Studies on development of novel diagnostic methods and discovery of chemotherapeutic agents against animal African trypanosomosis

2014

ZHOU, MO

Doctoral Program in Animal and Food Hygiene
Graduate School of Animal Husbandry
Obihiro University of Agriculture and Veterinary Medicine

動物アフリカトリパノソーマ症に対する新規診断法及び化学療法薬開発に関する研究

平成 26 年

(2014)

周　末

帯広畜産大学大学院畜産学研究科

博士後期課程　畜産衛生学専攻

Contents

Abbreviations

A AAT: animal African trypanosomosis

AAA(+): ATPases associated with diverse cellular activities

B BCT: buffy coat technique

B. bigemina: Babesia bigemina

B. bovis: Babesia bovis

BSF: bloodstream form

C CATT: card agglutination test for trypanosomiasis

D d.p.i. : Days post-infection

E EerI: Eeyarestatin I

ELISA: enzyme linked immunosorbent assay

EMF: epimastigote form

ER: endoplasmic reticulum

ERAD: endoplasmic reticulum associated protein degradation

EST: expressed sequence tags

F FBS: fetal bovine serum

G GST: glutathione S-transferase

H Hsp90: Heat shock protein 90

I ICT: immunochromatographic test

IFAT: indirect fluorescent antibody test

ISG75: invariant surface glycoprotein 75

ITS: internal transcribed spacer

L LAMP: loop-mediated isothermal amplification

M mAECT: minianion exchange centrifugation technique

MCF: metacyclic form

O OD: optical density

ORF: open reading frame

P PBS: phosphate-buffered saline

PCF: procyclic form

PCR: polymerase chain reaction

PDI: protein disulfide isomerase

PI: propidium iodide

P46-ELISA: rTcP46-based ELISA

R rTcP46: recombinant TcIL3000.0.25950 protein

S SDS-PAGE: sodium dodecyl sulfate-polyacrylamide gel electrophoresis

semi-nPCR: semi-nested PCR

SPP: signal peptide peptidase

T *T. annulata: Theileria annulata*

T. b. brucei: *Trypanosoma brucei brucei*

T. b. gambienese: Trypanosoma brucei gambiense

T. b. rhodesiense: *Trypanosoma brucei rhodesiense*

T. congolense: *Trypanosoma congolense*

TcP46: TcIL3000.0.25950 protein

T. evansi: *Trypanosoma evansi*

T. orientalis: Theileria orientalis

T. vivax: *Trypanosoma vivax*

V VATs: variable antigen types

VSG: variant surface glycoprotein

W WHO: world health organization

General introduction

1. Trypanosomosis in general

Trypanosomosis is a widespread protozoan disease that mainly affects poor and marginal population in Latin America, Africa, and Asia, and is caused by protozoan parasites of genus *Trypanosoma* infecting both humans and animals (Sülsen *et al.*, 2008). American trypanosomosis, or Chagas disease is caused by the parasite *Trypanosoma cruzi*, which is endemic throughout much of Mexico, Central and South America. In the 1980s, American trypanosomosis was believed to infect over 24 million people with another 100 million considered at risk, although these estimates have since been reduced (Schofield and Kabayo, 2008). African trypanosomosis causes sleeping sickness in humans and nagana in animals. This disease is prominent on the World Health Organization (WHO) list of neglected tropical diseases and is a major problem to the poorer countries in the world, especially throughout sub-Saharan Africa. Trypanosomosis is a serious constraint to both animal and human health and is one of the most important factors restricting economic development in Africa today (Steverding, 2008). Trypanosomosis caused by *T. evansi* remains an economically important disease in many developing countries of the world, affecting a wide range of wild species and livestock population. *T. evansi* has the widest geographical distribution among all pathogenic parasitic species prevalent in Africa, Asia and South America (Eyob and Matios, 2013).

2. Animal African trypanosomosis and the life cycle of trypanosomes

AAT or nagana disease is responsible for over 3 million cattle and other livestock deaths each year across sub-Saharan Africa with more than 46 million cattle at risk of infection with the disease leading to a considerable impact on the agricultural economy. Direct production

losses amount to approximately $1.2 billion each year. The estimates are as much as $4.7 billion a year when indirect losses from the inability to use land and livestock to their fullest potential are included (Grady *et al.,* 2011). AAT is spread by tsetse flies carrying a variety of trypanosomes, the most important species in this disease are *T. brucei brucei*, *T. congolense* and *T. vivax* (Nakayima *et al.,* 2012). Trypanosomes cause relatively mild infections in wild animals, while in domestic animals they cause a severe, often fatal disease. All domestic animals can be affected by nagana and the symptoms are fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia and paralysis (Steverding, 2008).

Trypanosomes are single-celled organisms. Like all parasitic protozoa, they display extreme adaptation to their environment that constitutively exhibit complex life cycles. They undergo both metabolic and morphological changes between the mammalian host and tsetse vector during their life cycle (Matthews and Gull, 1997; Leak, 1999; Matthews, 2005). In the mammalian host, bloodstream forms (BSF) are covered with a dense coat consisting of approximately 10^7 copies per cell of VSG that is famously involved in antigenic variation, allowing the trypanosome population to avoid elimination by the host immune system (Alsford *et al.,* 2012). When BSF trypanosomes are consumed by a tsetse during its blood meal, they enter the insect midgut and a subpopulation differentiates to procyclic forms (PCFs) that are adapted to life in the midgut (Eyford *et al.,* 2011). The dividing PCFs first establish in the tsetse midgut and then only a few of them migrate to proboscis (*T. congolense*) or salivary gland (*T. brucei* spp.) to mature as differentiated dividing epimastigote forms (EMFs). The EMFs continuously divide while adhering to the epithelial cells throughout the life span of the tsetse fly. Finally, the dividing, adherent EMFs undergo a cell differentiation process called metacyclogenesis differentiate into non-dividing, non-adherent, VSG-expressing and animal infectious metacyclic forms (MCF) (Vickerman, 1973; Hendry and Vickerman, 1988.). The MCF trypanosomes are injected into the host

bloodstream when tsetse takes a blood meal and complete the life cycle.

3. Diagnosis of animal African trypanosomosis

In the epidemiology and control of any disease, precise diagnosis and definitive identification of the causative agent is extremely important. However, there are no specific clinical signs of the infection, and the intermittent and usually low parasitaemias make the diagnosis of trypanosomosis difficult (Pillay *et al.,* 2013). The diagnosis of AAT has therefore been the subject of extensive research over the century following David Bruce's discovery of associations among the nagana, the tsetse fly and the trypanosome. The diagnosis of trypanosomosis is basically divided into direct and indirect detection techniques of the parasite.

Examination of blood smears is rapid and best suitable method to screening herds in the field. Although the examination of blood smears alone is a relatively insensitive way to detect infection, but the method is simple and with great practical significance (Reyna-Bello *et al.,* 1998). The more sensitive direct method to detect *T. congolense* and *T. vivax* infections is examination of wet preparations of the microhaematocrit buffy coat under microscope. In order to improve the sensitivity of parasitological diagnosis, the Maect was elaborated to concentrate the parasite in blood prior to microscopic examination (Chappuis *et al.,* 2005). This method has been used for the diagnosis of especially human sleeping sickness (Camara *et al.,* 2011). However, it is not yet widely used in animals under field conditions as the technique is impractical for routine veterinary purposes.

Because of the difficulty of detecting trypanosomes in the blood numerous indirect tests have been applied to the diagnosis of trypanosomosis. A range of serological tests has been applied to the diagnosis of animal trypanosomosis. However, the serological diagnosis could not differentiate the current from the past infections, and cross reactions often occur between

some trypanosome species. The CATT has been found useful in the diagnosis of *T. evansi* infections in water buffalo, but this method is less likely to be applicable to *T. congolense* and *T. vivax* than the *Trypanosoon* species because of difficulty in identifying suitable variable antigenic types (VATs) in these species (Luckins, 1992). The most successful serological adjuncts to diagnosis of trypanosomosis are the IFAT and the ELISA. IFAT has been shown to be both sensitive and specific in the bovine anti-trypanosome antibodies (Luckins and Mehlitz, 1978), although there is cross-reactivity among *T. b. brucei*, *T. congolense* and *T. vivax*. The antibody-detection ELISA has particularly been used for epidemiological surveys to detect trypanosome antibodies and it was shown to be capable of detecting specific antibodies in trypanosome-infected cattle. However, the detection techniques involve use of either whole parasite or crude parasite lysate as the antigen which are not often standardized (Hopkins *et al.*, 1998).

The development of sensitive and specific molecular detection techniques has facilitated epidemiological studies of trypanosomosis in many African countries including Uganda, Ivory Coast and Burkina Faso (Mugittu *et al.,* 2001). These techniques could rapidly detect and identify trypanosome species in both mammalian hosts and the tsetse vector with high sensitivity and specificity (Mwandiringana *et al.,* 2012). Various types of molecular methods have been used in the diagnosis of AAT. These applications have clearly demonstrated the potential use for wide-scale application of molecular diagnostic methods in the epidemiology of AAT.

4. Treatment of animal African trypanosomosis

The field control of AAT mainly relies on two broad strategies: vector control and using chemotherapeutic agents on infected animals. Attempts to control tsetse have been made for over 60 years. Initially, they included eradication of wildlife, clearing of fly barriers to prevent

the advance of the vector and widespread bush clearing to destroy breeding habitats. Following the introduction of modern chemicals, the principal method employed to control tsetse populations has been the use of insecticides alone or in conjunction with traps and screens. In the 1980's, application of the sterile male technique received considerable attention and this method has reduced fly populations in limited trails (Adamu *et al.,* 2011).

In general, the chemotherapeutic approach is used much more widely than vector control, because it is easier to kill the trypanosomes than the flies. Drug control of animal trypanosomosis relies essentially on three drugs, namely: Homidium (Homidium chloride-Novidium and Homidium bromide-Ethidium), Diminazine aceturate (Berenil) and Isometamidium chloride (Samorin, Trypamidium). Recently, Quinapyramine sulphate (Antrycide) has been reintroduced because of the need to combat camel trypanosomosis (Delespaux and de Koning, 2007). Diminazine aceturate (Berenil) is a widely used chemotherapeutic drug, which is effective against AAT. Isometamidium (Samorin, Trypa-midium) also the commonly employed trypanocides. Its main advantage is prophylactic use against trypanosomosis. The Homidium is the excellent chemotherapeutic agents for *T. congolense* and *T. vivax*. These three drugs have been in the market more than 40 years, drug resistance that started from one point has made them become ineffective over large area of Africa (Chitanga *et al.,* 2011). Furthermore, the toxicity and adverse effect of these compounds pose to human and animal health are also significant problems. Despite of this risk, the three established compounds remain available and popular and their use is well established in Africa.

5. Aims of the present study

AAT is a parasitic disease that causes serious economic losses in livestock due to anaemia, loss of condition and severe emaciation (Steverding, 2008). The effective control

and management of this disease relies on accurate diagnosis and subsequent treatment of the infected animals. Concerning serodiagnosis, the discovery and production of trypanosome specific antigens by recombinant DNA techniques is particularly important because such strategy produces pure and highly specific recombinant trypanosome antigens than the native preparations. Since recombinant antigens are standardized and have minimum batch-to-batch variation, they could be easily optimized for use in ELISA and ICT systems. Hence, complementing the other established diagnostic methods, such as microscopy and IFAT for the diagnosis of AAT. For the molecular diagnostic methods, PCR has been widely applied for the detection of trypanosomes and has been shown to be highly sensitive and specific. The use of PCR for detecting trypanosome DNA is the most reliable and accurate technique available for the specific identification of natural animal infections (Ahmed *et al.*, 2013). Thus, PCR-based diagnosis of trypanosomosis in livestock has now been used in a number of studies in Africa. Despite severe adverse effects in currently available trypanocidal drugs, treatment of HAT and AAT heavily depend on those drugs. Hence, the drug resistance is widely spread over Africa. Therefore, new trypanocidal drugs are urgently needed.

The overall objective of this study was to develop novel diagnostic methods of AAT and to evaluate new drug targets of African trypanosomes to facilitate drug discovery, with specific objectives as follows: 1) To identify and characterize *T. congolense* novel antigen for application in the serodiagnosis (chapter 1); 2) To establish and evaluate the potential use of TcIL3000.0.25950 gene from *T. congolense* in semi-nested PCR assay (chapter 2); and, 3) To evaluate the trypanocidal activities of the compounds targeting the different components of

endoplasmic reticulum associated protein degradation (ERAD) pathway (chapter 3).

Chapter 1

Identification and characterization of a *Trypanosoma congolense* 46 kDa protein as a candidate serodiagnostic antigen

1-1. Introduction

T. congolense is a major pathogen responsible for AAT (nagana), which is transmitted by the tsetse fly (*Glossina* spp.) (Coustou *et al.,* 2010). The disease can affect various species of mammals, and it is particularly important in cattle from the economic viewpoint (Kristjanson *et al*., 1999). The clinical manifestations of the acute stage after infection are fever, listlessness, emaciation, edema, anemia and eventually death (Mendoza-Palomares *et al*., 2008). The disease is the main constraint to livestock agriculture in large parts of the African continent, where it causes serious economic losses on an annual basis. The definitive diagnosis of acute AAT depends heavily on the direct detection of the parasite in blood by light-microscopy (Herber and Lumsden, 1976; Woo, 1970). However, microscopy has limitations in latent infections where the parasite is often difficult to detect in blood because of extremely low parasitemia. PCR and LAMP have recently been developed for detecting *T. congolense* infection with high sensitivity and specificity (Cox *et al*., 2010; Gillingwater *et al*., 2010; Laohasinnarong *et al*., 2011; Thekisoe *et al*., 2007). Although these molecular tests potentially improve the specificity and sensitivity of AAT diagnosis, the field application of these state-of-the-art tests is hampered by the requirement for a specialized laboratory setup and skilled personnel. Alternatively, a variety of serodiagnostic tests have been developed for trypanosomosis (Chappuis *et al*., 2005; Hilali *et al*., 2004; Nantulya *et al*., 1989). In order to

detect antibody responses against trypanosome infection, card agglutination test, antibody detection ELISA and IFAT are commonly utilized as recommended tests (OIE. 2012). These tests, however, use trypanosome cell lysate or fixed parasite antigens, whose qualities often vary from batch to batch (Goto *et al*., 2011; Rebeski *et al*., 2000). In contrast, recombinant antigens can easily be prepared in large scale, are relatively stable and have higher specificity than parasite cell lysate antigens (Tran *et al*., 2009). Thus, development of recombinant trypanosome antigen-based ELISA tests is urgently needed. *T. congolense* has at least four developmental stages in its life cycle, namely BSF, PCF, EMF and MCF (Peacock *et al*., 2012). Among these forms, BSF and MCF are animal-infective stages, which are the major targets for diagnosis and treatment. Both BSF and MCF express VSG, which allows antigenic variation to evade host antibody responses (Donelson *et al*., 1998). In general, VSGs are not suitable as diagnostic antigens, because of their antigenic variation. In contrast, the invariant antigens are likely to provoke protective immune responses including high antibody responses in the chronic phase of *T. congolense* infection (Agur and Mehr, 1997). Therefore, the invariant antigens are good candidates for diagnosis and vaccine development. The recombinant ISG75 has been successfully expressed in *E. coli*, and rISG75-ELISA showed high specificity and sensitivity for *T. evansi* infection in camels (Tran *et al*., 2009). In previous studies, Eyford reported EST analysis and differential protein expression in each life cycle stage of *T. congolense* (Eyford *et al*., 2011; Helm *et al*., 2009). The present study focused on identification of the proteins highly expressed in BSF and/or MCF stage from the EST and the proteome data sets and sought to evaluate novel invariable proteins as candidate serodiagnostic antigens for *T. congolense* infection.

1-2. Materials and methods

Parasites. *T. congolense* IL3000 strain is a savanna type parasite that was discovered

near the Kenya/Tanzania border in 1966 (according to the records of the Biological Services Unit at the International Livestock Research Institute, Nairobi, Kenya). Samples of this parasite were stored in liquid nitrogen at the National Research Center for Protozoan Diseases in Japan. The PCF and BSF were cultured using TVM-1 and complete Hirum's modified Iscove's medium (HMI)-9 medium, respectively (Hirumi and Hirumi, 1991). The EMF and MCF of these parasites were produced from *in vitro* PCF culture (Coustou *et al*., 2010; Hirumi and Hirumi, 1991; Sambrook and Russell, 2001). PCF were routinely maintained by diluting 3 ml of log-phase parasite suspension with 7 ml of fresh medium every 2 days. Adherent EMF appeared in PCF cultures 1-2 months after the initiation of PCF cultures. EMF colonies became confluent within 2 months. The plastic-adherent EMF cultures were maintained by replacing the entire culture supernatant with fresh medium every 2 days. Live PCF were obtained from cultures by centrifugation at 1,500 *g* for 10 min at 4^{o}C. Live EMF were prepared from culture flasks by washing adherent cells three times with 10 ml of PBS containing 1% glucose (PSG) to remove non-adherent cells, and the remaining cells were removed with a rubber cell scraper followed by centrifugation at 1,500 x*g* for 10 min at 4^{o}C. Since differentiation from EMF to MCF continuously occurs in EMF cultures, MCF accumulates in the culture supernatant. Hence, MCF was purified from EMF culture supernatants by DE 52 anion-exchange column chromatography (Whatman Plc., Buckinghamshire, U.K.) (Lanham and Godfrey, 1970).

Cloning of the TcP46. Total DNA was extracted from the parasite using a Puregene DNA Purification System Kit (Qiagen, Dusseldorf, Germany) according to the manufacturer's instructions and stored at -30^{o}C until used. The open reading frame of the TcP46 gene (Gene ID: TcIL3000.0.25950) was amplified by PCR from *T. congolense* total DNA using primers with the *Eco* RI and *Sal* I sites (underlined), namely P1 (5'-GCGAATTC ATG AAC GGA TCG GCT GT-3') and P2 (5'-GCGTCGAC TTA GTA ATT CGC CTC GC-3'). The PCR

products were inserted into the pCR2.1-TOPO vector and sequenced with M13 forward and M13 reverse primers. The hydrophilic and antigenic characteristics of TcP46 were predicted using the DNASTAR analyzer program (Netwell, Tokyo, Japan). The putative N-terminal signal peptide was analyzed using the SignalP server (http://www.cbs.dtu.dk/services/SignalP/).

