donkeys (Mukiria *et al*., 2010).

Equine piroplasmosis is an OIE notifiable disease in Sudan (Wilson, 2017), and it was first reported in Sudan in 1907 as biliary fever, with the conclusion that *Theileria equi* was more prevalent than *Babesia caballi* in Sudan (Salim *et al*., 2008; Oliver, 1907). Recent studies have reported the occurrence of equine piroplasmosis in different parts of Sudan (Salim *et al*., 2013). Microscopic examination of Giemsa-stained blood smears for the detection and identification of equine piroplasmosis- and trypanosomosis-causing protozoa has low sensitivity, particularly in cases with low parasitemia (Muieed *et al*, 2011; Krause, 2003; Seifi *et al*., 2000). Thus, serological and molecular techniques have been shown to be more accurate diagnostic methods for the detection of equine piroplasmosis (Persing and Conrad, 1995) and trypanosomosis (Gari *et al*., 2010).

Previous studies done on equine piroplasmosis in Sudan either were performed in horses only (Salim *et al*., 2008), or included a few donkeys from some different parts of Sudan, but not from Khartoum State (Salim *et al*., 2013). Few donkeys from Khartoum State were included in another previous study on the molecular detection of trypanosomosis in Sudan (Salim *et al*., 2014). Therefore, I conducted this study to provide an update on the prevalence of trypanosomosis and equine piroplasmosis in donkeys in Sudan, particularly in Khartoum State, by utilizing serological and molecular diagnostic techniques.

Materials and methods

Study area and sample collection

For this study, two batches of a total of 465 samples were collected. The first batch of samples was obtained from 198 donkeys in a local market in West Omdurman, Khartoum State, Sudan, in 2016. The second batch of samples was obtained from 267 donkeys in different parts of Sudan in 2019 (Figure 5). The numbers of samples that were obtained from different locations in Sudan are shown in Table 1 (these data are presented with consent from the donkey owners). Briefly, 8 ml of blood was drawn from the jugular vein of each donkey, of which 3 ml was stored in vacutainer tubes with ethylenediaminetetraacetic acid (EDTA) (Terumo, Japan) for DNA extraction, and 5 ml was stored in plain vacutainers (Terumo, Japan) for serum separation. Sera were separated by centrifugation into 1.5 ml tubes and kept at -20 °C until further use. The genomic DNA of each sample of the first batch was extracted from whole blood samples after loading onto Whatman™ FTA™ Elute Cards (GE Healthcare, USA), according to the manufacturer's instructions, while a DNAzol® extraction kit (Molecular Research Center, USA) was used for the second batch. This study was performed with permission from and in accordance with the standards for animal experimentation of Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan (Approval No. 29-2, 18-18, 19-19).

Card agglutination test for *Trypanosoma evansi* (CATT/*T. evansi*)

CATT/*T. evansi* was used for the detection of anti-salivarian trypanosome antibodies in the first batch of serum samples, according to the manufacturer's instructions (Institute of Tropical Medicine, Belgium) and the OIE manual (OIE, 2012). Briefly, following the method of Verloo *et al*., 2000, 25 µl of serum diluted by 100 µl of CATT diluent was allotted onto the reaction zone of a test card. Approximately 45 µl of CATT reagent was added to the serum and mixed. Using a stirring rod, the mixture was then spread, and the card was put on a CATT rotator (Institute of Tropical Medicine, Belgium) for 5 min at 70 rpm to allow the mixture to react. A sample was considered positive when a blue agglutination was observed (Songa and Hamers, 1988; Verloo *et al*., 2000).

Enzyme-linked immunosorbent assay (ELISA)

T. evansi crude antigen-based ELISA (TeCA-ELISA) and recombinant *T. evansi* GM6-based ELISA (rTeGM6-4r-ELISA) were employed for serological surveillance of trypanosome antibodies in all serum samples. For tests of trypanosomosis, according to OIE manual, *T. evansi* cell lysate crude antigen (TeCA) was prepared (OIE, 20018). And according to Nguyen *et al*., 2014, the rTeGM6-4r was produced and its ELISA was conducted (Nguyen *et al*., 2014). For tests of equine piroplasmosis, merozoite antigen 2 (EMA-2)- and 48 kDa merozoite rhoptry protein (BC48)-based ELISAs were performed as described previously (Xuan *et al*., 2001) for the detection of *Th. equi* and *B. caballi*, respectively.

The optical density at 450 nm (OD_{450}) was assessed 5 min after the addition of the substrate and immediately after stopping the reaction, using a Glomax Multi detection system microplate reader (Promega, Japan).

PCR-based identification of parasites

Two different PCR techniques were applied for the detection and species identification of trypanosome in the first batch of donkeys: (i) single-step ITS1 PCR, which amplified the internal transcribed spacer-1 (ITS1) region and identify three major trypanosome species (*Trypanozoon*, *T. congolense*, and *T. vivax*) (Njiru *et al*., 2005); and (ii) RoTat1.2 VSG PCR (*T. evansi* type A-specific), which specifically amplified the gene encoding the Rode *Trypanozoon* antigen type 1.2 (RoTat 1.2) variable surface glycoprotein (VSG) in *T. evansi* (Claes *et al*., 2004). For the detection and identification of trypanosome DNA in the second batch of donkeys, only ITS1 PCR was employed. All primer sequences used in PCR in this study are listed in Table 2.

ITS1 PCR was performed in a total reaction volume of 10 µl, including 2 µl of 5× Phusion® HF reaction buffer (with 1.5 mM $MgCl_2$ included in the final concentration), 0.8 µl of 250 µM dNTPs, 0.1 µl of Phusion® DNA polymerase (New England BioLabs, USA), 1 µl of each 10 µM forward and reverse primer, 5.1 µl of double-distilled water, and 1 µl of the DNA sample. The PCR conditions were as follows: an initial denaturation step at 98 °C for 30 s; followed by 35 cycles of

amplification with denaturation at 98 °C for 10 s, annealing at 64 °C for 30 s, and extension at 72 °C for 30 s; and a final extension step at 72 °C for 2 min.

RoTat1.2 VSG PCR was also performed in a total reaction volume of 10 µl containing 1 µl of 10× reaction buffer, 0.3 µl of 50 mM $MgCl_2$, 0.8 µl of 250 µM dNTPs, 0.1 µl of Taq DNA polymerase (Invitrogen, Thermo Fisher Scientific Inc., USA), 0.5 µl of each 10 mM forward and reverse primer, 5.8 µl of double-distilled water, and 1 µl of the DNA sample. The PCR conditions were as follows: an initial denaturation step at 94 °C for 3 min; followed by 40 cycles of amplification with denaturation at 94 °C for 30 s, annealing at 57 °C for 30 s, and extension at 72 °C for 60 s; and a final extension step at 72 °C for 10 min.

Two more PCR techniques were employed to detect and identify piroplasms in all donkeys: (i) EMA-1 PCR, which specifically amplified the gene encoding *Th. equi* merozoite antigen 1 (EMA-1) (Alhassan *et al*., 2005); and (ii) BC48 PCR, which amplified the gene encoding the 48 kDa rhoptry protein of *B. caballi* (BC48) (Alhassan *et al*., 2005). All primer sequences used in PCR in this study are listed in Table 2.

All reactions were carried out in a Veriti thermal cycler (Thermo Fisher Scientific, USA), and gel-electrophoresed using 2% agarose gels, which then stained with 0.1% ethidium bromide, and visualized under ultraviolet light.

DNA cloning and sequencing

For the first batch of samples, the ITS1, RoTat1.2 VSG, EMA-1, and BC48 amplicons were extracted from agarose gels using a QIAamp gel extraction kit (Qiagen, Germany), cloned into cloning vector using Zero Blunt TOPO (for ITS1) and TOPO TA (for RoTat1.2 VSG, EMA-1, and BC48) cloning kits (Invitrogen, Thermo Fisher Scientific Inc., USA), respectively, according to the manufacturer's instructions. These vectors transformed into chemically competent *Escherichia coli* cells (One Shot Mach1; Thermo Fisher Scientific Inc., USA). After checking several colonies for each PCR-cloned product by colony PCR, plasmid DNAs were purified from 18, 7, 4, and 1 positive clones of ITS1, RoTat1.2 VSG, EMA-1, and BC48, respectively, using a QIAprep Spin Miniprep kit (Qiagen, Germany). Approximately 100–200 ng/µl of purified plasmid DNAs were sequenced using a Big Dye Terminator kit (Applied Biosystems, USA). The sequencing PCR conditions consisted of an initial denaturation step at 96 ℃ for 1 min, followed by 25 cycles of amplification with denaturation at 96 ℃ for 10 s, annealing at 50 ℃ for 5 s, and extension at 60 ℃ for 2 min. The PCR products were ethanol-precipitated and dissolved in a 20 µl Hi-Di formamide solution before DNA sequencing, according to manufacturer's instructions.

The same procedure was followed for the ITS1 and BC48 amplicons from the second batch of samples, and 5 ITS1, and 2 BC48 clones from this batch were then selected for use in plasmid DNA purification and sequencing using the abovementioned protocols.

Sequencing analysis

The ITS1, RoTat1.2 VSG, EMA-1, and BC48 sequences obtained from the first batch of donkey DNA samples and the ITS1, and BC48 sequences obtained from the second batch of donkey DNA samples were aligned with published sequences from the GenBank database using the Basic Local Alignment Search Tool (BLAST), and their phylogenetic trees were then constructed using the maximum likelihood method implemented in Mega software (version 7). All sequences obtained in this study were deposited in GenBank under accession numbers LC492114 to LC492131, LC493166 to LC493172, LC514705 to LC715709, LC546936 to LC546940, and LC554413 to LC554414.

Results

Seroprevalence of hemoprotozoan parasites

CATT/*T. evansi*

Out of the 198 donkeys investigated in this study, anti-salivarian trypanosome antibodies were detected in 52 (26.3%) samples using CATT/*T. evansi*.

ELISA

Cutoff values (mean ± 3 standard deviations) for rTeGM6-4r-, TeCA-, EMA-2-, and BC48-based ELISAs for the first batch of samples were calculated from the OD_{450} values of four negative control sera, which were obtained from commercial sources (Sigma-Aldrich, USA; Jackson Immuno Research Laboratories, USA.; Southern Biotech, USA; Immuno Bio Science Corp., USA), yielding values of 0.30, 0.56, 0.69, and 1.27, respectively. Notably, out of the 198 donkeys investigated, 19 (9.6%) and 56 (28.3%) were found to be positive for anti-salivarian trypanosome antibodies using rTeGM6-4r- and TeCA-based ELISAs, respectively. In contrast, 156 (78.8%) and 10 (5.1%) were found to be positive for *Th. equi* and *B. caballi*, respectively (Figure 6).

For the second batch of samples, cutoff values (mean ± 3 standard deviations) for rTeGM6-4r-, TeCA-, EMA-2-, and BC48-based ELISAs were calculated from the OD_{450} values of the same four negative control sera as above, yielding values of 1.45, 0.71, 1.70, and 1.04, respectively. Out of 267 samples, 75 were found to be positive for trypanosome antibodies using

rTeGM6-based ELISA (28.1%), while TeCA-based ELISA detected trypanosome antibodies in 101 samples (37.8%). Meanwhile, 142 (53.2%) and 68 (25.5%) samples were found to be positive for *Th. equi* and *B. caballi* antibodies as detected by EMA-2- and BC48-based ELISAs, respectively (Figure 7). The seroprevalence of trypanosomes and piroplasms in different sampling locations is summarized in Table 3.

Molecular detection of hemoprotozoan parasites

Out of the 198 samples analyzed using ITS1 PCR, the DNA of *Trypanozoon* (~480 bp) and *T. congolense* (~700 bp) was detected in 77 (38.9%) and 18 (9.1%) samples, respectively. Moreover, 5 (2.5%) samples showed evidence of mixed infections with *Trypanozoon* and *T. congolense* (Figure 8). Additionally, 35 (45.5%) of the 77 *Trypanozoon*-positive samples were found to be positive using RoTat1.2 PCR, confirming that they represented *T. evansi* type A (~205 bp) (Figure 9). Regarding piroplasms, species-specific PCR assays showed that 18 (9.1%) and 8 (4%) out of 198 samples were positive for *Th. equi* (~750 bp) and *B. caballi* (~610 bp), respectively (Figure 10). On the other hand, out of the 267 samples of the second batch, the DNA of *Trypanozoon* (~480 bp) was detected in 68 (25.5%) samples using ITS1 PCR (Figure 11), while the DNA of *Th. equi* (~750 bp) and *B. caballi* (~610 bp) was detected in 4 (1.5%) and 11 (4.1%) samples, respectively (Figure 12). A summary of the PCR results of all 465 samples collected in different parts of Sudan is provided in Table 4.

