RECOMBINANT EXPRESSION AND ENZYMATIC CHARACTERISATION OF *TRYPANOSOMA VIVAX* CATHEPSIN L-LIKE PROTEASE (*Tvi*CATL) FOR SINGLE CHAIN VARIABLE FRAGMENT ANTIBODY PRODUCTION

by

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PREFACE

The experimental work described in this dissertation was carried out in Biochemistry, School of Life Sciences, University of KwaZulu-Natal, Pietermaritzburg, from January 2019 to January 2021, under the supervision of Professor THT Coetzer. The studies represent original work by the author and have not otherwise been submitted in any other form to another University. Where use has been made of the work of others, it has been duly acknowledged in the text.

Bhavana Ramjeawon

30 June 2022

As the candidate's Supervisor I agree to the submission of this dissertation.

Prof. THT Coetzer

30 June 2022

DECLARATION-PLAGARISM

I, Bhavana Ramjeawon, declare that:

1. The research reported in this dissertation, except where otherwise indicated, is my original research.

2. This dissertation has not been submitted for any degree or examination at any other university.

3. This dissertation does not contain other persons' data, pictures, graphs, or other information, unless specifically acknowledged as being sourced from other persons.

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   a) Their words have been re-written but the general information attributed to them has been referenced
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5. This dissertation does not contain text, graphics or tables copied and pasted from the Internet, unless specifically acknowledged, and the source being detailed in the dissertation and in the reference sections.

Bhavana Ramjeawon

30 June 2022

DEDICATION

This dissertation is dedicated to my parents:

Roshan and Vijay Ramjeawon

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ABSTRACT

Humans and animals in sub-Saharan Africa are at risk of African trypanosomiasis (AT), caused by tsetse fly-transmitted protozoan blood parasites of the *Trypanosoma* genus. Animal African trypanosomiasis (AAT), or nagana, is caused by *T. brucei*, *T. congolense* and *T. vivax* and negatively impacts livestock farming and consequently the economy of the continent. Since AAT occurs in rural areas, affordable rapid diagnostic tests (RDTs) and drugs are required. Diagnostic tests focus on antibody detection; however, antigen detection is more favorable since anti-trypanosome antibodies persist in blood for years following recovery. Due to the parasite's defense by antigenic variation, development of a vaccine is unlikely. Molecules that are essential for parasite survival, such as peptidases, are currently being targeted for diagnosis and chemotherapy. A cathepsin-L-like cysteine protease from *T. vivax*, *Tvi*CATL, is released by dying parasites in the host bloodstream and was shown to be a diagnostic target for detecting host antibodies. To achieve diagnosis of current infections, detection of *Tvi*CATL is being explored. The overall aim of this study was to enzymatically characterise *Tvi*CATL; and to study the interaction of antibodies against the *Tvi*CATL antigen which could be used as a chemotherapeutic drug for the diagnosis of *T. vivax* infections. The protease, *Tvi*CATL, was recombinantly expressed in *E. coli* using the pET-28a expression vector and purified using a nickel chelate affinity column. The resulting 47 kDa protein was identified using western blot and was shown to hydrolyse H-D-Ala-Leu-Lys-AMC and was inhibited by bestatin and E-64 and had optimal activity between pH 6.5 and 7.5. The cross-reactivity between *Tvi*CATL and antibodies produced against other *Trypanosoma* spp cysteine proteases was evaluated in western blots, and results confirmed cross-reactivity. In addition, chicken anti-*Tvi*CATL antibodies were able to detect *Tvi*CATL in *Tvi*CATL-spiked bovine serum. The production of antibodies using the Nkuku® phage library was employed as an alternative to the animal-based antibody production and single-chain variable fragment (scFvs) antibodies were selected by panning against the *Tvi*CATL antigen. After four rounds of panning, *Tvi*CATL-scFvs binders were enriched and four clones gave the highest signal when evaluated using a monospecific ELISA. Due to the low values obtained, optimisation of panning is necessary for improved results. Optimisation of recombinant expression and purification of the identified scFvs for use in a sandwich ELISA were explored to this end. This study showed that *Tvi*CATL is a promising chemotherapeutic and diagnostic target for African animal trypanosomiasis.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

2 x YT	2 x yeast extract, tryptone
AAT	animal African trypanosomiasis
ABTS	3-ethyl-3, dihydrobenzithiazole-6-sulfonate
AMC	7-amino-4-methylcoumarin
AMT	acetate-MES-Tris
ASSURED	affordability, sensitivity, specificity, user friendly, rapid and robust, equipment free, deliverable
AT	African trypanosomiasis
BP	BSA-PBS
BSA	bovine serum albumin
BSF	bloodstream form
CATB	cathepsin B
CATL	cathepsin L
CATT	card agglutination test
CDR	complementarity determining region
CMK	chloromethylketone
dH2O	distilled H_2O
CSF	cerebrospinal fluid
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E-64	L-trans-epoxysuccinyl-leucylamido(4-guanidino)butane
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme-linked immunosorbent assay
EMF	epimastigote form
g	relative centrifugal force
GPI-PLC	glycosyl-phosphatidyl-inositol-specific phospholipase C
HAT	human African trypanosomiasis
h	hour(s)
HRPO	horseradish peroxidase

IC_{50}	half maximal inhibitory concentration
IMAC	immobilised metal ion affinity chromatography
IPTG	Indolyl-β-D-galactopyronoside
kDa	kilo Dalton
LAMP	loop-mediated isothermal amplification
MCF	metacyclic form
MES	2-ethanesulfonic acid
min	minute(s)
MP	milk powder-PBS
NECT	nifurtimox-eflornithine combination therapy
PAGE	polyacrylamide gel electrophoresis
PARP	procyclic acidic repetitive proteins
PBS	phosphate buffered saline
PBS-T	Tween-20-PBS
PCF	procyclic form
PCR	polymerase chain reaction
PEG	polyethylene glycol
p*I*	isoelectric point
RDT	rapid diagnostic test
RT	room temperature
s	second(s)
sarkosyl	N-lauroylsarcosine sodium salt
SBTI	soyabean trypsin inhibitor
SDS	sodium dodecyl sulfate
scFv	single chain variable fragment
TBS	Tris buffered salin
Tris	2-amino-2-hydroxymethyl-propane-1,3-diol
TYE	tryptone, yeast extract
VAT	variant antigen type
VSG	variant surface glycoproteins
WHO	World Health Organisation

Chapter 1

Literature review

1.1 African trypanosomiasis

Close to 50 million inhabitants of sub-Saharan Africa are at risk of being infected with the fatal tropical disease, African trypanosomiasis (AT) (Franco *et al.*, 2020). It is caused by the protozoan blood parasite of the *Trypanosoma* genus and affects both man and animals (Stevens and Brisse, 2004). Human African trypanosomiasis (HAT), also referred to as sleeping sickness, is caused by two subspecies of *Trypanosoma brucei*: *T. b. gambiense* and *T. b. rhodesiense* and is transmitted from one host to another by means of a tsetse fly vector belonging to the *Glossina* genus, predominantly located in sub-Saharan African where greatly affected areas are that of poor and rural districts (Brun *et al.*, 2010). *T. b. gambiense* and *T. b. rhodesiense* each have a unique geographical location (western/central Africa and eastern/southern Africa, respectively) as well as pathogenicity (Simarro *et al.*, 2008). The main insect vector for *T. b. gambiense* and *T. b. rhodesiense* is the group of tsetse belonging to the *Palpalis* and the *Morsitans* group, respectively (Wertheim *et al.*, 2012) (Figure 1.1). While *T. b. rhodesiense* HAT is rapid and acute, the *T. b. gambiense* form is chronic and can last for years (Simarro *et al.*, 2008). Domestic and livestock animals serve as carriers and reservoirs from which a tsetse fly acquires a bloodmeal. Whereas cattle are the main reservoir for the zoonotic *rhodesiense* form, humans are the reservoir for *T. b. gambiense* (Brun *et al.*, 2010). Although 98% of HAT reported cases are caused by *T. b. gambiense* there has been a 92% decrease in the number of cases due to effective surveillance and treatment over the past 20 years (Franco *et al.*, 2020). Although the number of cases have decreased, good surveillance is important to prevent a resurgence of the disease from small pockets of asymptomatic carriers (Büscher *et al.*, 2018; Mehlitz and Molyneux, 2019; Alvar *et al.*, 2020). It is estimated that the economic losses due to this disease is close to $5 billion affecting approximately 8-11 million km^2 of land, occupied by close to 300 million people and between 40-50 million cattle (McDermott and Coleman, 2001). The spread of trypanosomiasis, however, does not only occur along the tsetse belt in Africa but is also transmitted in regions lacking this fly species. The distribution to areas such as South America is due to other biting fly species namely tabanids (or horse flies) or from the *Stomoxys* spp. (Gardiner and Wilson, 1987). Animal African Trypanosomiasis (AAT) (or nagana) is caused by three different species of *Trypanosoma, T. b. brucei, T. congolense* and *T. vivax,* affecting domestic animals and wildlife

(Morrison *et al.*, 2016).

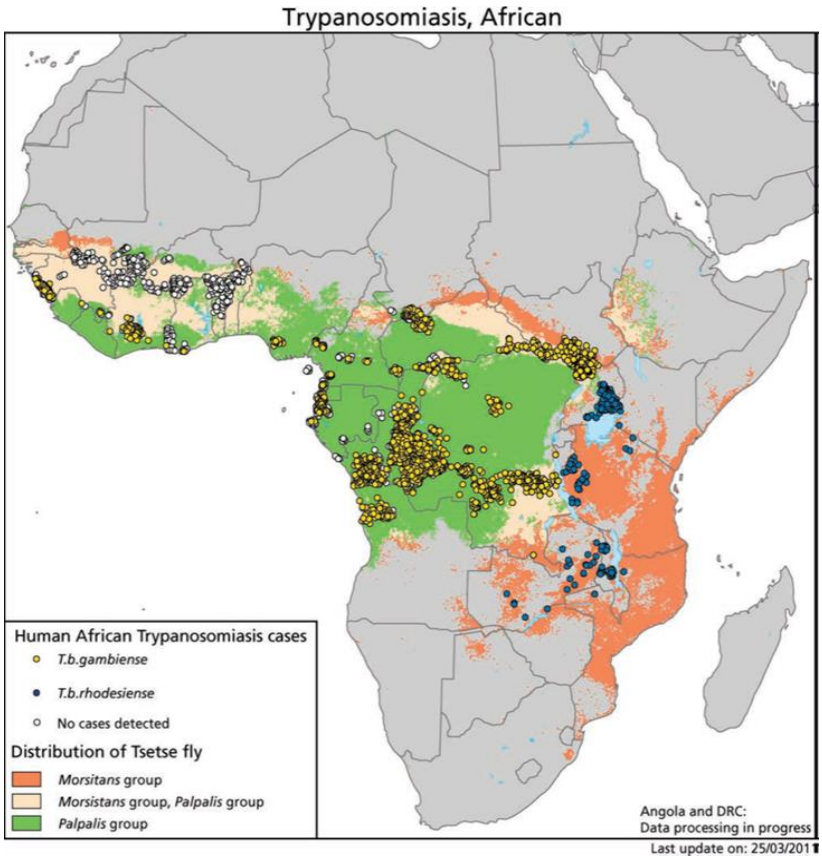

Figure 1.1: Distribution of HAT in Africa. The geographical distribution of the two human infective species: *T. b. gambiense* and *T. b. rhodesiense* and teste fly distribution. The majority of the cases are due to *T. b. gambiense* (Wertheim et al., 2012)

1.2 Trypanosomes

1.2.1 Classification of trypanosomes

Trypanosomes belong to the protozoa phylum and are categorised into the kinetoplastida order, where the kinetoplast contains the mitochondrial DNA of the parasite. The *Trypanosoma* genus can be further divided into two groups: *Stercoraria* and *Salivaria,* due to the different methods of transmitting the disease from the vector to the host (Hoare, 1966; Lemos *et al.*, 2020) (Figure 1.2). Parasites of the *Stercoraria* group develops within the digestive system of the insect vector, and the mammalian host is infected via faeces of the vector. An example of a *Stercoraria* group parasite is *T. cruzi,* which is responsible for Chagas disease (American trypanosomiasis). Parasites belonging to the *Salivaria* group develop in the midgut of the vector and travel to the salivary glands. Parasites are then injected into the host when the insect vector acquires a bloodmeal via saliva. The *Salivaria* group comprises *T. brucei, T. congolense* and *T. evansi Salivaria* (Radwanska *et al.*, 2018). The