Southern blot analysis. Total DNA was prepared from *T. congolense* by the phenol-chloroform method (Sambrook and Rusell, 2001). For Southern blot analysis, total DNA was digested overnight with *Hin*d III, *Kpn* I, *Sal* I, *Xba* I, *Bcg* I, *Bsp* MI, *Msc* I and *Xho* I restriction enzymes and electrophoresed on 1.0% (w/v) agarose gel. The electrophoresed DNA samples were transferred to a nylon membrane (Hybond-N^+, GE Healthcare, Pittsburgh, PA, U.S.A.) as previously described (Sakurai *et al*., 2008). Preparation of the labeled cDNA probe with the full-length TcP46 gene, DNA hybridization and labeling of the probe were performed using AlkPhos Direct Labeling Kit and Detection Systems (GE Healthcare). The result was visualized by using CDP-star (GE Healthcare) according to the manufacturer's instructions. Imaging was performed using X-ray film (Eastern Kodak Company, Rochester, NY, U.S.A.).

Expression and purification of rTcP46. The ORF of the TcP46 gene in the pCR2.1-TOPO vector was subcloned into a pGEX-4T-1 *Escherichia coli* expression vector (GE Healthcare). The correct orientation and sequence of the subcloned TcP46 gene was examined by sequence analysis and designated as the pGEX-4T-1/TcP46. rTcP46 was expressed as a GST-fusion protein in the *E. coli* BL21 strain according to the manufacturer's instructions (GE Healthcare). In order to purify rTcP46, *E. coli* was suspended in TNE buffer (50 mM Tris-HCl (pH 7.5), 100 mM NaCl, and 2 mM EDTA), sonicated and then centrifuged at 9,000 x *g* for 10 min at 4^{o}C. The rTcP46-GST fusion protein was affinity purified from the supernatant using glutathione-Sepharose 4B beads (GE Healthcare). Protein concentrations

were measured using a modified Lowry protein assay kit (Thermo Scientific, Pittsburgh, PA, U.S.A.).

Preparation of mouse anti-rTcP46 immune sera. Five six-week-old ICR mice (Clea, Tokyo, Japan) were immunized intraperitoneally with 100 μg of purified rTcP46-GST in an equal volume of TiterMax® Gold (TiterMax USA Inc., Norcross, GA, U.S.A.) for the primary immunization. Two booster immunizations were given at 14 day intervals using the same amount of the antigen emulsified in TiterMax® Gold. Serum samples were collected two weeks after the last immunization. The experiment was conducted in accordance with the Guiding Principles for the Care and Use of Research Animals of the Obihiro University of Agriculture and Veterinary Medicine (No. 24-135).

Indirect fluorescent antibody test and confocal laser scanning microscopy. Blood smears of *T. congolense* PCF, EMF, MCF and BSF stages were fixed with 100% methanol for 30 min. Anti-rTcP46 mouse serum, diluted 1:100 with PBS containing 0.5% bovine serum albumin (PBS-BSA), was applied to the fixed smears as the primary antibody and incubated for 1 hour at 37^{o}C. After three washings with PBS, Alexa-Fluor® 488 conjugated goat anti-mouse IgG secondary antibody (1:600 dilution in PBS-BSA, Molecular Probes, Eugene, OR, U.S.A.) was applied and incubated for 30 min at 37^{o}C. The slides were washed four times with PBS and incubated with 6.25 μg/ml PI (Molecular Probes) containing 100 μg/ml RNase A (Qiagen) for 10 min at 37^{o}C. After three washings with PBS, the glass slides were mounted by adding 50 μl of a 50% glycerol-PBS (v/v) solution and then covered with a cover glass. The slides were examined by confocal laser scanning microscopy (Leica, Solms, Germany).

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot analysis. To identify the molecular mass of native TcP46 throughout the life cycle stages of *T. congolense*, the mouse anti-rTcP46 serum was used to detect the native

TcP46 from trypanosome cell lysates by Western blot analysis. Each life cycle of the parasite was harvested from *in vitro* culture and washed three times with PBS. The parasite pellets were treated with cell lysis buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM $CaCl_2$, 10% glycerol and 1% Triton X-100) and incubated at 4^{o}C for 1 hour. The cell lysates were sonicated and centrifuged at 7,000 x*g* for 20 min. The supernatants were collected, and BCA protein assay was used for protein quantification (BCA Protein Assay Kit, PIERCE Chemical Company, Rockford, IL, U.S.A.). The supernatant (50 μg/lane) was then subjected to SDS-PAGE. The cell lysates were mixed with SDS-PAGE sample buffer (125 mM Tris-HCl (pH 6.8), 4% (w/v) SDS, 20% (v/v) glycerol, 3% (v/v) 2-ß-mercaptoethanol and 0.02% bromophenol blue. After incubating at 100^{o}C for 5 min, the lysates were separated by SDS-PAGE with 10% gel. The separated parasite proteins were then transferred to an Immobilon-P transfer membrane (Millipore, Billerica, MA, U.S.A.). Western blot analysis was carried out as previously described (Bannai, *et al*, 2003). To determine the antibody response against the 46.4 kDa protein (TcP46) in mice infected with *T. congolense*, the GST-rTcP46 (25 μg/lane) and GST protein (25 μg/lane) were subjected to SDS-PAGE, and transferred to a membrane, and then probed with 100 times diluted infected mouse sera and pre-infected mouse sera, respectively, by Western blot analysis.

Mice infections. After collection of blood to obtain pre-infection mouse sera, three ICR mice (female, 8 weeks old) were inoculated intraperitoneally with *in vitro* prepared BSF (10^3 parasites/mouse). The parasitemia of each mouse was examined every day for 78 days. Thereafter, it was examined weekly for another month. The level of parasitemia was estimated according to the matching method (Hilali, *et al.,* 2004). This experiment was conducted in accordance with the Guiding Principles for the Care and Use of Research Animals promulgated by Obihiro University of Agriculture and Veterinary Medicine (No. 24-135).

Enzyme-linked immunosorbent assay (ELISA). Individual wells of a microtiter plate

(Thermo Scientific) were coated with the purified GST-rTcP46 protein (0.5 μg / well) or the control GST protein (0.5 μg/well) in an antigen coating buffer (0.05 M carbonate-bicarbonate buffer, pH 9.6) overnight at 4°C. The plates were then blocked with PBS containing 3% (w/v) skim milk for 1 hour at 37 °C. After washing, the plates were incubated with 200 times diluted mice serum samples or cattle serum samples. The plates were washed six times with PBST and then incubated with horseradish peroxidase (HRP) conjugated to goat anti-mouse IgG or HRP conjugated to goat anti-bovine IgG (Invitrogen, Carlsbad, CA, U.S.A.) diluted to 1:5,000 with the blocking solution for 1 hour at 37℃. Thereafter, the enzyme reaction was developed with 3,3',5,5'-tetramethylbenzidine (TMB, Kirkegaard & Perry Laboratories, Gaithersburg, MD, U.S.A.) at room temperature. Finally, 50 μl of stop solution (1 M phosphoric acid) was added, and the absorbance was read at 450 nm (Nguyen *et al*., 2012). The cut-off value was defined as the mean value plus 3 standard deviations of the mean optical density (OD) obtained from 9 SPF mice serum samples and 26 normal cattle serum samples, respectively. At the same time, ELISA was also performed according to the OIE Manual of Diagnostic Tests and Vaccines for Terrestrial Animals (2012) using PCF cell lysate antigen. Each microplate well (Thermo Scientific) was coated with 160 ng of antigen and incubated overnight at 4 °C. The subsequent protocols were performed as described above.

Serum samples. The serum samples used in this study include 6 serum samples from mice experimentally infected with *T. congolense* IL3000 strain, 9 samples from SPF mice, 26 samples from healthy cattle obtained in Japan, 9 samples from cattle experimentally infected with *Babesia bovis*, 15 samples from cattle experimentally infected with *Babesia bigemina* and 5 samples from cattle experimentally infected with *Anaplasma marginale.*

1-3. Results

Identification and characterization of the TcP46 gene. Four proteins which were

found to have greater expression in MCF and/or BSF stages were selected from the previously reported differential protein expression data set (Table 1) (Eyford *et al*., 2011). All of these proteins were successfully expressed by the bacterial expression system and purified for preliminary evaluation as diagnostic antigens. Western blot analysis of *T. congolense*-infected mouse sera was used for this evaluation. As a result, only the recombinant TcIL3000.0.25950 protein was recognized in the infected mouse sera (Data not shown). Analysis of the putative N-terminal signal peptide in the TcIL3000.0.25950 protein (TcP46) sequence using the SignalP server showed that this sequence had no signal peptide. The TcP46 consisted of highly hydrophilic amino acid residues and its predicted antigenic index was high throughout the entire sequence (Fig. 1A).

Southern blot analysis. Southern blot analysis was performed to determine the copy number of the TcP46 gene in the parasite genome. The genomic DNA probed with TcP46 cDNA showed a single hybridization band after DNA digestion by *Hin*d III, *Kpn* I, *Sal* I and *Xba* I, which did not cut the TcP46 open reading frame (Fig. 1B, lanes 1-4). However, *Bcg* I, *Bsp* MI, *Msc* I and *Xho* I cut a single site within the TcP46 gene, yielding three bands with a common fragment at 4 kbp (Fig. 1B, lanes 5-8). These results suggest that genomic DNA of *T. congolense* contains at least 2 copies of tandemly arranged TcP46 genes. The distance between the tandemly arranged TcP46 genes is 2.8 kbp.

Detection of anti-rTcP46 antibody from *T. congolense*-infected mouse sera. The full-length TcP46 gene was cloned into prokaryotic expression vector pGEX-4T-1 and expressed in *E. coli* as a soluble GST-fusion protein with a molecular mass of approximately 72 kDa, including the 26 kDa GST tag (Fig. 2, lane 1). The rTcP46 protein was recognized in sera from mice experimentally infected with *T. congolense* by Western blot analysis (Fig. 2, lane 3), whereas there was no reaction with the GST protein (Fig. 2, lane 4). Neither GST-rTcP46 nor GST was recognized in pre-immune sera (Fig. 2, lanes 5 and 6).

Characterization of the native TcP46. Mouse anti-rTcP46 sera were prepared, and used to identify native TcP46 in all life cycle stages of *T. congolense* by means of Western blot analysis and confocal laser scanning microscopy. The anti-rTcP46 sera specifically reacted with the approximately 46 kDa protein in parasite lysates of all life cycle stages by Western blot analysis. Although the bands were broad, the molecular mass of native TcP46 was consistent with the expected mass (Fig. 3A). This indicates that TcP46 is an invariable protein constitutively-expressed throughout the life cycle stages. Meanwhile, specific and stronger reactions were detected in BSF and MCF-stage parasites in comparison with the weak reactions in the EMF and PCF-stage parasites (Fig. 3A). To determine the cellular localization of TcP46, all stages of *T. congolense* parasites were probed with the mouse anti-rTcP46 serum. Confocal laser scanning microscopy demonstrated that the expression of native TcP46 was mainly in the cytoplasm in all of the developmental stages (Fig. 3B).

Specificity and sensitivity of the rTcP46-based ELISA. The specificity of the rTcP46-based ELISA (P46-ELISA) was evaluated using the sera of mice experimentally infected with *T. congolense* and the sera of non-infected SPF mice. The cut-off value of the P46-ELISA was 0.07. This was calculated by the OD value from the serum samples of 9 SPF mice. Furthermore, the six serum samples from mice experimentally infected with *T. congolense* showed a high absorbance value. There was no cross-reaction with 26 serum samples from healthy cattle, 9 samples from *B. bovis*-infected cattle, 15 samples from *B. bigemina*-infected cattle or 5 samples from *A. marginale*-infected cattle (Fig. 4). The cut-off value of the rP46-ELISA for cattle serum samples was 0.46 (Fig. 4B). The sensitivity of rP46-ELISA was examined using sera sequentially obtained from 3 mice infected with *T. congolense* IL3000. Specific antibodies against TcP46 were detected from sera of the 3 mice as early as 8 days post-infection. High antibody titers were maintained until the chronic stage of infection, which was characterized by undetectable levels of parasitemia (Fig. 5A). This

P46-ELISA could detect the infection 6 days earlier than PCF cell lysate ELISA (Fig. 5B).

1-4. Discussion

T. congolense infection (nagana) causes significant losses in livestock production in Africa. To control this disease, it is important to develop sensitive and reliable serological tests for the detection of *T. congolense* infection in animals. So far, only a few recombinant antigens have been identified to develop serological diagnostic methods of trypanosome infection (Bannai *et al*., 2003; Boulangé *et al*., 2002; Sengupta *et al*., 2012; Tran *et al*., 2009). Therefore, there is a need to seek more candidate diagnostic antigens in order to develop accurate and sensitive serodiagnostics for nagana. Since *T. congolense* undergoes a complex developmental cycle, each developmental stage of the parasite expresses both stage-specific and constitutive proteins. In this study, I focused on the proteins with high expression levels in the MCF and BSF stages, because of their importance in serodiagnosis. Four proteins (TcIL3000.0.25950, TcIL3000.0.10.3480, TcIL3000.8.629, and TcIL3000.7.1980) were selected from the data for differential protein expression in all life cycle stages of *T. congolense* (Table 1) (Eyord *et al*., 2011). All of these proteins were successfully expressed by the bacterial expression system and purified for preliminary evaluation as a diagnostic antigen by Western blot analysis using *T. congolense*-infected mouse sera (Data not shown). As a result, only the recombinant TcIL3000.0.25950 protein was recognized by *T. congolense*-infected mouse sera (Fig. 2). Thus, I decided to further investigate the TcIL3000.0.25950 protein as a candidate serodiagnostic antigen. The TcIL3000.0.25950 gene contained an ORF of 1,218 bp encoding a 46.4 kDa protein (TcP46). Southern blot analysis revealed that at least 2 copies of the TcP46 gene are tandemly arranged in the parasite genome (Fig. 1B). The TcP46 protein is expressed throughout the life cycle stages of the parasite as an approximately 46 kDa protein (Fig. 3A). As the TcP46 was predicted to be a soluble protein, TcP46 was localized in the

parasite cytosol (Fig. 3B). Meanwhile, a strong reaction was observed in the BSF and MCF parasite stages compared with EMF and PCF. This result, in part, consists with the previously reported proteome analysis which revealed that TcP46 showed 5.36 times higher expression levels in MCF than EMF parasite levels (Eyord *et al*., 2011). The potential of rTcP46 as a serodiagnostic antigen was evaluated by ELISA with sequentially collected serum samples from *T. congolense* experimentally infected mice. The results showed that rTcP46-based ELISA was able to detect a specific antibody response from 8 days post-infection until the end of the experiment (92 days post-infection) (Fig. 5). This would imply that rTcP46-based ELISA may be an applicable diagnostic test of both the acute and chronic stages of the infection. In addition, the high antigenicity suggested that TcP46 may play an important role in the host immune response during *T. congolense* infection. No false-positive samples due to cross-reaction with sera derived from cattle infected with *B. bovis*, *B. bigemina* or *A. marginale* were detected by P46-ELISA. Since mixed infection of these protozoan parasites and *T. congolense* possibly occurs, the result indicates that ELISA could be a specific test.

In conclusion, the TcP46 gene was identified and characterized as an immunodominant antigen that was a candidate serodiagnostic antigen of *T. congolense* infection. The GST-rTcP46-based ELISA had high specificity and was applicable for both the acute and chronic stages of infection. Overall, TcP46 might be a useful serodiagnostic antigen for *T. congolense* infection. A further study will require the use of a number of serum samples from *T. congolense*-infected cattle in order to evaluate its practical use in the field.

1-5. Summary

T. congolense is a major livestock pathogen in Africa, causing large economic losses with serious effects on animal health. Reliable serodiagnostic tests are therefore urgently needed to control *T. congolense* infection. In this study, we have identified one *T. congolense* protein as a new candidate serodiagnostic antigen. The TcP46 is expressed 5.36 times higher in metacyclic forms than epimastigote forms. The complete nucleotide sequences of TcP46 contained an open reading frame of 1,218 bp. Southern blot analysis indicated that at least two copies of the TcP46 gene were tandemly-arranged in the *T. congolense* genome. The recombinant rTcP46 was expressed in *Escherichia coli* as a GST fusion protein. Western blot analysis and confocal laser scanning microscopy revealed that the native TcP46 protein is expressed in the cytoplasm during all life-cycle stages of the parasite. Moreover, an ELISA based on rTcP46 detected the specific antibodies as early as 8 days post-infection from mice experimentally infected with *T. congolense*. No cross-reactivity was observed in the rTcP46-based ELISA against serum samples from cattle experimentally infected with *Babesia bigemina*, *B. bovis* and *Anaplasma marginale*. These results suggest that rTcP46 could be used as a serodiagnostic antigen for *T. congolense* infection.

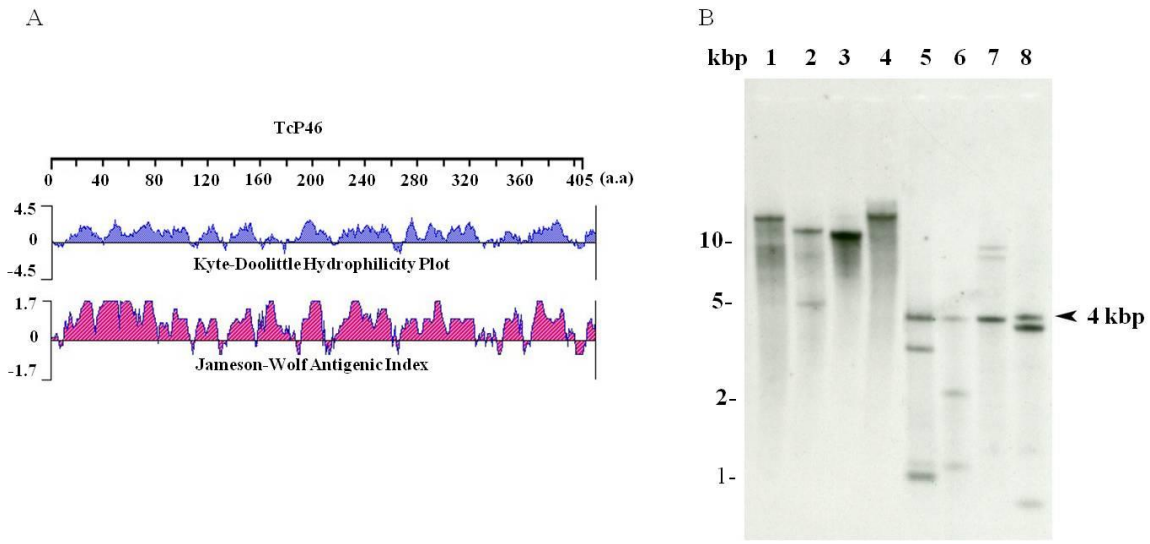

Fig. 1. Genetic and molecular characterizations of TcIL3000.0.25950 protein (TcP46). (A) Software analysis of hydrophilicity and antigenicity of TcP46. (B) Southern blot analysis of TcP46. Genomic DNA was treated with *Hin*d III (lane 1), *Kpn* I (lane 2), *Sal* I (lane 3), *Xba* I (lane 4), *Bcg* I (lane 5), *Bsp* MI (lane 6), *Msc* I (lane 7) and *Xho* I (lane 8). The restriction enzymes used for lanes 1-4 did not cut the TcP46 open reading frame (ORF), while the enzymes used for lanes 5-8 cut a single position within the ORF.