Sequencing analysis

In the first batch of samples, confirmation of the ~480 and ~700 bp PCR products as *Trypanozoon* and *T. congolense* savannah type, respectively, was achieved by the selection, cloning, and sequencing of the ITS1 region of 18 positive samples (nine samples of each species). The sequence similarity and pairwise distance values among trypanosomes obtained in this study and reference sequences of trypanosomes from GenBank confirmed the presence of the subgenus *Trypanozoon* (Figure 13; GenBank accession numbers LC492114, LC492115, LC492116, LC492117, LC492118, LC492119, LC492120, LC492121, and LC492122) and *T. congolense* savannah (Figure 14; GenBank accession numbers LC492123, LC492124, LC492125, LC492126, LC492127, LC492128, LC492129, LC492130, and LC492131). Seven positive ~205 bp PCR products were selected for use in the cloning and sequencing of the RoTat1.2 VSG region, which confirmed that they represented *T. evansi* type A (GenBank accession numbers LC493166, LC493167, LC493168, LC493169, LC493170, LC493171, and LC493172). In addition, selection, cloning, and sequencing of the EMA-1 region of 4 samples positive for the ~750 bp PCR product and the BC48 region of one sample positive for the ~610 bp PCR product confirmed the infection etiology as *Th. equi* (GenBank accession numbers LC514705, LC514706, LC514707, and LC715708) and *B. caballi* (GenBank accession number LC715709), respectively.

Moreover, selection, cloning, and sequencing of the ITS1 and BC48 regions of the 5 and 2 positive samples from the second batch, respectively, confirmed that the ~480 and ~610 bp PCR products represented the sequences of *Trypanozoon* (GenBank accession numbers LC546936,

LC546937, LC546938, LC546939, and LC546940), and *B. caballi* (LC554413, and LC554414), respectively.

Discussion

Donkeys are common livestock animals in Sudan, with there being an estimated 7.51 million donkeys in the country, with an annual increase in their numbers there of approximately 3% (MARF, 2009). Donkeys are used for transportation, packing, pulling carts, farming, raising water, and milling (Starkey and Starkey, 1997). Although donkeys can acquire a variety of diseases, they are hardy animals and usually remain asymptomatic carriers of infections (Angara *et al*., 2011). The number of donkeys in Khartoum, the capital city of Sudan, is declining with urbanization (Rabeh, 2009). However, donkeys remain essential for transportation, particularly in rural areas and on the margins of the three cities of Khartoum State (Angara *et al*., 2011).

In this study, I evaluated donkeys in different parts of Sudan, including Khartoum State, due to the general neglect of the health of these animals therein despite their considerable economic contributions to human life, as they ultimately allow individuals and families to increase their income and avoid poverty.

PCR of the ITS1 DNA sequence was used in this study for parasite detection. The ITS1 region of rDNA is a preferred target for the detection of trypanosomes because of its highly conserved flanking regions and variable size among trypanosome species (Njiru *et al*., 2005). Trypanosomosis has been reported to be more prevalent in horses than in donkeys in Sudan because only *T. vivax* was detected in donkeys, whereas *Trypanozoon*, *T. congolense*, *T. vivax*, and *T. simiae* were detected in horses in different parts of the country (Salim *et al*., 2014). Herein, I observed a prevalence of 38.9% for *Trypanozoon* (77/198) and 9.1% for *T. congolense* (18/198) in donkeys in

West Omdurman, Khartoum State, Sudan, and a prevalence of 25.5% for *Trypanozoon* (68/267) in donkeys in different parts of Sudan. Other PCR products (~250, ~280, ~400, and ~600 bp in size, suspected to represent *T. vivax*, *T. godfreyi*, *T. simiae*, and *T. congolense kilifi*, respectively) were also detected by ITS1 PCR. Only the prevalence of *Trypanozoon* and *T. congolense* savannah was confirmed by DNA sequence analysis in the present study. Sequencing of other PCR products did not result in confirmation of the presence of the suspected trypanosomes.

In the second batch of samples, which were collected from different parts of Sudan, the highest prevalence of *Trypanozoon* in donkeys was found in Nyala, Western Sudan, and was 85% (34/40), followed by 20% in Atbara, Northern Sudan (5/25), 14.4% in Kassala, Eastern Sudan (18/125), and 14.3% in Khartoum State (11/77). This follows the same pattern in prevalence that was detected previously in horses in Sudan in a study that reported that the highest prevalence of trypanosome DNA in horses occurred in Western Sudan, followed by Eastern Sudan, and then Khartoum State (Salim *et al*., 2014).

T. congolense and *T. vivax* were previously detected in equines, camels, and dogs in non-tsetse-infested areas in Sudan (Salim *et al*., 2014; Mossaad *et al*., 2017a, 2017b), suggesting that these parasites have a mechanical transmission mechanism there. Mechanical transmission of *T. congolense* by African tabanids has been demonstrated experimentally under natural conditions (Desquesnes and Dia, 2003b). Moreover, after the referendum separating South Sudan from Sudan in 2011, the movement of animals between the two countries has contributed to the spread of trypanosomiasis in tsetse-free areas (Ahmed *et al.*, 2016). These factors may explain the presence of

T. congolense in donkeys from Khartoum, which I reported for the first time herein, as this is a region that is hundreds of kilometers away from the nearest tsetse-infested areas. In Sudan, RoTat1.2 VSG PCR has been used for the confirmation of *T. evansi* infection in camels (Salim *et al*., 2011; Mossaad *et al*., 2017a). This is the first report of the detection of *T. evansi* type A in donkeys using RoTat1.2 VSG PCR in Sudan. Notably, the RoTat1.2-negative samples found could contain *T. evansi* type B or *T. brucei*.

Various serological tests, including indirect fluorescent antibody tests, ELISA, and CATT/*T. evansi*, have been introduced into laboratory and field use for the detection of trypanosome-specific antibodies. Only CATT/*T. evansi* can be used in the field. This method classifies truly infected animals, implying that it can be applied in targeting individual animals for treatment with trypanocidal drugs. ELISA is suitable for verifying the disease-free status of animals because it can correctly classify uninfected animals (OIE, 2018). The OIE recommends serial testing with CATT and ELISA, followed by the retesting of suspected animals, before they can be declared to have disease-free status. Furthermore, it is preferable to confirm these findings by PCR. In the present study, out of the 198 samples collected in the first batch, 52 (26.3%), 19 (9.6%), and 56 (28.3%) serum samples were found to be positive using CATT/*T. evansi*, rTeGM6-4r-based ELISA, and TeCA-based ELISA, respectively. Further, out of the 267 samples in the second batch, 75 (28.1%) and 101 (37.8%) serum samples were found to be positive for trypanosome antibodies using rTeGM6- and TeCA-based ELISAs, respectively. The detection performance of rTeGM6- and TeCA-based ELISAs for the detection of trypanosome antibodies in animals was previously reported

to be relatively similar (Nguyen *et al.*, 2015). However, crude antigen-based ELISA showed high cross-reactivity with *Theileria*- and *Babesia*- infected serum samples from water buffaloes (Nguyen *et al.*, 2014). In this study, the number of positive trypanosome-infected sera detected with TeCA-based ELISA in donkeys was higher than the number detected with rTeGM6-4r-based ELISA. In contrast, antibodies and DNA of *Th. equi* and *B. caballi* were detected in these donkeys. Thus, the possibility of cross-reactivity may explain the differences found in the detection performances of different ELISAs.

In the first batch of samples investigated in this study, I found that 8/198 (4%) and 10/198 (5.1%) samples were positive for *B. caballi* using PCR and ELISA, respectively, whereas 18/198 (9.1%) and 156/198 (78.8%) samples were positive for *Th. equi* in West Omdurman, Khartoum State. These findings indicated that the prevalence of *Th. equi* was higher than that of *B. caballi* in donkeys in Khartoum, as detected by both serological and molecular techniques. However, among the second batch of samples, the DNA of *Th. equi* and *B. caballi* was detected in 4/267 (1.5%) and 11/267 (4.1%) samples, respectively, indicating a higher prevalence of *B. caballi* in donkeys in different parts of Sudan, which is different from the prevalence of 25.2% and 0% of *Th. equi* and *B. caballi*, respectively, that was previously detected in horses in different parts of Sudan (Salim *et al.*, 2008). In contrast, 142/267 (53.2%) and 68/267 (25.5%) samples were found to be positive for *Th. equi* and *B. caballi* antibodies as detected by EMA-2- and BC48-based ELISAs, respectively. The highest seroprevalence of *Th. equi* was found in Atbara, Northern Sudan, and was 100% (25/25), followed by 72.5% in Nyala, Western Sudan (29/40), 45.5% in Khartoum State (35/77), and 42.4%

in Kassala, Eastern Sudan (53/125). A 100% prevalence of *Th. equi* antibodies was reported in horses in Atbara, Northern Sudan, in a previous study that also found the highest prevalence of *B. caballi* antibodies in horses in Atbara, Northern Sudan (Salim *et al.*, 2008). According to the present study, the highest prevalence of *B. caballi* antibodies in donkeys (87.5%) was detected in Nyala, Western Sudan (35/40), followed by 84% in Atbara, Northern Sudan (21/25), 9.1% in Khartoum State (7/77), and 4% in Kassala, Eastern Sudan (5/125). These findings indicated the high prevalence of piroplasm antibodies in donkeys in all sampling areas, from which the widespread of equine piroplasmosis in donkeys in Sudan can be concluded. On the other hand, it was found that the prevalence of *Th. equi* antibodies is higher than that of *B. caballi* antibodies, which could be due to two reasons. First, the distribution of vector, as *Hyalomma anatolicum anatolicum* is the most abundant species of ticks associated with equines in Sudan, and could be more important as a transmission vector of *Th. equi* than *B. caballi* (Salim *et al.*, 2008). The second possible reason for the lower prevalence of *B. caballi* could be the quick dissemination of this parasite after acute infection (Frerichs *et al.*, 1969; Salim *et al.*, 2008), which is also supported by the difficulty of detection of *B. caballi* in blood smears at all stages of the infection except the early acute phase of the disease (Todorovic and Carson, 1981).

In general, the seroprevalence of piroplasms in donkeys in Sudan, as detected by ELISA, was higher than the prevalence of these parasites detected by PCR, potentially because of chronic infections causing the parasitemia to be below the detection limit of PCR. Similar findings have been reported in Mongolia, where piroplasms were detected in 51.2% and 81.6% of horses by PCR

and ELISA, respectively (Munkhjargal *et al*., 2013). The low correlation between the results of serological and molecular methods for the detection of equine piroplasms could be explained by the observation that these methods detect different entities, and therefore differ in principle. Thus, PCR is considered a reliable diagnostic method for active infections, whereas serological tests are more reliable for the detection of persistent and past infections (Kappmeyer *et al*., 1999; Mahmoud *et al*., 2015). A similar pattern of *Th. equi* infection rates being higher than those of *B. caballi* was previously reported in horses in different parts of Sudan, and the prevalence was higher when using ELISA than when using PCR, although no donkeys were included in that study (Salim *et al*., 2008). Another molecular surveillance study was conducted in five states in Central, Eastern, and Western Sudan, including Khartoum State, using blood samples from horses and donkeys. In that study, 22% of horses in Khartoum State were PCR-positive for equine piroplasmosis, but no donkeys from Khartoum State were examined (Salim *et al*., 2013). The present study targeted donkeys only, and I provided basic information on the prevalence of equine piroplasmosis among them. These findings could help in the development of future disease control strategies.

Summary

The high prevalence of trypanosomosis and equine piroplasmosis in donkeys was documented in the study area. Different causative agents of these diseases were identified, and some agents, such as *Trypanosoma congolense* savannah and *T. evansi* type A, were reported for the first time in donkeys in Sudan. Moreover, the detection of the tsetse-transmitted *T. congolense* savannah in donkeys in West Omdurman, Khartoum State, should alert veterinary authorities of the possibility that this parasite could be mechanically transmitted to other susceptible animal species. Thus, donkeys should be included in any control strategies for trypanosomosis and piroplasmosis in the future, and should be given more veterinary care in general in Sudan.

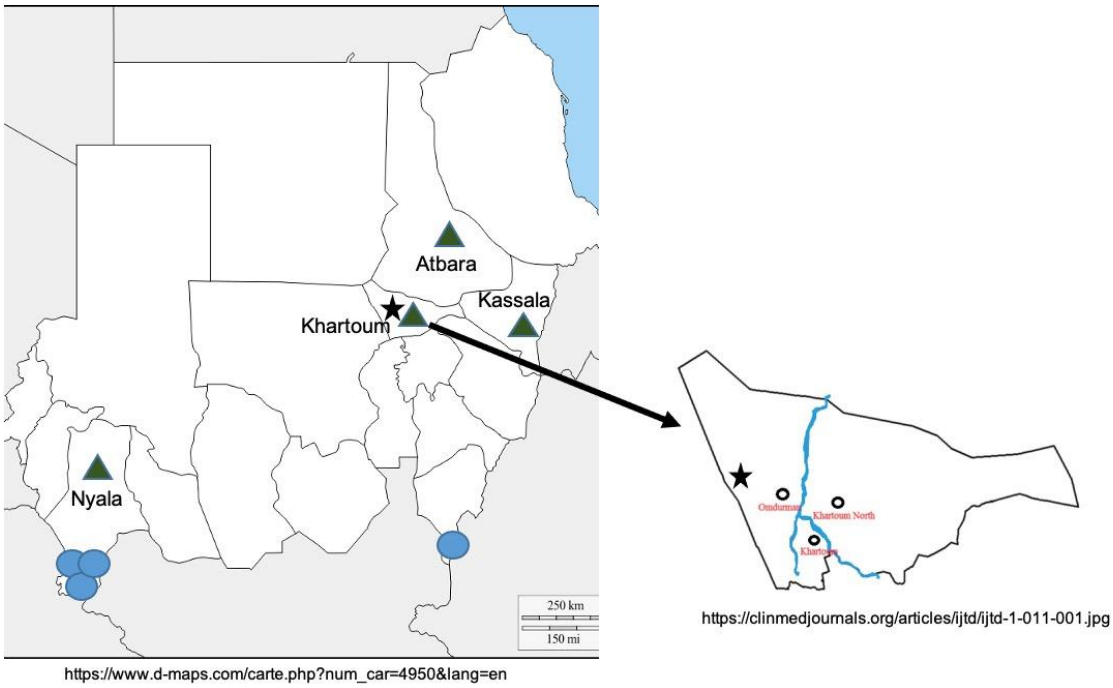

Figure 5: Map of Sudan showing the sampling locations in different parts of the country where blood samples were obtained from donkeys. The location of the local market in West Omdurman, Khartoum State, where the first batch of samples was collected, is indicated by the black star. The blue circles represent the tsetse-infested areas in Sudan.