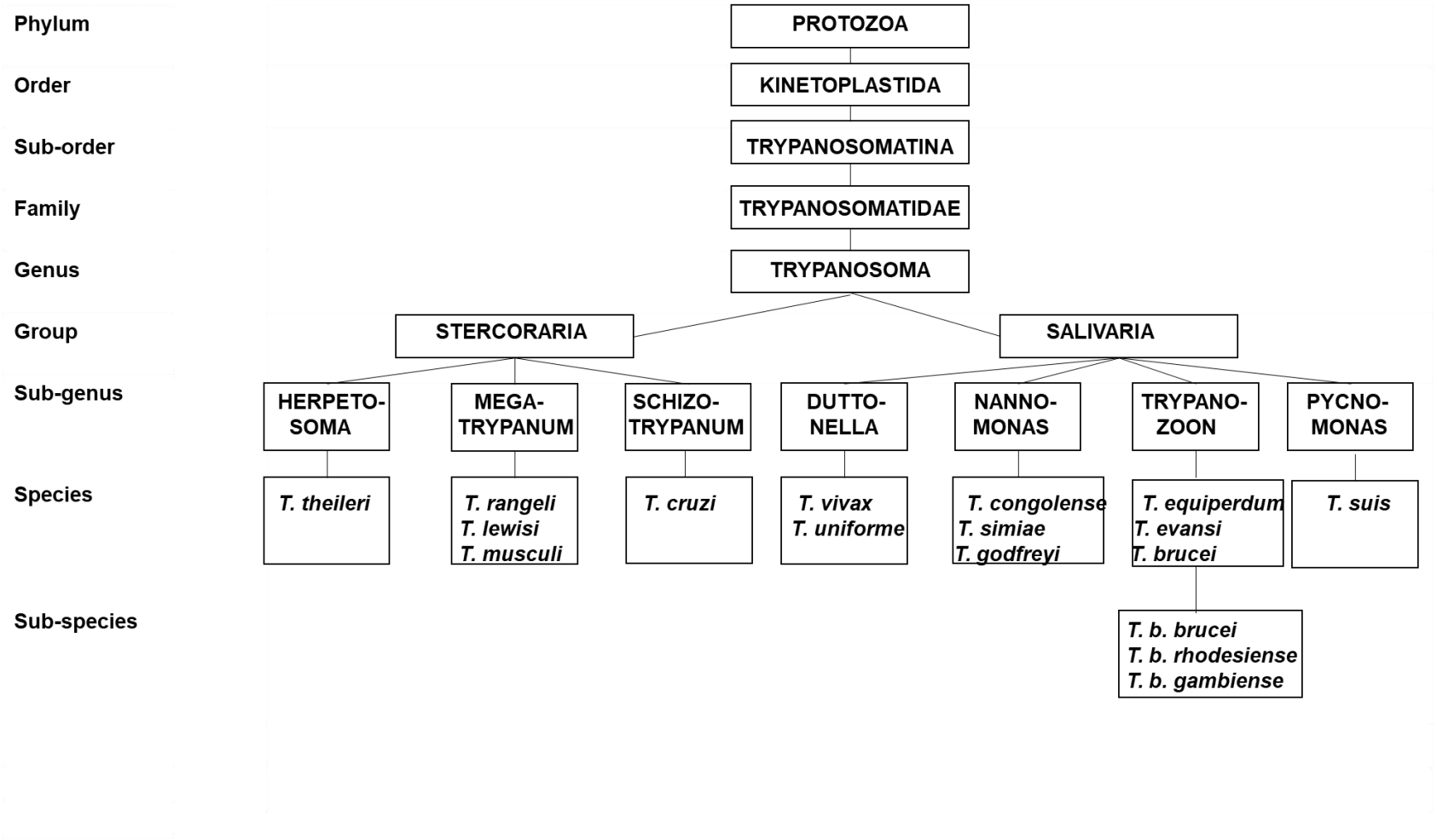

Figure 1.2: Classification of trypanosomes from phylum to subspecies categories. Adapted from (Baral, 2010)

Stercoraria and *Salivaria* can be further divided into sub-genera, namely *Herpetosoma, Megatrypanum* and *Schizotrypanum* of the *Stercoraria* group and *Duttonella, Nannomonas, Trypanozoon* and *Pycnomonas* of the *Salvaria* group. Each sub-genus section can also be divided into species and subspecies, however only the *T. brucei* species is separated into subspecies (*T. b. brucei, T. b. rhodesiense and T. b. gambiense*) (Baral, 2010; Radwanska *et al.*, 2018).

1.2.2 Morphology of trypanosomes

Trypanosomes are unicellular, spindle-shaped parasitic organisms varying in size between 8-50 µm, containing a microtubule cytoskeleton responsible for its shape (Matthews, 2005) (Figure 1.3). As they are eukaryotic, trypanosomes possess the common features of other eukaryotic organisms such as a nucleus, endoplasmic reticulum, Golgi apparatus, endo- and exoplasmic systems and mitochondria (Clayton *et al.*, 1995). Due to a dual host life cycle, these kinetoplastid organisms often require additional specific features including a flagellum, flagellar pocket and kinetoplastid DNA (Baral, 2010).

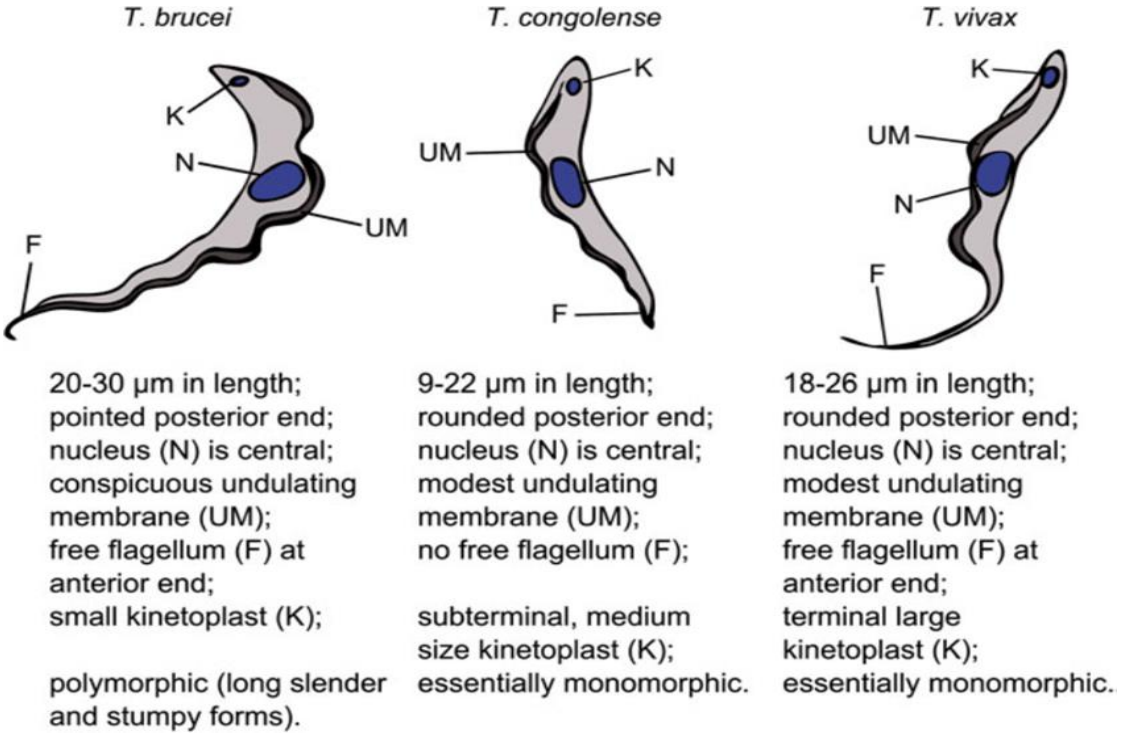

Figure 1.3: Morphology of the bloodstream form trypomastigotes of the three trypanosomes affecting animals in Africa. Bloodstream form trypomastigotes are the disease-causing form and thus targeted for therapy (Giordani *et al.*, 2016).

Located at the posterior site is the flagellar pocket which is the exit point of the flagellum, that spans almost the entirety of the parasite, and the site of endo- and exocytosis (Field and

Carrington, 2009). The flagellum is involved in multiple functions: motility, attachment to host surfaces and cell division (Baral, 2010). The basal body from which the flagellum stems is attached to a substructure called the kinetoplast (Sunter and Gull, 2016) which contains mitochondrial DNA that makes up 10-20% of parasitic cellular DNA (Hajduk *et al.*, 1986). This cellular structure is maintained in other kinetoplastids such *T. cruzi* and *Leishmania* species (Field and Carrington, 2009).

1.2.3 Life cycle of trypanosomes

The tsetse fly is responsible for the transmission of most African trypanosomes from one host to another. Approximately 30 species of tsetse fly, both male and female, can bring about transmission (Jordan, 1993). During the life cycle, the parasite undergoes multiple physical changes such as morphology and gene expression, both of which are essential for a complete parasite life cycle (Matthews, 1999).

The tsetse fly vector, already infected with trypanosomal parasites, infects the mammalian host by depositing metacyclic form (MCF) parasites during a bloodmeal (Figure 1.4). These MCF undergo a morphology change into bloodstream form (BSF) trypomastigotes, which are coated with variant surface glycoproteins (VSGs) and are long and slender. They are carried to other sites of the body via the bloodstream and replicate due to binary fission (Matthews, 2005).

Upon taking a bloodmeal from an infected host, the tsetse fly vector gets infected with now short stumpy BSF, which migrates to the fly's midgut and transforms into procyclic form (PCF) parasites, where they replicate (binary fission). Upon transition to the PCF, a loss of VSG occurs, however new proteins are expressed: procyclic acidic repetitive proteins (PARP) or procyclins, a cell surface glycoprotein. The loss of VSG is due to glycosyl-phosphatidyl-inositol-specific phospholipase C (GPI-PLC) and a zinc metalloprotease which assists in the cleavage of VSG. Not only does a loss in VSG occur, but energy generation from glycolysis to oxidative phosphorylation also takes place (Matthews, 2005). The life cycle of *T. vivax,* however, differs from other trypanosomes; this may be due to *T. vivax* expressing different proteins on the cell surface allowing this species to have a different surface compared to other trypanosomal parasites, as well as developing in a different part in the tsetse fly compared to other trypanosomes (Jackson *et al.*, 2015). *T. vivax* lacks the migration to the midgut and ultimately the transition into PCF; and instead develops directly into epimastigote form (EMF) parasites within the insect's proboscis (Jackson *et al.*, 2015).

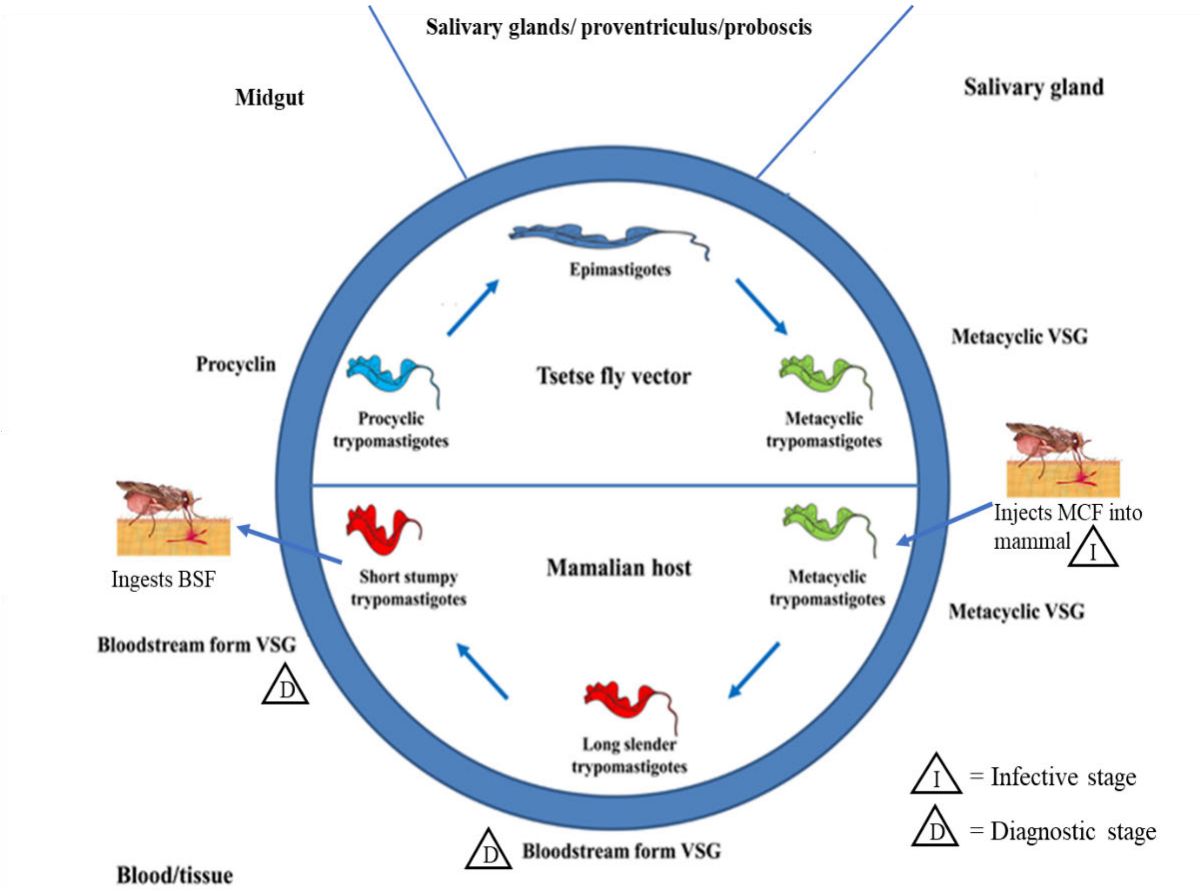

Figure 1.4: Trypanosome life cycle consisting of two hosts, insect and human, in various regions of the insect and human body accompanied by different morphological changes throughout the life cycle. Adapted from Stijlemans *et al.* (2017).

Epimastigotes, for trypanosomes other than *T. vivax,* are then formed once the parasite leaves the vector midgut and migrate towards the salivary glands for *T. brucei* and proventriculus for *T. congolense*, where they mature and multiply. Epimastigotes (all forms of trypanosomal parasites) transform back into MCF, regain the VSG coat and are able to infect a mammalian host (Matthews, 2005).

Due to *T. vivax* parasites lacking the procyclic stage and developing directly into EMF parasites in the insect's mouthparts, this may also explain why *T. vivax* spread to other continents since they can spread by other biting insects and are not strictly dependent on going through the tsetse fly gut (Jackson *et al.*, 2015).

1.2.4 Antigenic variation

Bloodstream form trypanosomes are able to avoid the host's immune system due to antigenic variation, aiding in their survival (Taylor and Rudenko, 2006). Trypanosomes are covered by a dense VSG coat that protects them from death when the host develops an antibody response. Only one VSG is expressed at any given time and is termed variant antigen type (VAT), which is the epitope of VSG that is exposed on the surface of a living trypanosome and is recognised by antibodies (Taylor and Rudenko, 2006; Büscher, 2014). Although there are more than 1000 VSG genes and pseudogenes each version of VSG replaced is antigenically distinct. The surface of each trypanosome is covered with $5x10^6$ identical VSG homodimers, which accounts for approximately 90% of the surface. Host antibody recognition and capture of the VSG epitope is unlikely due to the close spacing of the VSG domains (Overath and Engstler, 2004). Humans and several non-human primate species contain trypanosome lytic factors in their blood and therefore cannot become infected by most trypanosomal species (Thomson et al., 2009*)*.