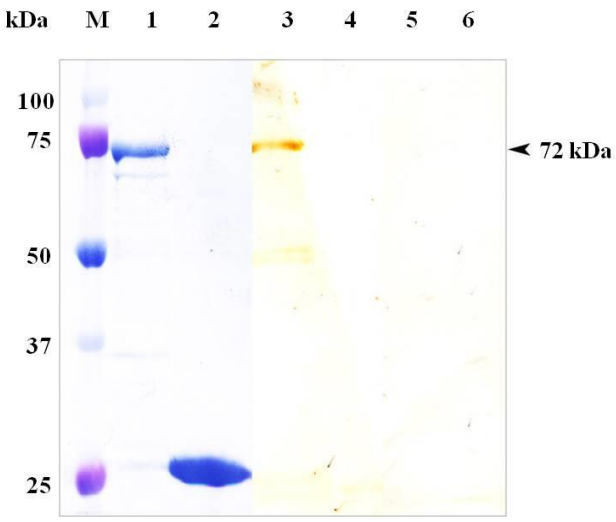

Fig. 2. SDS-PAGE and Western blot analysis of the recombinant TcP46. M: Molecular size marker. The rTcP46 fused with GST (lane 1) and purified rGST (lane 2) were stained by amide black. The GST-rTcP46 (lane 3) and the rGST (lane 4) were reacted with serum from mice infected with *T. congolense*. The rTcP46 (lane 5) and the rGST (lane 6) were reacted with pre-immune mouse sera.

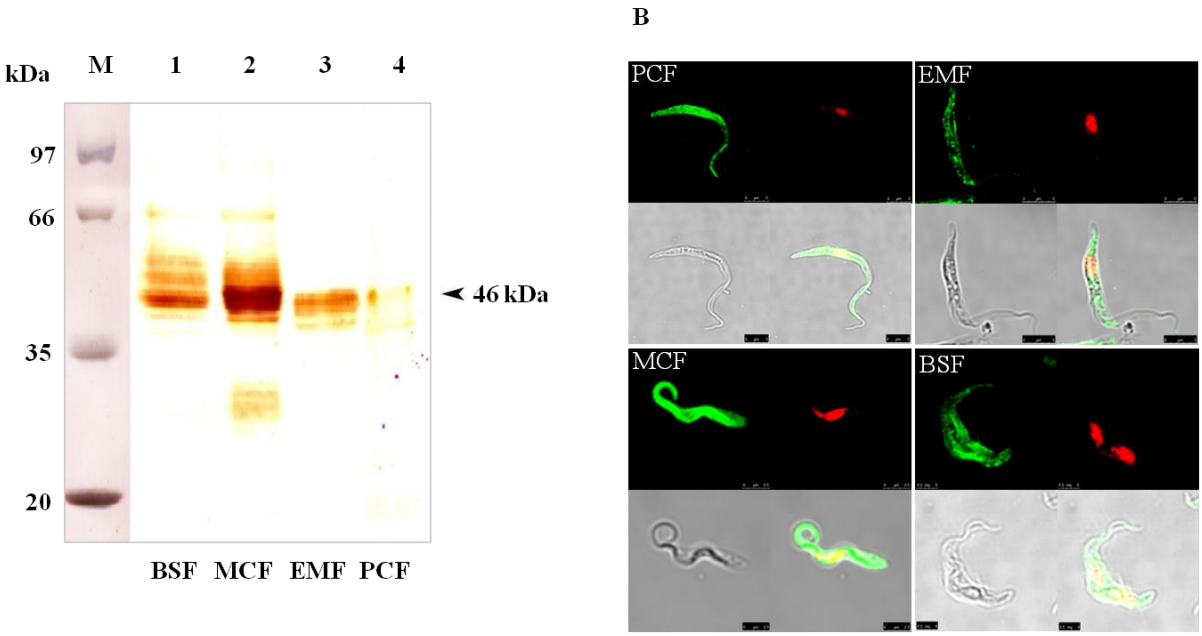

Fig. 3. Detection of the native TcP46 from all life cycle stages of the parasite. (A) Lane M: Molecular size marker. Western blot analysis of the native TcP46 was carried out using the cell lysate from BSF, MCF, EMF and PCF stages of *T. congolense* and anti-rTcP46 mouse serum. (B) Cellular localizations of the TcP46 in all four life cycle stages of *T. congolense* (PCF, EMF, MCF and BSF) were examined by immunofluorescence staining and confocal laser scanning microscopy. Green indicates immunofluorescence staining of TcP46, and red indicates nucleus and kinetoplast.

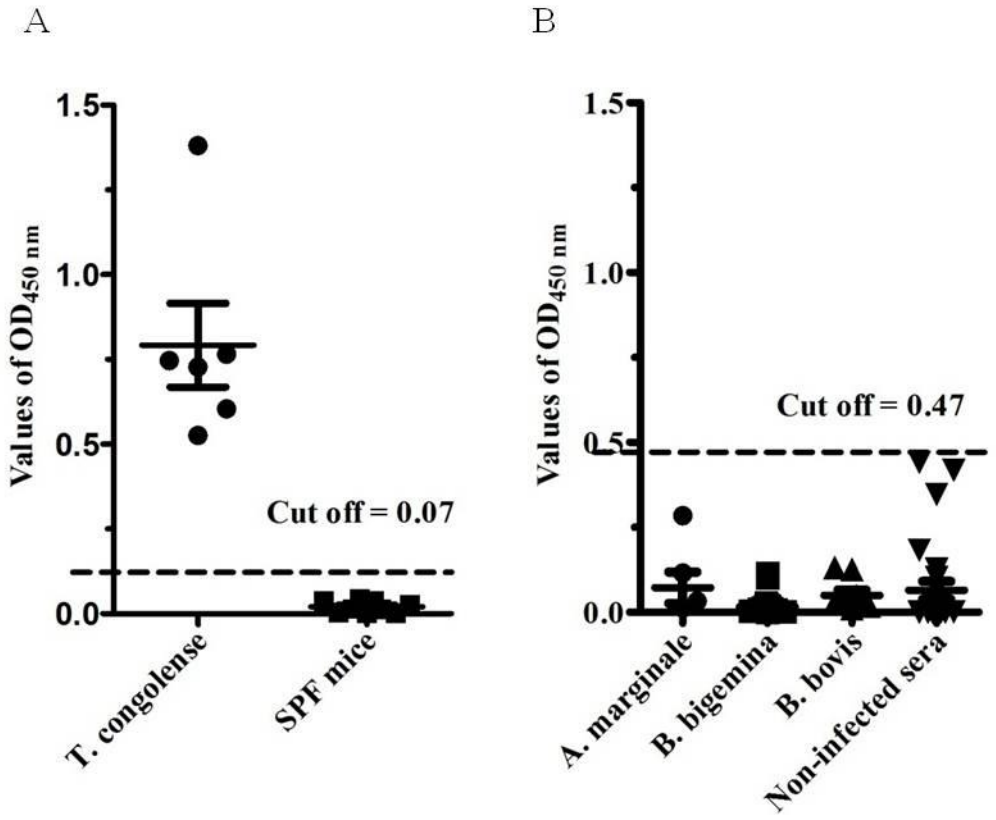

Fig. 4. Evaluation of the specificity of rTcP46-based ELISA. (A) Evaluation of specificity with *T. congolense* experimentally infected mouse sera (n=6); SPF mouse sera (n=9), dashed line indicates the cutoff value (0.07). (B) Evaluation of specificity with *Babesia bovis* experimentally infected cattle sera (n=9); *Babesia bigemina* experimentally infected cattle sera (n=15); *Anaplasma marginale* experimentally infected cattle sera (n=5), dashed line indicates the cutoff value (0.46).

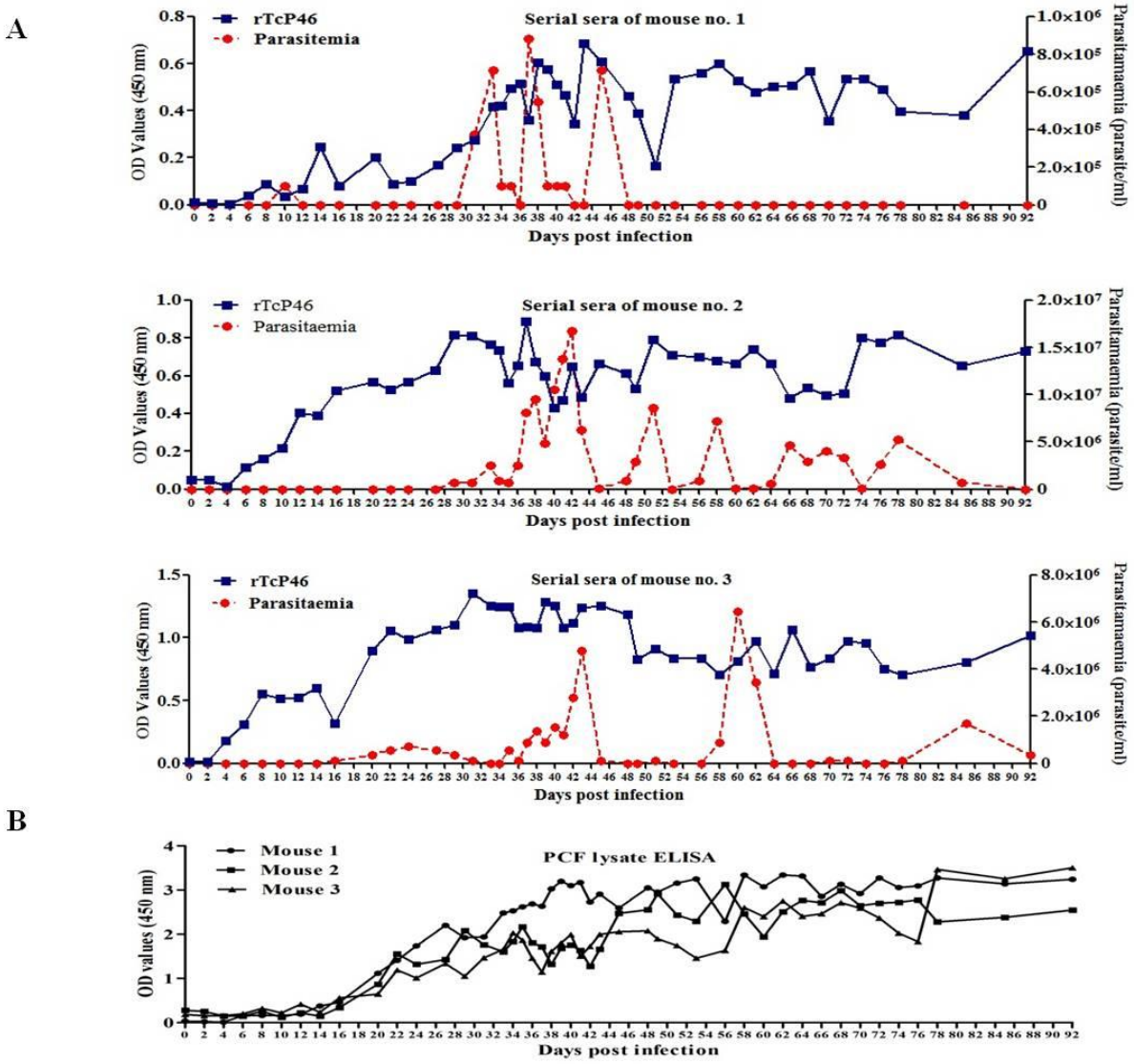

Fig. 5. (A) Detection of the specific antibody response against TcP46 in three mice experimentally infected with *T. congolense* by rTcP46-based ELISA. (B) Detection of the antibody responses in three mice experimentally infected with *T. congolense* by means of PCF cell lysate antigen ELISA.

Table 1. Selected genes express higher in BSF and/or MCF than PCF and EMF.

Gene ID	Length (bp)	BSF→PCF	PCF→EMF	EMF→ MCF	MCF→BSF
TcIL3000.0.25950	1,218	0.26	DNR	5.36	DNR
TcIL3000.10.3480	744	0.12	DNR	8.98	DNR
TcIL3000.8.6290	318	DNR	0.37	6.94	DNR
TcIL3000.7.1980	1,179	DNR	DNR	DNR	7.96

Number indicates fold expression level of each protein in the two life-cycle stages [Eyford *et al.*, 2011].

"DNR" means the data not reliable.

Chapter 2

Field evaluation and validation of a semi-nested PCR for the diagnosis of *Trypanosoma congolense* infection in cattle and dog in South Africa

2-1. Introduction

T. congolense is a tsetse fly-trasmitted protozoan parasites responsible for AAT (nagana) in cattle and other vertebrates including dogs, pigs, sheep, goats, and laboratory rodents (Coustou *et al*., 2010). The disease leads to serious economic losses of livestock production in 37 countries of sub-Saharan Africa (Inoue *et al*., 2000; Swallow, 2000; Shaw, 2009). Signs of acute infection include anemia, intermittent fever, parasitemia, lymphadenopathy, jaundice, progressive emaciation, loss of production, weakness, and death if it is untreated. Chemotherapy and vaccination have not proved to be quite effective against the disease due to the development of drug resistance and the switching their dense protective coat of immunodominant VSG, respectively (Horn and McCulloch, 2010; Mungube *et al*., 2012). Therefore, an early and correct diagnosis is essential to control the disease.

The definitive diagnosis of acute AAT heavily depends on the direct detection using direct observation of wet blood films under light microscope, or concentration techniques such as the buffy coat technique (BCT) by light microscope (Woo, 1970; Herbert and Lumsden, 1976; Murray *et al*., 1977). However, microscopy has limitations in latent infections where the parasite is often difficult to detect in blood because of extremely low parasitemia. Consequently, the PCR based techniques have been widely used in the course of epidemiological studies of trypanosomosis by several investigators (Reifenberg *et al*., 1997;

Clausen *et al*., 1998; Mugittu *et al*., 2001). Recently, the PCR test based on the ITS of ribosome DNA (Kin PCR) showed a multiple species diagnosis of the main livestock trypanosomes (Desquesnes *et al.*, 2001). Although recent study suggests that the ITS- PCR is less sensitive than the species-specific primers, it is able to simultaneously screen samples for other pathogenic trypanosomes, and it may be a better choice of test in multi-disease studies (de Clare Bronsvoort *et al.*, 2010). Therefore, it is required to develop a specific assay to *T. congolense* particularly for improving the sensitivity in field application. In my previous study, Southern blot analysis of the TcP46 gene indicated that at least two copies were included in the *T. congolense* genome as a tandemly arranged orientation (Zhou *et al.*, 2014). The TcP46 gene shared a low nucleotide sequences identity with *T. b. brucei* and no match with the other *Trypanosoma* spp. and apicomplex paresites when detected by BLAST. Therefore, the TcP46 gene may be a candidate target gene for developing a specific and sensitive diagnostic method to screen the *T. congolense* infection in the field.

The objective of the study was to evaluate the diagnostic efficiency of the TcP46 gene for the diagnosis of *T. congolense* infection in a semi-nPCR and compare it with that Kin PCR commonly used in to screen trypanosomes infection in the field. Despite AAT being a very important disease of domestic animal in South Africa, there is insufficient data on the epidemiology and genotypes of *T. congolense* isolate in the area. Therefore, this new method was used to detect the prevalence of *T. congolense* infection in South Africa.

2-2. Materials and methods

Sample collections and DNA extraction. During March and April 2013, 96 bovine and 29 canine blood samples were collected from 6 districts in Kwazulu-Natal province of South Africa (Fig. 6). The bovine blood DNA was extracted using a QIAamp DNA Blood Mini Kit according to the manufacturer's protocol (Qiagen, Germany). The extracted DNA was eluted

with 100 μl of the kit elution buffer, and then stored at -30℃. On the other hand, the each canine blood sample was blotted and dried on the FTA card (Whatman, United Kingdom). The card was washed by FTA purification reagent (Whatman, United Kingdom), and then eluted by TE (10mM Tris-HCI, 0.1 mM EDTA, pH 8) buffer, DNA on the washed punch was ready to be used as the source of template DNA for PCR.

Parasites cultivation and DNA extraction. *T. congolense* IL3000 savannah strain isolated near Kenya/Tanzania border was used. Detail of *in vitro* cultivation and genomic DNA extraction were described in Materials and Methods for Chapter 1.

Development of a semi-nested PCR. Primers for the semi-nPCR were designed based on the target sequence of TcP46 gene from *T. conglense* (TcIL3000.0.25950) (Table 2). PCR were carried out under standard and enhanced conditions. For first-round amplification, a 1 μl aliquot of extracted DNA was added to a PCR reaction mix, containing 2 μl of 10 × Ex *Taq* Buffer (TaKaRa, Japan), 2 μl dNTPs (2.5 mM), 1 μl of each of the primers (10 mM), 0.1μl of Ex *Taq* polymerase (TaKaRa, 5 unite/μl), and 12.9 μl of ultra-pure water to yield a final volume of 20 μl. The amplification was carried out by set up PCR with initial denaturation at 95℃ for 5 min, followed by 35 cycles of 45s denaturation at 94℃ for 45s, 45s primer annealing at 59℃ and 1 min primer extension at 72℃. The final primer extension step was carried out for 10 min at 72℃. Thereafter, second round amplification (semi-nPCR) was carried out in an exactly similar manner except that the DNA sample was a 0.2 μl aliquot of the first-round PCR product and ultra-pure water was 13.7 μl. The primers and the annealing temperature employed were shown in the Table 2. Finally, a 10 μl aliquot of the semi-nPCR product was separated by agarose gel (1.5%) electrophoresis and stained with ethidium bromide solution.

Specificity and sensitivity testing. The specificity of the developed nPCR method was tested using the genomic DNA extracted from *B. bovis, B. bigemina, B. canis, T. evansi, T.*

annulata, T. orientalis, N. caninum, and *T. gondii* and healthy cattle blood. Futhermore, the sensitivity of the semi-nPCR for *T. congolense* was estimated using 10-fold serial dilution of the cultured parasites (Start from 1×10^6 trypanosomes/ml). Then, DNAs were extracted from different trypanosome dilutions (500 μl) and eluted into 50 μl of ultra-pure water. The semi-nPCR assay was carried out using each DNA sample. In addition, the detection limit of the semi-nPCR was determined using the serially diluted DNA of *T. congolense*, and the semi-nPCR assay was carry out using the different concentration DNA samples.