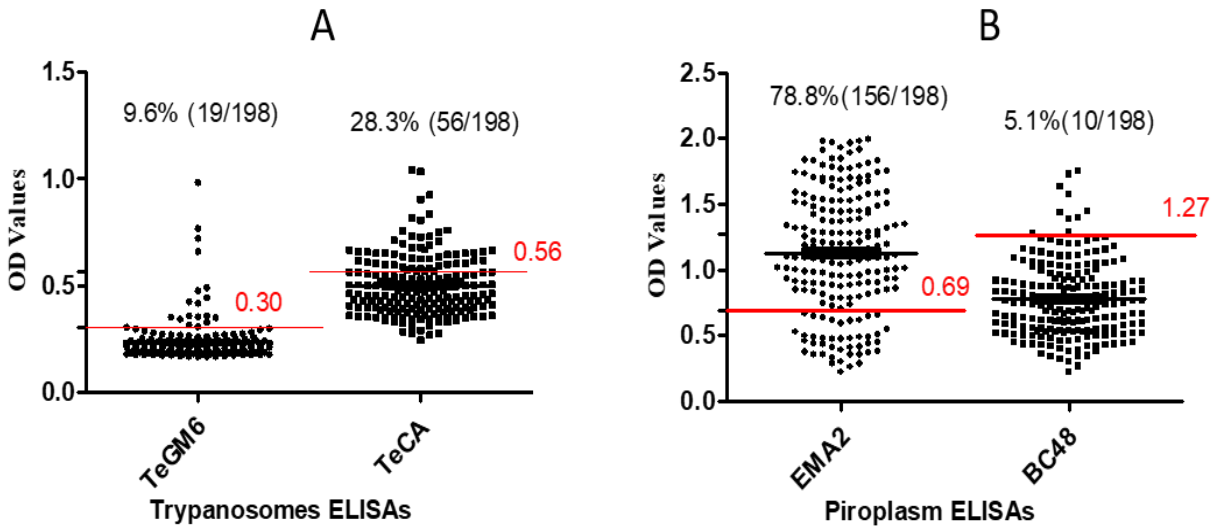

Figure 6: Detection of anti-salivarian trypanosome antibodies (A) and anti-piroplasm antibodies (B) in the first batch of donkey samples using ELISA. Cutoff values for the ELISAs are indicated in red.

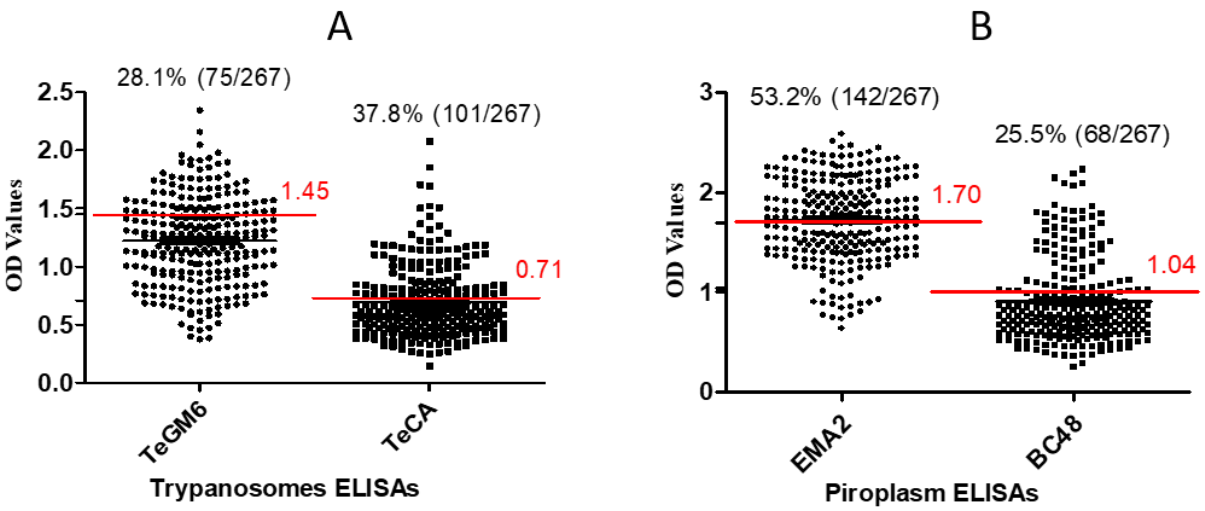

Figure 7: Detection of anti-salivarian trypanosome antibodies (A) and anti-piroplasm antibodies (B) in the second batch of donkey samples using ELISA. Cutoff values for the ELISAs are indicated in red.

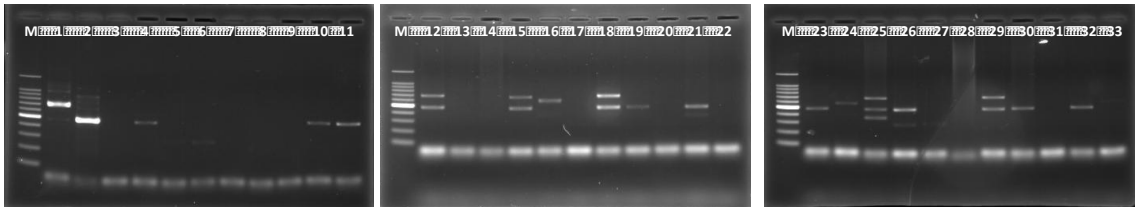

Figure 8: Detection of *Trypanosoma* spp. in DNA samples from donkeys using ITS1 PCR. Results were analyzed on 2% agarose gels stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*T. congolense*); lane 2, positive control (*T. evansi*); lane 3, negative control; lanes 4, 10, 11, 19, 21, 23, 26, 30, and 32, samples positive for *Trypanozoon* (~480 bp); lanes 12, 15, 18, and 29, mixed infections with *Trypanozoon* and *T. congolense* (~700 bp); lanes 16 and 24, suspected *T. congolense kilifi* (~600 bp); faint bands in lanes 5, 6, 26, and 27 (~250 bp) and in lane 21 (~400 bp), suspected *T. vivax* and *T. simiae*, respectively; lane 25, mixed infection with *T. congolense*, *Trypanozoon*, and suspected *T. simiae*; lanes 7, 8, 9, 13, 14, 17, 20, 22, 28, 31, and 33, negative samples.

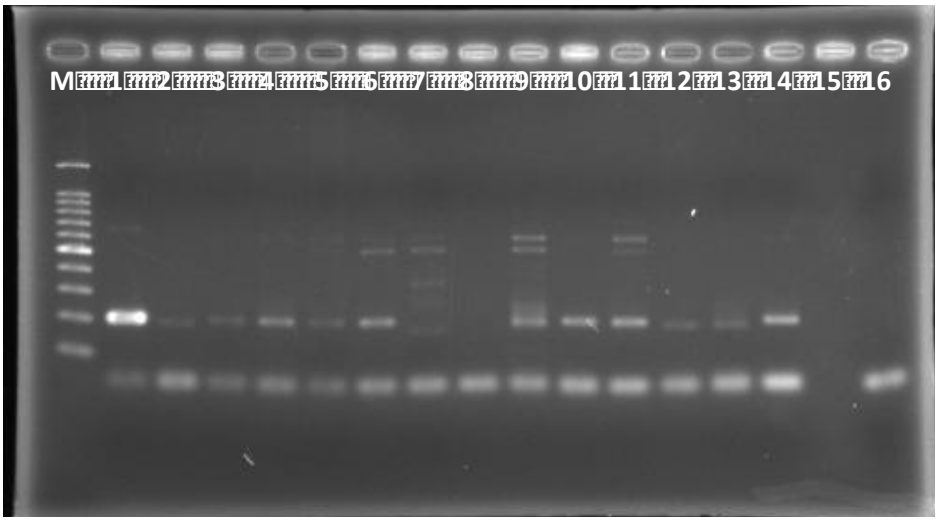

Figure 9: Detection of *T. evansi* type A in *Trypanozoon*-positive DNA samples from donkeys using RoTat1.2 PCR. Results were analyzed on 2% agarose gels stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*T. evansi* type A); lane 16, negative control; lanes 2–6 and 9–14, positive samples; lanes 7 and 8, negative samples. Other bands are nonspecific.

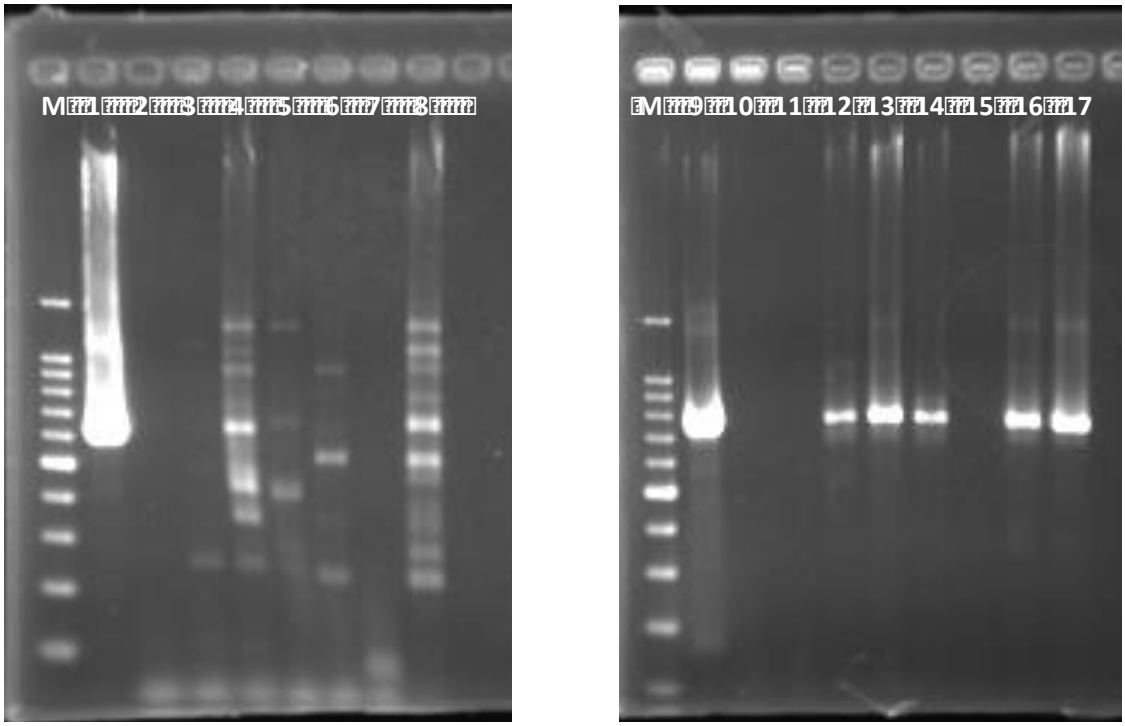

Figure 10: Detection of piroplasms in DNA samples from donkeys using species-specific PCR. Results were analyzed on 2% agarose gels stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*B. caballi*); lane 9, positive control (*Th. equi*); lanes 2 and 10, negative controls; lanes 4, 5, and 8, samples positive for *B. caballi* (~610 bp); lanes 12, 13, 14, 16, and 17, samples positive for *Th. equi* (~750 bp); lanes 3, 6, 7, 11, and 15, negative samples. Other bands are nonspecific.

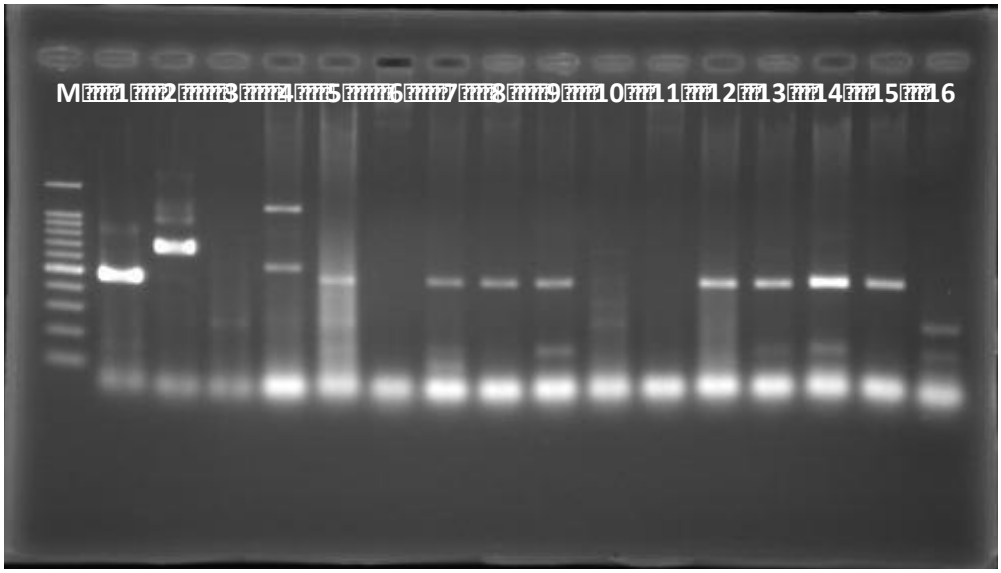

Figure 11: Detection of *Trypanozoon* in DNA samples from donkeys using ITS1 PCR. Results were analyzed on 2% agarose gels stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*T. evansi*); lane 2, positive control (*T. congolense*); lane 3, negative control; lanes 5, 7–9, and 12–15, positive samples; lanes 4, 6, 10, 11, and 16, negative samples. Other bands are nonspecific.