The new population of trypanosomes expressing a different VSG coat is a small group low in numbers, which reproduce and replace the VAT recognised by the immune system of infected individuals (Chung *et al.*, 2004). Even though a new VAT assists in the protection of trypanosomes, it is highly immunogenic and anti-VAT IgM and IgG antibody responses is developed against this domain causing parasite lysis (Higgins and Carrington, 2014).

1.3 Clinical features

A unique feature of HAT is the occurrence in two stages: the first haemolymphatic and second meningoencephalic (or neurological) stage. Each stage of the disease appears in distinctive locations of the human body in addition to having distinct symptoms. During the first stage, haemolytic trypanosomes are located and multiply in tissue, blood and lymph systems. Predominant symptoms include headache and fever among other signs. The meningoencephalic stage (second stage) is the invasion of the central nervous system due to parasites crossing the blood-brain barrier. Poor coordination, confusion and irregular sleeping patterns in patients are the typical characteristics of the second stage. The two strains to cause human sleeping sickness, *T. b. gambiense* and *T. b. rhodesiense* have various progressive durations, however death could occur if HAT caused by either parasite is left untreated (Checchi *et al.*, 2008; Büscher *et al.*, 2017).

Animal African trypanosomes can either be haematic or humoral. Haematic trypanosomes are *T. congolense* and *T. vivax*, which are restricted to the blood vessels; whereas humoral trypanosomes, *T. b. brucei,* and the human-infective *T. rhodesiense* and *T. gambiense* are found

within tissue and can cross the blood-brain barrier, therefore causing neurological diseases (Uilenberg and Boyt, 1998; Lonsdale-Eccles and Grab, 2002). Due to the range of trypanosome species causing AAT, domesticated animals, livestock and wildlife are all affected (Jordan, 1976).

Animal African trypanosomiasis occurs in two stages, namely early and chronic, with anaemia been a mutual clinical feature in both stages. High levels of parasitaemia are present in the blood during the early stages leading to the development of anaemia. In addition, enlarged lymph nodes and spleen, fatigue and decreased milk production are also characteristic traits of the early stage of the disease (Taylor and Authié, 2004). Alternately, during the chronic stage, anaemia is not due to high parasitaemia levels and levels of parasitaemia are considerably lower in general. Lymph nodes and spleen return to normal size; however, heart failure and infertility are a common occurrence during the chronic stage, resulting in death (Taylor and Authié, 2004).

1.4 Diagnosis

Accurate diagnosis of African trypanosomiasis in both humans and animals is essential particularly because of the complexity of treatment in humans and the cost to poor subsistence farmers in the case of the animal disease (Franco *et al.*, 2022). In the case of HAT, it is also essential to determine the stage of the disease (whether the parasites are in the bloodstream or have crossed the blood-brain barrier) because the treatment is stage-specific with that for the latter being very toxic (Magez *et al.*, 2021). Laboratory diagnosis of AT is fundamental as clinical signs and symptoms alone may not provide an assumptive decision upon diagnosing AAT or HAT (Nantulya, 1990). Confirming the presence of trypanosomes in the blood of an infected animal or human is a mandatory method for diagnosis of trypanosomiasis, however diagnosis based on molecular and serological techniques also exist.

1.4.1 Parasitological diagnostics

Parasitological diagnosis using microscopy is a direct technique for diagnosing patients and animals infected with trypanosomes. Parasites can be viewed under microscopic examination when present in lymph nodes, blood or cerebrospinal fluid (CSF). Identifying trypanosomal parasites in blood is achieved through light microscopy using wet and thick blood films. In wet blood films, finger-pricked blood is examined microscopically at 400 x magnification. This technique is still used due to its simplicity and low cost despite its low sensitivity, having a detection limit of 10 000 trypanosomes/ml (Chappuis *et al.*, 2005). Giemsa- or Field's-stained blood films also results in low sensitivity results. Microscopic examination using thick blood film has a slightly higher sensitivity, with a detection limit of approximately 5000 trypanosomes/ml.

However, the technique is time-consuming and requires trained staff to conduct the analysis (Chappuis *et al.*, 2005).

Although a definite diagnosis is achieved using microscopy, parasite detection is often laborious and a negative demonstration of parasites does not disregard infection, which may be due to low parasite concentration, *T. b. gambiense* in particular (Chappuis *et al.*, 2005). Therefore, centrifugation techniques have been developed to increase the sensitivity of parasitological techniques. The microhaematocrit centrifugation technique concentrates trypanosomes in the buffy coat by high speed centrifugation of blood followed by microscopic examination at 200 x magnification (Woo, 1970). Sensitivity increased when Lanham and Godfrey (1970) developed the mini-anion-exchange centrifugation technique that separates trypanosomes from blood based on charge by anion-exchange chromatography and is viewed microscopically, detecting fewer than 100 trypanosomes/ml.

Originally developed for *Plasmodium* species, the quantitative buffy coat technique can also be applied for detecting other parasite species, including *Trypanosoma* (Levine *et al.*, 1989; Bailey and Smith, 1992; Chatel *et al.*, 1999). Parasites are concentrated; and nucleus and kinetoplast stained with acridine orange fluorescent dye. Microscopic examination of motile trypanosomes is visualised under UV light and distinction of trypanosomes is made due to the stained nucleus and kinetoplast. This technique is highly sensitive, 95% sensitivity and detecting 450 trypanosomes/ml (Chappuis *et al.*, 2005).

1.4.2 Stage determination

Due to unspecific clinical signs, costly treatment and different medication used to treat the different stages (first and second stage) and species (*T. b. gambiense* and *T. b. rhodesiense*) causing HAT, disease staging in patients is vital and is achieved by examination of CSF obtained by lumbar puncture. As per guidelines of the World Health Organisation (WHO, 2013), first-stage HAT is diagnosed when five or fewer white blood cells per µl and no trypanosomes are present in the CSF of patients; and second-stage patients are those with more than five white blood cells per µl or there is a demonstration of trypanosomes in the CSF.

1.4.3 Molecular diagnostics

Detection of trypanosomal DNA or RNA is the basis of molecular diagnostics which involves two major assays: polymerase chain reaction (PCR) and loop-mediated isothermal amplification (LAMP). Although highly sensitive, molecular diagnostic techniques are complicated to perform

and require highly skilled and trained professionals (Büscher and Lejon, 2004). Patient lymph node aspirate, blood or CSF, all of which contain trypanosomal DNA, are used in PCR and DNA detection tests to identify trypanosomes to genus, species and subspecies levels using specific primers (Clausen *et al.*, 1999). The advantages of using PCR is due to its increased sensitivity and specificity, however it is not readily available to be used in the field (Mugasa *et al.*, 2012).

An alternate DNA amplification method is LAMP, which is promising in the molecular diagnostic field of African trypanosomiasis. In LAMP, DNA is amplified with high sensitivity and visualised fluorescently (Notomi *et al.*, 2000). This method was developed to diagnose AAT in livestock; however, it was also used to diagnose HAT caused by *T. brucei* species (Mitashi *et al.*, 2012).

To decrease and eventually eradicate the occurrence of HAT, two goals were set by WHO: the elimination of HAT as a public health problem by 2020; and the interruption of HAT caused by *T. b. gambiense* by 2030 (WHO, 2012). Due to control strategies such as screening, treatment and vector control, the objective to eliminate HAT as a public health problem is being achieved (Franco *et al.*, 2020); however, the avoidance of resurgence is of utmost importance to reach the next goal set by WHO. Therefore, case detection and testing will be crucial to monitor infections caused by *T. b. gambiense*, which accounts for 98% of total HAT cases. As most cases occur in disadvantaged settings, testing is often done in regional reference laboratories where test specimens are transported to. As a result, these specimens have to be easily collected and distributed safely, such as dried blood spots; and the diagnostic test conducted should be cheap and allow a large volume of samples to be analysed while also been reliable. Recent studies conducted by Compaoré *et al.* (2020) were carried out to compare the sensitives of three molecular based diagnostic tests: Loopamp, M18S quantitative real-time PCR (M18S qPCR) and TgsGP qPCR, applied on dried blood spots. Loopamp showed the best sensitivity but had its challenges as only eight specimens could be tested per run. This study demonstrates that a development towards a molecular diagnostic test still remains. In addition, a novel procedure developed by Hayashida *et al.* (2020) for the diagnosis of HAT used a bio-inkjet printer machine to produce a dried format kit, displayed good results: stable at room temperature for up to 6 months, detection of parasites using crude lysate and parasite DNA and compatible test results to conventional PCR.

1.4.4 Serological diagnostics

Detection of antigens released by parasites in the host or the antibodies produced by the host in response to trypanosome infection forms the basis of serological diagnostics, which constitutes a

variety of formats including agglutination assays, complement fixation, enzyme-linked immunosorbent assay (ELISA), western blot and immune trypanolysis (Büscher, 2014). Antibodies are detectable a month after infection but may persist in serum for up to three years following recovery, providing false positive results as the infection may have cleared (Paquet *et al.*, 1992). Due to other methods of diagnosis such as microscopy and PCR which are laborious and expensive, respectively, there is a need for point of care diagnostic tests which are available to the end-user.

The serodiagnosis of HAT using antibody detection methods have been employed successfully to accommodate mass population screening, albeit reliable tests only exist for *T. b. gambiense* infection. Such method is the card agglutination test (CATT)/ *T. b. gambiense*, the first rapid diagnostic test (RDT) for the diagnosis of HAT caused by *T. b. gambiense* by detecting specific antibodies (Büscher *et al.*, 2017) (Figure 1.5). The technique consists of screening antibodies (found in the blood, plasma, or serum of an infected individual) against VAT LiTat 1.3, the *T. b. gambiense* surface antigen of lyophilised bloodstream form parasites. The sensitivity and specificity of CATT is approximately 78% to 100% and 97%, respectively (WHO, 2013), however, false negative results occur when trypanosomal strains lack the LiTat 1.3 gene (Dukes *et al.*, 1992). Although screening with CATT/ *T. b. gambiense* has helped to decrease infections caused by *T. b. gambiense*, no such test exists for the diagnosis of the other human-infectious parasite, *T. b. rhodesiense*. This, however, can be propitiated due to *T. b. rhodesiense* cases presenting high levels of parasitaemia which can be detected using parasitological techniques (Lejon *et al.*, 2019).

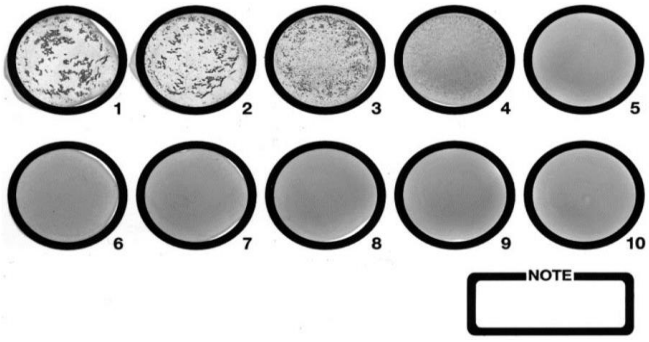

Figure 1.5: An example of card agglutination test of blood samples using a dilution of 1:4. Samples 1 to 3 show strongly positive reaction, sample 4 is weakly positive whilst the remaining samples (5-10) exhibit negative results for infection (Chappuis *et al.*, 2005).

An alternative to CATT for massing screening of the population is the latex agglutination test (LATEX/*T. b. gambiense*) for the detection of antibodies in individuals infected with *T. b. gambiense*. An improvement of the first test which uses a single antigen VSG *T. b. gambiense* VAT LiTat 1.6 (Büscher *et al.*, 1991), the modified LATEX/ *T. b. gambiense* test consists of a mixture of three variable surface glycoproteins of *T. b. gambiense* VAT LiTat 1.3, LiTat 1.5 and LiTat 1.6 coated onto lyophilised latex particles. The latex agglutination test does not only offer higher sensitivity and specificity compared to using a single antigen, but also simplicity (using a mixture of VSG, therefore performing only one test) and additionally, stability of the reagent (long-term and at high temperatures) (Büscher *et al.*, 1999).

Due to the occurrence of trypanosomiasis in predominately rural areas where there is a lack of electricity and infrastructure, RDTs need to comply with the ASSURED (7-amino-4-methylcoumarin) criteria. Recently, two such RDTs for the serodiagnosis of *T. b. gambiense*-HAT have been developed for detecting specific antibodies: HAT Sero *K*-SeT (http://www.corisbio.com/Products/Human-Field/Human-African-Trypanosomiasis.php) and SD Bioline HAT (https://www.alere.com/en/home/product-details/sd-bioline-hat.html). Both tests use purified native antigens from *T. b. gambiense*, VSG LiTat 1.3 and LiTat 1.5. Studies have shown that a mixture of at least two antigens provides adequate sensitivity (Büscher *et al.*, 2014; Bisser *et al.*, 2016); however, an antibody test may result in a positive case identification because of the presence of antibodies in blood even after infection has cleared off due to either self-cure or treatment.

Whole blood or plasma can be used to detect antibodies using the HAT Sero *K*-SeT. The test contains an equal mix of the (LiTat 1.3 and LiTat 1.5) glycoproteins on a single test line, precoated onto a nitrocellulose membrane and a control test line. Blood and buffer are mixed, allowed to migrate and interact with target antigens. Test results are read after 15 minutes. A test positive for HAT will have both lines visible, the test line and control; a negative result will have only the control line visible and no visible line on the control will result in an invalid test (Büscher *et al.*, 2014).