Evaluation of the semi-nested PCR in mouse models. Five 6-week-old female BALB/c mice (CLEA, Japan) were infected intraperitoneally with 1×10^3 *T. congolense* BSF. Every day, 60 μl of blood was collected into hematocrit capillaries from the tail vein. Then 10 μl blood was centrifuged for 5 min at 10,000 ×*g* to obtain the buffy coat. A drop of the buffy coat was placed on a glass slide and examined for motile parasites under a light microscope. The remaining (50 μl) whole blood was used for total DNA preparation with the QIAamp DNA Blood Mini Kit (Qiagen). The DNA was detected by both the semi-nPCR and Kin PCR (Desquesnes *et al*., 2001).

Detection of *T. congolense* infection from field samples. Field blood samples collected from cattle and dogs were screened for *T. congolense* using the semi-nPCR as described above and the Kin PCR as described by Desquesnes *et al* (2001). The obtained results were compared to evaluate their practical utility for field detecting of *T. congolense* infection.

Cloning and sequencing of nPCR products. Three *T. congolense*-positive bovine or canine blood DNA were randomly selected from all the examined districts, and subjected to DNA cloning and sequencing of TcP46 genes. The PCR product purification, cloning, and sequencing were performed according to previous description (Cao *et al.*, 2012).

2-3. Results

Specificity and sensitivity of the semi-nPCR. The specific outer and inner primers of semi-nPCR based on TcP46 specially produced the amplifications of 816 bp and 590 bp from the *T. congolense* genomic DNA, while no amplification was detected from other parasites used as the negative control. (Fig. 7 A and B).

The sensitivity of the semi-nPCR. I evaluate the sensitivity of this semi-nested with the extract DNAs from dilutions of *T. congolense* in the *in vitro* culture. In the first round PCR, positive band of 816 bp was detected from DNA extracted from dilutions of *T. congolense* with 1×10^3 trypanosome ml^{-1} (Fig. 8A, lane 4.). In the second round PCR, a positive band of 590 bp was detected from DNAs extracted from dilutions of *T. congolense* with 1×10^2 trypanosome ml^{-1} (Fig. 8B, lane 5.). In the merged PCR, a positive band of 590 bp was detected from DNAs extracted from dilutions of *T. congolense* with 10 trypanosome ml^{-1} (Fig. 8C, lane 6.). In addition, I also evaluated the sensitivity of this semi-nested with 10-fold diluted genomic DNA. The detection limit for the first round PCR was 0.07 ng/µl (Fig. 9A, lane 4.), for the second round PCR was 7 pg/µl (Fig. 9A, lane 5.), for the merged PCR was 0.7 pg/µl (Fig. 9A, lane 7.), respectively.

Detection of *T. congolense* infection in mouse models. Diagnostic experimentations in the mouse models revealed that specific amplifications were first observed at 24 h post infection by the semi-nested PCR in all the mice. However, the BCT could detect the parasite on the 6^{th} d. p. i. in one of the five mice. The Kin-PCR could detect the parasite on the 3^{rd} d. p. i. in one of five mice (Table 3).

Screening for *T. congolense* infection. The field DNA samples collected from cattle and dogs in South Africa were investigated using the semi-nPCR and Kin PCR assays in order to demonstrate the field utility of these methods as a diagnostic tool for epidemiological studies. The semi-nPCR detected 27 (28.13%) cattle and 5 (17.24%) dogs as positive for *T. congolense*, while the Kin PCR assay indicated that 8 (8.34%) cattle and 0 (0%) dog samples

were positive for *T. congolense* (Table 4).

Cloning and sequencing of TcP47 genes of *T. congolense.* Two bovine samples from each farm and 5 canine positive samples were amplified for subsequent cloning and DNA sequence analyses. After ligation and subsequent cloning, the nucleotide sequences of the target gene fragments were determined. Homology search and alignment of the sequenced TcP46 fragments revealed that the gene was conserved among the *T. congolense* isolates from cattle and dogs in the five farms of South Africa. The partial TcP46 sequence of South African isolates shared 98.47% nucleotide sequence identity and 97.45% amino acid identity with that of *T. congolense* IL3000 savannah strain (Gene ID: TcIL3000.0.25950).

2-4. Discussion

T. congolense is widespread in many countries of sub-Sahara Africa, posing a real threat to the livestock industry (Gillingwater *et al.* 2010). The presence of *T. congolense* may require early and urgent diagnosis and veterinary intervention for minimizing the disease burden. As previously reported, PCR has advantages over the conventional methods used for the diagnosis of trypanosomosis (Desquesnes *et al*., 2001; Masake *et al.,* 2002; Geysen *et al.,* 2003; Li *et al.,* 2007). However, the usual one-step PCR amplification from the field sample may not achieve sufficient sensitivity (Mekuria and Gadissa, 2011; Nakayima *et al.,* 2012; Takeet *et al.,* 2013). Therefore, in present study, in order to increase the detection threshold, I developed a semi-nested PCR assay. The sensitivity of the semi-nPCR was evaluated using the 10-fold diluted *T. congolense* genomic DNA. The result indicated that this semi-nPCR method can detect the template as little as 0.7 pg, which was more sensitive than the widely used Kin PCR (Njiru *et al.,* 2005). Thus, the semi-nPCR may be suitable for screening the parasite DNA in the subclinically infected cattle or other host during early infection.

Diagnosis of trypanosomoses requires highly sensitive and specific tests. In many cases

the identification of parasites concerns their epidemiology and it is important to distinguish species and subspecies. Therefore, highly specific tests are important in the diagnosis of trypanosomosis. In the present study, I found that the TcP46 gene, at least the parts used for PCR target was conserved within the trypanosome species through *in silico* analysis using NCBI BLAST server. Moreover, the semi-nPCR assay with *B. bovis, B. bigemina, B. canis, T. evansi, T. annulata, T. orientalis, N. caninum,* and *T. gondii* DNA also did not shown amplification, which demonstrated that the specific region of TcP46 gene might be suitable for molecular identification of *T. conglense* with maximal specificity.

Comparison of the methods for detection of *T. congolense* showed that this semi-nPCR was more sensitive than the conventional microscopy and commonly used Kin PCR method. Experimental infection in mice revealed the presence of parasite only on the 6^{th} d. p. i. by buffy coat examination and on the 3^{rd} d. p. i. by Kin PCR amplification. However, semi-nPCR could detect the parasitic DNA as early as 1^{st} d. p. i. The DNA sample from one mouse failed to amplify any DNA on the 8^{th} d. p. i. which may have been due to sampling problems or arising from low number of parasites in the circulation. Furthermore, the records of the Kin-PCR detection also demonstrated that the Kin-PCR was uncontinuous and unstable for detecting the parasite. Although I recoded the difference in detecting the infection in mice, but the semi-nPCR was capable of detecting the parasite earlier than BCT and Kin-PCR. This result was consistent with previous reports (Thumbi *et al.*, 2008).

In South Africa, tsetse-transmitted trypanosomosis has been the cause of stock losses in past and present, and a major problem to livestock owners in certain areas of KwaZulu-Natal (Motloang *et al.,* 2012). Recent study indicated that AAT was still prevalent in KwaZulu-Natal and that *T. congolense* was the dominant causative organism (Van den Bossche *et al*., 2006). Therefore, the field samples from KwaZulu-Natal of South Africa were examined by the semi-nPCR and Kin-PCR methods for assessing the suitability of the

semi-nested PCR for use with field samples. The results suggest that the semi-nested PCR method was suitable for diagnosis of the field samples. It revealed higher prevalence for *T. congolense* infection in cattle (28.13%) and dogs (17.24%) than Kin-PCR.

In conclusion, these results demonstrate that the semi-nPCR based on the TcP46 gene specifically detected *T. congolense* at low parasitermias in parasite cultivation and mouse models. The semi-nPCR can also be used as a reliable and potential tool for epidemiological survey of the *T. congolense*-infected animals. Moreover, the present study provideds a new evidence to confirm that the *T. congolense* is a widely epidemic species in cattle and dogs in South Africa. The identical sequences of TcP46 gene were detected among the *T. congolense*-infected cattle and dogs in South Africa, and slightly different with *T. congolense* IL3000 savannah strain. Therefore, further large and wide-scale investigations are necessary to provide essential information about the geographical distributions, host specificities, and genotypes of *T. congolense* isolates.

2-5. Summary

T. congolense is a major pathogen responsible for nagana in cattle and other vertebrate animals including dogs, pigs, sheep, and goats, as well as laboratory rodents. Therefore, sensitive and specific molecular diagnostic tests are urgently required for appropriate control of *T. congolense* infection. The aim of this study was to develop a semi-nPCR for the detection of *T. congolense* using oligonucleotide primers designed from a tandemly-arranged gene of *T. congolense* (TcP46). Two sets of primers produced the expected amplifications of 816 bp (external primers) and 590 bp (internal primers) with the *T. congolense* DNA, whereas no amplifications were observed with the genomic DNA extracted from the other hemoparasites including the *B. bovis*, *B. bigemina*, *B. canis*, *T. evansi*, *T. annulata*, *T. orientalis*, *N. caninum*, and *T. gondii*. Thereafter, the sensitivity of the method was determined as 0.7 pg purified DNA or 10 trypanosomes ml^{-1}. Furthermore, the semi-nPCR could detect the presence of the parasites as early as one d. p. i. in experimentally *T. congolense*-infected mice blood, which was more sensitive than the conventional buffy coat technique as 6 d. p. i. and the previously reported PCR test based on the ITS1 of rDNA (Kin-CR) as 3 d. p.i. In addition, I screened 96 bovine and 29 canine blood DNA samples from South Africa by the present semi-nPCR method. The results revealed that the 28.13% and 17.24% were positive for cattle and dog samples, respectively. Sequence analysis using partial TcP46 gene revealed that the South Africa isolates shared 98.5% nucleotide sequence identity with that of *T. congolense* IL3000 strain. Altogether, the primer pairs described in this study will be useful for epidemiological studies on *T. congolense* infection. The result also demonstrated a high prevalence of *T. congolense* in South Africa calling for the need to design effective control programs in the further study.

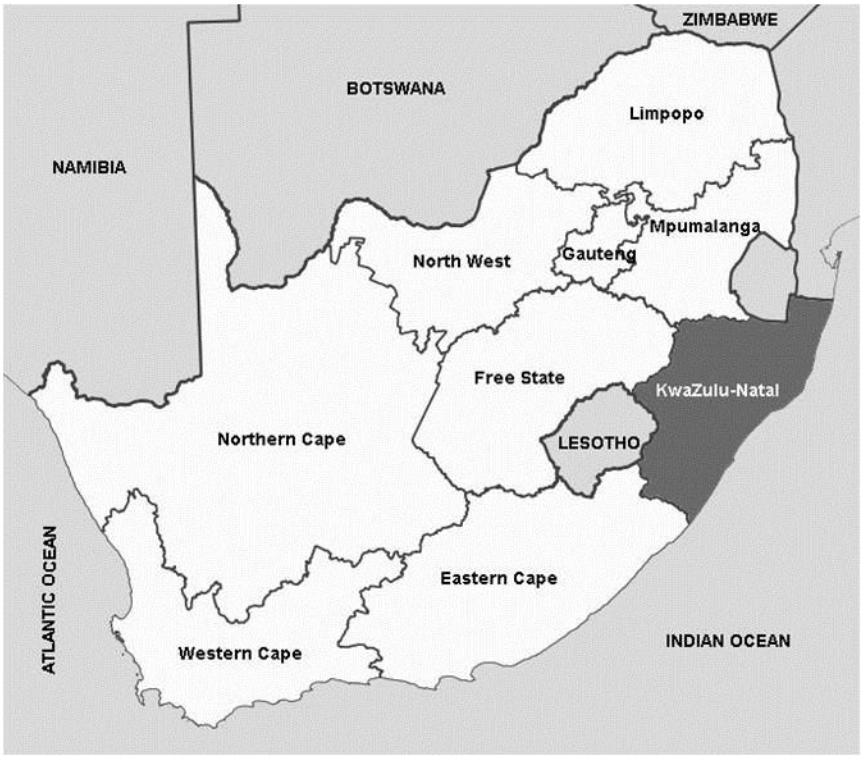

Fig. 6. Regions for collecting samples in South Africa.

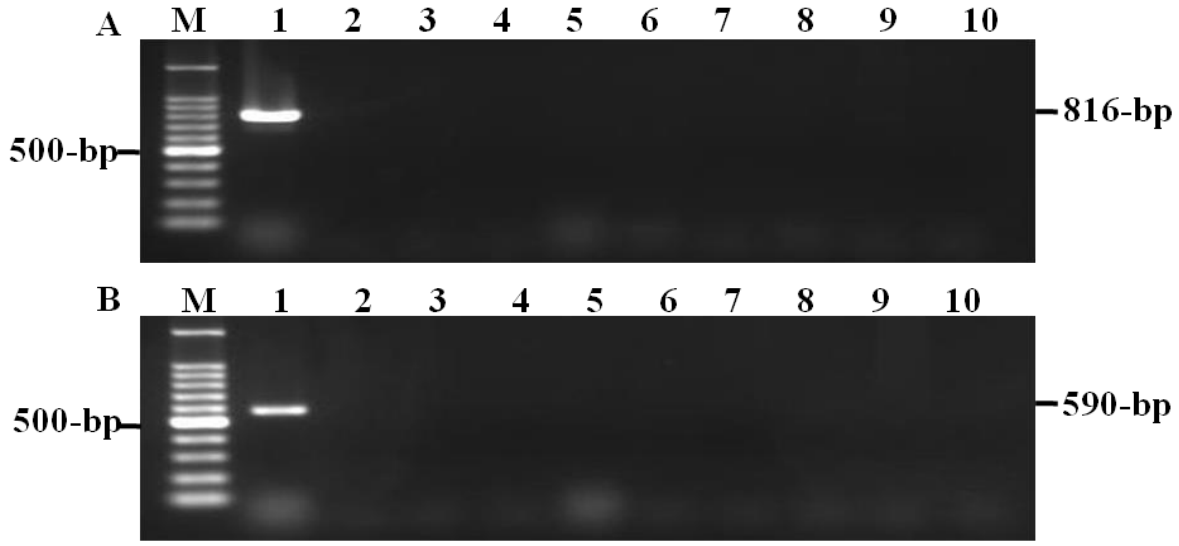

Fig. 7. Specificities of semi-nPCR method. (A) First round of semi-nested PCR. (B) Second round of semi-nPCR. In all panels, Lane M is 100-bp ladder DNA marker. Lane 1, 2, 3, 4, 5, 6, 7, 8, 9 and10 represent the *T. congolense, B. bovis, B. bigemina, B. canis, T. evansi, T. annulata, T. orientalis, N. caninum, T. gondii,* and bovine blood DNA.

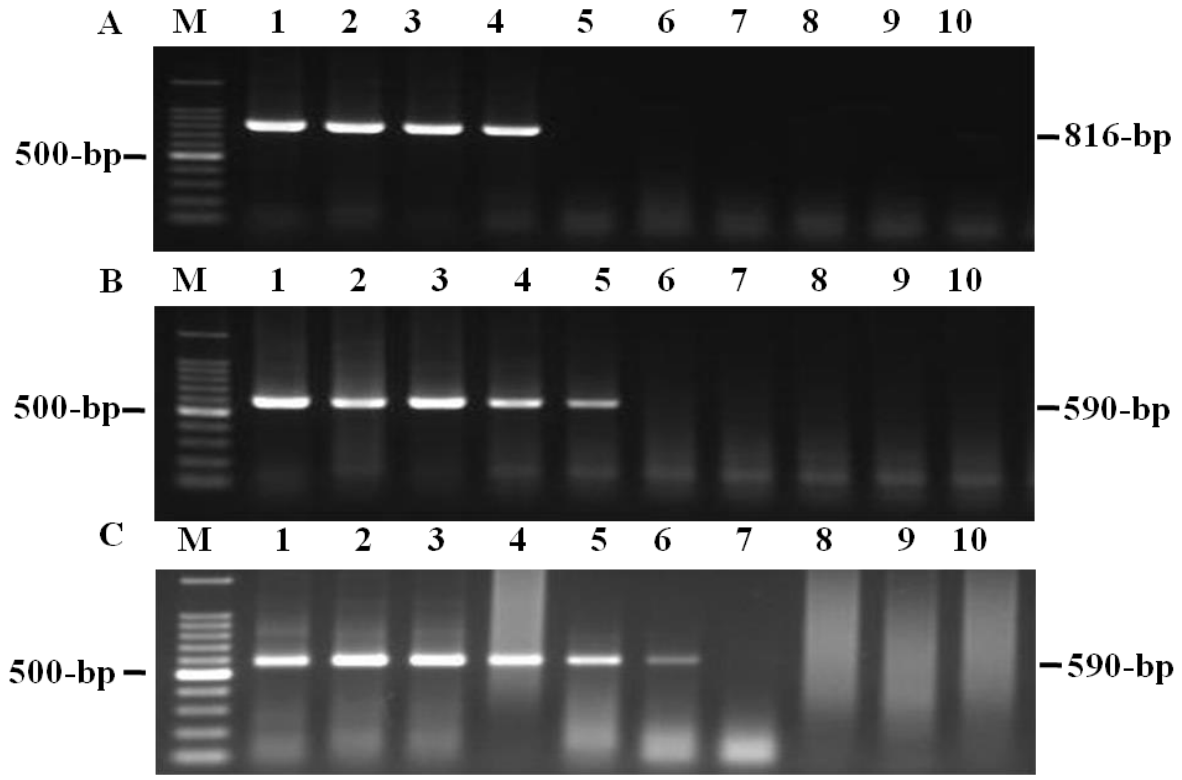

Fig. 8. Sensitivity of the semi-nPCR method. The semi-nPCR method was carried out using the DNAs from *T. congolense*. (A) First round of semi-nested PCR. (B) Second round of semi-nPCR. (C) Merged semi-nested PCR. In all panels, Lane M is 100-bp ladder DNA marker. Lane 1, 2, 3, 4, 5, 6, 7, 8, 9 and10 represent the different concentration of purified *T. congolense* in each dilution, 1×10^{6}, 1×10^{5}, 1×10^{4}, 1×10^{3}, 1×10^{2}, 1×10^{1}; 1×10^{0}, 1×10^{-1}, 1×10^{-2} and 0 trypanosomes ml^{-1}.