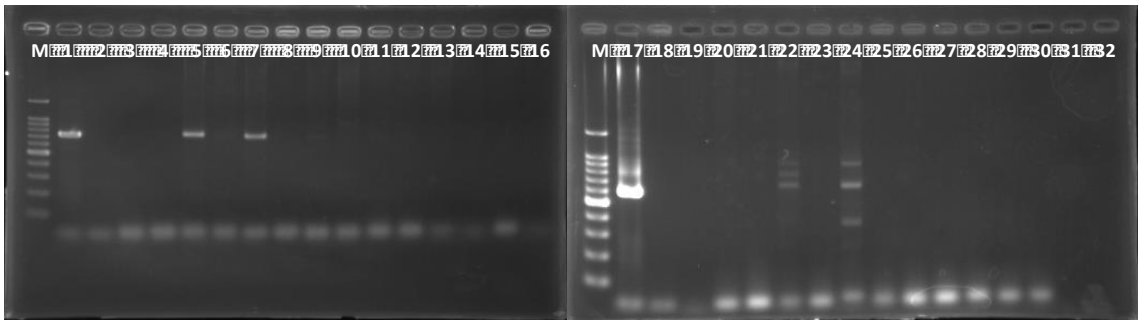

Figure 12: Detection of piroplasms in DNA samples from donkeys using species-specific PCR. Results were analyzed on 2% agarose gels stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*Th. equi*); lane 17, positive control (*B. caballi*); lanes 2 and 18, negative controls; lanes 5 and 7, samples positive for *Th. equi* (~750 bp); lanes 22 and 24, samples positive for *B. caballi* (~610 bp). Other bands are nonspecific, and all other lanes are negative samples.

	Accession no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17
1	**LC492114**																	
2	**LC492115**	0.016																
3	**LC492116**	0.041	0.044															
4	**LC492117**	0.060	0.050	0.047														
5	**LC492118**	0.057	0.047	0.044	0.016													
6	**LC492119**	0.057	0.047	0.044	0.009	0.013												
7	**LC492120**	0.057	0.047	0.044	0.016	0.006	0.013											
8	**LC492121**	0.053	0.044	0.047	0.019	0.003	0.016	0.009										
9	**LC492122**	0.016	0.006	0.044	0.05	0.047	0.047	0.047	0.044									
10	AB551922 *T. evansi* Egypt	0.013	0.003	0.041	0.047	0.044	0.044	0.044	0.041	0.003								
11	KR858267 *T. evansi* India	0.060	0.050	0.047	0.006	0.016	0.009	0.016	0.019	0.050	0.047							
12	AF306771 *T. brucei* Tanzania	0.044	0.035	0.031	0.016	0.013	0.013	0.013	0.016	0.035	0.031	0.016						
13	AF306772 *T. brucei* Nigeria	0.022	0.013	0.041	0.047	0.044	0.044	0.044	0.044	0.013	0.009	0.047	0.031					
14	KU552352 *T. equiperdum*	0.019	0.009	0.047	0.053	0.050	0.050	0.050	0.047	0.009	0.006	0.053	0.038	0.016				
15	KU552354 *T. equiperdum*	0.050	0.041	0.031	0.022	0.019	0.019	0.019	0.022	0.041	0.038	0.022	0.006	0.038	0.044			
16	JN673389 *T. congolense*	0.635	0.645	0.642	0.660	0.660	0.651	0.660	0.657	0.645	0.645	0.654	0.654	0.645	0.638	0.657		
17	JN673388 *T. congolense*	0.632	0.642	0.638	0.657	0.657	0.651	0.657	0.654	0.642	0.642	0.657	0.651	0.642	0.642	0.654	0.063	
18	KY412803 *T. theileri*	0.550	0.547	0.557	0.547	0.560	0.550	0.560	0.557	0.547	0.547	0.550	0.553	0.557	0.547	0.560	0.676	0.682

Figure 13: Sequence analysis and pairwise distance analysis of ~480 bp amplicons detected in the samples taken from donkeys in Khartoum State based on their ITS1 sequences, confirming the identity of the parasites detected as *Trypanozoon*. Accession numbers of the *Trypanozoon* isolates detected in this study are indicated in blue, boldface font.

	Accession no.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23
1	**LC492123**																							
2	**LC492124**	1.84																						
3	**LC492125**	4.36	4.04																					
4	**LC492126**	0.61	2.14	3.72																				
5	**LC492127**	0.00	1.84	4.36	0.61																			
6	**LC492128**	5.65	5.33	2.46	4.99	5.65																		
7	**LC492129**	6.31	6.63	5.35	5.64	6.31	4.06																	
8	**LC492130**	5.34	5.02	4.05	4.68	5.34	3.10	5.36																
9	**LC492131**	1.52	3.39	5.96	2.14	1.52	7.27	7.94	6.96															
10	JN673389 *T. congolense* Savannah Tanzania	0.61	2.46	5.00	1.22	0.61	6.30	6.30	5.99	2.14														
11	JN673388 *T. congolense* Savannah Tanzania	3.40	2.77	3.09	3.71	3.40	3.72	5.98	4.36	4.34	4.03													
12	MN213749 *T. congolense* Savannah unknown	23.20	23.60	22.50	22.30	23.20	24.20	25.50	25.10	25.30	23.60	24.10												
13	JX910374 *T. congolense* Burkina Faso	9.06	9.75	9.72	9.03	9.06	10.40	11.20	11.20	10.10	9.74	10.40	30.00											
14	MG255203 *T. congolense* Cameroon	5.00	5.32	5.66	4.99	5.00	6.98	7.31	6.67	6.61	4.99	5.01	24.60	10.50										
15	MG255204 *T. congolense* Cameroon	3.71	3.40	2.47	3.08	3.71	3.73	4.69	3.42	5.30	4.35	3.72	22.40	9.07	5.00									
16	MG283145 *T. congolense* Cameroon	5.30	5.62	6.62	5.29	5.30	7.94	8.27	7.63	6.91	5.30	5.96	25.30	11.50	2.14	5.95								
17	FJ712718 *T. congolense* Kenya	3.09	3.40	2.46	3.08	3.09	3.71	5.33	3.40	4.66	3.72	2.45	23.20	9.07	4.37	2.46	5.31							
18	MK495745 *T. congolense* Cote dIvoire	5.30	5.62	6.62	5.29	5.30	7.94	8.27	7.63	6.91	5.30	5.96	25.30	11.50	2.14	5.95	0.00	5.31						
19	MK651119 *T. congolense* Algeria	7.38	7.05	4.08	6.70	7.38	5.05	8.44	6.73	9.05	8.05	6.04	25.80	12.00	8.76	5.40	9.75	5.38	9.75					
20	AB742531 *T. congolense* Ghana	24.90	24.00	25.30	24.80	24.90	23.60	27.10	23.60	26.90	25.70	22.30	49.70	31.20	24.10	25.30	25.70	24.00	25.70	27.80				
21	U22319 *T. congolense* Riverine-forest Kenya	25.80	24.90	26.20	25.70	25.80	24.50	28.10	24.50	27.80	26.60	23.20	51.10	32.20	25.00	26.20	26.60	24.90	26.60	28.80	0.61			
22	MK756201 *T. congolense* Nigeria	28.70	27.90	28.40	28.70	28.70	25.80	29.40	26.60	30.00	29.60	26.10	52.90	35.00	28.00	28.40	29.60	27.80	29.60	30.10	2.77	2.77		
23	U22317 *T. congolense* Kilifi Kenya	60.70	61.40	64.40	61.00	60.70	62.40	64.30	63.80	60.70	60.10	62.70	91.00	67.70	63.50	62.00	63.80	64.00	63.80	67.00	70.80	71.80	71.00	
24	MK756200 *T. congolense* Kilifi Nigeria	63.30	62.60	64.10	63.60	63.30	62.10	64.10	63.60	63.30	62.60	63.80	90.00	69.00	64.70	61.80	65.00	63.70	65.00	66.80	70.00	71.00	70.30	1.84

Figure 14: Sequence analysis and pairwise distance analysis of ~700 bp amplicons detected in the samples taken from donkeys in Khartoum State based on their ITS1 sequences, confirming the detection of the parasite *T. congolense* savannah. Accession numbers of *T. congolense* savannah detected in this study are indicated in red, boldface font.

Table 1: The number of samples obtained from donkeys in different parts of Sudan.

Sampling	City	Location	No. of samples
First batch	Khartoum	Central Sudan	**198**
Second batch	Khartoum	Central Sudan	77
	Atbara	Northern Sudan	25
	Kassala	Eastern Sudan	125
	Nyala	Western Sudan	40
	Total		**267**
Total			**465**

Table 2: PCR techniques and primers used in this study for the detection of trypanosome and piroplasm DNA in donkeys from Sudan.

PCR	Target gene	Primers	Sequence (5'-3')	Specificity	Size (bp)	References
ITS 1	ITS1	ITS1CF ITS1BR	CCGGAAGTTCACCGATATTG TTGCTGCGTTCTTCAACGAA	*Trypanozoon* *T. congolense* *T. vivax*	480 620–700 250	Njiru *et al*., 2005
RoTat 1.2	RoTat 1.2 VSG	RoTat 1.2F RoTat 1.2R	GCGGGGTGTTTAAAGCAATA ATTAGTGCTGCGTGTGTTCG	*T. evansi* (type A)	205	Claes *et al*., 2004
Th. equi	EMA-1	EMA-1F EMA-1R	GCATCCATTGCCATTTCGAG TGCGCCATAGACGGAGAAGC	*Th. equi*	750	Alhassan *et al*., 2005
B. caballi	BC48	BC48F BC48R	GGCTCCCAGCGACTCTGTGG CTTAAGTGCCCTCTTGATGC	*B. caballi*	610	Alhassan *et al*., 2005

Table 3: Seroprevalence of hemoprotozoan parasites in donkeys sampled in different parts of Sudan as determined using ELISAs.

	Trypanosomes		**Piroplasms**	
	No. of positive samples (%)		No. of positive samples (%)	
Location	**rTeGM6**	**TeCA**	**EMA-2**	**BC48**
Khartoum (First batch)	19/198 (9.6)	56/198 (28.3)	156/198 (78.8)	10/198 (5.1)
Atbara	18/25 (72.0)	24/25 (96.0)	25/25 (100.0)	21/25 (84)
Khartoum	16/77 (20.8)	36 /77(46.8)	35/77 (45.5)	7/77 (9.1)
Kassala	25/125 (20)	13/125 (10.4)	53/125 (42.4)	5/125 (4)
Nyala	16/40 (40)	28/40 (70)	29/40 (72.5)	35/40 (87.5)
Total of second batch	75/267 (28.1)	101/267 (37.8)	142/267 (53.2)	68/267 (25.5)
Total	**94/465 (20.2)**	**157/465 (33.8)**	**298/465 (64.1)**	**78/465 (16.8)**

Table 4: Prevalence of hemoprotozoan parasite DNA in donkeys as determined by PCR assays.

NA: Not analyzed

Area \ Spp.	Trypanosomes: No. of positive samples (%)				Piroplasms: No. of positive samples (%)	
	ITS1 PCR			RoTat1.2 PCR	EMA-1 PCR	BC48 PCR
	Trypanozoon (~480 bp)	*T. congolense* (~700 bp)	Mixed *Trypanozoon* and *T. congolense*	*T. evansi* type A (~205 bp)	*Th. equi* (~750 bp)	*B. caballi* (~610 bp)
Khartoum (First batch)	77/198 (38.9)	18/198 (9.1)	5/198 (2.5)	35/77 (45.5)	18/198 (9.1)	8/198 (4.0)
Atbara	5/25 (20)	0/25 (0)	0/25 (0)	NA	0/25 (0)	1/25 (4.0)
Khartoum	11/77 (14.3)	0/77 (0)	0/77 (0)	NA	0/77 (0)	1/77 (1.3)
Kassala	18/125 (14.4)	0/125 (0)	0/125 (0)	NA	1/125 (0.8)	3/125 (2.4)
Nyala	34/40 (85.0)	0/40 (0)	0/40 (0)	NA	3/40 (7.5)	6/40 (15.0)
Total of second batch	68/267 (25.5)	0/267 (0)	0/267 (0)	NA	4/267 (1.5)	11/267 (4.1)
Total	**131/465 (31.2)**	**18/465 (3.9)**	**5/465 (1.1)**	**35/77 (45.5)**	**22/465 (4.7)**	**19/465 (4.1)**

Chapter 2

Epidemiological studies on trypanosomosis in different animal species in the Visayas region of the Philippines

Introduction

Several species of trypanosomes are known to be pathogenic to livestock. *Trypanosoma evansi*, the causative agent of surra, has the widest range of hosts among trypanosomes, including domestic and wild animals, and the largest geographical distribution. It is endemic to Central and South America, Africa, and Asia (Dargantes *et al*., 2009; Dargantes, 2010). Except for *T. evansi*, no other trypanosome species have been reported in the Philippines, with the exception of a report by Ybañez *et al*. (2013) of the detection of *T. theileri* in cattle on Cebu Island. However, the possibility of the introduction of other trypanosome species into the country should be considered due to the importation of animals into Philippines (Baticados *et al*., 2011a).