SD Bioline HAT 1.0 detects all antibodies (IgG, IgM, IgA) to *T. b. gambiense* and consists of two separate test lines, one each for LiTat 1.3 and LiTat 1.5, and a control line precoated onto a nitrocellulose membrane strip. Blood is mixed with buffer and the sample travels across the strip and results are read after 15-20 minutes. If at least one test line is visible as well as the control, the patient is tested positive for HAT. A negative test for HAT results from just the control line visible; and the test is deemed invalid if the control line failed visibility (Bisser *et al.*, 2016).

Although tests using whole trypanosome lysates as antigens are successful, producing these native antigens in a standardised way and in large quantities is difficult and expensive and therefore, much research has been carried out to find alternate antigens. Such method is the identification and expression of potential diagnostic trypanosomal proteins using a variety of expression systems. Lejon *et al.* (2005) expressed VSG RoTat 1.2 of *T. evansi* in *Spodoptera frugiperdis.* Other potential diagnostic antigens targeted for *T. congolense* infection, congopain and a cathepsin B-like protease were identified and expression was done in *Pichia pastoris* (Mendoza-Palomares *et al*., 2008; Boulangé *et al*., 2011). More common is the bacterial expression system*, E. coli*, used for the expression of proteins such as *Tvi*GM6 (Pillay *et al.*, 2013) and Invariant Surface Glycoprotein 65 (ISG65). Most of these antigens have been tested in various ELISA formats for the serodiagnosis of trypanosomiasis using sera from infected animals or humans. Recombinant ISG65 has been used to develop a test for the diagnosis of HAT (Sullivan *et al.*, 2013). Recombinantly expressed N-terminal of VSG LiTat 1.5 and of ISG65 was used as the antigen in SD Bioline HAT 2.0 and had shown higher sensitivity (71.2%) and similar specificity (98.1%) than the native VSGs in SD Bioline HAT 1.0 (59.0% and 98.9%, respectively) (Lumbala *et al.*, 2018).

The current gold standard for the serodiagnosis of AAT are ELISA-based tests such as sandwich ELISA (antigen detection ELISA), indirect ELISA (antibody detection ELISA) or inhibition ELISA (antibody detection ELISA). The major downside of the sandwich and indirect ELISAs is that they use as the antigen, whole trypanosomal lysate which is difficult and hazardous to prepare. The recently developed inhibition ELISA (Geerts *et al.*, 2021) measures the presence of antibodies directed against *T.b. gambiense* VSG LiTat 1.3 and LiTat 1.5 in serum, plasma or dried blood spots. This test displays many advantages: it is cost-effective, can be performed in national and regional laboratories in countries greatly affected by HAT and in large numbers, and uses recombinant antigens instead of native antigens. It is also highly specific and sensitive, which is important in areas where case numbers are low to prevent false-positive results which may cause distress. These features of the test are also required for the small number of cases of *g*-HAT that need to be detected in the current elimination of HAT phase.

Applying the same concept as an ELISA, an RDT (a lateral test flow strip within a cassette) was developed using two trypanosomal proteins *Tvi*GM6 and *Tco*CB1. Both proteins were previously recombinantly expressed and tested using infected sera when used in an indirect ELISA and showed potential as a target for the serodiagnosis of *T. vivax* and *T. congolense* infection, respectively. A newly adapted RDT designed for diagnosing *T. vivax* and *T. congolense* infections

using both proteins exhibited high sensitivity and specificity (Boulangé *et al.*, 2017) (Figure 1.6). The RDT contains three lines: a control line and two test lines containing the antigens *Tvi*GM6 (Tv) specific for *T. vivax* and *Tco*CB1 (Tc) specific for *T. congolense.*

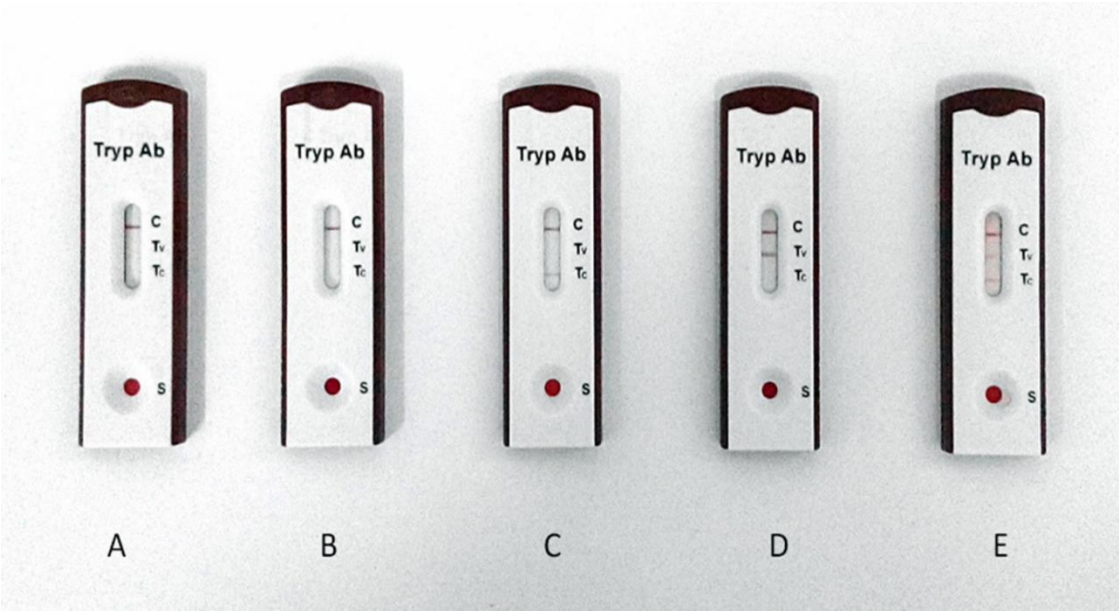

Figure 1.6: A rapid diagnostic test for the detection of *T. congolense* (Tc) and *T. vivax* (Tv) infection. (A) Blood from uninfected cow. Negative result for *T. congolense* and *T. vivax* infection, however valid due to presence of control line (c). (B) Blood from a *T. theilieria*-infected cow. Negative for Tc and Tv infection. (C) Blood from a cow infected with *T. congolense.* Positive for Tc and negative for Tv. (D) Blood from a cow infected with *T. vivax*. A positive reaction for Tv and negative for Tc. (E) Blood from a cow infected with both, *T. congolense* and *T. vivax*. Positive for both, Tc and Tv (Boulangé *et al.*, 2017).

1.5 Control strategies

Due to the massive economic and agricultural burden as well as the impact on human and animal health, controlling (and ultimately eradicating) trypanosomiasis is of upmost importance. There are currently three control approaches for tsetse fly-transmitting trypanosomiasis: vector control, trypanotolerance and drug therapy (McDermott and Coleman, 2001).

1.5.1 Vector control

Vector control measures such as insecticides, sterile insect technique, fly traps and the bait technique were employed in the past to eliminate infected tsetse flies thus reducing the spread of trypanosomes (McDermott and Coleman, 2001). Ground and aerial spraying of insecticides (e.g., dithiothreitol (DTT)) have been used for more than 50 years with great success in clearing tsetse flies. However, there are disadvantages involving the use of these insecticides such as insects

developing resistance against the insecticides, death of non-target insects and the negative impact on the environment (Vreysen *et al.*, 2013). An alternative to insecticide spraying is the sterile insect technique, where male flies are sterilized by radiation and released into the wild. Females are therefore unable to preproduce, which eventually results in a decrease in the population (Hendrichs and Robinson, 2009). The technique is advantageous as it does not impact the environment negatively and has been used successfully in several African countries where trypanosomiasis persists (Feldmann and Hendrichs, 1998).

1.5.2 Trypanotolerance

Local African cattle breeds such as N'Dama, Mututu and Dahomey are known as trypanotolerant cattle, which is defined as 'the capacity to survive and remain productive after trypanosome infection' (Murray *et al.*, 1982). These cattle are more resistant to infection and control anaemia and parasitaemia levels, should infection occur (Naessens, 2006). Trypanotolerant cattle are helpful in the control of trypanosomiasis as livestock productivity can still exist, especially in areas where trypanosomiasis prevails (Giordani *et al.*, 2016).

1.5.3 Vaccination

Due to increasing AAT drug resistance, toxicity and limited availability, the obvious alternative would be to develop a vaccine for use in animals. The development of vaccines to prevent trypanosomiasis is challenging due to antigenic variation in trypanosomes and therefore vaccination against the VSG surface coat protein would result in poor immunity and protection against trypanosomal parasites (Magez *et al.*, 2010). In addition to avoiding an antibody response from the host due to antigenic variation, trypanosomes can eliminate the host B-cell memory thereby causing immune-suppression (Radwanska *et al.*, 2008). To combat these challenges, a different approach was taken which involved targeting pathogenic factors produced by the parasite, and not focusing on the parasite itself to produce an anti-disease, rather than an anti-parasite, vaccine (Playfair *et al.*, 1990).

The observation that trypanotolerant cattle breeds, in contrast to susceptible breeds, exhibited high levels of IgG in response to *T. congolense* infection and that these antibodies also inhibited the activity of congopain (the major cysteine protease of *T. congolense*), congopain was explored as an anti-disease vaccine candidate (Authié *et al.*, 2001). Immunised cattle maintained or gained weight during infection and showed less severe anaemia than non-immunised controls in the chronic phase of the disease. However, this vaccination strategy was not sufficiently protective to be practical and other anti-disease vaccine candidates such as sialidases, endogenous peptidase

inhibitors and complement activating factors have been proposed (Antoine-Moussiaux *et al.*, 2009).

1.5.4 Trypanocidal drugs

The drugs currently available for AAT are diminazene aceturate, homidium chloride, isometamidium chloride, quinapyramine sulfate and suramin; with diminazene aceturate, homidium chloride, and isometamidium chloride making up more than 90% of drugs used to treat infected animals (McDermott and Coleman, 2001). These drugs, however have several limitations: they often cause irritation at the site of injection, they have been in use for more than 60 years and counterfeit drugs are readily available in certain African countries, hence leading to drug resistance (Sutcliffe *et al.*, 2014; Giordani *et al.*, 2016; Tchamdja *et al.*, 2016).

Diminazene aceturate is the most commonly used compound for livestock treatment against *T. congolense* and *T. vivax* due to its low toxicity and it has been on the market for 65 years (Giordani *et al.*, 2016). Homidium chloride had been introduced into the market in 1952 due to its chemoprophylactic properties to treat *T. congolense* and *T. vivax* infected livestock. However, due to its toxicity the usage decreased, from when it once occupied 26% of the market, to now only occupying 10% (Giordani *et al.*, 2016).

Isometamidium chloride, used initially for *T. congolense* and *T. vivax* infections in African cattle, goat and sheep, is also active against infections caused by *T. brucei* spp. and *T. evansi.* Utilised together with diminazene aceturate, these drugs possess the ability to minimise drug resistance (Leach and Roberts, 1981), however, there have been reports still suggesting parasite resistance against trypanocides, especially in *T. congolense* but also in *T. vivax*, and *T. brucei* spp. (Giordani *et al.*, 2016).

Treatment for HAT is slightly more complex as identity of the infecting parasite along with stage of infection, needs to be established due to the toxicity of the drugs used to treat HAT. Suramin and pentamidine are the first-line treatments used for first stage *T. b. rhodesiense* and *T. b. gambiense* infections respectively; while the second stage is treated with melarsoprol for *T. b. rhodesiense* (Kennedy, 2013*)*. An improved version of eflornithine has been developed called nifurtimox-eflornithine combination therapy (NECT) which is the proposed treatment for *T. b. gambiense* infected patients in the second stage of the disease (Priotto *et al.*, 2009).

Two novel oral drugs have recently been introduced; fexinidazole and benzoxaborole SCYX-7158. Fexinidazole is undergoing phase 3 trials and studies have shown that HAT patients with

first and second stage *T. b. gambiense* are best suitable for treatment with fexinidazole (Deeks, 2019).

1.6 Peptidases as drug and diagnostic targets

Many parasitic organisms require peptidases for their life cycle and many of these parasitic peptidases contribute to the pathogenicity by their involvement in host cell and tissue invasion and immune system evasion (Sajid and McKerrow, 2002). Due to their role in the biology of trypanosomes, cysteine peptidases are promising candidates for chemotherapeutic drug design. Examples are reduction of *T. cruzi* infectivity in a mouse model of acute Chagas' disease by the cysteine protease vinyl sulfone inhibitor, WRR-483 (Chen *et al.*, 2010) and inhibition of *T. bruce*i Lister427 bloodstream form parasite growth by benzimidazoles with EC_{50} values ≤ 20 μM and inhibition of the activity of cysteine protease, rhodesian (*Tbr*CATL) with IC_{50}-values of 0.25 μM (Pereira *et al.*, 2019). In addition, as potential drug candidates, the use of peptidases in diagnostics is plausible. As discussed in Section 1.4.4, peptidases can be employed as prospective antigens in an ELISA or RDT format for the detection of antibodies found in infected sera.

Peptidases (proteolytic enzymes/proteases) are enzymes that catalyse the hydrolysis (or cleavage) of peptide bonds in proteins (Neurath, 1984). These enzymes are grouped according to substrate specificity and catalytic mechanism. Peptidases exhibit either endopeptidase or exopeptidase activity based on the cleavage site of the protein within or at the N- or C-terminal ends of the polypeptide chain respectively. Exopeptidases that cleave N-terminally are called aminopeptidases and those hydrolysing the C-terminal peptide bond are carboxypeptidases (Lecaille *et al.*, 2002).

The nomenclature proposed by Schechter and Berger (1967) is commonly used to describe the interaction between the binding sub-sites of the enzymes (S) and the corresponding amino acid residues of the peptide (P) substrate. S_1, S_2, S_3 (N-terminal direction from the sessile bond) and S_1', S_2', S_3' (C-terminal direction) denote the substrate binding pockets of the enzyme; and P_1, P_2 P_3 and P_1', P_2', P_3', the substrate amino acid residues (Schechter and Berger, 1967) (Figure 1.7).