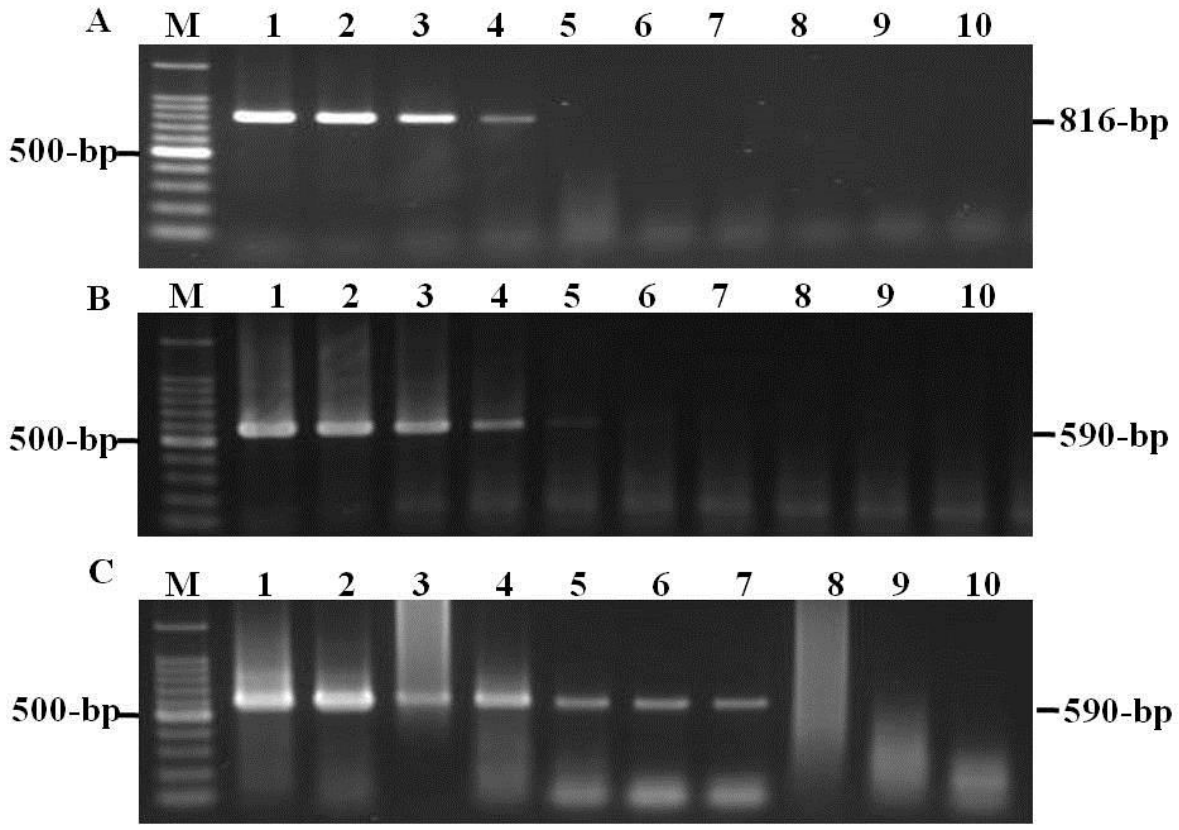

Fig. 9. Sensitivity of the semi-nPCR method. The semi-nPCR method was carried out using the 10-fold diluted *T. congolense* genomic DNA. (A) First round of semi-nested PCR. (B) Second round of semi-nPCR. (C) Merged semi-nested PCR. In all panels, Lane M is 100-bp ladder DNA marker. Lane 1: 70 ng/μl; 2: 7 ng/μl; 3: 0.7 ng/μl; 4: 0.07 ng/μl; 5: 7 pg/μl; 6: 0.7 pg/μl; 7: 0.07 pg/μl; 8: 7 fg/μl; 9: 0.7 fg/μl; 10: 0 fg/μl.

Table 2. Oligonucleotide primers of *T. congolense* based on the TcP46 (TCIL3000.0.25950) gene.

Code	Sequence(5'-3')	Location in gene	Fragment	Annealing Tm
Outer primer				
Forward primer	GAGGTACGGAAACCGATT	138-155	816-bp	59℃
Reverse primer	GCACCAAACTGCCTACTC	935-953		
Inner primer				
Forward primer	GAGGTACGGAAACCGATT	138-155	590-bp	60℃
Reverse primer	CATTCACCTTCGCCTCGT	710-727		

Table 3. Sequence analysis of blood samples from mice infected with *T. congolense*

Mouse no.	Method	Result on day post infection										
		0	1	2	3	4	5	6	7	8	9	10
1	Buffy coat	-	-	-	-	-	-	-	+	+	-	-
	Kin PCR	-	-	-	-	-	-	-	-	+	-	+
	Semi-nested PCR	-	+	+	+	+	+	+	+	+	+	+
2	Buffy coat	-	-	-	-	-	-	-	+	-	-	+
	Kin PCR	-	-	-	-	-	-	-	-	+	+	+
	Semi-nested PCR	-	+	+	+	+	+	+	+	+	+	+
3	Buffy coat	-	-	-	-	-	-	-	-	-	-	-
	Kin PCR	-	-	-	-	-	-	-	+	+	+	-
	Semi-nested PCR	-	+	+	+	+	+	+	+	+	+	+
4	Buffy coat	-	-	-	-	-	-	-	-	-	-	-
	Kin PCR	-	-	-	-	-	-	+	+	+	-	+
	Semi-nested PCR	-	-	+	+	+	+	+	+	-	+	+
5	Buffy coat	-	-	-	-	-	-	-	+	+	-	-
	Kin PCR	-	-	-	+	+	-	-	+	+	-	+
	Semi-nested PCR	-	+	+	+	+	+	+	+	+	+	+

IL3000.

Table 4. Screening the South African cattle and dogs for *T. congolense* infection by the Kin-PCR and the semi-nested PCR assays.

Animal	Total number of collected samples	Positive numbers (%)	
		Kin PCR	semi-nested PCR
Cattle	96	8 (8.34)	27 (28.13)
Dog	29	0 (0)	5 (17.24)

Chapter 3

Validation of endoplasmic reticulum-associated degradation pathway as a chemotherapeutic target for African trypanosomosis

3-1. Introduction

African trypanosomes, protozoan parasites of the genus *Trypanosoma* are the etiological agents of HAT and AAT, which are transmitted through the bite of tsetse flies (*Glossina* spp.) (Michels, 1988, Steverding and Sextone, 2013). The most important trypanosome species in sub-Saharan Africa are *Trypanosoma brucei gambiense* and *T. b. rhodesiense* in human and *T. b. brucei, T. congolense, T. vivax* in animal (Awulu *et al., 2013*). Every year, over 60 million people and 46 million cattle living in 36 sub-Saharan countries are threatened with sleeping sickness and nagana (Steverding *et al.,* 2005). Drugs are the only therapeutic option for the treatment as there is no vaccine and no prospects that one will be developed (MacGregor *et al*., 2012). Currently, chemotherapy of African trypanosomosis still relies on a few drugs developed decades ago, these drugs display undesirable toxic side effects and the emergence of drug-resistant trypanosomes has been reported (Merschjohann and Steverding, 2006; Phillips, 2012). Therefore, new drugs are urgently needed for chemotherapy of sleeping sickness and nagana disease.

ERAD is responsible for the degradation of aberrant or misfolded proteins in the ER, providing an important protein folding "quality control" mechanism, which has emerged as a potential target for pharmacological intervention with cancer (Li and Li, 2011; Verfaillie *et al.,* 2013) and parasitic diseases (Chung, *et al.,* 2012). ERAD requires a number of molecular chaperones for identification of the misfolded proteins and dedicated ER resident reductases

for cleaving disulfide bonds in these proteins to facilitate retrograde transport to the cytosol. Heat shock protein 90 (Hsp90) is a phylogenetically conserved, abundant, and essential molecular chaperone, it has been shown to maintain membrane-embedded ERAD substrates in a soluble state and to help extract and determine the fate of ERAD substrates (Youker *et al.,* 2004; Taylor *et al.,* 2010). Several tumor types study demonstrated that Hsp90 inhibitor, 17-DMAG possesses marked *in vitro* antitumor activity (Smith *et al.,* 2005). PDI is one of the most abundant ER proteins, which maintains a sentinel function in organizing accurate protein folding and recognition and repair of aberrant protein assembly (Lovat *et al.,* 2008; Hoffstrom, *et al.,* 2010). 16F16 is the irreversible inhibitor of the PDI, it has provided necessary tools to further understand the role of PDI in human disease (Hussain *et al.,* 1982). ERAD substrates are retro-translocated across the ER membrane into the cytosol for degradation. SPP plays an important role in this process (Li *et al.,* 2009). Inhibition of SPP activity resulted in the accumulation of the cleaved signal peptide in the membrane (Hussain *et al.,* 1982). Gamma-secretase and SPP inhibitors have been demonstrated in impairing the development of *P. berghei in vitro* in hepatoma cells as well as *in vivo* in mouse liver (Parvanova *et al.,* 2009). Furthermore, the AAA(+) adenosine triphosphatase withdraws ERAD substrates from the retrotranslocation channel to the cytosol where they are degraded by the ubiquitin/ proteasome system (Meyer, 2012). The AAA+ ATPase p97 is a key element of ubiquitin regulated processes. EerI, a chemical inhibitor that can block ERAD, has been shown to have preferential cytotoxic activity against cancer cells (Wang *et al.,* 2008). EerI targets p97 complex to inhibit deubiquitination of p97-associated ERAD substrates, which is required for the degradation process. In addition, DBeQ a reversible inhibitor of the p97 ATPase activity, was found to be a potent activator of caspases in cancer cells and to inhibit both ERAD and autophagosome maturation (Chou *et al.,* 2011; Chou and Deshaies, 2011). Ubiquitin-tagged substrates are degraded by the 26S proteasome that is a multisubunit complex comprising a

proteolytic 20S core particle capped by 19S regulatory particle. Epoxomicin targeting 20s proteasome has been identified as a proteasome inhibitor in previously study (Hanada *et al.*, 1992; Meng *et al.*, 1999*).*

ERAD pathway has also been identified as a potential drug targets for treating parasitic protozoan infections (Chung *et al.*, 2012). To explore the potential of African trypanosome ERAD pathway as a drug target, I employed both bioinformatics and biochemical techniques. My approach began with bioinformatics analysis of the ERAD pathways of *T. b. brucei* and *T. congolense*. ERAD pathway of these parasites was highly simplified relative to that of mammalian cells. Subsequently, a panel of known inhibitors was screened against the components of the ERAD pathway using *in vitro* cultures with the aim of identifying novel targets for AAT. *T. b. brucei* and *T. congolense* were vulnerable to the small molecules that have been established to inhibit proteins within the ERAD system. The screening and follow-up studies resulted in the identification of a series of inhibitors as novel *T. b. brucei* and *T. congolense* inhibitors, the properties of which are described in this study.

3-2. Materials and methods

Homology searching. *T. b. brucei* and *T. congolense* genome sequence data were searched at TriTrypDB (http://tritrypdb.org/tritrypdb). The database was screened for the components of ERAD using the extensive list of query sequence corresponding to the components identified in human ERAD networks (Christianson *et al.*, 2011).

Chemicals and reagents. 17-DMAG, 16F16, LY-411575, L-658,485, LY-374973, DBeQ and Eeyarestatin I were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). $(Z\text{-}LL)_2$-Ketone and Epoxomicin purchased from Millipore (Watford, UK) and Cayman Chemical (Ann Arbor, Michigan, USA), respectively. Cell Counting Kit-8 (CCK-8) was purchased from Dojindo Molecular Technologies (Dojindo, Japan). All reagents were of

highest quality commercially available and the chemical structure of these compounds was showed in Fig. 10. The compounds were prepared as 100 mM stock solutions in dimethyl sulphoxide (DMSO) (16F16, LY-374973, DBeQ, Eeyarestatin I), 50 mM stock solutions in DMSO (LY-411575, $(Z\text{-}LL)_2$-Ketone) and 20 mM stock solutions in DMSO (17-DMAG, L-658,485). Epoxomicin was purchased from the company and already prepared as 180 uM stock in DMSO. These stock solutions were then stored at -30 or 4 ℃. From these stock solutions, further drug dilutions were made for use in the *in vitro* drug sensitivity assays, using DMSO as a solvent. Drug dilutions were made fresh on the day of each experimental assay procedure.

In vitro* cultivation of *T. b. brucei* and *T. congolense. The bloodstream form *T. b. brucei* GUTat 3.1 strain and *T. congolense* IL3000 strain were stored in the liquid nitrogen at National Research Center for Protozoan Diseases in Japan and used in assays for assessment of compound sensitivity *in vitro*. The *T. b. brucei* and *T. congolense* were routinely cultured in T-75 vented cap flasks and kept in humidified incubators at 5% CO_2, 37°C and 33°C, respectively. The parasite culture media was complete Hirum's modified Iscove's medium (HMI)-9 medium containing 20% fetal bovine serum (FBS) and 100 units penicillin and 0.1 mg/mL streptomycin (Invitrogen, Carlsbad, California, USA) (Hirumi and Hirumi, 1991). Parasites were utilized when in the log phase of growth.

Mammalian cell cultures. The HFF cells were stored in the liquid nitrogen at National Research Center for Protozoan Diseases in Japan. And the HFF cells were maintained in Dulbecco's modified Eagle's medium (DMEM, Sigma, USA) supplemented with 10% fetal FBS and 100 units penicillin and 0.1 mg/mL (Invitrogen, Carlsbad, California, USA) with incubation at 37 °C and 5% CO_2 in humidified conditions.

Limit of detection. CCK-8 assays were performed according to the manufacturer's protocol (Dojindo). Prior to each assay, cells were grown to mid-logarithmic phase and were

pelleted by centrifugation. The cells were suspended in their culture medium. 100 ul of 2-fold serial dilutions *T. b. brucei* cells culture were dispensed into each well, range from 17-4.5×10^6 cells/well. 10 μl of CCK-8 solution was added to each well and the plates were incubated for 4 h at 37℃. For the *T. congolense*, 100 μl of 2-fold serial dilutions *T. congolense* cells were dispensed into each well, range from 43-5.625×10^6 cells/well. 10 μl of CCK-8 solution was added to each well and the plates were incubated for 4 h at 33℃. For the HFF cells, when the cell culture was confluent, the cell monolayer was washed with culture medium, trypsinized and 100 μl of 2-fold serial dilutions were distributed in flat-bottomed 96-well plates, range from 137-1.75×10^5 cells/well. The plate was incubated for 16 h at 37℃ for cell adherence. 10 μl of the CCK-8 solution was added to each well of the plate, followed by another 2 h incubation. Absorbance of each plate was measured using MTP-500 micro-plate reader (450 nm). The limit of detection was calculated as the number of cells giving a signal greater than the mean signal of the blank wells plus 3 times the standard deviation of the blank wells.

***In vitro* growth inhibition assay for *T. b. brucei*.** To prepare for growth inhibition assay, *T. b. brucei* parasites in log phase growth were counted and diluted to 2×10^5/ml in HMI-9 media. Compounds to be tested were serially diluted in DMSO and 0.5 μl added to 49.5 μl media in 96-well plates. 50 μl diluted parasite culture was added to each well to give a final concentration of 1×10^4 parasites / well, and the final DMSO concentration was 0.5%. After 68 h of incubation, 10 μl of the CCK-8 solution was added to each well, and the plates were incubated for an additional 4 h. To assess cell viability, absorbance of each plate was measured using MTP-500 micro-plate reader (450 nm). The data points were analyzed and Graphpad Software was used to generate sigmoidal dose-response curves for the determination of IC_{50} values.

***In vitro* growth inhibition assay for *T. congolense*.** *T. congolense* parasites in the log phase of growth were diluted with HMI-9. Parasites were diluted to 2 × 10^5 cells/ml in HMI-9

to evaluate trypanocidal activity. For IC_{50} determination, compounds were serially diluted in DMSO and 0.5 μl added to 49.5 μl HMI-9 into 96-well plates. Parasites from the diluted culture were added to each well (50 μl) to give a final concentration of 10^5 parasites / well and a final DMSO concentration of 0.5%. After 68 h incubation, 10 μl of CCK-8 solution was added to each well of the plate, and the plates were incubated for 4 hours in the incubator and read on a microplate reader using a test wavelength of 450 nm, and data evaluation as described for the *T. b. brucei* assay.

Mammalian cell cytotoxicity asssy and selectivity index. To prepare for assay, HFF cells in log growth phase were removed from the T-75 flask using 5 ml of 0.05% trypsin and a 10 μl aliquot was counted using a hemocytometer to determine cell concentration. Cells were diluted to 4×10^4 cells / ml in DMEM to prepare for plating, and 100 μl of cells was added to a 96-well plate. Cells were allowed to adhere for 16 h prior to removal of plating media and replacement with 99.5 μl of fresh medium into each of the wells. Compounds to be tested were serially diluted in DMSO and 0.5 μl of the dilutions added to the 99.5 μl of DMEM already in the plates. After 70 h of incubation at 37 ℃ and 5% CO_2, 10 μl of CCK-8 solution was added to each well of the plate, and the plates were incubated for 2 h in the incubator and read on a microplate reader using a test wavelength of 450 nm, and data evaluation as described for the *T. b. brucei* assay. The selectivity index (SI) value was calculated to select compound that selective to trypanosome parasites and had low toxicity effects on mammlian cells by using the formula: Selectivity index (SI) = IC_{50} value (cytotoxicity)/ IC50 value (trypanocidal activity).

3-3. Results

Homology searching. My study using standard orthologue detection tools revealed highly simplified ERAD pathway in *T. b. brucei* and *T. congolense* relative to mammalian

cells. All functional modules of the ERAD pathway (Christianson *et al.*, 2011), including protein recognition, translocation, ubiquitin ligation and protein extraction, showed far fewer orthologues in *T. b. brucei* and *T. congolense* relative to the corresponding mammalian pathway. Moreover, the identity of each protein level was lower relative to mammalian cells, which may result in the relatively high selectivity of the inhibitors for the components in ERAD pathway for *T. b. brucei* and *T. congolense* (Table 5).

Detection limit. I assessed if there was a direct correlation between absorbance and cell number by CCK-8 assays on serial dilutions of the trypanosome cells and HFF cells (Fig. 11). Linear regression analysis showed a strong positive correlation between the level of absorbance and the number of parasite and HFF cells (*T. b. brucei*, R^2=0.98; *T. congolense*, R^2=0.97, HFF, R^2=0.97). The detection limits of this assay were 81,519 *T. b. brucei* cells and 45,832 *T. congolense* cells, respectively. I also sought to determine the limit of detection achievable with the HFF cells. The detection limit was 141 cells for HFF.

***In vitro* growth inhibition assay of *T. b. brucei*.** All the compounds display trypanocidal activities against *T. b. brucei* GUTat 3.1 strain. 17-DMAG, epoxomicine and EerI exhibibit outstanding activity for *T. b. brucei* with IC_{50} of 0.03 ± 0.01 nM, 8.54 ±1.76 nM and 79.30 ± 0.13 nM, respectively. 16F16, $(Z\text{-}LL)_2$ Ketone, DBeQ and L-658, 458 were less active for *T. b. brucei* with IC_{50} values in the micromolar range, the IC_{50} values were 2,959.33 ± 332.36 nM, 1,250.33 ± 22.59 nM , 2,004.00 ± 161.17 nM and 5,392.33 ± 752.83, respectively. LY-411575 and LY-374973 were the least active for *T. b. brucei* with IC_{50} of 16,169.00 ± 1,495.44 nM and 24,400.33±14,619.30 nM (Table 6).