Surra in the Philippines is believed to have been introduced through the importation of infected horses from China by American army during the American-Spanish War in the early 19th Century (Dargantes, 2010). In the Philippines, surra is considered by the Philippine government as the second most important and economically significant parasitic animal disease, after fasciolosis (Dargantes, 2010). As a result, the government has planned to implement a national control program to treat or prevent infection by trypanocidal drugs in areas with a high prevalence of the clinical

cases. *T. evansi* has been reported in all regions of the Philippines, and over the past few decades several large epidemics of the disease caused by this parasite have occurred, particularly on the islands of Visayas and Mindanao, and these have been characterized by high morbidity and mortality in water buffaloes which are considered the main draft animals in the country, as well as in horses, cattle, and goats (Manuel, 1998; Reid, 2002; Dargantes, 2010).

The order of 4.9 million Philippine pesos (130,000 US dollars) per year was estimated as the financial loss caused by surra from 1989 to 1997 (Manuel, 1998; Dargantes, 2010). However, it is difficult to assess the true incidence of surra in the Philippines due to conflicting reports and a lack of reliable statistics on the extents of such losses (Reid, 2002). Surra causes economic losses due to high mortality, loss of weight, reduced milk yield and draft capacity, and decrease reproductive performance (Losos, 1980; Mahmoud and Gray, 1980; Dargantes, 2010).

Buffaloes are considered reservoir hosts of *T. evansi*, usually with asymptomatic infections that cause suppression of the immune system. However, outbreaks of acute disease may occur (Baticados *et al*., 2011a). The water buffalo, commonly known as the carabao, is the national animal of the Philippines, and is used by farmers as livestock, a pack animal, and as a helper in land cultivation, and its waste is used as fertilizer and fuel. In 2012, a population of 2.96 million buffaloes was recorded in the country, 99% of which are held in backyards or on farms belong to small farmers who have limited resources and low income (Reyes *et al*., 2013). In 1993, to conserve, propagate, and promote the carabao as a source of draft animal power, meat, milk, and hide, the Philippine Carabao Center (PCC) was established. The PCC also aimed to benefit rural farmers

through carabao genetic improvement, technological development and distribution, and the establishment of carabao-based initiatives, thus ensuring higher incomes and better nutrition (https://www.pcc.gov.ph/). The movement of imported water buffaloes from the PCC to rural villages, as part of the government's goal to improve the productivity of local carabaos, was associated with some surra outbreaks in the country. These animals could have acted as amplifiers for infections of other domestic animals in the area. The prevalence of surra peaks during the agricultural planting season, which is the time of maximum work stress for animals (Reid, 2002). The stress of overwork could be associated with mortalities in draft water buffalo with *T. evansi* infection. Moreover, also other factors including malnutrition, coexisting infections, and adverse climatic conditions, may contribute in reducing resistance and increasing susceptibility to the infection (Löhr *et al*., 1985; Manuel, 1998; Dargantes *et al*., 2010).

Philippine goats are multipurpose animals utilized for their meat, milk, and hides, but receive far less attention than water buffaloes, especially in terms of feed, water, and shelter (Abilay, 1983). Goats have developed into 'handy animals', and are now distributed throughout the country (Ibarra, 1988) with a total population of 3.75 million goats (Philippine Statistics Authority, 2019). They are commonly known as the 'poor man's cow' in the Philippines because only a small investment is needed to raise them, making their farming attractive for households and subsistence-farming families (Dar and Fylon, 1996).

There are conflicting reports on the susceptibility of goats to infection by *T. evansi*, possibly owing to differences in the size of the infective dose, virulence of the strain of *T. evansi*, or

susceptibility of the breed of goat (Otieno and Gacanja, 1976; Ngeranwa *et al*., 1993). Goats have been reported to be important reservoirs for trypanosomes because they are highly trypanotolerant, usually showing subclinical trypanosomosis, with low and persistent parasitemia (Musinguzi *et al*., 2016; Mossaad *et al*., 2020). However, an experimental infection with *T. evansi* in goats in the Philippines resulted in the development of a clinical disease, which suggested that the infective dose influences the clinical outcome of the infection (Dargantes *et al*., 2005).

The Visayas region of the Philippines is composed of several small islands, including Cebu and Bohol. Cebu City, located on Cebu Island, is the second largest metropolitan city in the country, and is the usual gateway for livestock trading in the area (Ybañez *et al*., 2013). On the other hand, the PCC, which was established to improve the livestock industry in the country, is located at Ubay Stock Farm in Ubay City, Bohol. Ubay Stock Farm in Bohol is the oldest and largest government-run livestock facility in the Philippines, and contains several types of farm animals, such as carabao, cattle, horses, goats, and many more (https://steemit.com/farm/@lapilipinas/ubay-stock-farm-bohol-oldest-and-largest-government-livestock-facility-in-the-philippines). Thus, the survey of trypanosome infection in the Visayas region is highly desirable for the livestock sector of the whole country.

T. evansi was previously reported in several animal species throughout different regions of the Philippines (Reid, 2002; Baticados *et al*., 2011a, 2011b; McInnes *et al*., 2012), including water buffaloes in Bohol (Villareal *et al*., 2013). However, it has not yet been reported in Cebu, where only *T. theileri* was previously reported in cattle (Ybañez *et al*., 2013). Thus, the aim of this study

was to perform a PCR-based molecular survey for trypanosomes in Bohol and Cebu, the Philippines, to check the prevalence of trypanosomal diseases in different animal species in the area.

Materials and methods

Study area and sample collection

Cebu samples

A total of 251 goats were randomly sampled from four farms in Cebu (Barili, *n* = 214; Danao City, *n* = 19; Dumanjug, *n* = 16; and Minglanilla, *n* = 2) (Figure 15), with sex and age information.

Bohol samples

Two batches of samples were collected from Ubay Stock Farm in Ubay City, Bohol (Figure 16). The first batch of samples was obtained from 109 water buffaloes in the PCC in May-June 2018. The second batch of samples was collected in September 2018, and included a total of 160 samples from different animal species at Ubay Stock Farm as follows: cattle (*n* = 76), water buffaloes (*n* = 42), goats (*n* = 35), and horses (*n* = 7).

Two ml of blood were collected from the jugular vein of each animal in tubes containing anticoagulant. DNA was subsequently extracted from these samples using the QIAamp DNA Blood Mini Kit (Qiagen, Germany), according to the manufacturer's instructions. All procedures conducted adhered to the principles of the Animal Welfare Act of the Philippines (R.A. 8485, as amended by R.A. 10631) and the Bureau of Animal Industry Administrative Order 40, and the protocol was

reviewed and approved by the Gullas College of Medicine Institutional Animal Care and Use Committee. Permission for animal experiments (permit no. 19-21) and permission for the use of DNA (permit no. 1703-3) were obtained from Obihiro University of Agriculture and Veterinary Medicine, Obihiro, Hokkaido, Japan.

PCR-based detection of trypanosomes

Two different PCR techniques were employed to detect and identify trypanosomes DNA: (i) single-step ITS1 PCR (Njiru *et al*., 2005); and (ii) CatL PCR, which specifically amplified the cathepsin L-like (CatL) gene in *T. theileri* (Rodrigues *et al*., 2010). All primer sequences used in PCR in this study are listed in Table 5. Both PCR assays were performed using Phusion® DNA polymerase (New England BioLabs, USA), as described in Chapter 1.

DNA cloning and sequencing

The ITS1 amplicons were extracted from agarose gels, cloned, and transformed into chemically competent *Escherichia coli* cells (One Shot Mach1; Thermo Fisher Scientific, USA) using Zero Blunt TOPO cloning kits (Invitrogen, Thermo Fisher Scientific Inc., USA), as described in Chapter 1. After checking several colonies for each PCR-cloned product by colony PCR, positive clones of ITS1 and CatL were selected for use in plasmid DNA purification with a QIAprep Spin Miniprep kit (Qiagen, Germany) as follows: for goats from Cebu, 10 ITS1 clones were obtained; for

water buffaloes from Bohol (first batch), 14 ITS1 and 10 CatL clones were obtained; and for water buffaloes, cattle, goats, and horses from Bohol (second batch), 5, 9, 8, and 2 ITS1 clones were obtained, respectively. Approximately 100–200 ng/µl pure plasmid DNA was used for sequencing, as described in Chapter 1.

Sequencing analysis

The ITS1 and CatL sequences obtained from different animal species were aligned with published sequences from GenBank using the Basic Local Alignment Search Tool (BLAST), and their phylogenetic trees were constructed using the maximum likelihood method implemented in Mega software (version 7). All sequences obtained in this study were deposited in GenBank under accession numbers LC521909 to LC521918, and LC546887 to LC546934.

Results

Cebu samples

Of the 251 goat DNA samples collected from Cebu, 83 (33.1%) were positive for *T. evansi*, as shown by them having the PCR product with a band length of ~480 bp, which is indicative of the amplification of the ITS1 gene of *T. evansi*. Two other samples (0.8%) were positive for *T. evansi* and showed additional bands, ~400 bp in size, corresponding to the expected size of the ITS1 sequence of *T. theileri*, using ITS1 PCR (Figure 17). When animals were divided up according to their sex and age, the detection rate of *T. evansi* was 38.3% (23/60) in male goats, 32.5% (62/191) in females, 34.5% (30/87) in younger goats (< 1-year-old), and 33.5% (55/164) in adult goats (> 1-year-old).

Confirmation of the identity of the ~480 and ~400 bp PCR products as those of *T. evansi* and *T. theileri*, respectively, was achieved by the selection, cloning, and sequencing of the ITS1 region of 10 positive samples. The sequence similarity found among the trypanosomes obtained in this study and reference sequences from GenBank confirmed the presence of *T. evansi* in eight positive samples (GenBank accession numbers LC521909, LC521910, LC521911, LC521912, LC521913, LC521914, LC521915, and LC521916), and the presence of *T. theileri* (Clade TthII) in the two positive samples that showed the additional ~400 bp PCR bands (GenBank accession numbers LC521917 and LC521918) (Table 6).

Bohol samples

First batch

When using ITS1 PCR for the detection of trypanosome DNA, 27 (24.8%) of the 109 water buffaloes that were first sampled in Bohol were positive for *T. evansi* (~480 bp), and 19 (17.4%) were positive for *T. theileri* (~400 bp). In addition, 4 (3.7%) samples showed signs of a mixed infection of *T. evansi* with *T. theileri* (Figure 18). The 19 *T. theileri*-positive samples were screened using CatL PCR, which confirmed that 12 (63.2%) of them represented detection of *T. theileri* (Figure 19).

Selection, cloning, and sequencing of the ITS1 region of 14 positive samples, and the CatL region of 10 positive samples, as well as the sequence similarity found among them and reference sequences from GenBank, confirmed the presence of *T. evansi* in 5 (GenBank accession numbers LC546887, LC546888, LC546889, LC546890, and LC546891) and *T. theileri* in 9 (GenBank accession numbers LC546892, LC546893, LC546894, LC546895, LC546896, LC546897, LC546898, LC546899, and LC546900) ITS1-positive samples, and the presence of *T. theileri* in 10 CatL-positive samples (GenBank accession numbers LC546925, LC546926, LC546927, LC546928, LC546929, LC546930, LC546931, LC546932, LC546933, and LC546934).

Second batch

The screening of a total of 160 samples collected from different animal species in Bohol using ITS1 PCR for the detection of trypanosome DNA resulted in an overall estimated trypanosome prevalence of 44.7% in cattle (34/76), followed by 34.3% in goats (12/35), 28.6% in horses (2/7), and 16.7% in water buffaloes (7/42). In cattle, 22 (28.9%) samples were positive for *T. evansi*, 7 (9.2%) samples were positive for *T. theileri*, and 5 (6.6%) samples showed signs of a mixed infection of *T. evansi* with *T. theileri*. In goats, 7 (20%) samples were positive for *T. evansi*, 4 (11.4%) samples were positive for *T. theileri*, and only one (2.9%) sample was positive for a mixed infection of *T. evansi* with *T. theileri*. In horses and water buffaloes, only *T. evansi* was detected in 2 (28.6%) and 7 (16.7%) samples, respectively. The results of ITS1-PCR of all samples are summarized in Table 7.

Selection, cloning and sequencing of the ITS1 region of positive samples and the sequence similarities found among them and reference sequences from GenBank confirmed the presence of *T. evansi* in 4 samples from cattle (GenBank accession numbers LC546901, LC546902, LC546903, and LC546904), 5 samples from water buffaloes (GenBank accession numbers LC546905, LC546906, LC546907, LC546908, and LC546909), 5 samples from goats (GenBank accession numbers LC546910, LC546911, LC546912, LC546913, and LC546914), and 2 samples from horses (GenBank accession numbers LC546915, and LC546916); and the presence of *T. theileri* in 5 samples from cattle (GenBank accession numbers LC546917, LC546918, LC546919,

LC546920, and LC546921), and 3 samples from goats (GenBank accession numbers LC546922, LC546923, and LC546924).