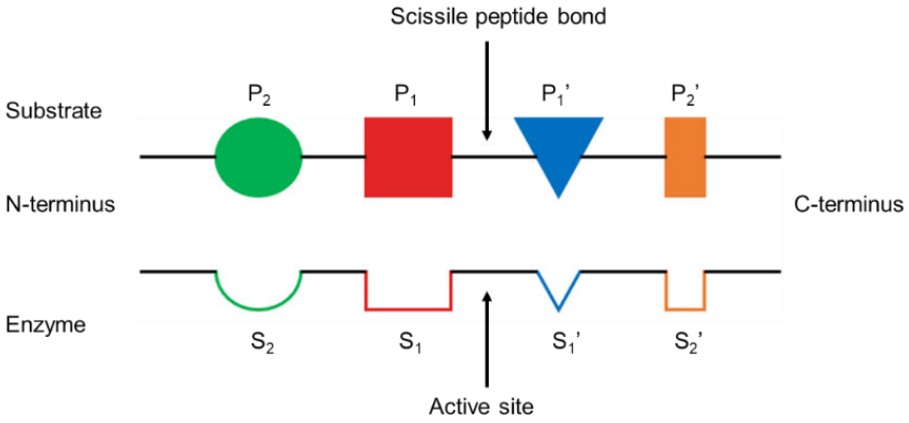

Figure 1.7: Representation of the interaction between peptide substrate and enzyme active site as proposed by Schechter and Berger. Interaction occurs between the peptide (P) substrate with the enzyme sub-sites (S). The complementary pairing occurring between the peptide substrate and enzyme sub-sites allow for correct sub-site fitting. Adapted from Schechter and Berger (1967).

Peptidases are grouped into seven classes namely, the hydrolases: metallo, cysteine, serine, aspartic, glutamic and threonine, and a peptide lyase, asparagine peptide lyase, based on the amino acid residues located in the catalytic site of the enzyme (Rawlings *et al.*, 2018). Enzymes belonging to the hydrolase group cleave peptide bonds by means of hydrolysis and different nucleophiles are used for the hydrolysis. Asparagine peptide lyases are the only group of enzymes that does not undergo hydrolysis, rather cleavage of peptide bonds occurs by re-arrangement of asparagine to succinimide (Rawlings and Bateman, 2021). Each of these classes are further divided into clans and families (Section 1.6.2).

1.6.1 Catalytic mechanism of cysteine peptidases

Cysteine proteases contain Cys^{25}, His^{159} and Asn^{175} residues that form the catalytic site, where the thiol group of the cysteine residue serves as the nucleophile as it is deprotonated by histidine, forming a thiolate cysteine. An intermediate tetrahedral complex is then formed due to the nucleophilic attack of the thiolate cysteine on the carbonyl carbon of the substrate scissile bond forming a covalent bond, which is stabilised by asparagine. This step is followed by acylation, the release of the C-terminal portion of the substrate and formation of an enzyme-substrate thiol ester complex. This complex undergoes hydrolysis by water forming a second tetrahedral complex, which breaks into the free-enzyme and N-terminal portion of the substrate, due to deacylation (Lecaille *et al.*, 2002) (Figure 1.8).

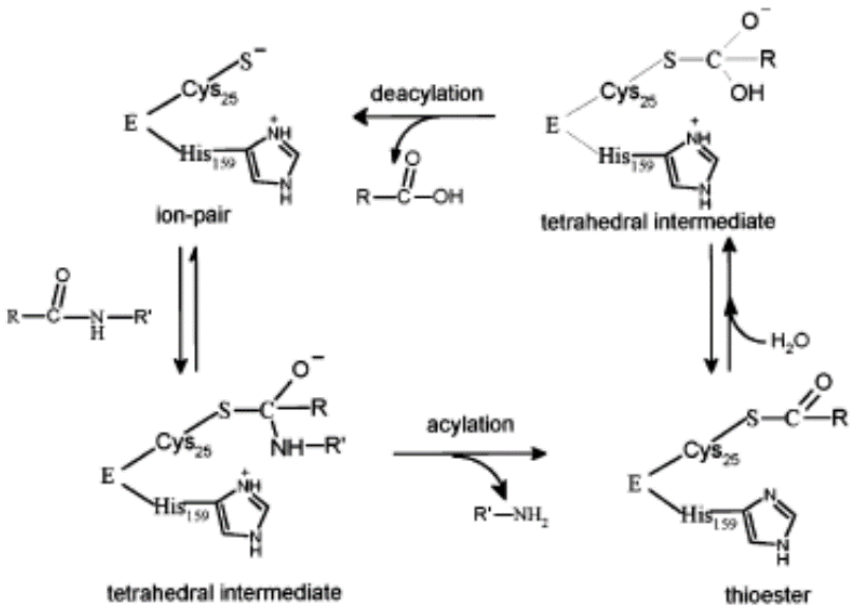

Figure 1.8: Catalytic mechanism of cysteine peptidases. Cysteine peptidases contain a catalytic triad consisting of cysteine, histidine and asparagine, where cysteine is the nucleophile (Lecaille *et al.*, 2002).

1.6.2 Classification of cysteine peptidases

Cysteine peptidases are grouped into eight clans, namely CA, CD, CE, CF, CL, CM, CN and CO (Barrett and Rawlings, 2004). The clan CA consists of more than 20 families, of which the C1 family (papain family) is the largest (Barrett and Rawlings, 1996). This family contains mammalian cathepsins B, C, K, L and S as well as cysteine proteases of protozoan parasites such as *Tco*CATL (congopain) from *T. congolense*, *Tvi*CATL (vivapain) from *T. vivax* and *Tcr*CATL (cruzipain) from *T. cruzi*, CDA, CPC and CPB from *Leishmania mexicana* and falcipains from *Plasmodium falciparum* (Mottram *et al.*, 1998; Rosenthal, 1999; Klemba and Goldberg, 2002; Sajid and McKerrow, 2002; Caffrey and Steverding, 2009). Cysteine proteases belonging to clan CA are irreversibly inhibited by trans-epoxysuccinyl-L-leucyl-amido (4-guanidino) butane (E-64) and have a S_2 pocket substrate specificity and a preference for bulky, hydrophobic residues (Barrett and Kirschke, 1981; Barrett *et al.*, 1982).

1.6.3 Cysteine peptidases of parasites

Cysteine peptidases are also found in a variety of protozoan parasites namely *Trypanosoma* spp., *Entamoeba histolytica*, *Leishmania* spp., *Cryptosporidium* spp., *Plasmodium* spp. and *Toxoplasma gondii*. The importance of cysteine peptidases is the role that they play in the pathogenesis of protozoan parasites e.g. cell hydrolysis of host/parasite proteins, cell and/or

tissue penetration, autophagy and invasion of the host immune system (Siqueira-Neto *et al.*, 2018).

The structure of papain-like cysteine peptidases consists of an active site flanked by two domains: a bundle of α-helices at the N-terminal and C-terminally a β-barrel (Figure 1.9). The catalytic cysteine is positioned at the start of a long helix that runs through the middle of the molecule (Barrett and Rawlings, 2004).

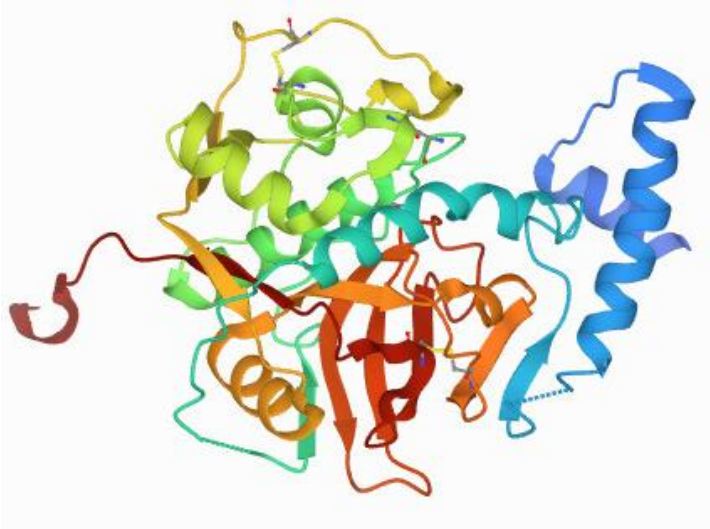

Figure 1.9: The crystal structure of a cysteine protease from *T. b. brucei*. The N-terminal domain is represented by α-helices and the C-terminal by β-sheets (Johé *et al.*, 2021).

Cysteine proteases that are similar to mammalian cathepsins B and L are termed cathepsin B-like and cathepsin L-like peptidases (CATB and CATL, respectively), and belong to the C1 family from the clan CA. The amino acids cysteine and histidine occupy the active site forming a catalytic dyad for this group of peptidases (Caffrey and Steverding, 2009).

Kinetoplastid cathepsin-like proteases are comprised of a pre-domain, pro-domain and catalytic domain, which are initially synthesised as an inactive precursor (Lecaille *et al.*, 2002). In addition, characteristic to only kinetoplastid CATL, these enzymes possess an 11-13 kDa highly immunogenic C-terminal extension, linked to the catalytic domain by a poly-threonine (for *T. cruzi*) or -proline-rich hinge region (*T. congolense*), the function of which is still unknown (Caffrey and Steverding, 2009).

Cathepsin L-like peptidases (CATL) expressed by *T. b. brucei* and *T. b. rhodesiense*, named *Tbb*CATL and *Tbr*CATL respectively, were observed in all life-cycle stages, particularly in the bloodstream form where it is found in lysosomes (Mbawa *et al.*, 1991; Scory *et al.*, 1999). The

major cathepsin L-like peptidases in *T. congolense* and *T. vivax* (*Tco*CATL and *Tvi*CATL, respectively) are expressed in the bloodstream form parasite and is released into the host bloodstream after parasite lysis (Cortez *et al.*, 2009; Pillay *et al.*, 2010). *Tcr*CATL, the homologue found in *T. cruzi* and etiological agent for Chagas disease in Latin America, was also identified throughout the parasite's life-cycle (Siqueira-Neto *et al.*, 2018), whereas the cathepsin B-like peptidase (CATB) of *T. congolense*, *Tco*CATB, showed high levels of expression in the bloodstream form where it is localised in the lysosomes (Mendoza-Palomares *et al.*, 2008).

The amino acid sequence alignment (Figure 1.10) shows that, along with other cysteine proteases, cysteine, histidine and asparagine residues within the catalytic domain are conserved in trypanosome cathepsin L-like proteases. The poly-threonine or -proline-rich region linking the catalytic domain to the C-terminal extension is also conserved.

Antibodies produced against trypanosomal cysteine proteases was shown to have an inhibitory effect towards these proteases when either produced by trypanotolerant cattle when infected, or by production when immunised in susceptible cattle (Authié *et al.*, 2001). Troeberg *et al.* (1997) investigated the effect of anti-trypanopain (*Tbb*CATL) antibodies on *Tbb*CATL activity and found that antibodies were able to either enhance or inhibit *Tbb*CATL activity. The authors proposed that this is due to the polyclonal nature of the antibodies elicited during the immune response.

Cysteine proteases were shown to be attractive diagnostic candidates particularly in an antigen detection diagnostic test, allowing differentiation between current and past infections. A potential antigen for the diagnosis of *T. vivax* infection is the cathepsin L-like protease, *Tvi*CATL. Antigen-specific antibodies are therefore needed to develop an antigen detection-based ELISA test. To this end, in addition to high-titre polyclonal antibodies produced in chickens against *Tvi*CATL, this antigen was also used to pan a phage display library for the identification of single chain variable fragment (scFv) *Tvi*CATL binders.