***In vitro* growth inhibition assay of *T. congolense*.** In the *in vitro* assay of *T. congolense* IL3000, all the compounds showed trypanocidal activities. Among which, 17-DMAG, epoxomicine and EerI demonstrated high efficacy, showing IC_{50} values of 0.11 ± 0.01 nM, 15.65±1.31 nM and 904.80 ± 40.53 nM, respectively. DBeQ, L-658,458, 16F16 and

LY-374973 showed lower trypanocidal activity with IC_{50} of 1,100.53 ± 122.72 nM , 2,259.67 ± 107.90 nM, 2,414.67 ± 388.07 and 8,711.67 ± 773.27, respectively. $(Z\text{-}LL)_2$Ketone and LY-411575 resulted in very low IC_{50} values for *T. congolense* compared to other compounds with IC_{50} of 11,767.33 ± 572.07 nM and 18,366.67 ± 2,903.96 nM.

Cytotoxicity and selectivity of these compounds. Except for the compounds targeting SPP, all other compounds were also active against HFF cells, with IC_{50} values ranging from 11.22 ± 4.40 nM to 4,353.00 ± 419.81 nM (Table 6). Some compound showed good selectivity against these trypanosome strains. 17-DMAG, EerI and $(Z\text{-}LL)_2$ Ketone were more selective towards *T. b. brucei* than towards HFF cells (SI: 134,976.74, 10.50 and >24.99), whereas epoxomicine and DBeQ were more toxic (SI: 1.31 and 1.95). The lowest selectivity for *T. b. brucei* was seen for 16F16 with SI of 0.51. On the other hand, 17-DMAG was also highly selective towards *T. congolense* than towards HFF cells (SI: 38,923.99). The SPP inhibitors ($(Z\text{-}LL)_2$ Ketone, LY-411575, L-658, 458, LY-374973) showed lower selectivity against *T. congolense* with IC_{50}values of >2.66, >3.40, >5.53, >7.17, respectively. DBeQ also showed lower selectivity against *T. congolense* (SI: 3.55). The epoxomicine, 16F16 and EerI showed the very weak selectivity against *T. congolense* with SI values of 0.72, 0.63 and 0.92, respectively.

3-4. Discussion

Chemotherapy of African trypanosomosis still relies on a few drugs developed decades ago, most of which show poor efficacy and significant toxicity, and are being increasingly subject to drug resistance (Delespaux and de Koning, 2007). The identification of novel targets and antiparasitic compounds is a pressing need for chemotherapy of sleeping sickness and nagana diseases. Here I show that the ERAD pathway represents an exploitable vulnerability as a drug target of African trypanosomes.

ERAD pathway is a complex network that comprises restricted, partially redundant protein complexes (Vembar and Brodsky, 2008). And many of the individual components thought to underlie ERAD have been identified through genetic and biochemical analyses in *S. cerevisiae* and mammals (Hoseki *et al.*, 2010; Bagola *et al.*, 2011*;* Christianson *et al.*, 2011). Although African trypanosomes are highly divergent from yeast and mammals, the general features of the trypanosome endomembrane system are conserved (Engstler *et al.*, 2007). Both *in silico* and functional data indicated a complex ER quality control system in trypanosome that is required in many higher eukaryotes, and recently research also indicated the presence of conventional ERAD pathway in trypanosomes (Banerjee *et al.*, 2007; Field *et al.*, 2010). My finding was consistent with these researches. In this study, based on the components of human ERAD pathway, homology BLAST was done with TriTryp DB. All functional modules of the mammalian ERAD pathway showed fewer orthologues in *T. b. brucei* and *T. congolense*, even the identity level of these components at protein level was lower when compared with human.

In order to identify the novel drug targets of African trypanosome, I performed a small scale screen of well characterized inhibitors that target an array of ERAD pathway proteins. I focused on ERAD components identified in *T. b. brucei* and *T. congolense* with known associated inhibitors, including Hsp90 (Smith *et al.*, 2005; Pratt *et al.*, 2010), PDI (Hoffstrom *et al.*, 2010), ER intramembrane aspartyl protease SPP (Li *et al.*, 2009; Parvanova *et al.*, 2009), ATPase p97 (Chou *et al.*, 2011) and proteasome (Hanada *et al.*, 1992; Meng *et al.*, 1999). *T. b. brucei* and *T. congolense* were indeed susceptible to each of the inhibitors to varying degrees. In addition, each inhibitor was assayed against the HFF cells, and a selectivity index was produced to determine the fold increase in potency for the inhibitor toward *T. b. brucei* and *T. congolense* vs. the human cell line. As predicted, the majority of compounds were more potent for African trypanosomes vs. the human cell line.

My screening revealed remarkable *in vitro* trypanocidal activity for both *T. b. brucei* and *T. congolense* for 17-DMAG, and also with a high selectivity index. Resent finding indicated that Hsp90 has important biological roles in some protozoan parasites like *Plasmodium* spp, *Toxoplasma gondii* and trypanosomatid (Angel *et al.,* 2013). These results demonstrated that Hsp90 might be the very important chaperone in *T. b. brucei* and *T. congolense* ERAD pathway. Inhibiting PDI activity in *T. b. brucei* and *T. congolense* suppresses the growth of both parasites. However, the cytotoxicity of 16F16 determined for HL-60 cells was too high. The SPP and the gamma-secretase inhibitor impaired the development of trypanosome strains *in vitro* with very low cytotoxicity with HFF cells, which indicated a potential high selective index for this class of compounds. DBeQ has been identified as a selective, potent, reversible inhibitor of the p97ATPase, was found to be a potent activator of caspases in cancer cell and to inhibit both ERAD and autophagosome maturation (Chou *et al.,* 2011). Eeyarestatin I is a chemical inhibitor that can bind both p97 and ER membrane to influence the downstream deubiquitination process. However, EerI treatment cannot lower the ATPase activity of p97 (Wang *et al.,* 2008; Wang *et al.,* 2010). In the current study, DBeQ and EerI showed good trypanocidal activity against both trypanosome species, which indicated that p97 inhibition maybe a potential chemotherapeutic strategy. Epoxomicin is a potent anti-tumor agent isolated from *Actinomycetes* that is used as a selective and irreversible inhibitor of the 20S proteasome. It show dramatic effects on the growth of *T. b. brucei* and *T. congolense*, which suggesting the essential roles of proteasome in protein degradation.

This data suggest that African trypanosomes are susceptible to inhibition of the components of ERAD pathway. These results were consist with the previous study that ERAD is a vectorial process, in which the ERAD components are organised into a functional network, inhibition of any individual component of ERAD will disrupt the homeostatic balance of the ER (Hoseki *et al.,* 2010; Bagola *et al.,* 2011; Christianson *et al.,* 2011).

Furthermore, target validation and early lead identification represent the initial stages of a potential drug development program. In the ERAD case, there is the possibility of taking advantage of the much more advanced programs in the cancer field. Parasites and cancer cells share basic characters related to the metobalic requiments associated with their high proliferation rate (Hooft van Huijsduijnen *et al.*, 2013). Therefore, tapping into the therapeutics of cancer based on ERAD pathway might have a great value in the development of drugs against African trypanosomes.

In conclusion, these compounds targeting the components have been shown to display substantial trypanocidal activity. Targeting ERAD is probably applicable for treatment of AAT. However, a potential weakness in using the known associated inhibitors designed against human targets to search for anti-parasitic compounds is that the starting chemical points are not selective. Therefore, these compounds may serve as lead compounds for the development of analogues with improved selectivity. Another possibility would be combination of some of these compounds. Such drug combination regimes may lead to synergistic effects, in which lower amounts of drugs sufficient to kill the parasites will lead to a reduction in toxicity.

3-5. Summary

The current pharmacopoeia to treat the lethal human and animal diseases caused by the African trypanosomes remain limited. The parasite's ability antigenic variation represents a considerable barrier to vaccine development, making the identification of new drug targets extremely important. In order to find new drug targets, in this study, 9 inhibitors that target the different components of ERAD pathway were tested for their *in vitro* activity against BSF of *T. b. brucei*, *T. congolense* and normal HFF cells. All the compounds exhibited trypanocidal activity, 17-DMAG was the most potent inhibitor in both *T. b. brucei* (IC_{50}=0.03 ± 0.01 nM) and *T. congolense* (IC_{50}=0.11 ± 0.01 nM), while LY-374973 was the least potent inhibitor for *T. b. bruce*i (IC50=24,400.33 ± 14,619.30 nM) and LY-411575 (IC50=18,366.67 ± 2,903.96 nM) was the least potent inhibitor for *T. congolense.* However, some of these inhibitors also displayed cytotoxicity towards HFF cells. The tested inhibitors showed different selectivity indeies (SI) for *T. b. brucei* and *T. congolense.* 17 DMAG exhibited higher selectivity for both *T. b. brucei* (SI=134,976.74) and *T. congolense* (SI=38,923.99). 16F16 exhibited lowest selectivity for both *T. b. brucei* (SI=0.63) and *T. congolense* (SI=0.51). According to these results, targeting the ERAD pathway may be a new strategy in the treatment of trypanosome infection, in particular, these inhibitors may serve as lead compounds for development of novel trypanocidal drugs.

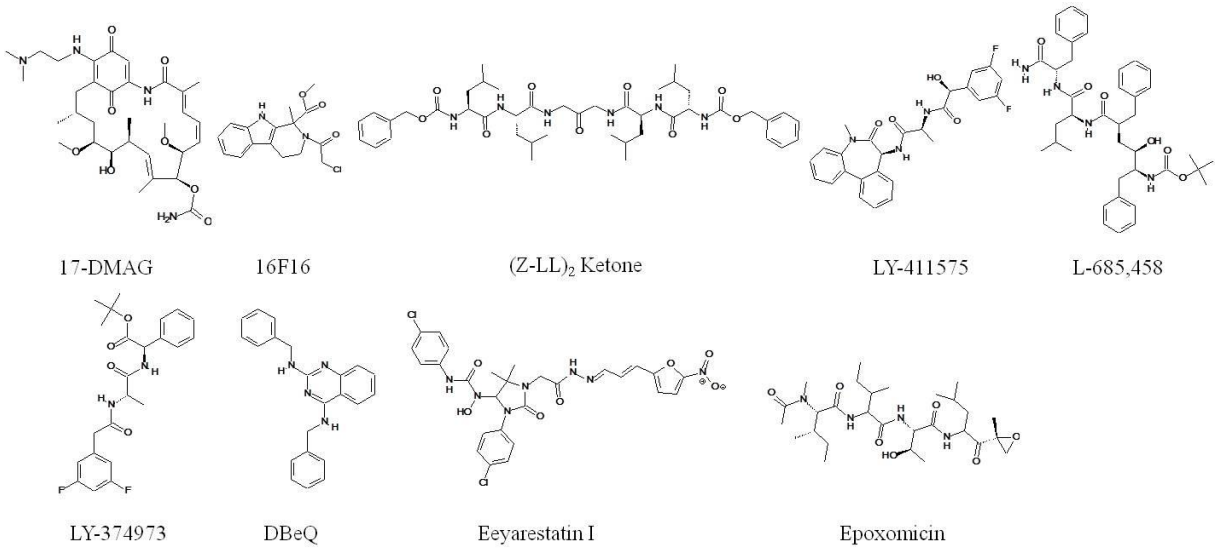

Fig.10. Chemical structures of tested compounds.

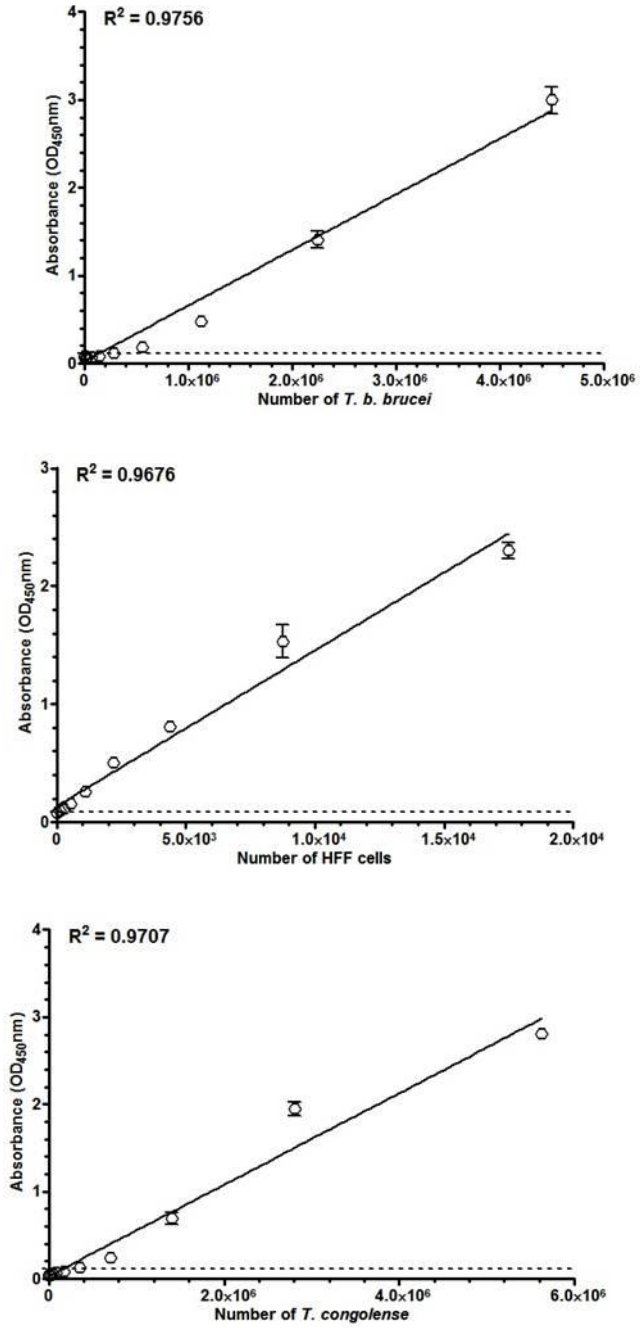

Fig. 11. Detection limit in CCK-8 screening assay for *T. b. brucei, T. congolense* and HFF cells. Solid line shows linear regression for the cell number versus absorbance. Broken line shows detection limit for the assay, calculate as 3 times of the standard deviation of the blank.

Table 5. The predicted components of ERAD orthologues in *T. brucei* and *T. congolense* organized by functional modules.

Functional module	Enzymes	Gene	*T. brucei* TREU927	Identity at protein level (%)	*T. congolense* IL3000	Identity at protein level (%)
Substrate recognition	HSP70 family	BiP(GRP78)	Tb927.11.7460	63.18	TcIL3000_0_02950	63.03
and processing	HSP90 family	**GRP94**	**Tb927.3.3580**	**33.22**	**TcIL3000_3_2270**	**33.25**
	Reductase	ERDJ5(HSP40)	Tb927.2.5160	11.10	TcIL3000_2_1270	11.97
		ERO1	Tb927.8.4890	24.24	TcIL3000_0_00060	25.05
	Dithiol Isomerase	**PDI**	**Tb927.4.2450**	**17.32**	**TcIL3000_4_2300**	**17.79**
	Carboxypeptidase	CPVL	Tb927.10.1050	22.98	TcIL3000_10_560	22.60
	Lectin	OS9	Tb927.11.10700	8.39	TcIL3000.11.11360	9.13
Dislocation	Processing protease	**SPP**	**Tb927.3.4910**	**22.63**	**TcIL3000_3_2960**	**25.30**
	Derlin	Derlin1	Tb927.11.12590	27.80	TcIL3000.11.13160	27.03
		Derlin2	Tb927.11.3170	23.55	TcIL3000.11.2970	23.87
Uiquitin ligation		gp78	Tb927.9.5260	14.00	TcIL3000_9_1750	7.15
complexes		UBAC2	Tb927.8.6620	14.56	TcIL3000_8_6500	12.64
		Hrd1	Tb927.9.5260	17.10	TcIL3000_9_1750	7.09
		UBE2G2	Tb927.11.14200	25.40	TcIL3000.11.14520	25.40
		SEL1L	Tb927.8.1250	15.48	TcIL3000_8_920	16.53
		RNF5	Tb927.10.6380	21.34	TcIL3000_10_5450	21.12
		DOA10	Tb927.11.16240	14.67	TcIL3000_0_12420	12.59
Substrate extraction	Recruitment factors	UFD1L	Tb927.10.7790	24.30	TcIL3000_10_6650	22.82
		p97	**Tb927.10.5770**	**11.07**	**TcIL3000_10_4820**	**11.50**
		RAD23	Tb927.6.4650	22.31	TcIL3000_6_4100	20.47
		DOA1	Tb927.8.6330	20.04	TcIL3000_0_44870	10.57
	Deubiquitylating	YOD1/OTU1	Tb927.10.8790	21.25	TcIL3000_10_7560	23.71
		USP13	Tb927.3.4840	25.63	TcIL3000_0_42590	25.74
	Ubiquitin extension	UBE4B	Tb927.6.2460	12.08	TcIL3000_6_1960	12.72

Table 6. Trypanocidal activity of the inhibitors and their cytotoxicity against HFF cell line.

Inhibitors	IC_{50} ± SD			Selective index (SI)	
	T .b. brucei (nM)	*T. congolense* (nM)	HFF (nM)	HFF/ *T. b. brucei*	HFF/ *T. congolense*
17-DAMG	0.03 ± 0.01	0.11 ± 0.01	4,353.00 ± 419.81	1,34976.74	38,923.99
16F16	2,959.33 ± 332.36	2,414.67 ± 388.07	1,518.667 ± 331.13	0.51	0.63
$(Z\text{-}LL)_2$ Ketone	1,250.33 ± 22.59	1,1767.33 ± 572.07	>31,250.00*	>24.99	>2.66
LY-411575	16,169.00 ± 1495.44	18,366.67 ± 2903.96	>62,500.00*	>3.87	>3.40
L-658,458	5,392.33 ± 752.83	2,259.67 ± 107.90	>12,500.00*	>2.32	>5.53
LY-374973	24,400.33 ± 14619.30	8,711.67 ± 773.27	>62,500.00*	>2.56	>7.17
DBeQ	2,004.00 ± 161.17	1,100.53 ± 122.72	3,907.67 ± 658.10	1.95	3.55
Eeyarestatin I	79.30 ± 0.13	904.80 ± 40.53	832.70 ± 143.04	10.50	0.92
Epoxomicin	8.54 ± 1.76	15.65 ± 1.31	11.22 ± 4.40	1.31	0.72

HFF: human foreskin fibroblast cells.

*: The highest concentration tested.