Discussion

The Visayas region of the Philippines is the usual gateway for the Philippine livestock trade through Cebu City, the second largest metropolitan city in the country (Ybañez *et al*., 2013). Moreover, Ubay Stock Farm is located in this region, which is the oldest and largest government-run livestock facility in the Philippines and contains many different types of farm animals. Further, this region contains the PCC, which was established on Bohol Island. Thus, confirmation of the presence or absence of pathogens in the Visayas region is of high importance for the livestock industry in the country as a whole.

The occurrence of trypanosomosis caused by *T. evansi* (surra) has been reported in several animal species throughout different regions of the Philippines (Reid, 2002; Baticados *et al*., 2011a, 2011b; McInnes *et al*., 2012). In the Visayas region, it was previously reported in water buffaloes in Bohol (Villareal *et al*., 2013). However, it has not yet been reported in Cebu, where only *T. theileri* was previously reported to have been found in cattle (Ybañez *et al*., 2013).

For this study, samples were collected from different animal species in Cebu and Bohol for the detection of *Trypanosoma* spp. using PCR-based assays. Of the 251 goats sampled in Cebu, 85 (33.9%) were positive for *T. evansi* using ITS1 PCR. Two of these positive samples showed additional bands of ~400 bp in size, which were confirmed as representing *T. theileri*. One of these two samples was collected from a goat farm in Barili, which is a transitory commercial farm that maintains goats obtained from various places for a few weeks before they are distributed to different areas in Cebu and nearby provinces (Galon *et al*., 2019). The other positive sample came

from a farm in Danao City that raises goats and cattle and offers quality goats and cattle to other areas for use in breeding and meat production.

It is commonly believed that caprine trypanosomosis is only a sporadic disease because goats are highly resistant to infections. However, current epidemiological information indicates that goats can play a vital role in the transmission of such diseases (Gutierrez *et al*., 2006; Musinguzi *et al*., 2016; Mossaad *et al*., 2020). There have been no previous reports that associated the sex and age of goats with their susceptibility to infection by *T. evansi*. In the present study, the detection rate of *T. evansi* in male goats (38.3%; 23/60) was higher than the detection rate in females (32.5%; 62/191). A slightly higher detection rate was found in younger goats (< 1-year-old) (34.5%; 30/87) relative to that in adult goats (> 1-year-old) (33.5%; 55/164). However, these findings do not suggest that sex (p = 0.4) and age (p = 0.8) are significantly associated with *T. evansi* infection in goats. Furthermore, the higher numbers of female and adult goats that were tested in this study should be taken into consideration when interpreting these detection rates.

Sequencing of the ITS1 region of the positive samples (those with ~480 and ~400 bp PCR products) confirmed them to contain *T. evansi* and *T. theileri*, respectively, based on the sequence similarities found among the trypanosome sequences obtained in this study and reference sequences from GenBank. Although goats are known to be non-susceptible to *T. theileri*, they have been reported to be susceptible to *T. theodori*, a *T. theileri*-like trypanosome that belongs to the same subgenus, *Megatrypanum* (Hoare, 1972). On the contrary, *T. theileri* has previously been reported to occur in cattle in Cebu (Ybañez *et al*., 2013). More investigations are needed to confirm the

status of *T. theileri* in goats in Cebu, while taking into consideration the fact that tabanid flies, which are the vectors of *T. theileri* (Böse and Heister, 1993), are abundant in the Philippines (Baticados *et al*., 2011b).

Goat farming in the Philippines is mostly carried out at the backyard level, with goats commonly raised by smallholder farmers or backyard livestock producers with other animal species, including cattle, buffalo, and poultry (Lapar *et al*., 2003). Most of the samples in this study were collected from a goat farm in Barili, a commercial farm that maintains goats obtained from different areas (Galon *et al*., 2019). These goats may have been initially raised with other animal species, which could be the source of the *T. evansi* infections detected in them, as this parasite has already been detected in other regions of the country (Reid, 2002; Baticados *et al*., 2011a, 2011b; McInnes *et al*., 2012; Villareal *et al*., 2013). Nonetheless, goats might play a role in the epidemiology of such diseases, and should not be neglected in any control strategy targeting them. To my knowledge, this is the first report of *T. evansi* being detected in Cebu in the Philippines.

The water buffalo is the national animal of the Philippines, and 99% of buffaloes in the country are raised in backyards or farms belonging to smallholder farmers with limited resources and low incomes (Reyes *et al*., 2013). For this study, samples were collected from buffaloes in the PCC, which was established at Ubay Stock Farm in Ubay City, Bohol, to improve the livestock industry in the country by importing water buffaloes and then distributing them to villages to improve the productivity of local carabaos. Other samples were collected from different animal species at Ubay Stock Farm in Bohol, which is the oldest and largest government-run livestock

facility in the Philippines and contains several types of farm animals, such as carabao, cattle, horses, goats, and others.

Of the water buffaloes that were first sampled in Bohol, 24.8% (27/109) were positive for *T. evansi*, 17.4% (19/109) were positive for *T. theileri*, and 3.7% (4/109) were positive for a mixed infection of *T. evansi* and *T. theileri*. Twelve (63.2%) of the 19 *T. theileri*-positive samples were confirmed to contain *T. theileri*. In the second batch of samples, only *T. evansi* was detected, and was present in 16.7% (7/42) of sampled water buffaloes. The prevalence of these trypanosomes detected by PCR-based assays was high compared to the prevalence of 0.13% that was previously reported for *T. evansi* in water buffaloes in Luzon in the northern part of the Philippines using both microscopy and PCR methods, which was attributable to the previous treatment of animals with the trypanocidal drug Suramin® in that study (Baticados *et al*., 2011a). However, *T. evansi* was detected using a PCR-based detection assay in all 79 samples collected from water buffaloes in Luzon and Bohol, which confirms the persistence, high incidence, and widespread distribution of this parasite in the country, despite the prevention and control measures implemented by the government to eradicate such diseases (Villareal *et al*., 2013).

Water buffaloes are usually reservoirs for *T. evansi*, with asymptomatic infections (Baticados *et al*., 2011a). Buffaloes in the PCC are imported and distributed to villages to improve the productivity of local carabaos. Detection of *T. evansi* in these apparently healthy animals suggested that they could be sources of infection to other livestock, particularly given the fact that

tabanid flies, the transmission vectors of *T. evansi*, are abundant in the Philippines (Baticados *et al*., 2011b).

Ubay Stock Farm, where the PCC is located, contains different species of farm animals, which were also sampled for the detection of *T. evansi* in this study. An overall trypanosome prevalence of 44.7% in cattle (34/76), followed by 34.3% in goats (12/35), and 28.6% in horses (2/7) was found. In cattle, 22 (28.9%) samples were positive for *T. evansi*, 7 (9.2%) samples were positive for *T. theileri*, and 5 (6.6%) samples contained signs of a mixed infection of *T. evansi* and *T. theileri*, while in goats 7 (20%) samples were positive for *T. evansi*, 4 (11.4%) samples were positive for *T. theileri*, and only one (2.9%) sample was positive for a mixed infection of *T. evansi* and *T. theileri*. In horses, only *T. evansi* was detected, and it was only found in 2 (28.6%) samples. A prevalence of 1.22% of *T. evansi* was previously reported in cattle in Luzon based on the use of PCR detection assays (Baticados *et al*., 2011b), which was obviously lower than that I found in this study in Bohol. On the other hand, *T. theileri* has only previously been reported in the Philippines in cattle studied in Cebu (Ybañez *et al*., 2013). In this study, this trypanosome species was found in cattle and goats in Bohol, as well as in water buffaloes in the PCC. Different animal species have been affected by surra outbreaks in the Philippines in the past few decades, showing signs of clinical disease with high mortality. Some of these outbreaks were suggested to have been associated with the movement of imported buffaloes from the PCC to outlying villages, where they played a role as amplifier hosts for infections of other local livestock (Reid, 2002). Thus, the presence of infected buffaloes from the

PCC in the same area with other farm animals at Ubay Stock Farm in Bohol could provide an explanation of the high prevalence of *T. evansi* and *T. theileri* in those animals.

Summary

A high prevalence of *T. evansi* was found in different animal species in the Visayas region, the gateway for the livestock trade in the Philippines. These animals are usually distributed from the Visayas region to other regions of the country, so they could be sources of infection that might lead to the development of new outbreaks. Despite the government's disease control and eradication measures, which depend mainly on providing trypanocidal drugs for treatment and prevention to all animals in surra endemic areas, these parasites are still detected in the present study. Moreover, the detection of *T. theileri* in different animal species in this livestock trade-related area means that the possibility of the introduction of other trypanosome species into the country due to the importation of animals into the Philippines should be seriously considered. Thus, the government should revalidate its livestock importation policies to allow only the introduction of disease-free animals into the country. In addition, the regular treatments with trypanocidal drugs applied to animals should also be evaluated by taking into the consideration the possibility of the development of drug resistance.

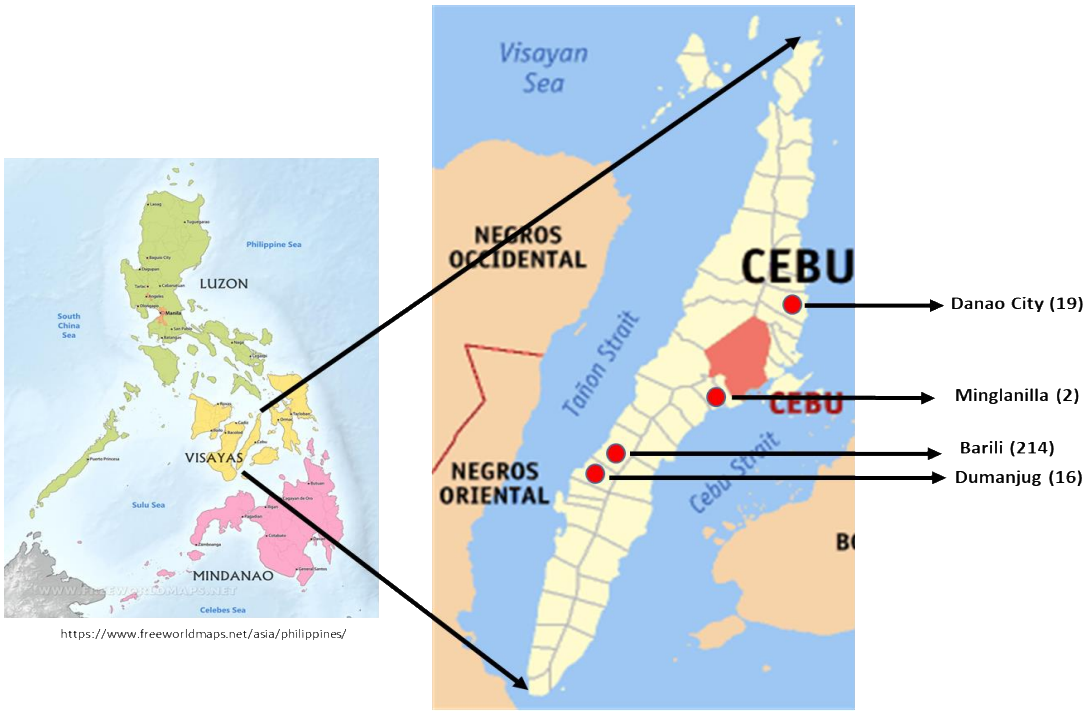

Figure 15: Map of the Philippines showing the sampling locations on Cebu Island, and the number of samples collected in each area.

Figure 16: Map of the Philippines showing Ubay City indicated by the black star, where the sampling area of Ubay Stock Farm is located, in Bohol Island.

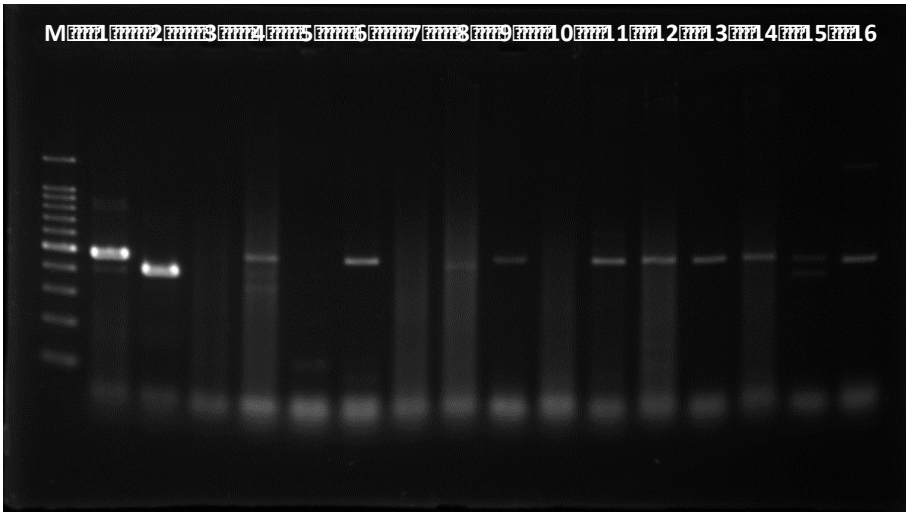

Figure 17: Detection of *Trypanosoma* spp. in DNA samples from goats in Cebu using ITS1 PCR. Results are shown on 2% agarose gel stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*T. evansi*); lane 2, positive control (*T. theileri*); lane 3, negative control; lanes 4, 6, 9, 11, 12, 13, 14, and 16, samples positive for *T. evansi* (~480 bp); lane 15, mixed infection with *T. evansi* and *T. theileri* (~400 bp); lanes 5, 7, 8, and 10, negative samples. Other bands are nonspecific.