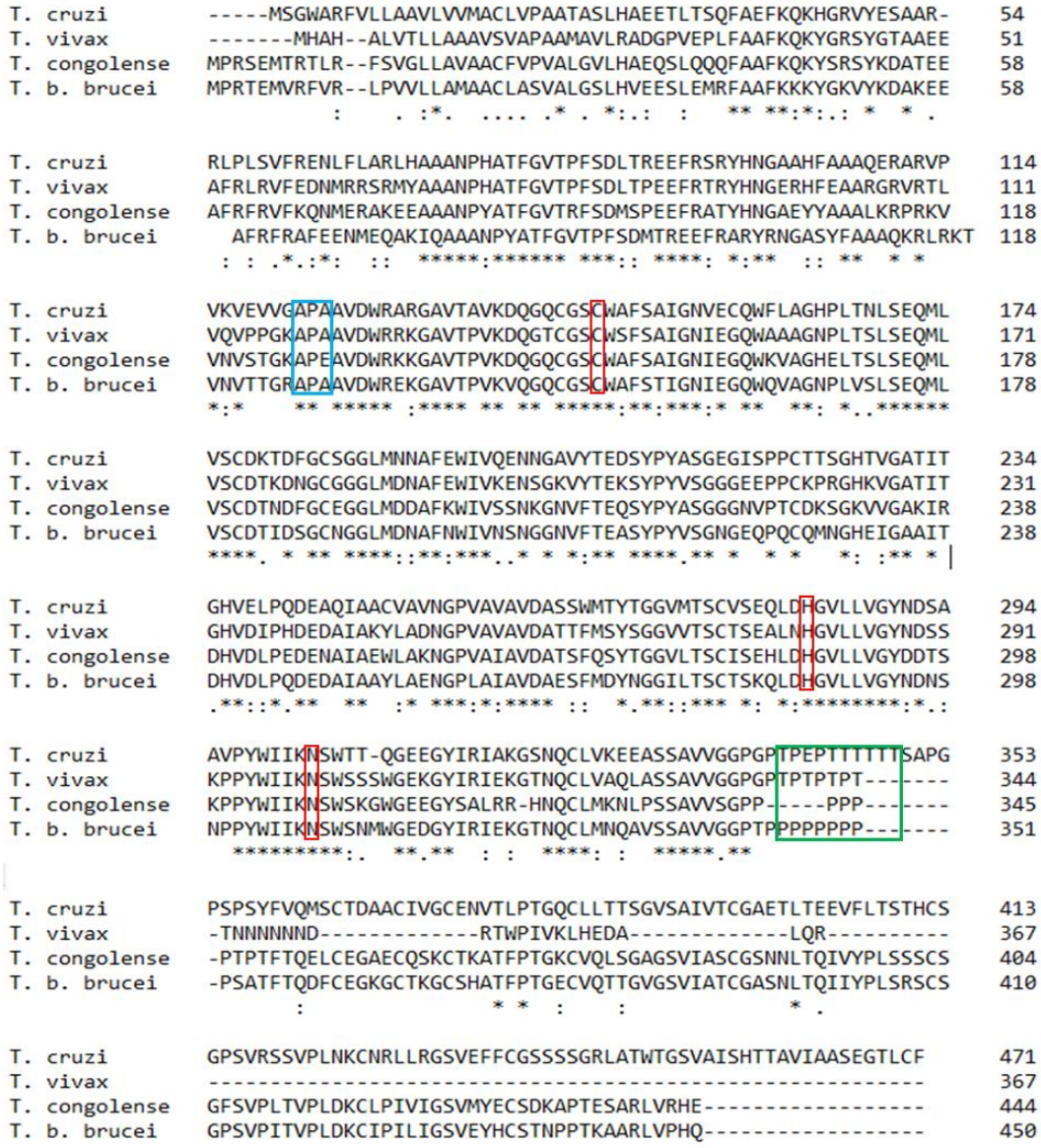

Figure 1.10: The amino acid sequence alignment of cathepsin L-like peptidases from *T. cruzi, T. vivax, T. congolense* and *T. b. brucei*. The start of the mature protease is represented by a blue box, the catalytic site amino acid residues by red boxes and the poly-threonine or -proline-rich region in green. The alignment is denoted as following: conserved residues (*), amino acids with strongly similar properties (:), or weakly similar properties (.). Accession numbers: *T. cruzi*: AAC00067.1*, T. vivax*: CCD21670.1, *T. congolense*: AAA18215.1 and *T. b. brucei*: CAA34485.1

1.7 Producing antibodies using phage display

The production of monoclonal antibodies (mAb) using hybridoma technology has been the forefront technique for many years to produce non-specific antibodies. However, alternate means of producing highly specific antibodies and antibody fragments are constantly being developed, one being the production of recombinant antibodies expressed in bacterial cells using phage display technology. The concept of phage display was first exploited by Smith (1985) and is based on the display of specific libraries (proteins or antibody fragments) on the surface of filamentous phages. Genetic material encoding these proteins (genotype) are fused within the phage particle and the resulting protein is displayed as a fusion (phenotype) to the phage proteins. This creates a link between genotype and phenotype, a key feature of phage display, whereby the gene coding sequence is known and therefore the protein produced is also known (Sambrook and Russel, 2001) and antibodies are produced within a couple of weeks instead of months- as when immunisation is employed (Carmen and Jermutus, 2002).

Filamentous phages, most commonly used in phage display technology, are a group of viruses that infect Gram-negative bacteria via F-pili and is the group of which f1, fd and M13 phages belong to (Russel, 1991). All filamentous phages share a common morphology (6-7 nm diameter, cylindrical shape) and a circular, single-strand DNA genome encoding 11 genes (Sambrook and Russel, 2001). Five of the 11 genes encode coat proteins: minor gene protein pIII, pVI, pVII, pIX, and major coat protein pVIII, inside which phage DNA is located; minor proteins are located at either ends of the phage particle (Figure 1.11). The pIII protein is responsible for infecting bacterial cells and this is mediated by the interaction between the pIII protein's N-terminal and F-pilus of bacteria. Following infection by bacteriophages, lysis of host cells does not occur rather they replicate while host cell continues with growth (McCafferty *et al.*, 1990; McLaughlin GL *et al.*, 1996). Although all five proteins were used as fusion coat proteins for phage display, pIII is more typically used (Carmen and Jermutus, 2002).

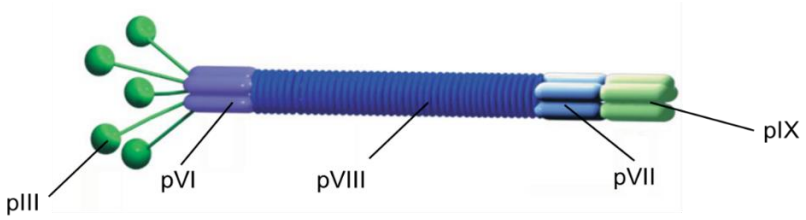

Figure 1.11: Schematic representation of the M13 filamentous phage. The M13 phage is composed of five coat proteins pIII, pVI, pVII, pIX, pVIII. Single-stranded DNA is encapsulated inside pVIII and exogenous peptides are usually displayed on pIII (Arap, 2005).

A phagemid vector codes for both coat protein (i.e. pIII) of the phage particle and protein to be expressed on the phage particle (i.e. antibody) resulting in a fusion protein displayed on the filamentous phage and no other phage protein (Schmitz *et al.*, 2000). In addition, these vectors also carry origin of replications of both *E. coli* and phage, and an antibiotic resistance marker (Schmitz *et al.*, 2000). Transformation of *E. coli* with this vector results in the expression of the fusion protein (antibody included). The phagemids are small in size and consequently have higher transformation efficiencies, therefore allowing the construction of large libraries of protein or antibodies (Hoogenboom *et al.*, 1991). Phagemids alone cannot infect bacteria and therefore to allow for enrichment of selected antibodies, *E. coli* is infected with a helper phage (Vieira and Messing, 1989) such as M13KO7. These helper phages are essential as they provide genes required for assembly of recombinant phage DNA and the fusion protein displayed on the bacterial cell surface (Carmen and Jermutus, 2002).

1.7.1 Antibody fragments used in phage display

The conventional antibody structure consists of two identical heavy (H) and light (L) chains of 50 kDa and 25 kDa respectively (Figure 1.12), joined via an inter-chain disulfide bond. Each chain consists of an N-terminal variable domain (V_H in the heavy chain and V_L in the light chain) and a constant domain (C_H and C_L for heavy and light chain, respectively) (Davies and Chacko, 1993). Variable (V), joining (J) and constant (C) genes encode both heavy and light chains, however the heavy chain has an additional diversity (D) gene. The light chains (V_L and C_L) and the N-terminal domains of the heavy chains (V_H and C_H), where the antigen binding site is located, is called the Fab fragment and the remaining domains of the heavy chain comprise the Fc region (Figure 1.12). The variable domains of the light and heavy chain domains of the Fab region contain hypervariable sequences that make up the complementarity determining region (CDR) or antigen binding site (Davies and Chacko, 1993).

The most commonly produced antibody fragments using phage display are the Fab and single chain fragment (scFv). The scFv consists of V_H joined to a V_L. Several factors contribute to the use of scFv over Fab fragments such as the number of Fab fragments displayed is less than scFvs (Carmen and Jermutus, 2002) and has a lower immunogenicity than that of scFv. Due to its small size, areas inaccessible to traditional mAb can be reached by scFv (Yokota *et al.*, 1992), furthermore scFv fragments displayed on phage particles are shown to retain their ability to bind antigen (Finlay *et al.*, 2011) and is less toxic than Fab molecules to the host cell when expressed (Arndt *et al.*, 2001).

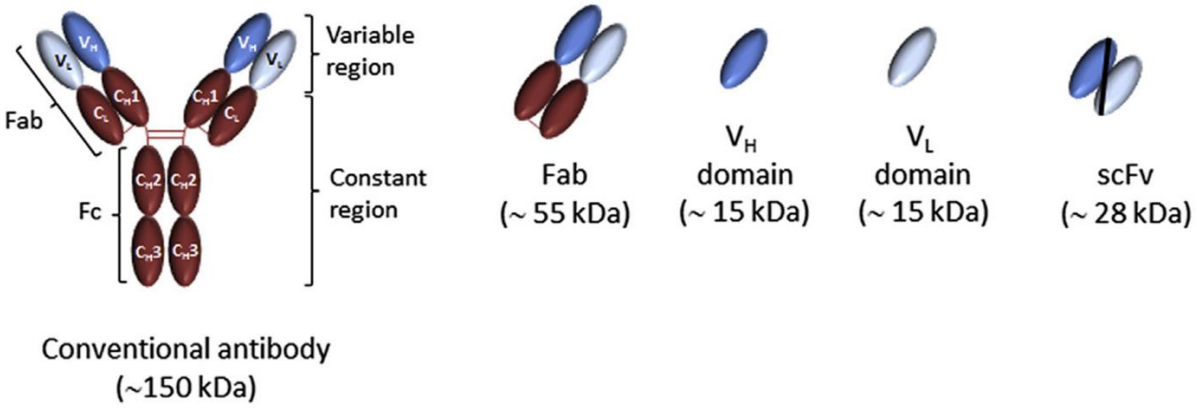

Figure 1.12: Representation of antibody and antibody fragments commonly used in phage display technology. The conventional antibody structure is made up of a heavy and light chain, each of which contains a variable and constant region joined together by disulfide bonds (Marcotte and Hammarström, 2015).

Antibody libraries are constructed using genes encoding the variable domains from either immune or non-immune donors (Clackson *et al.*, 1991). Using genetic information derived from immunisation is generally limited due to the production of antibodies against the antigen used for immunisation, whereas non-immune libraries could be used against a range of antigens (Carmen and Jermutus, 2002).

Combinatorial libraries based on human or mouse immunoglobulin genes are cumbersome to produce due to the number of PCR primers required for amplification. Using antibodies retrieved from birds is much less complicated due to how antibody diversity is created. V and J genes of H and L chains undergo VDJ and VJ rearrangement, respectively. Introduction of pseudo V region genes results in diversity by gene conversion (Reynaud *et al.*, 1985; Reynaud *et al.*, 1987; Thompson and Neiman, 1987; Reynaud *et al.*, 1989). Consequently, all V regions will have identical amino acid sequences at both termini and therefore only one primer set is required for

amplification: one for the heavy chain and one for the light chain. This method using chicken immunoglobulin genes was first introduced by Davies *et al.* (1995). The *Nkuku*® phage library was constructed in 2004 using such a method (Van Wyngaardt *et al.*, 2004) and is the phagemid library used in the present study.

1.7.2 Bio-panning process for selection of binders

Constructing a phagemid library is the first step of the phage display process, where V_H and V_L genes are amplified and ligated into the phagemid plasmid (Figure 1.13, A). *E. coli* cells are then transfected with the plasmid (B), resulting with the scFv::pIII fusion protein displayed on the bacterial cell surface (C). The phagemids are then rescued by M13KO7 helper phages (D). A mixed population of cells now exists, with *E. coli* cells containing either the phagemid or the helper phage (E). This mixed population is then panned against the immobilised antigen, where specific scFvs are bound to antigen and non-specific binders are washed away (F). Bound phages are eluted, and phagemids are transfected back into *E. coli* (G). The resulting population is rescued again (H), and the next round of panning starts again.

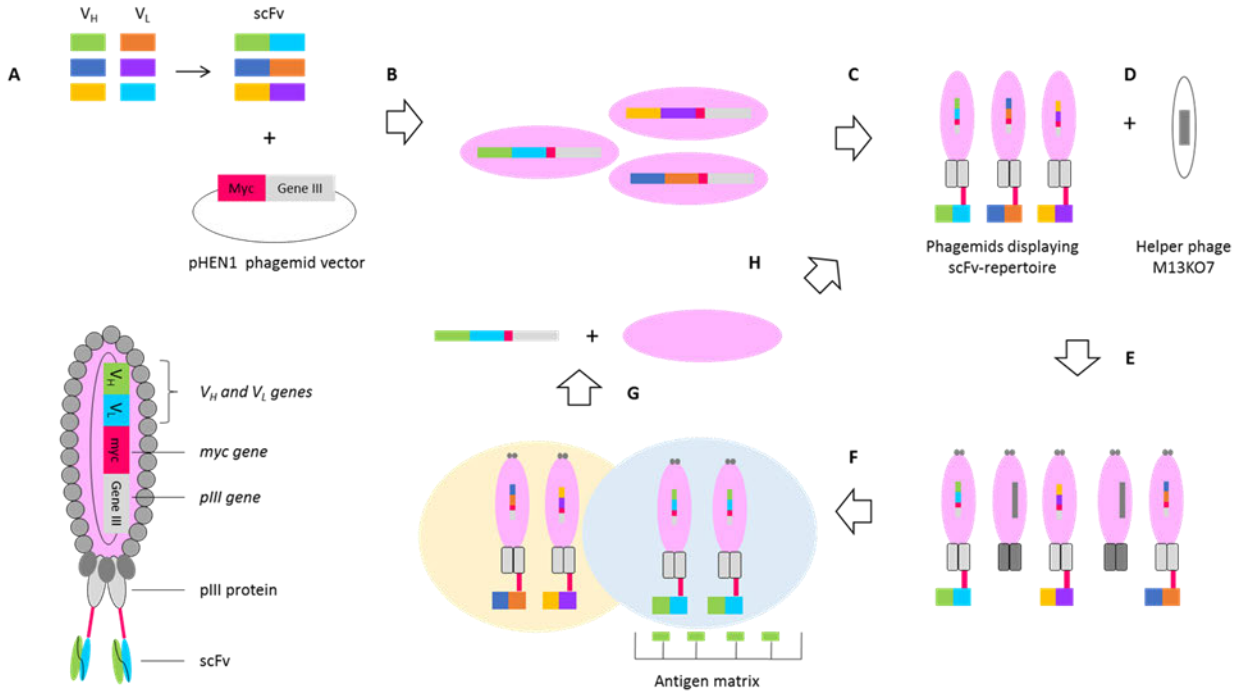

Figure 1.13: Schematic representation of incubation of phage display library with coated antigen and the phage display panning process (Eyssen, 2018).