Selective index = IC_{50} (HFF) / IC_{50} (*T. b. brucei* or *T. congolense)*

General discussion

AAT is mainly caused by *T. congolense, T. vivax* and *T. brucei*. In wild animals, these parasites cause relatively mild infections while in domestic animals they cause a severe, often fatal disease. All domestic animals can be affected by AAT and the symptoms are fever, listlessness, emaciation, hair loss, discharge from the eyes, oedema, anaemia, and paralysis. For control of AAT, it is important to block the transmission routes for controlling the spread of infection. Therefore, the development of reliable and sensitive diagnostic tests and effective drugs for trypanosome infection is essential for the control of AAT.

The main objectives of this study were to develop the novel diagnostic methods of AAT and subsequently to discover the potent drug target and drugs for African trypanosomosis. An ELISA is widely applied for the demonstration of antibodies against a variety of infectious agents, including African trypanosomes. Currently, ELISA used for trypanosomosis was developed by using the trypanosome cell lysate antigen as a recommended test (OIE. 2012). However, the qualities of these ELISA tests often vary from batch to batch (Goto *et al*., 2011; Rebeski *et al*., 2000). In contrast, recombinant antigens can easily be prepared on a large scale, are relatively stable and have higher specificity than parasite cell lysate antigens (Tran *et al*., 2009). Consequently, the development of sensitive and specific ELISA tests based on the recombinant protein is necessary.

In chapter 1, in order to evaluate novel invariable proteins as candidate serodiagnostic antigens for *T. congolense* infection, I focused on the proteins with high expression levels in the MCF and BSF stages, because of their importance in serodiagnosis. Four proteins

(TcIL3000.0.25950, TcIL3000.0.10.3480, TcIL3000.8.629, and TcIL3000.7.1980) were selected from the data for differential protein expression in all life cycle stages of *T. congolense* (Table 1) (Eyord *et al*., 2011). As a result, only the recombinant TcP46 protein was chosen and characterized.

In order to characterize this candidate antigen, I used the bioinformatics parameters such as antigenicity plot and hydrophobicity plot of predicted peptide as benchmark for preliminary screeing of putative antigenic peptides. Subsequently, the results suggested that this recombinant protein could be the prospective diagnostic candidate. Furthermore, Southern blot analysis revealed that at least 2 copies of the TcP46 gene are tandemly arranged in the parasite genome. In order to confirm whether rTcP46 is indeed antigenic, Western blot analysis was done and this recombinant protein reacted to the sera from mice infected with *T. congolense*. Moreover, the Western blot analysis and IFAT with anti-rTcP46 mice sera indicated that TcP46 protein is expressed in the cytosol throughout the life cycle stages of the parasite as an approximately 46 kDa protein. Meanwhile, a strong reaction was observed in the BSF and MCF parasite stages compared with EMF and PCF. Based on this data, I confirmed that TcP46 are endogenous proteins of *T. congolense* and may be able to detect the antibodies induced by this antigen when *T. congolense* infection happens. Subsequently, rTcP46-ELISA was performed to ascertain whether this antigen could be used for the serodiagnosis of *T. congolense* infection. The results revealed that this antigen is specific for *T. congolense* infection only, indicating that it could be a potential antigen for detection of antibodies against *T. congolense*. Indeed, the TcP46 gene shared a low nucleotide sequences identity with *T. b. brucei* and no match with the other *Trypanosoma*

spp. and apicomplex paresites detected by BLAST. Therefore, the TcP46 gene may be a candidate target for developing a specific and sensitive molecular diagnostic method to screen the *T. congolense* infection.

In chapter 2, I assess a semi-nPCR assay based on the TcP46 gene for detecting of *T. congolense* infection. The sensitivity of the semi-nPCR assay was evaluated and the results showed that, this semi-nPCR method could detect the template as few as 0.7 pg, which was more sensitive than the widely used Kin-PCR (Njiru *et al.,* 2005). Furthermore, comparison of the methods for the detection of *T. congolense* showed that this semi-nPCR was more sensitive than the conventional microscopy and commonly used Kin-PCR method. In addition, the specificity analysis also demonstrated that the specific region of TcP46 gene might be suitable for molecular identification of *T. conglense* with maximal specificity. Finally, the method was used to detect the prevalence of *T. congolense* occurring in South Africa. These results suggest that the semi-nPCR method was suitable for detection the field samples. It revealed higher prevalence for *T. congolense* infection in cattle and dogs than the Kin PCR method. Moreover, the present study provided new evidence to confirm that the *T. congolense* is a wide epidemic species that infected cattle and dogs in South Africa.

In chapter 3, I validated ERAD pathway of *T. b. brucei* and *T. congolense* as drug target to facilitate discovery of novel drugs for treatment of AAT. The ERAD pathway of cancer cells and parasitic protozoa is an attractive chemotherapeutic target, because ERAD is responsible for the degradation of aberrant or misfolded proteins in the ER, providing an important protein folding “quality control” mechanism (Li and Li, 2011; Chung, *et al.,* 2012; Verfaillie *et al.,* 2013). This pathway requires a number of molecular chaperones for

identification of the misfolded proteins and dedicated ER resident reductases for cleaving disulfide bonds in these proteins to facilitate retrograde transport to the cytosol, inhibition of any individual component of ERAD will affect the ERAD system. Therefore, my approach began with a bioinformatics analysis of the ERAD pathways of *T. b. brucei* and *T. congolense*. Based on the components of human ERAD pathway, homology BLAST was done with TriTryp DB. A simplified ERAD pathway was found in *T. b. brucei* and *T. congolense* comparing with mammalian ERAD pathway. Subsequently, a panel of known inhibitors was screened against the components of ERAD pathway using *in vitro* cultures with the aim of identifying novel targets for AAT. *T. b. brucei* and *T. congolense* were indeed susceptible to each of the inhibitors in varying degrees. In addition, each inhibitor was assayed against the HFF cells, and a selectivity index was produced to determine the fold increase in potency for the inhibitor toward *T. b. brucei* and *T. congolense* vs. the human cell line. As predicted, the majority of compounds were more potent against African trypanosomes than the human cell line. These results suggested that, targeting ERAD is probably applicable for treatment of AAT. Thus, this study provides a basis for development of new trypanocidal drugs targeting ERAD pathways of the parasites.

In conclusion, I identified one recombinant antigen, TcP46, for developing serodiagnostic methods to detect *T. congolense* infection and showed that this antigen is specific to *T. congolense* infection. ELISA based on the rTcP46 showed that it could be a marker for both early and chronic stages of *T. congolense* infection. Therefore, rTcP46-ELISA could be a promising diagnostic method. Subsequently, the semi-nPCR assay based on the TcP46 gene specifically detected *T. congolense* at low parasitermias in

parasite culture and mouse models. The semi-nPCR also could be used as a reliable and potential tool for epidemiological survey of the *T. congolense*-infected animals. Additionally, growth inhibition effects of a panel of known inhibitors targeting the components of ERAD pathway were evaluated in tryapanosome species. The effective growth inhibitions of parasites were observed in *T. b. brucei* and *T. congolense in vitro*. Therefore, the ERAD pathway is a promising drug target of anti-trypanosome drugs.

General summary

The main objective of this study was to develop diagnostic method of AAT, and to evaluate drug target of trypanosome parasites to facilitate drug discovery.

In chapter 1, I have identified and characterized a novel antigen of *T. congolense* and evaluated its potentiality for serodiagnosis. There was no cross-reactivity observed in the rTcP46-based ELISA with other parasites and rTcP46-based ELISA could detect both early and chronic stage infection. The results demonstrated that the rTcP46 was a promising serodiagnostic antigen to detect *T. congolense* infection.

In chapter 2, I developed a semi-nPCR for the detection of *T. congolense* using oligonucleotide primers designed from a tandemly-arranged TcP46 gene. This semi-nPCR method with promising specificity and high sensitivity, could be used as a reliable and potential tool for epidemiological survey of the *T. congolense*-infected animals. Moreover, the present study provided new evidence to confirm that the *T. congolense* is a wide epidemic species that infected cattle and dogs in South Africa.

In chapter 3, I validated ERAD pathway in *T. b. brucei*, *T. congolense* parasites as a drug target. I evaluated the *in vitro* activity of 9 inhibitors that targeted the different components of ERAD pathway against bloodstream forms of *T. b. brucei*, *T. congolense* and normal HFF cells. All the compounds exhibited trypanocidal activity at different levels. Therefore, these results suggested that targeting the ERAD pathway may be a new strategy in the treatment of trypanosome infection and these inhibitors may serve as lead compounds for development of novel trypanocidal drugs.

In conclusion, the TcP46 is a novel immunodominant antigen that could be used for serodiagnosis of *T.congolense* infection. The tandemly-arranged TcP46 gene was also a good candidate for molecular diagnosis of *T. congolense* infection. Furthermore, ERAD pathway is a promising drug target of anti-trypanosome drugs. Inhibitors that target the different components of ERAD pathway could inhibit the growth of the trypanosome parasite.

Acknowledgments

This research work was carried out at National Research Center for Protozoan Diseases (NRCPD), Obihiro University of Agriculture and Veterinary Medicine (Obihiro Univ. Agric. Vet. Med.), and supported by a grant from the Global COE Program and a Grant-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science, and Technology of Japan. This research work was also supported by a doctoral fellowship from the Japanese Society for the Promotion of Science (JSPS).

I sincerely thank my supervisor Prof. Noboru Inoue, for his intellectual guidance, constructive criticisms, invaluable suggestions, patiently revising this dissertation, and tireless support during all the period of my study.

I sincerely thank Prof. Shin-ichiro Kawazu, Prof. Xuenan Xuan, Prof. Ikuo Igarashi, Assoc Prof. Yoshifumi Nishikawa, for their constructive criticisms, invaluable suggestions, and revising this dissertation.

I also would like to acknowledge Prof. Hiroshi Suzuki, Prof. Naoaki Yokoyama, Assoc. Prof. Shinya Fukumoto and Assist. Prof. Rika Shirafuji, for their valuable advice, critical suggestions and comments.

I extend my gratitude to Ruttayaporn Ngassaman, Keisuke Suganuma, Thu-Thuy Nguyen, Shino Yamasaki and Simon Peter Musinguzi for their kind assistance, valuable suggestions and comments. I would like to thank all of the former and present staff and students in vector biology and vaccine groups for their kind assistance and company during my PhD study.

Finally, I would like to express my special gratitude to my family for their devoted support. I would like in a special way to express my gratitude to my husband Dr. Cao for his endless help and support.

References

Adamu, U. O., Haruna, M. K., Ovbagbedia, R. P., Bizi, R., Benjamin, W., Malala, U. A., Nwezor, F. N. C., Muhammed, M., 2011. Control of african trypanosomiasis in Nigeria: time to strengthening integrated approaches. Int. J. Anim. Vet. Adv. 3, 138-143.

Agur, Z. and Mehr, R., 1997. Modelling *Trypanosoma congolense* parasitaemia patterns during the chronic phase of infection in N'Dama cattle. Parasite Immunol. 19, 171-182.

Ahmed, H. A., Picozzi, K., Welburn, S.C., MacLeod, E. T., 2013. A comparative evaluation of PCR-based methods for species-specific determination of African animal trypanosomes in Ugandan cattle. Parasites & Vectors. 6, 316.

Alsford, S., Eckert, S., Baker, N., Glover, L., Sanchez-Flores, A., Leung, K. F., Turner, D. J., Field, M. C., Berriman, M., Horn, D., 2012. High-throughput decoding of antitrypanosomal drug efficacy and resistance. Nature. 482, 232-236.

Angel, S. O., Matrajt, M., Echeverria, P. C., 2013. A review of recent patents on the protozoan parasite HSP90 as a drug target. Recent Pat. Biotechnol. 7, 2-8.

Awulu, E. A., Oniye, S. J., Adelanwa, M. A., 2013. Phytochemical screening and *in vivo* antitrypanosomal activity of methanol extracts of *peristrophe bicalyculata* in mice infected with *Trypanosoma evansi*. Int. J. Sci. Tech. 3, 34-39.

Bagola, K., Mehnert, M., Jarosch, E., Sommer, T., 2011. Protein dislocation from the ER. Biochim. Biophys. Acta. 1808, 925-936.

Banerjee, S., Vishwanath, P., Cui, J., Kelleher, D. J., Gilmore, R., 2007. The evolution of N-glycan-dependent endoplasmic reticulum quality control factors for glycoprotein folding and degradation. Proc. Natl. Acad. Sci. 104, 11676-11681.

Bannai, H., Sakurai, T., Inoue, N., Sugimoto, C. and Igarashi, I., 2003. Cloning and

expression of mitochondrial heat shock protein 70 of *Trypanosoma congolense* and potential use as a diagnostic antigen. Clin. Diagn. Lab. Immunol. 10, 926-933.

Boulangé, A., Katende, J. and Authié, E., 2002. *Trypanosoma congolense*: expression of a heat shock protein 70 and initial evaluation as a diagnostic antigen for bovine trypanosomosis. Exp. Parasitol. 100, 6-11.

Cao, S., Aboge, G. O., Terkawi, M. A., Yu, L., Kamyingkird, K., Luo, Y., Li, Y., Goo, Y. K., Yamagishi, J., Nishikawa, Y., Yokoyama, N., Suzuki, H., Igarashi, I., Maeda, R., Inpankaew, T., Jittapalapong, S., Xuan, X., 2012. Molecular detection and identification of *Babesia bovis* and *Babesia bigemina* in cattle in northern Thailand. Parasitol. Res. 111, 1259-1266.

Camara, M., Camara, O., Ilboudo, H., Sakande, H., Kaboré, J., N'Dri, L., Jamonneau, V., Bucheton, B., 2011. Sleeping sickness diagnosis: use of buffy coats improves the sensitivity of the mini anion exchange centrifugation test. Trop. Med. Int. Health. 15, 796-799.

Chappuis, F., Loutan, L., Simarro, P., Lejon, V. and Büscher, P., 2005. Options for field diagnosis of human african trypanosomiasis. Clin. Microbiol. Rev. 18, 133-146.

Chitanga, S., Marcotty, T., Namangala, B., Van den Bossche, P., Van Den Abbeele, J., Delespaux, V., 2011. High prevalence of drug resistance in animal trypanosomes without a history of drug exposure. PLoS Negl Trop Dis. 5, e1454.

Chou, T. F., Brown, S. J., Minond, D., Nordin, B. E., Li, K., Jones, A. C., Chase, P., Porubsky, P. R., Stoltz, B. M., Schoenen, F. J., Patricelli, M. P., Hodder, P., Rosen, H., Deshaies, R. J., 2011. Reversible inhibitor of p97, DBeQ, impairs both ubiquitin-dependent and autophagic protein clearance pathways. Proc. Natl. Acad. Sci. U. S. A. 108, 4834-4839.

Chou, T. F., Deshaies, R. J., 2011. Development of p97 AAA ATPase inhibitors. Autophagy. 7, 1091-1092.

Christianson, J. C., Olzmann, J. A., Shaler, T. A., Sowa, M. E., Bennett, E. J., Richter, C. M., Tyler, R. E., Greenblatt, E. J., Harper, J. W., Kopito, R. R., 2011. Defining human ERAD networks through an integrative mapping strategy. Nat. Cell Biol. 14, 93-105.

Chung, D. W., Ponts, N., Prudhomme, J., Rodrigues, E. M., Le Roch, K. G., 2012. Characterization of the ubiquitylating components of the human malaria parasite's protein degradation pathway. PLoS One. 7, e43477.

Clausen, P. H., Wiemann, A., Patzelt, R., Kakaire, D., Poetzsch, C., Peregrine, A., Mehlitz, D., 1998. Use of a PCR assay for the specific and sensitive detection of *Trypanosoma* spp. in naturally infected dairy cattle in peri-urban Kampala, Uganda. Ann. N. Y. Acad. Sci. 849, 21-31.

Coustou, V., Guegan, F., Plazolles, N. and Baltz, T., 2010. Complete *in vitro* life cycle of *Trypanosoma congolense*: development of genetic tools. PLoS Negl. Trop. Dis. 4: e618.

Cox, A. P., Tosas, O., Tilley, A., Picozzi, K., Coleman, P., Hide, G. and Welburn, S. C., 2010. Constraints to estimating the prevalence of trypanosome infections in East African zebu cattle. Parasites & Vectors. 3, 82.

de Clare Bronsvoort, B. M., von Wissmann, B., Fèvre, E. M., Handel, I. G., Picozzi, K., Welburn, S. C., 2010. No gold standard estimation of the sensitivity and specificity of two molecular diagnostic protocols for *Trypanosoma brucei* spp. in Western Kenya. PLoS One. 5, e8628.

Delespaux, V., de Koning, H. P., 2007. Drugs and drug resistance in African trypanosomiasis. Drug Resist Updat. 10, 30-50.

Desquesnes, M., McLaughlin, G., Zoungrana, A., Dávila, A. M., 2001. Detection and identification of Trypanosoma of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. Int J Parasitol. 31, 610-614.

Donelson, J. E., Hill, K. L. and El-Sayed, N. M., 1998. Multiple mechanisms of immune

evasion by African trypanosomes. Mol. Biochem. Parasitol. 91, 51-66.

Engstler M., Bangs J. D., Field M. C. 2007. Intracellular transport systems in trypanosomes: function, evolution and virulence, p. 281-317. In Barry J. D., Mottram J., McCulloch R., Acosta-Serrano A., editors. (ed.), Trypanosomes-after the genome. Horizon Press, Norwich, United Kingdom.

Eyford, B. A., Sakurai, T., Smith, D., Loveless, B., Hertz-Fowler, C., Donelson, J. E., Inoue, N., Pearson, T. W., 2011. Differential protein expression throughout the life cycle of *Trypanosoma congolense*, a major parasite of cattle in Africa. Mol. Biochem. Parasitol. 177, 116-125.

Eyob, E., Matios, L., 2013. Review on camel trypanosomosis (surra) due to *Trypanosoma evansi*: Epidemiology and host response. J. Vet. Med. Anim. Health. 5, 334-343.

Field, M. C., Sergeenko, T., Wang, Y.N., Böhm, S., Carrington, M., 2010. Chaperone requirements for biosynthesis of the trypanosome variant surface glycoprotein. PLoS One. 5, e8468.

Geysen, D., Delespaux, V., Geerts, S., 2003. PCR-RFLP using Ssu-rDNA amplification as an easy method for species-specific diagnosis of *Trypanosoma* species in cattle. Vet Parasitol. 110, 171-180.

Gillingwater, K., Mamabolo, M. V. and Majiwa, P. A., 2010. Prevalence of mixed *Trypanosoma congolense* infections in livestock and tsetse in KwaZulu-Natal, South Africa. J. S. Afr. Vet. Assoc. 81, 219-223.

Goto, Y., Duthie, M. S., Nguyen, T. T., Asada, M., Kawazu, S., Carter, D. and Inoue, N., 2011. Serological characterizations of tandem repeat proteins for detection of African trypanosome infection in cattle. Parasitol. Int. 60, 538-540.

Grady, S. C., Messina, J. P., McCord, P. F., 2011. Population vulnerability and disability in Kenya's tsetse fly habitats. PLoS Negl Trop Dis. 8, e957.