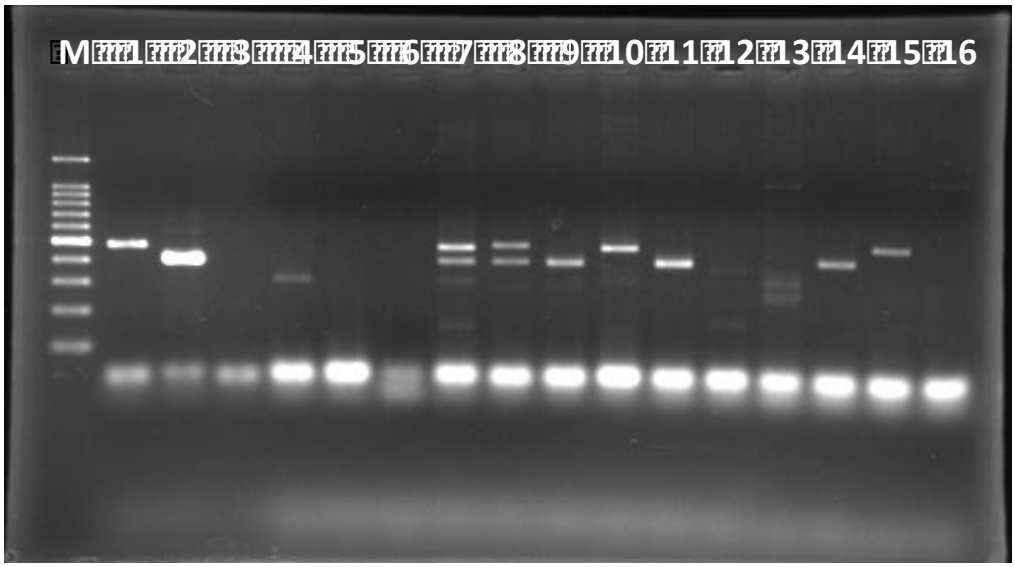

Figure 18: Detection of *Trypanosoma* spp. in DNA samples from water buffaloes in Bohol using ITS1 PCR. Results are shown on 2% agarose gel stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*T. evansi*); lane 2, positive control (*T. theileri*); lane 3, negative control; lanes 10 and 15, samples positive for *T. evansi* (~480 bp); lanes 9, 11, and 14, samples positive for *T. theileri* (~400 bp); lanes 7 and 8, mixed infection with *T. evansi* and *T. theileri*; lanes 4, 5, 6, 12, 13, and 16, negative samples. Other bands are nonspecific.

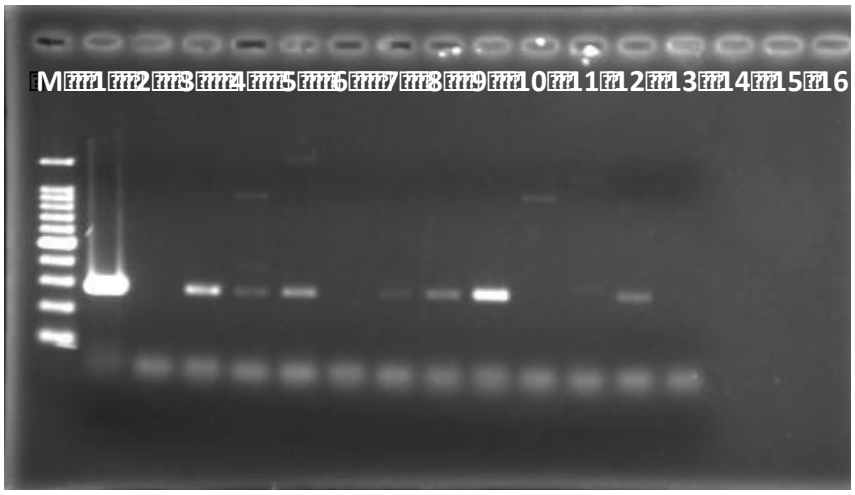

Figure 19: Confirmation of the presence of *Trypanosoma theileri* in *T. theileri* ITS1-positive DNA samples from water buffaloes in Bohol using CatL PCR. Results are shown on 2% agarose gel stained with ethidium bromide. M, 100 bp marker; lane 1, positive control (*T. theileri*); lane 2, negative control; lanes 3–5, 7–9, and 12, samples positive for *T. theileri* (~300 bp); lanes 6, 10, 11, and 13, negative samples. Other bands are nonspecific.

Table 5: PCR techniques and primers used in this study for the detection of trypanosome DNA in different animal species in the Philippines.

PCR	Target gene	Primers	Sequence (5'-3')	Parasites	Size (bp)	References
ITS 1	ITS1	ITS1CF	CCGGAAGTTCACCGATATTG	*Trypanozoon*	480	Njiru *et al*., 2005
		ITS1BR	TTGCTGCGTTCTTCAACGAA	*T. theileri*	400	Suganuma *et al*., 2019
CatL	CathepsinL-like	TthCATL1	CGTCTCTGGCTCCGGTCAAAC	*T. theileri*	300	Rodrigues *et al.*, 2010
		DTO155	TTAAAGCTTCCACGAGTTCT TGATGATCCAGTA			

Table 6: Sequence similarity among trypanosomes obtained from goats in Cebu, the Philippines, and reference sequences of trypanosomes from GenBank.

		NCBI BLAST highest identity			
Target gene	**GenBank accession No.**	**Isolate**	**Accession No.**	**Identity (%)**	**References**
ITS1	LC521909	*T. evansi*	D89527.1	99	-
	LC521910	*T. evansi* isolate haB	MN446740.1	99	-
	LC521911	*T. evansi*	KR858267.1	99	-
	LC521912	*T. evansi* isolate B 19 clone A	MN121259.1	99	-
	LC521913	*T. evansi*	KR858268.1	99	-
	LC521914	*T. evansi* isolate Egy.2	AB551920.1	98	Amer *et al.*, 2011
	LC521915	*T. evansi*	FJ712714.1	99	Tian *et al*., 2011
	LC521916	*T. evansi*	KR858268.1	99	-
	LC521917	*T. theileri* strain: Esashi 12	AB569250.1	98	Hatama *et al*., 2007
	LC521918	*T. theileri* strain: Esashi 12	AB569250.1	99	Hatama *et al*., 2007

Table 7: Detection of trypanosome DNA in different animal species in the Philippines using ITS1 PCR.

Area	Animal species (No. of samples)	Trypanosomes			Total
		No. of positive samples (%)			
		T. evansi	*T. theileri*/ *T. theileri*-like	Mixed infection	
Cebu	Goat (251)	83 (33.1)	0	2 (0.8)	85 (33.9)
Bohol	Water buffalo (109)	27 (24.8)	19 (17.4)	4 (3.7)	50 (45.9)
	Cattle (76)	22 (28.9)	7 (9.2)	5 (6.6)	34 (44.7)
	Goat (35)	7 (20)	4 (11.4)	1 (2.9)	12 (34.3)
	Horse (7)	2 (28.6)	0	0	2 (28.6)
	Water buffalo (42)	7 (16.7)	0	0	7 (16.7)
Total	**520**	**148 (28.5)**	**30 (5.8)**	**12 (2.3)**	**190 (36.5)**

General discussion

Vector-borne hemoprotozoan diseases, such as animal trypanosomosis, theileriosis, and babesiosis, are considered major threats that can cause devastating losses to the livestock industry throughout the world, especially in developing countries, where livestock play an essential role in the economy. The epidemiology and distribution of animal hemoprotozoan diseases depend on many factors, such as their vectors and preferred hosts, as well as the susceptibility of the host species and breed, which is of particular importance since some breeds may be susceptible to a disease while others of the same species are not.

Different animal species are known to serve as reservoir hosts for hemoprotozoan diseases, with individuals of such species having asymptomatic infections. In this study, I evaluated the roles of some reservoir hosts in the epidemiology of selected hemoprotozoan diseases, namely trypanosomosis and equine piroplasmosis, in two developing countries with different livestock systems.

Donkeys are common livestock animals in Sudan. They are economically important animals and considered a source of income. They can acquire a wide variety of diseases, of which they usually remain asymptomatic carriers (Angara *et al.*, 2011). In this study, I tested donkeys in different parts of Sudan due to the general neglect of these animals' care there, despite their significant economic contributions to human livelihoods. Using PCR-based detection assays, the high prevalence of various different trypanosomes was detected in donkeys in different parts of Sudan, including

Trypanozoon, *T. evansi* type A, and *T. congolense*, which confirms the widespread occurrence of trypanosomosis in apparently healthy donkeys in Sudan. This is the first report of the tsetse-transmitted *T. congolense* being detected in donkeys in a non-tsetse-infested area in Sudan. This finding has been attributed to the fact that, after the referendum separating South Sudan from Sudan in 2011, the movement of animals between the two countries has contributed to the spread of trypanosomosis in tsetse-free areas therein (Mossaad *et al*., 2017a). In addition, *T. congolense* and *T. vivax* have previously been detected in equines, camels, and dogs in non-tsetse-infested areas in Sudan (Salim *et al*., 2014; Mossaad *et al*., 2017a, 2017b), which suggested the existence of a mechanical transmission mechanism for *T. congolense*. *T. evansi* type A was previously reported to have been found in camels in Sudan (Salim *et al*., 2011; Mossaad *et al*., 2017a). The present study is the first to report the application of RoTat1.2 VSG PCR in the detection of *T. evansi* type A in donkeys in Sudan.

Different serological techniques were employed in this study for the detection of anti-trypanosome antibodies, including CATT/*T. evansi*, rTeGM6-4r-based ELISA, and TeCA-based ELISA, which detected the high seroprevalence of trypanosomes in donkeys in Sudan.

Regarding piroplasms, the seroprevalence of antibodies for these parasites in donkeys in Sudan, as detected by ELISA, was higher than the prevalence of these parasites detected by PCR, which might be because of chronic infections by them causing the parasitemia to be below the detection limit of PCR. Thus, PCR is considered a reliable diagnostic method for active infections,

whereas serological tests are more reliable for the detection of persistent and past infections (Kappmeyer *et al*., 1999; Mahmoud *et al*., 2015).

The high detected prevalence of trypanosomosis and piroplasmosis in apparently healthy donkeys indicated that they have great resistance to these diseases and remain asymptomatic carriers of them. This suggested that donkeys can serve as reservoir hosts for infections of other animals. Thus, even though donkeys are not usually included in the control measures targeting such diseases because they do not show clinical signs of them, they can still play an important role in the transmission of these diseases to other livestock, especially more susceptible species, given the fact that the sequence analysis revealed the similarity among the parasites obtained from donkeys in this study and reference sequences (from GenBank) of parasites obtained from other susceptible animal species. Moreover, *T. congolense*, a tsetse-transmitted trypanosome, was detected in this study in Khartoum State, which is a non-tsetse-infested area of Sudan, and is located very far from the tsetse-infested areas in the Southeast and Southwest parts of Sudan. This finding suggested the possibility that this parasite could be mechanically transmitted by biting insects. Therefore, donkeys urgently need to be included in the control measures applied by veterinary authorities in Sudan against hemoprotozoan diseases, and special attention should be paid to their ability to distribute tsetse-transmitted trypanosomes to other susceptible animals in non-tsetse-infested areas.

The water buffalo is the national animal of the Philippines, where it is commonly known as the carabao and is raised in backyards or on small farms and used by farmers for many purposes, including production, packing, and agriculture work (Reyes *et al*., 2013). The Philippine Carabao

Center (PCC) was established to improve the productivity of local carabaos (https://www.pcc.gov.ph/). It is located at Ubay Stock Farm in Ubay City, Bohol, which is the oldest and largest government-run livestock facility in the Philippines and contains several types of farm animals. Philippine goats are multipurpose animals raised for their meat, milk, and hides (Abilay, 1983), and are commonly known as the 'poor man's cow' because only a small investment is needed to raise them (Dar and Fylon, 1996). The water buffalo is considered a reservoir host of *T. evansi*, and usually has asymptomatic infections with this parasite (Baticados *et al*., 2011a). Goats have been reported to be an important reservoir for trypanosomes because they are very trypanotolerant, usually showing subclinical trypanosomosis, with low and persistent parasitemia, and previous studies have indicated that they can play a vital role in the transmission of this disease (Gutierrez *et al*., 2006; Musinguzi *et al*., 2016; Mossaad *et al*., 2020). In the Philippines, trypanosomosis caused by *T. evansi* (surra) is considered as one of the most economically significant animal parasitic diseases, and over the past few decades several large epidemics of this disease have occurred, which were characterized by causing high morbidity and mortality in buffaloes, as well as in horses, cattle, and goats (Manuel, 1998; Reid, 2002). This study aimed to perform the PCR-based detection of trypanosomes in goats, water buffaloes, and other animal species in the Visayas region of the Philippines to determine the prevalence of this disease in the area, and to evaluate the role of reservoir animals in its epidemiology. The Visayas region of the Philippines is important because it includes Cebu City, the second largest metropolitan city and the usual gateway for livestock trade in the country (Ybañez *et al*., 2013), and Ubay City, where the PCC is located at Ubay Stock Farm.