1.7.3 Applications of phage display

The study of protein (antibody)-protein interactions can be achieved using phage display technology, from which the region that the antibody recognises on the protein (the epitope) can be determined (Scott and Smith, 1990); and of agonists and antagonists to determine receptor specificity (Hammer *et al.*, 1992); in addition, protein-DNA interactions (Cheng *et al.*, 1996) can also be resolved using phage display technology. Using phage display technology, antibodies have been produced against CR6261 (influenza virus) and KB001 (*Pseudomonas aeruginosa*) (Nelson *et al.*, 2010). The *Nkuku*® phage library has been used to determine epitopes of foot-and-mouth disease virus (Opperman *et al.*, 2012) and to generate antibodies for African horse sickness virus to use in diagnostic tests (Van Wyngaardt *et al.*, 2004; Van Wyngaardt *et al.*, 2013)**.**

1.8 Rationale of study

African trypanosomiasis affects millions of humans and animals in Africa, the worst affected residing in rural and underdeveloped parts of the continent where diagnosis and treatment are poor and often result in death. Animal African trypanosomiasis is caused by three protozoan parasites belonging to the *Trypanosoma* genus namely, *T. b. brucei*, *T. congolense* and *T. vivax*. Human African trypanosomiasis is caused by *T. b. gambiense* and *T. b. rhodesiense*. Trypanosomes live in their hosts' bloodstream, and they are able to avoid the hosts' immune system by switching to different VSGs and through disabling the host's memory B-cell compartment. Consequently, there is little hope for developing an effective vaccine for trypanosomiasis. The parasite can survive within the host without an antibody response and also contributes to drug resistance. Current control strategies, especially for AAT are not effective because drug resistance is on the increase since no new drugs have been developed in the past 60 years. Proteases have diverse roles in parasites and have been seen as promising candidates for diagnostic and drug development for human and animal African trypanosomiasis. Studying the enzymatic characterisations of trypanosome proteases will provide knowledge and understanding for drug development. In addition, the potential of these proteases can also be applied in the field of diagnostics. A cathepsin L-like cysteine protease of T. vivax is a potential drug and diagnostic target, and studies involving this particular parasite can provide insight for drug and/or diagnostic development.

1.9 Aims and Objective of present study

The first overarching aim of the study was to enzymatically characterise the cysteine protease from *T. vivax*, *Tvi*CATL, which has been identified as a potential antigen for the diagnosis of *T.*

vivax infection in an antigen diagnostic test. The second broad aim was to investigate the cross-reactivity of *Tvi*CATL with other trypanosomal species-specific antibodies as well as phage-display-produced antibodies towards developing an antigen detection diagnostic test for trypanosomiasis.

Aim 1: To recombinantly express, purify and enzymatically characterise the cysteine protease, *Tvi*CATL, from *T. vivax*

The first specific aim of the study was to recombinantly express and purify *Tvi*CATL so that enzymatic studies could be carried out. Aim 1 can be divided into the following objectives:

Objective 1: Recombinantly express TviCATL using the E. coli BL21 (DE3) expression system

The *TviCATL*-pET28a (full length) construct, previously cloned from *T. vivax* genomic DNA, will be used for the expression of *Tvi*CATL in *E. coli* cells

Objective 2: Solubilisation and purification of TviCATL

Should the recombinantly expressed *Tvi*CATL be found in insoluble inclusion bodies, the non-chaotropic solubilisation agent N-lauroylsarcosine (sarkosyl) will be used for solubilisation prior to nickel chelate affinity purification of the His-tagged *Tvi*CATL

Objective 3: Enzymatic characterisation: active site titration, substrate specificity, inhibition studies, pH optimum

Following purification of *Tvi*CATL, the amount of active cysteine protease will be determined by active-site titration with E-64, before the hydrolysis of fluorescently labelled substrates with different amino acid residues in the P_1 and P_2 positions will be determined to find the optimal substrate for *Tvi*CATL. This will allow determining the optimal pH for substrate hydrolysis using constant ionic strength buffers, and assays with catalytic class-specific inhibitors to confirm *Tvi*CATL as a cysteine protease.

Aim 2: Antibody analysis and selection of single-chain variable fragments (scFvs) from the Nkuku® library against recombinant *Tvi*CATL

The second aim of the study was to evaluate previously-produced chicken anti-trypanosomal cysteine protease antibodies for their potential as diagnostic targets; and to produce scFvs against recombinant *Tvi*CATL. Aim 2 can be divided into the following objectives:

Objective 1: Evaluation of chicken anti-TviCATL antibodies by ELISA

Different coating concentrations of *Tvi*CATL will be compared in an enzyme linked immunosorbent assay (ELISA) to determine if the antigen is recognised by chicken anti-*Tvi*CATL N-terminal peptide IgY and chicken anti-FL*Tvi*CATL$_{C25A}$ IgY.

Objective 2: Evaluation of cross-reactivity of other Trypanosoma spp. antibodies with TviCATL

The possible cross-reactivity of *Tvi*CATL with antibodies produced against cysteine proteases of other *Trypanosoma* spp. will be evaluated in western blots. In addition to the anti-*Tvi*CATL IgY tested in the ELISA (positive controls), chicken anti-*Tco*CATL N-terminal peptide IgY and chicken anti-*Tbb*CATL IgY will be evaluated.

Objective 3: Detection of TviCATL in TviCATL-spiked bovine serum

Towards designing an antigen-detection ELISA diagnostic test, bovine serum will be spiked with *Tvi*CATL and the ability of the anti-*Tvi*CATL antibodies to detect the antigen determined.

Objective 4: Production and evaluation of TviCATL scFvs using phage display technology

A further step towards designing an antigen detection diagnostic test, scFvs targeting *Tvi*CATL will be produced using the *Nkuku*® phagemid library.

Chapter 2

Materials and methods

2.1 Materials

Table 2.1 Manufacturers of reagents and products used in this study

Buffer salts and other common chemicals were purchased from Merck (Germany) and Sigma (USA) and were of the highest purity available. All protocols involving animals were approved by the University of KwaZulu-Natal Animal Research Ethics committee approval number 051/15/Animal.

Method	Materials	Manufacturer
Recombinant expression	pET-28a expression vector	Novagen, Germany
	E. coli BL21 (DE3) cells	New England Biolabs, USA
	Bacterial agar, Tryptone, Yeast extract	Merck Biolab, Germany
	Kanamycin Lysozyme Whatman No. 1 filter paper	Gibco, UK Sigma, USA Whatman International Ltd, UK
SDS-PAGE molecular mass markers	Phosphorylase B (97.4 kDa) Bovine serum albumin (BSA, 68 kDa), Ovalbumin (45 kDa), Carbonic anhydrase (30 kDa), Soyabean trypsin inhibitor (SBTI, 21.5 kDa), Lysozyme (14 kDa) (homemade molecular weight marker, MWM)	Sigma, USA
Western blot	Nitrocellulose Fat free milk powder 4- chloro-1 naphthol	PALL Corp, USA Amresco, USA Sigma, USA
Protein purification	Dithiothreitol (DTT) His-select® nickel affinity resin Polyethylene glycol (PEG) M_r 20 000 14 kDa molecular weight cut-off dialysis tubing	Fermentas, Lithuania Sigma, USA Sigma, USA Pierce, USA
Enzymatic characterisation (substrates and inhibitors)	H-D-Ala-Leu-Lys-7-amino-4-methylcoumarin (AMC), H-D-Val-Leu-Lys-AMC, Benzyloxycarbonyl (Z)-Phe-Arg-AMC, Z-Pro-Arg-AMC, H-Pro-Phe-Arg-AMC,	Sigma, USA

	H-Ala-Phe-Lys-AMC, Z-Gly-Gly-Arg-AMC, H-Leu-AMC	
	trans-Epoxysuccinyl-L-leucylamido(4-guanidino) butane (E-64), Bestatin, Chymostatin, Z-Gly-Leu-Phe- chloromethylketone (CMK), Antipain, Leupeptin, Iodoacetate	Sigma, USA
	Black FluoroNunc™ 96-well plates	Nunc Intermed, Denmark
ELISA	2, 2'-azinobis (3-ethyl-3, dihydrobenzithiazole-6-sulfonate) (ABTS)	Roche, Germany
	Nunc-Immuno™ Maxisorp 96-well plates	Nunc Intermed, Denmark
	Bovine serum	Triple A Beef Abattoir, SA
Phage display	*E. coli* TG1 cells, *Nkuku*® phagemid library and M13KO7 wild type helper phages	Onderstepoort Veterinary Institute, SA
	Indolyl-β-D-galactopyronoside (IPTG)	Fermentas, Lithuania
	Ampicillin	Amresco, USA
	Sterile 0.22 µM filters	PALL Corp, USA
Antibodies used in western blots, ELISAs and phage display	Mouse anti-6xHis IgG-horseradish peroxidase (HRPO)	Sigma, USA
	Rabbit anti-chicken IgY-HRPO, Goat anti-mouse IgG-HRPO	Jackson ImmunoResearch Inc, USA
	Chicken anti-*Tvi*CATL N-terminal peptide IgY, Chicken anti-FL*Tvi*CATL$_{C25A}$ IgY, Chicken anti-*Tco*CATL N-terminal peptide IgY, Chicken anti-*Tbb*CATL IgY	Previously produced in-house (details and references provided in the relevant sections)
	Mouse anti-M13 IgG	ThermoFisher Scientific USA

2.2 Recombinant expression of *Tvi*CATL

TviCATL was previously cloned into the pET-28a expression vector in our laboratory and preserved as a glycerol stock of *E. coli* BL21 (DE3) (Eyssen *et al.*, 2018) cells. Once the target gene is cloned into the pET vector under the control of the T7 promoter, the plasmid can be

transformed into *E. coli* BL21 (DE3) expression host, which carries the T7 RNA polymerase gene. The T7 RNA polymerase binds to the T7 promoter which allows for transcription of the target gene and expression occurs upon addition of IPTG (Novagen, 2002). The glycerol stock sample of the *TviCATL*-pET28a (full length) construct was streaked onto 2 x YT plates [1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) bacteriological agar] containing kanamycin (34 µg/mL) and incubated at 37°C for 16 h. A single colony was inoculated in 50 mL terrific broth (TB) medium [1.2% (w/v) tryptone, 2.4% (w/v) yeast extract, 0.4% (v/v) glycerol, 170 mM KH_2PO_4, 720 mM K_2HPO_4] containing 34 µg/mL kanamycin at 37°C for 16 h with agitation (200 rpm). Following incubation, cells were harvested by centrifugation (6 000 *x g*, 10 min, 4°C), resuspended in phosphate buffered saline (PBS) [100 mM Na_2HPO_4, 2 mM KH_2PO_4, 2.7 mM KCl, 137 mM NaCl, pH 7.2] (5 mL) containing 1% (v/v) Triton X-100 with lysozyme (1 mg/mL final concertation) and incubated at 37°C for 30 min. The cell suspension was frozen at -20°C for 16 h, thereafter thawed at RT, sonicated on ice (30 s x 5) each and the cellular debris was pelleted from the soluble protein lysate by centrifugation (10 000 x *g*, 10 min, 4°C). The protein lysate was subsequently filtered through Whatman No. 1 filter paper and stored at -20°C.

Samples of the supernatant and pellet containing the soluble and insoluble fractions, respectively, were electrophoresed on two 12.5% reducing SDS-PAGE gels (Laemmli, 1970) (Section 2.3). One was stained with Coomassie blue R-250 and the other used for transfer of proteins onto nitrocellulose for western blot analysis (Section 2.4).

2.3 SDS-PAGE analysis

Protein analysis was conducted by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) described by Laemmli (1970). The anionic detergent, SDS, converts proteins from a native state to a negatively charged one by binding tightly to the protein. The now negatively charged protein will migrate to the positive electrode, and will break down into their constituents, giving an estimate of relative molecular mass. This technique together with Western blot (Section 2.4) were used to identify proteins using specific antibodies. A discontinuous buffer system was used consisting of a running gel buffer (1.5 M Tris-HCl, pH 8.8) and a stacking gel buffer (500 mM Tris-HCl, pH 6.8). Protein samples were combined with an equal volume of reducing treatment buffer [125 mM Tris-HCl, 4% (w/v) SDS, 20% (v/v) glycerol, 10% (v/v) 2-mercaptoethanol, pH 6.8] and boiled at 100 °C for 5 min. SDS-PAGE was conducted using the BioRad Mini protein III electrophoresis apparatus at 20 mA per gel in tank buffer [250 mM Tris-HCl buffer, 192 mM glycine, 0.1% (w/v) SDS, pH 8.3]. After electrophoresis, the gels were placed in Coomassie blue R-250 staining solution [0.125% (w/v) Coomassie blue R-250, 50% (v/v) methanol, 10% (v/v)

acetic acid] for 16 h and destained in several changes of destaining solution [50% (v/v) methanol, 10% (v/v) acetic acid]. The gels were viewed, and images captured using the G-box system (Syngene, Cambridge, UK). To determine the molecular weight of a protein of unknown size, a set of proteins of known sizes were separated on the same reducing 12.5 % SDS-PAGE gel and a calibration curve was constructed plotting the relative migration distance (R_f) of the standard proteins against the log M_r of each (Figure 2.1).