Hanada, M., Sugawara, K., Kaneta, K., Toda, S., Nishiyama, Y., Tomita, K., Yamamoto, H., Konishi, M., Oki, T., 1992. Epoxomicin, a new antitumor agent of microbial origin. J Antibiot. 45, 1746-1752.

Helm, J. R., Hertz-Fowler, C., Aslett, M., Berriman, M., Sanders, M., Quail, M. A., Soares, M. B., Bonaldo, M. F., Sakurai, T., Inoue, N. and Donelson, J. E., 2009. Analysis of expressed sequence tags from the four main developmental stages of *Trypanosoma congolense*. Mol. Biochem. Parasitol. 168, 34-42.

Hendry, K. A., Vickerman, K., 1988. The requirement for epimastigote attachment during division and metacyclogenesis in *Trypanosoma congolense*. Parasitol Res. 74, 403-408.

Herbert, W. J., Lumsden, W. H., 1976. *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitemia. Exp Parasitol. 40, 427-431.

Hilali, M., Abdel-Gawad, A., Nassar, A., Abdel-Wahab, A., Magnus, E. and Büscher, P., 2004. Evaluation of the card agglutination test (CATT/T. evansi) for detection of *Trypanosoma evansi* infection in water buffaloes (Bubalus bubalis) in Egypt. Vet. Parasitol. 121, 45-51.

Hirumi, H. and Hirumi, K., 1991. In vitro cultivation of *Trypanosoma congolense* bloodstream forms in the absence of feeder cell layers. Parasitology. 102, 225-236.

Hoffstrom, B. G., Kaplan, A., Letso, R., Schmid, R. S., Turmel, G. J., Lo, D. C., Stockwell, B. R., 2010. Inhibitors of protein disulfide isomerase suppress apoptosis induced by misfolded proteins. Nat Chem Biol. 6, 900-906.

Hooft van Huijsduijnen, R., Guy, R. K., Chibale, K., Haynes, R.K., Peitz, I., Kelter, G., Phillips, M. A., Vennerstrom, J. L., Yuthavong, Y., Wells, T. N., 2013. Anticancer properties of distinct antimalarial drug classes. PLoS One. 8, e82962.

Hopkins, J. S., Chitambo, H., Machila, N., Luckins, A. G., Rae, P. F., van den Bossche, P., Eisler, M. C., 1998. Adaptation and validation of antibody-ELISA using dried blood spots on filter paper for epidemiological surveys of tsetse-transmitted trypanosomosis in cattle.

Prev Vet Med. 37, 91-99.

Horn, D., McCulloch, R., 2010. Molecular mechanisms underlying the control of antigenic variation in African trypanosomes. Curr Opin Microbiol. 13, 700-705.

Hoseki, J., Ushioda R., Nagata, K., 2010. Mechanism and components of endoplasmic reticulum-associated degradation. J. Biochem. 147, 19-25.

Hussain, M., Ozawa, Y., Ichihara, S., Mizushima, S., 1982. Signal peptide digestion in *Escherichia coli*. Effect of protease inhibitors on hydrolysis of the cleaved signal peptide of the major outer-membrane lipoprotein. Eur J Biochem. 129, 233-239.

Inoue, N., Lluz, A. T., Mori, T., Nagasawa, H., Fujisaki, K., Mikami, T., 2000. Novel species specific antigens of *Trypanosoma congolense* and their different localization among life-cycle stages. J. Vet. Med. Sci. 62, 1041-1045.

Kristjanson, P. M., Swallow, B. M., Rowlands, G. J., Kruska, R. L. and de Leeuw, P. N., 1999. Measuring the costs of African animal trypanosomosis, the potential benefits of control and returns to research. Agr. Syst. 59, 79-98.

Lanham, S. M. and Godfrey, D. G., 1970. Isolation of salivarian trypanosomes from man and other mammals using DEAE-cellulose. Exp. Parasitol. 28, 521-534.

Laohasinnarong, D., Thekisoe, O. M., Malele, I., Namangala, B., Ishii, A., Goto, Y., Kawazu, S., Sugimoto, C. and Inoue, N., 2011. Prevalence of Trypanosoma sp. in cattle from Tanzania estimated by conventional PCR and loop-mediated isothermal amplification (LAMP). Parasitol. Res. 109, 1735-1739.

Leak, S. G. A., 1999. Tsetse biology and ecology: their role in the epidemiology and control of trypanosomiasis. Wallingford, UK: CABI Publishing.

Li, F. J., Gasser, R. B., Lai, D. H., Claes, F., Zhu, X. Q., Lun, Z. R., 2007. PCR approach for the detection of *Trypanosoma brucei* and *T. equiperdum* and their differentiation from *T. evansi* based on maxicircle kinetoplast DNA. Mol Cell Probes. 21, 1-7.

Li, X., Chen, H., Bahamontes-Rosa, N., Kun, J. F., Traore, B., Crompton, P. D., Chishti, A. H., 2009. *Plasmodium falciparum* signal peptide peptidase is a promising drug target against blood stage malaria. Biochem Biophys Res Commun. 380, 454-459.

Li, X., Zhang, K., Li, Z., 2011. Unfolded protein response in cancer: the physician's perspective. J Hematol Oncol. 4, 8.

Lovat, P. E., Corazzari, M., Armstrong, J. L., Martin, S., Pagliarini, V., Hill, D., Brown, A. M., Piacentini, M., Birch-Machin, M. A., Redfern, C. P., 2008. Increasing melanoma cell death using inhibitors of protein disulfide isomerases to abrogate survival responses to endoplasmic reticulum stress. Cancer Res. 68, 5363-5569.

Luckins, A. G., Mehlitz, D., 1978. Evaluation of an indirect fluorescent antibody test, enzyme-linked immunosorbent assay, and quantification of bovine immunoglobulins in the diagnosis of bovine trypanosomiasis. Trop Anim Health Prod. 10, 149-159.

Luckins, A. G., 1992. Methods for diagnosis of trypanosomiasis in livestock. Word Animal Review. 70/71, 15-20.

MacGregor, P., Szoor, B., Savill, N. J., Matthews, K. R., 2012. Trypanosomal immune evasion, chronicity and transmission: an elegant balancing act. Nat. Rev. Microbiol. 10, 431-438.

Masake, R. A., Njuguna, J. T., Brown, C. C., Majiwa, P. A., 2002. The application of PCR-ELISA to the detection of *Trypanosoma brucei* and *T. vivax* infections in livestock. Vet. Parasitol. 105, 179-189.

Matthews, K. R., Gull, K., 1997. Commitment to differentiation and cell cycle re-entry are coincident but separable events in the transformation of African trypanosomes from their bloodstream to their insect form. J. Cell Sci. 110, 2609-2618.

Matthews, K. R., 2005. The developmental cell biology of *Trypanosoma brucei*. J. Cell Sci. 118, 283-290.

Mekuria, S., Gadissa, F., 2011. Survey on bovine trypanosomosis and its vector in Metekel and Awi zones of Northwest Ethiopia. Acta Trop. 117, 146-151.

Mendoza-Palomares, C., Biteau, N., Giroud, C., Coustou, V., Coetzer, T., Authié, E., Boulangé, A. and Baltz, T., 2008. Molecular and biochemical characterization of a cathepsin B-like protease family unique to *Trypanosoma congolense*. Eukaryot. Cell. 7, 684-697.

Meng, L., Mohan, R., Kwok, B. H., Elofsson, M., Sin, N., Crews, C. M., 1999. Epoxomicin, a potent and selective proteasome inhibitor, exhibits *in vivo* antiinflammatory activity. Proc Natl Acad Sci. 96, 10403-10408.

Merschjohann, K, Steverding, D., 2006. In vitro growth inhibition of bloodstream forms of *Trypanosoma brucei* and *Trypanosoma congolense* by iron chelators. Kinetoplastid Biol Dis. 16, 1-5.

Meyer, H., 2012. P97 complexes as signal integration hubs. BMC Biol. 10, 48.

Michels, P.A., 1988. Compartmentation of glycolysis in trypanosomes: a potential target for new trypanocidal drugs. Biol Cell. 64, 157-164.

Motloang, M., Masumu, J., Mans, B., Van den Bossche, P., Latif, A., 2012. Vector competence of *Glossina austeni* and *Glossina brevipalpis* for *Trypanosoma congolense* in KwaZulu-Natal, South Africa. Onderstepoort J Vet Res. 79, E1-6.

Mugittu, K. N., Silayo, R. S., Majiwa, P. A., Kimbita, E. K., Mutayoba, B. M., Maselle, R., 2001. Application of PCR and DNA probes in the characterisation of trypanosomes in the blood of cattle in farms in Morogoro, Tanzania. Vet Parasitol. 94, 177-189.

Mungube, E. O., Vitouley, H. S., Allegye-Cudjoe, E., Diall, O., Boucoum, Z., Diarra, B., Sanogo, Y., Randolph, T., Bauer, B., Zessin, K. H., Clausen, P. H., 2012. Detection of multiple drug-resistant *Trypanosoma congolense* populations in village cattle of south-east Mali. Parasites & Vectors. 5, 155.

Murray, M., Murray, P. K., McIntyre, W. I., 1977. An improved parasitological technique for the diagnosis of African trypanosomiasis. Trans R Soc Trop Med Hyg. 71, 325-326.

Mwandiringana, E., Gori, E., Nyengerai, T., Chidzwondo, F., 2012. Molecular detection techniques have been developed for diagnosis of infections with African trypanosomes in humans, animals and tsetse flies. Afr J Biotechnol. 11, 14490-14497.

Nakayima, J., Nakao, R., Alhassan, A., Mahama, C., Afakye, K., Sugimoto, C., 2012. Molecular epidemiological studies on animal trypanosomiases in Ghana. Parasites & Vectors. 5, 217.

Nantulya, V. M., Songa, E. B. and Hamers, R., 1989. Detection of circulating trypanosomal antigens in *Trypanosoma evansi*-infected animals using a *T. brucei* group-specific monoclonal-antibody. Trop. Med. Parasitol. 40, 263-266.

Nguyen, T. T., Goto, Y., Lun, Z. R., Kawazu, S. and Inoue, N., 2012. Tandem repeat protein as potential diagnostic antigen for *Trypanosoma evansi* infection. Parasitol. Res. 110, 733-739.

Njiru Z. K., Constantine, C. C., Guya, S., Crowther, J., Kiragu, J. M., Thompson, R. C., Dávila, A. M., 2005. The use of ITS1 rDNA PCR in detecting pathogenic African trypanosomes. Parasitol Res. 95, 186-192.

OIE, 2012. Manual of Diagnostic Tests and Vaccines for Terrestrial Animals, 7 Edition. OIE, Paris.

Parvanova, I., Epiphanio, S., Fauq, A., Golde, T. E., Prudêncio, M., Mota, M. M., 2009. A small molecule inhibitor of signal peptide peptidase inhibits. Plasmodium development in the liver and decreases malaria severity. PLoS One. 4, e5078.

Peacock, L., Cook, S., Ferris, V., Bailey, M. and Gibson, W., 2012. The life cycle of *Trypanosoma (Nannomonas) congolense* in the tsetse fly. Parasites & Vectors. 5, 109.

Phillips, M. A., 2012. Stoking the drug target pipeline for human African trypanosomiasis.

Mol Microbiol. 86, 10-14.

Pillay, D., Izotte, J., Fikru, R., Büscher, P., Mucache, H., Neves, L, Boulangé, A., Seck, M. T., Bouyer, J., Napier, G. B., Chevtzoff, C., Coustou, V., Baltz, T., 2013. *Trypanosoma vivax* GM6 antigen: a candidate antigen for diagnosis of African animal trypanosomosis in cattle. PLoS One. 8, e78565.

Pratt, W. B., Morishima, Y., Peng, H. M., Osawa, Y., 2010. Proposal for a role of the Hsp90/Hsp70-based chaperone machinery in making triage decisions when proteins undergo oxidative and toxic damage. Exp Biol Med. 235, 278-289.

Rebeski, D. E., Winger, E. M., Okoro, H., Kowalik, S., Bürger, H. J., Walters, D. E., Robinson, M. M., Dwinger, R. H. and Crowther, J. R., 2000. Detection of *Trypanosoma congolense* antibodies with indirect ELISAs using antigen-precoated microtitre plates. Vet. Parasitol. 89, 187-198.

Reifenberg, J. M., Solano, P., Duvallet, G., Cuisance, D., Simpore, J., Cuny, G., 1997. Molecular characterization of trypanosome isolates from naturally infected domestic animals in Burkina, Faso. Vet Parasitol. 71, 251-262.

Reyna-Bello, A., García, F. A., Rivera M, Sansó, B., Aso, P. M., 1998. Enzyme-linked immunosorbent assay (ELISA) for detection of anti-*Trypanosoma evansi* equine antibodies. Vet Parasitol. 80, 149-157.

Sakurai, T., Sugimoto, C. and Inoue, N., 2008. Identification and molecular characterization of a novel stage-specific surface protein of *Trypanosoma congolense* epimastigotes. Mol. Biochem. Parasitol. 161, 1-11.

Sambrook, J. and Russell, D. W., 2001. Preparation and analysis of eukaryotic genomic DNA, In: Sambrook, J. and Russell, D. W., Molecular Cloning. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, pp. 6.1-6.64.

Sengupta, P. P., Balumahendiran, M., Balamurugan, V., Rudramurthy, G. R. and Prabhudas, K.,

2012. Expressed truncated N-terminal variable surface glycoprotein (VSG) of *Trypanosoma evansi* in E. coli exhibits immuno-reactivity. Vet. Parasitol. 187, 1-8.

Schofield, C. J., Kabayo, J. P., 2008. Trypanosomiasis vector control in Africa and Latin America. Parasites & Vectors. 1, 24.

Shaw, A. P., 2009. Assessing the economics of animal trypanosomosis in Africa-history and current perspectives. Onderstepoort J Vet Res. 76, 27-32.

Smith, V., Sausville, E. A., Camalier, R. F., Fiebig, H. H., Burger, A. M., 2005. Comparison of 17-dimethylaminoethylamino-17-demethoxygeldanamycin (17DMAG) and 17-allylamino-17-demethoxygeldanamycin (17AAG) in vitro: effects on Hsp90 and client proteins in melanoma models. Cancer Chemother Pharmacol. 56, 126-137.

Steverding, D., Spackman, R. W., Royle, H. J., Glenn, R. J., 2005. Trypanocidal activities of trileucine methyl vinyl sulfone proteasome inhibitors. Parasitol Res. 95, 73-76.

Steverding, D., 2008. The history of African trypanosomiasis. Parasites & Vectors. 1, 3.

Steverding, D., Sexton, D. W., 2013. Trypanocidal activity of salinomycin is due to sodium influx followed by cell swelling. Parasites & Vectors. 6, 78.

Swallow, B. M., 2000. Impacts of Trypanosomosis on African Agriculture. FAO, Rome.

Sülsen, V. P., Frank, F. M., Cazorla, S. I., Anesini, C. A., Malchiodi, E. L., Freixa, B., Vila, R., Muschietti, L. V., Martino, V. S., 2008. Trypanocidal and leishmanicidal activities of sesquiterpene lactones from *Ambrosia tenuifolia* Sprengel (Asteraceae). Antimicrob Agents Chemother. 52, 2415-2419.

Takeet, M. I., Fagbemi, B. O., Donato, M. D., Yakubu, A., Rodulfo, H. E., Peters, S. O., Wheto, M., Imumorin, I. G., 2013, Molecular survey of pathogenic trypanosomes in naturally infected Nigerian cattle. Res Vet Sci. 94, 555-561.

Taylor, M., Navarro-Garcia, F., Huerta, J., Burress, H., Massey, S., Ireton, K., Teter, K., 2010. Hsp90 is required for transfer of the cholera toxin A1 subunit from the endoplasmic

reticulum to the cytosol. J Biol Chem. 285, 31261-31267.

Thekisoe, O. M., Kuboki, N., Nambota, A., Fujisaki, K., Sugimoto, C., Igarashi, I., Yasuda, J. and Inoue, N., 2007. Species-specific loop-mediated isothermal amplification (LAMP) for diagnosis of trypanosomosis. Acta Trop. 102, 182-189.

Thumbi, S. M., McOdimba, F. A., Mosi, R.O., Jung'a, J.O., 2008. Comparative evaluation of three PCR base diagnostic assays for the detection of pathogenic trypanosomes in cattle blood. Parasites & Vectors. 1, 46.

Tran, T., Claes, F., Verloo, D., De Greve, H. and Büscher, P., 2009. Towards a new reference test for surra in camels. Clin. Vaccine Immunol. 16, 999-1002.

Van den Bossche, P., Esterhuizen, J., Nkuna, R., Matjila, P. T., Penzhorn, B. L., Geerts, S., Marcotty. T., 2006. An update of the bovine trypanosomosis situation at the edge of Hluhluwe-iMfolozi Park, KwaZulu-Natal Province, South Africa. Onderstepoort J Vet Res. 73, 77-79.

Vembar, S. S., Brodsky, J. L., 2008. One step at a time: endoplasmic reticulum-associated degradation. Nat Rev Mol Cell Biol. 9, 944-957.

Verfaillie, T., Garg, A. D., Agostinis, P., 2013. Targeting ER stress induced apoptosis and inflammation in cancer. Cancer Lett. 28, 249-264.

Vickerman, K., 1973. The mode of attachment of *Trypanosoma vivax* in the proboscis of the tsetse fly *Glossina fuscipes*: an ultrastructural study of the epimastigote stage of the trypanosome. J Protozool. 20, 394-404.

Wang, Q., Li, L., Ye, Y., 2008. Inhibition of p97-dependent protein degradation by Eeyarestatin I. J Biol Chem. 283, 7445-7454.

Wang, Q., Shinkre, B. A., Lee, J. G., Weniger, M. A., Liu, Y., Chen, W., Wiestner, A., Trenkle, W. C., Ye, Y., 2010. The ERAD inhibitor Eeyarestatin I is a bifunctional compound with a membrane-binding domain and a p97/VCP inhibitory group. PLoS One. 11, e15479.

Woo, P. T., 1970. The haematocrit centrifuge technique for the diagnosis of African trypanosomiasis. Acta Trop. 27, 384-386.

Youker, R. T., Walsh, P., Beilharz, T., Lithgow, T., Brodsky, J. L., 2004. Distinct roles for the Hsp40 and Hsp90 molecular chaperones during cystic fibrosis transmembrane conductance regulator degradation in yeast. Mol. Biol. Cell. 15, 4787-4797.

Zhou M., Suganuma K., Ruttayaporn N., Nguyen T. T., Yamasaki, S., Igarashi, I., Kawazu, S.I., Suzuki, Y., Inoue, N., 2014. Identification and characterization of a *Trypanosoma congolense* 46 kDa protein as a candidate serodiagnostic antigen. J. Vet. Med. Sci. 76，799-806.