Thus, the survey of trypanosome infection in the Visayas region is of great importance for the livestock sector of the whole country.

Samples were obtained from goats in Cebu, and from different animal species at Ubay Stock Farm, including water buffaloes (in the PCC), cattle, goats, and horses. PCR-based assays were performed for the detection of trypanosome DNA in these samples. *T. evansi* and *T. theileri* were detected in goats in Cebu. This is the first reported detection of *T. evansi* in Cebu, which could be explained by the fact that the goats in the sampling locations are usually obtained from various places, and *T. evansi* has already been detected in different regions in the country (Reid, 2002; Baticados *et al*., 2011a, 2011b; McInnes *et al*., 2012; Villareal *et al*., 2013). Given that these goats are typically distributed from Cebu to other provinces in the country, and are known reservoir hosts for trypanosomes, this suggests that goats could be the source of infections by trypanosomes to other animal species. Goats are non-susceptible to *T. theileri*, but *T. theileri* was detected in two samples. One was a sample from a transitory commercial goat farm that maintains goats obtained from various places for a few weeks before they are distributed to different areas in Cebu and nearby provinces (Galon *et al*., 2019), and thus these goats may have been initially raised with other animal species. The second was a sample from a farm that raises goats and cattle for breeding and meat production. It is worth mentioning that tabanid flies, which are the transmission vectors of *T. theileri* (Böse and Heister, 1993), are abundant in the Philippines, where they serve as vectors of *T. evansi* (Baticados *et al*., 2011b). Moreover, *T. theileri* was previously reported in cattle in Cebu (Ybañez *et al*., 2013). More investigations are needed to confirm the status of *T. theileri* in goats in

Cebu. Such future studies should take into consideration the fact that goats have been reported to be susceptible to *T. theodori*, a *T. theileri*-like trypanosome that belongs to the same subgenus, *Megatrypanum* (Hoare, 1972).

T. evansi and *T. theileri* were detected in water buffaloes in Bohol by PCR-based assays, with high prevalence. This confirms the endemicity of these parasites in the Philippines, despite the government attempts to eradicate animal trypanosomosis by prevention and control measures (Villareal *et al*., 2013).

Water buffaloes are usually reservoirs for *T. evansi*, with asymptomatic infections (Baticados *et al*., 2011a). The detection of *T. evansi* in apparently healthy buffaloes in the PCC suggested that they could be the sources of infections to other livestock, as they are imported and distributed to rural villages to improve the productivity of local carabaos.

Using PCR-based assays, *T. evansi* and *T. theileri* were also detected in cattle and goats, while only *T. evansi* was detected in horses at Ubay Stock Farm, where the PCC is located. The only previous report of *T. theileri* in the Philippines was that of its detection in cattle in Cebu (Ybañez *et al*., 2013). In this study, I detected this species in cattle and goats in Bohol and Cebu, as well as in water buffaloes in the PCC in Bohol, which suggested the possibility of this trypanosome species having been introduced to the country through the livestock trade area of the Visayas region

Surra outbreaks in the Philippines in the past few decades have been characterized by the manifestation of clinical diseases with high mortality in different animal species. Some of these

outbreaks were suggested to have been associated with the movement of imported buffaloes from the PCC to outlying villages, where they acted as amplifier hosts for infections of other local livestock species (Reid, 2002). Thus, the presence of infected buffaloes in the PCC in the same area as other farm animals at Ubay Stock Farm in Bohol could explain the high prevalence of *T. evansi* and *T. theileri* in these animals.

General summary

In many developing countries, agriculture plays a central role in economies. Moreover, livestock plays important roles, such as draft power, production of milk and meat, transportation, and their property for farmers. Endemic vector born protozoan parasites, especially animal trypanosomosis, babesiosis and theileriosis, are major sources of economic losses in animal industry, especially in developing countries. The donkeys in North African countries and the water buffaloes in South East Asian countries have characteristic roles in each area. However, the level of those losses is not yet exactly specified, and the information about the economics of the treatment of these diseases is insufficient. This is mostly because no update on the efficacy of available drugs in the treatment of those pathogens taking into account the emergence of drug resistance against some of these drugs. This study has provided epidemiological data on the prevalence of two of the most important hemoprotozoan parasitic diseases in animals in two different agricultural developing countries using molecular and serological approaches. Sudan in north east of Africa and the Philippines in south east of Asia were chosen as they represent developing countries in two different continents with different livestock systems and climate zones.

In Chapter 1, I examined the prevalence of equine trypanosomosis and equine piroplasmosis in donkeys in Sudan, wherein donkeys are economically important animals, providing transportation and considered as a source of income to the individuals in rural areas and margins of the cities. The results revealed that trypanosomes (*T. congolense* and *Trypanozoon* spp.) and piroplasms (*Theileria*

equi and *Baesia caballi*) are highly prevalent in the study area. In general, and since the studied animals were apparently healthy, this indicates that donkeys seem to have the greatest resistance to these protozoan diseases and don't show symptoms though high prevalence of the diseases. The sero-prevalence recorded by ELISAs and card agglutination test for *T. evansi* was higher than the prevalence recorded by PCR. This suggested that the parasitemia was lower than the detectable levels by PCR confirming that donkeys are somehow tolerant to infection maintaining very low parasitemia. From this perspective, donkeys might play an important role as a reservoir host in the transmission of those diseases to the more susceptible hosts, especially horse, because no control measures are applied against these diseases in donkeys as they don't show any clinical symptoms. In addition, despite Sudan is known as a non-tsetse infested country, except some tsetse-infested areas in the Southeast and Southwest parts, and the study area is a tsetse-free, *T. congolense*, which is known as a tsetse-transmitted trypanosome spp., was detected by PCR in Khartoum State, which is very far from the tsetse-infested areas of the country, with a prevalence of 9.1%. This result suggested the mechanical transmission of this parasite by blood-sucking insects (e.g. tabanus flies), the same route of transmission of *T. vivax* and *T. evansi*. Therefore, special attention is required since the tsetse transmitted trypanosome is reported in tsetse-free area and special control measures are urgently needed.

In Chapter 2, I focused on the prevalence of trypanosomosis in four domestic animal species (water buffalo, goats, cattle and horses) in the Philippines. Water buffalo is the national animal in the Philippines. Several outbreaks of surra, caused by *T. evansi*, were reported in domestic animals

including water buffalo in different areas in the Philippines. Thus, animal trypanosomosis is one of the most important parasitic diseases that significantly affect the economy of the country, and is considered by the government to be the second most important disease of livestock, after fasciolosis. To control trypanosomosis, the Philippines government has planned to implement a national control program to provide regular trypanocidal treatments in areas with high prevalence of clinical disease. However, *T. evansi* infections were reported in this study in several animal species including water buffalo, goat, cattle, and horse. In general, *T. evansi* infection in water buffaloes and horses causes severe clinical symptoms; however, none of the clinical signs was observed in this study. This suggested the possibility of emergence of drug resistance due to improper treatment causing the animal to be tested positive without clinical symptoms. Moreover, I reported, for the first time, *T. evansi* infection in goats in Cebu Island which is considered as surra-free. This could be explained by the fact that Cebu City, the second largest metropolitan city located in Cebu Island, is the usual gateway of livestock trade in the Visayas region. Therefore, it is an area of a high risk of infection. It is commonly believed that caprine trypanosomosis is only sporadic because goats are highly resistant to infection. This study suggested that *T. evansi* is prevalent in Cebu Island and goats might play a vital role, as reservoir animals, in the transmission of *T. evansi* infection to other domestic animals in this area.

The two chapters show the endemicity of animal trypanosomosis and piroplasmosis in North Africa and the endemicity of animal trypanosomosis in South Eastern Asia. The study also highlights the importance of domestic animals that can be infected with no symptoms. These

animals, like donkeys and goats in this study, may act as reservoir animals for these protozoan diseases, and can transmit these diseases to the susceptible animals. This study proves the importance of regular surveillance to estimate the prevalence of these diseases, which highlights their various dynamics in the affected areas, and supports the efficient measures of intervention.

学位論文要旨

トリパノソーマ症とピロプラズマ症の流行において家畜のレゼルボアが果たしている役割に関する研究

発展途上国の多くは農畜産業が主要産業であり、また家畜は農民たちの農耕、家畜製品の生産手段、輸送手段、さらには資産として重要な地位を占めている。トリパノソーマ症とピロプラズマ症をはじめとする節足動物媒介性住血原虫病の多くは、慢性感染することで感染した家畜の生産性を著しく低下させることから、発展途上国の家畜産業振興の妨げとなっている。発展途上国の家畜産業の中でも、北部アフリカ地域ではロバが、東南アジアではスイギュウが、それぞれの地域で重要な地位を有している。しかしこれらの家畜に対する原虫病の実態は正確には推計されていない。そこで本研究では、環境要因と家畜飼養形態が大きく異なる北部アフリカ（スーダン）及び東南アジア（フィリピン）においてPCR 法を用いた分子生物学的疫学調査と ELISA 法などの血清学的疫学調査を行い、

これらの地域における家畜のトリパノソーマとピロプラズマの感染率を明らかにすることを目的として研究を実施した。

第 1 章では、スーダンをはじめとする北部アフリカ諸国におけるロバの経済的、輸送手段としての重要性を鑑み、同国においてロバにおけるトリパノソーマ症とピロプラズマ症の疫学調査を実施した。その結果、スーダン全土でトリパノソーマおよびウマピロプラズマの感染が蔓延していることが明らかとなった。ロバは一般的に、ウマに比べて原虫病に対する耐性が高く、特徴的な臨床上を示さないとされている。また PCR 法による分子生物学的疫学調査に基く陽性率に比べ、ELISA 法などの血清学的疫学検査での陽性率が高かった。このことは、これらの原虫感染に対してロバが耐性を示し、PCR 法の検出限界以下の低い血中原虫数で推移することによって、これらの原虫病のレゼルボアとなりうることを示唆している。さらに低い原虫数で臨床症状を示さないため、治療や淘汰などの清浄化対策が行われていないことは、ロバがレゼルボアとなってより感受性の高いウマへの感染源となりうる危険性を示している。またスーダンの大部分はツェツェバエ非生息地域であるにもかかわらず、ツェツェバエ生息地域から遠く離れたハルツーム市のロバにおけるツェツェバエ媒介性動物トリパノソーマ（*Trypanosoma congolense*）の感染（9.1 %）が明らかとなった。これは *T.*

*congolense*が *T. evansi* や *T. vivax* と同じくアブなどの吸血性昆虫の機械的伝播によって感染環が維持されていることを示唆している。すなわち、今後アフリカ諸国でのトリパノソーマ症対策を構築する際には、ツェツェバエ非生息地域であっても、ツェツェバエ媒介性動物トリパノソーマ症の存在を考慮した疫学調査ならびに対策が必要であることが示唆された。

第 2 章では、フィリピンをはじめとする東南アジア諸国において象徴的な家畜であるスイギュウに加え、ウシ・ウマ・ヤギの 4 種類の家畜におけるトリパノソーマ症の分子生物学的疫学調査を実施した。フィリピンにおいては過去にスーラ病のアウトブレイクが報告されており、トリパノソーマ症は肝蛭症に続いて 2 番目に重要視されている家畜疾病である。スイギュウにおけるスーラ病の対策として、フィリピン国政府が主体となってトリパノソーマ症高度流行地域を中心とした定期的なトリパノソーマ症治療薬の投与が実施されている。しかし本研究においてフィリピンカラバオセンターとその周囲で飼養されている家畜における *T. evansi* の感染（スイギュウ 16. %, ヤギ 32.2 %,ウシ 28.9%,ウマ 28.6%）が明らかとなった。また調査対象のうち、スイギュウとウマは一般的にスーラ病に対する感受性が高く臨床症状を呈することが知られている。しかし本研究の対象となった家畜には著しい臨床症状は確認されなかった。このことは、薬剤投与による清浄化が不十分なため薬剤耐性 *T. evansi* が出現し、

それらが低い血中原虫数で維持されている可能性を示唆している。さらにこれまで *T. evansi* の感染が報告されていなかったセブ島のヤギにおいて、初めて *T. evansi* の感染（33.9 %）が明らかとなった。セブ島はビサヤ諸島地域における家畜取引の中心となる地域である。またヤギもロバと同じくトリパノソーマ症に対する耐性が高い。すなわち、セブ島のヤギがレゼルボアとなって周辺地域へのトリパノソーマ症が拡散している可能性が示唆された。

本研究により北部アフリカ地域および東南アジア地域の国において節足動物媒介性原虫病が蔓延していることが明らかとなった。特にロバやヤギなど家畜原虫病に対する耐性が高く、臨床症状を呈することが少ないためこれまでに家畜原虫病の実態が顧みられていなかったこれらの家畜が、より感受性の高い家畜への住血原虫病のレゼルボアとしての役割を果たしている可能性が高いことから、今後本研究成果をもとにした効果的な節足動物媒介性住血原虫病対策の実施が期待される。

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Dedication

To my father, Tajelsir Mohamed Elata, and my younger brother Mohamed, may their souls rest in peace. I dedicate this work to both of them. May their memories forever be a comfort and blessing.

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