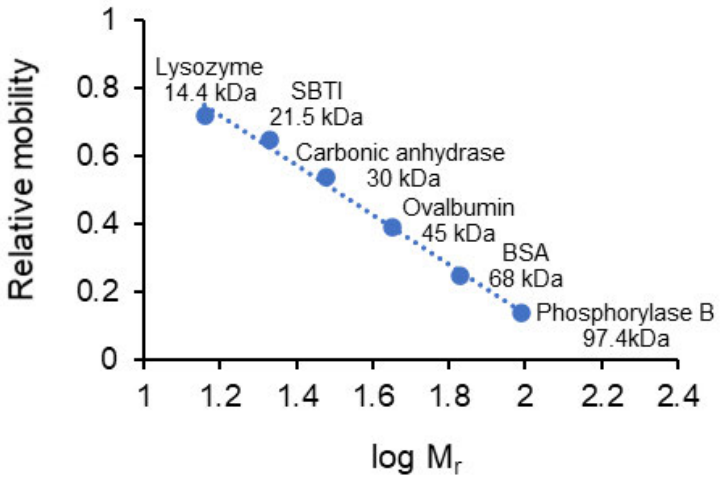

Figure 2.1: A standard curve showing the correlation between relative mobility and the log of molecular weight markers used in a reducing 12.5% SDS-PAGE gel. Standard proteins of known sizes, as indicated, were used as a molecular weight marker for a reducing SDS-PAGE gel (Section 2.3). The equation of the trendline is given by Relative mobility = -0.7303 (log M_r) + 1.598, with a correlation coefficient of 0.9916.

2.4 Western blot analysis

Analysis of proteins by western blot technique was conducted as described by Towbin *et al.* (1979).Following separation by SDS-PAGE (Section 2.3), proteins were electrophoretically transferred from the gel onto a nitrocellulose membrane pre-soaked in blotting buffer [45 mM Tris-HCl buffer, pH 8.3, 173 mM glycine, 0.1% (w/v) SDS] using a Semi-phor® semi-dry blotting apparatus (Sigma, St. Louis, USA) at constant voltage (20 V) and maximum current for 55 min. Upon completion, confirmation of protein transfer was conducted by transient staining with Ponceau S solution [0.1 % (w/v) Ponceau S in 1% (v/v) glacial acetic acid]. The nitrocellulose membrane was destained with several washes using distilled water to remove residual Ponceau S stain with the addition of a few drops of 500 mM NaOH. All unoccupied sites on the nitrocellulose membrane were blocked with blocking solution [5% (w/v) fat free powder milk in TBS (Tris

buffered saline) (20 mM Tris-HCl buffer, 200 mM NaCl, pH 7.4)] for 1 h at RT, followed by washing the membrane with TBS (3 x 5 min). The membrane was incubated with an optimised dilution of the appropriate primary antibody [diluted in 0.5% (w/v) BSA-TBS] for 16 h at 4°C. The blot was washed with TBS (2 x 5 min) and incubated with an optimised dilution of the appropriate horseradish peroxidase (HRPO) labelled detection antibody [diluted in 0.5% (w/v) BSA-TBS] at RT for 1 h and washed again with TBS (3 x 5 min). The blot was then immersed in substrate solution [0.06% (w/v) 4- chloro-1 naphthol (0.1% (w/v) methanol, 0.0015% (v/v) H_2O_2] and allowed to develop in the dark until bands were visible. The image was captured using the G-box system (Syngene, Cambridge, UK).

2.5 Solubilisation, refolding and purification of recombinant *Tvi*CATL

The expression of recombinant proteins in a heterologous host often results in protein aggregation in inclusion bodies. The recovery of active protein and complete renaturation requires solubilisation and refolding of the recombinant protein. Solubilisation of inclusion bodies is achieved by using chaotropes such as guanidine hydrochloride and urea and non-chaotropic agents like SDS and the sodium salt form of N-lauroylsarcosine (sarkosyl) (Burgess, 2009). Solubilised proteins are refolded into their native conformation upon removal of denaturants by dialysis, dilution in denaturation buffer or various chromatographic techniques (Clark, 1998). The removal of denaturants and refolding of the solubilised recombinant protein can be achieved by using immobilised metal ion affinity chromatography (IMAC), such as a nickel chelate column (Petty, 1998). The solubilised protein is treated with 0.1 to 1 mM DTT, added to the inclusion body buffer or refolding buffers, to re-oxidise the protein and prevent disulfide bonds forming between cysteine residues (Burgess, 2009).

Following expression using auto-induction in terrific broth, solubilisation and refolding was adapted and modified as described by (Schlager *et al.*, 2012). The cells were centrifuged (10 000 x *g*, 10 min, 4°C) and lysis buffer (PCL) [8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 1% (w/v) SDS, pH 7.4, 1 mM DTT] (5 mL for a 50 mL culture) was added to resuspend the bacterial pellet. The suspension was sonicated on ice (2 x 2 min), incubated for 1 h on ice and soluble proteins separated from insoluble debris by centrifugation (10 000 x *g*, 20 min, 4°C). Samples of the supernatant and the pellet containing the solubilised protein lysate and insoluble fractions, respectively, were analysed on a 12.5 % reducing SDS-PAGE gel (Section 2.3) and stained with Coomassie blue R-250.

Purification was performed by aliquoting His-select® nickel affinity resin (1 mL) into a 10 mL chromatography column. The column was first washed with two column volumes dH_2O and then with five column volumes of wash buffer (PCW) [8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 0.1% (w/v) sarkosyl, pH 7.4]. The solubilised protein lysate (5 mL) was incubated with the nickel resin and mixed using an end-over-end rotator at 4°C for 3 h. The recombinant protein has a high affinity for the nickel resin due to the polyhistidine tag and will therefore attach to the column. The unbound proteins were collected, and the column washed with PCW until an absorbance at 280 nm value of 0.02 was reached. Bound proteins were eluted with 5 mL elution buffer (PCE) [8 mM Na_2HPO_4, 286 mM NaCl, 1.4 mM KH_2PO_4, 2.6 mM KCl, 0.1% (w/v) sarkosyl, 250 mM imidazole, pH 7.4] in 1 mL fractions. The column was regenerated with 2 column volumes dH_2O, 5 column volumes of 6 M guanidine hydrochloride, 3 column volumes of dH_2O, and 3 column volumes of equilibration buffer [50 mM NaH_2PO_4, 500 mM NaCl, 10 mM imidazole, pH 6.8] and stored in 30% (v/v) ethanol at 4°C. Unbound and eluted fraction samples were analysed by 12.5 % reducing SDS-PAGE (Section 2.3) and western blotting (Section 2.4).

2.6 Dialysis

Fractions containing the target protein (fractions 1-5) were pooled and placed in dialysis tubing (14 kDa molecular weight cut-off) and dialysed against buffer [100 mM Bis-Tris buffer, pH 6.5, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 8 mM DTT]. The volume of dialysis buffer used was 100x the protein solution volume. Dialysis was carried out at 4°C for 16 h, thereafter buffer was changed and dialysed for a further 2 h at 4°C. The protein solution was then concentrated against polyethylene glycol (PEG) M_r 20 000 until the final volume was approximately 1-2 mL. Protein concentration was determined by performing a Bradford assay (Section 2.7) and purified samples were electrophoresed on a 12.5% reducing SDS-PAGE gel (Section 2.3) and stained with Coomassie blue R-250.

2.7 Protein quantification

Protein concentration was determined by using the Bradford (1976) assay. The Bradford assay is based on the binding of Coomassie brilliant blue G-250 dye to positively charged amino acids such as arginine, lysine and histidine on a protein, which results in a colour change from brown to blue. Interference can be caused by a high concentration (1%) of detergents such as SDS and Triton X-100 (Bradford, 1976).

A standard curve (Figure 2.2) was constructed by diluting a 1 mg/mL BSA stock to obtain a concentration range from 20 to 100 µg/mL. Triplicate samples of each dilution were made up to

100 µl with distilled water and 900 µl Bradford reagent [0.12% (w/v) Coomassie brilliant blue G-250 dissolved in 2% (v/v) perchloric acid] was added. The absorbance was measured at 595 nm using a Jenway 6/15 UV/ Vis. 24 spectrophotometer following 5 min of incubation. The standard curve was then constructed by plotting absorbance at 595 nm against a concentration range of BSA (20-100 µg/mL).

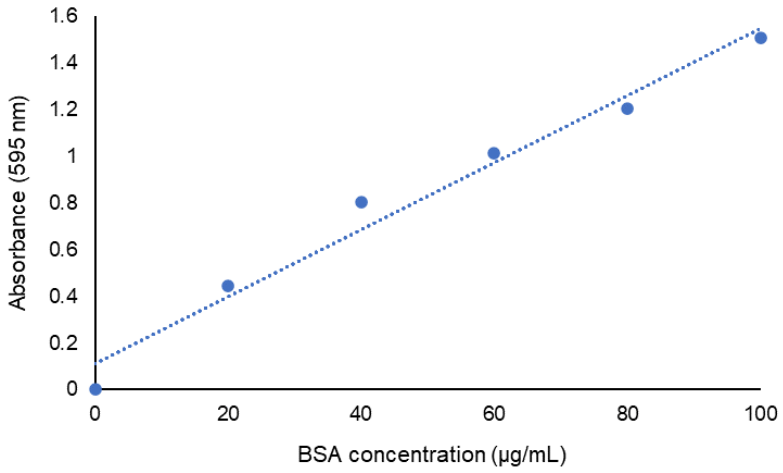

Figure 2.2: Bradford assay standard curve for protein concentration. Bovine serum albumin concentrations ranging from 20 to 100 µg/mL were added to Bradford reagent and incubated at room temperature for 5 min. The resulting absorbance values were then measured at 595 nm. The equation of the trendline is given by Absorbance at 595 nm = 0.0143 [BSA] + 0.1135 with a correlation coefficient of 0.976.

2.8 Enzymatic characteristation of *Tvi*CATL

Initial activity and optimal enzyme concentration for the hydrolysis of a fluorogenic peptide substrate was established by varying the concentration of enzyme (0.5-10 µg/mL, 10 µl), in duplicates, using assay buffer [100 mM Bis-Tris buffer, pH 6.5, 4 mM Na_2EDTA, 0.02% (w/v) NaN_3, 8 mM DTT] (88 µl). The H-D-Ala-Leu-Lys-AMC (20 µM) substrate was added to the different concentrations of enzyme and the fluorescence was measured (Ex_{360nm} and Em_{460nm}) using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany).

2.8.1 AMC standard curve

A 7-amino-4-methylcoumarin (AMC) standard curve was constructed to quantify the relationship between the amount of fluorescence released during the hydrolysis of a fluorescently-labelled synthetic peptide substrate and concentration of AMC (Figure 2.3). The AMC standards ranging from 5 to 10 000 nM (50 µl) were incubated, in triplicates, with 50 µL assay buffer at 37°C for 10

min and the fluorescence (Ex_{360nm} and Em_{460nm}) measured using the FLUORStar Optima Spectrophotometer and AMC concentration was plotted against fluorescence for construction of the AMC standard curve.

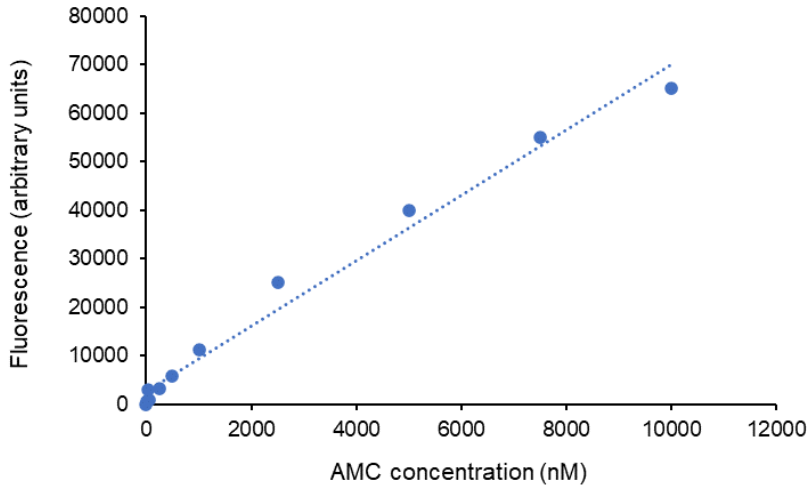

Figure 2.3: AMC standard curve showing the correlation between fluorescence and AMC concentration. Dilutions of the AMC stock solution were incubated with assay buffer at 37°C and the fluorescence measured at Ex_{360nm} and Em_{460nm}. The equation of the trendline is given by Fluorescence = 6.7139 [AMC] + 2695.5 with a correlation coefficient of 0.9838.

2.8.2 Active site titration

Active site titration with the irreversible cysteine protease inhibitor, E-64, was conducted according to Barrett and Kirschke (1981) to determine the active concentration of *Tvi*CATL. E-64 is an irreversible inhibitor which binds rapidly to the active thiol group of cysteine proteases. *Tvi*CATL (1 µM) was incubated, in duplicates, with E-64 (0-1 µM) in assay buffer at 37°C for 30 minutes. Subsequently, H-D-Ala-Leu-Lys-AMC (20 µM) substate was added. The fluorescence was measured (Ex_{360nm} and Em_{460nm}) using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany). The active concentration of *Tvi*CATL was determined by plotting fluorescence against E-64 concentration.

2.8.3 Substrate specificity

Various peptide substrates, in which they differ due to different residues in P_1, P_2 etc as indicated in Figure 1.7 of Chapter 1, were used to determine the substrate specificity of recombinant *Tvi*CATL. Briefly, recombinant *Tvi*CATL (6 µg; 14.8 µM active enzyme) was pre-incubated, in duplicates, in assay buffer for 10 minutes at 37°C. Thereafter, the various peptide substrates (20

µM) were added, and the fluorescence was measured using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany) at Ex_{360nm} and $Em_{460nm.}$

2.8.4 pH optimum

The pH optimum for hydrolysis of H-D-Ala-Leu-Lys-AMC by recombinant *Tvi*CATL (6 µg; 14.8 µM active enzyme) was determined using the acetate/Mes/Tris (AMT) constant ionic strength buffer (100 mM acetate, 100 mM MES, 200 mM Tris, 4 mM Na_2EDTA, 8 mM DTT) across a pH range of 4-9. Constant ionic strength buffers provide a constant ionic environment for enzyme activity across a range of pH values (Ellis and Morrison, 1982; Dehrmann *et al.*, 1995). Enzyme (17 µl; 6 µM active enzyme) was incubated with the AMT buffers for 10 minutes at 37°C, followed by the addition of H-D-Ala-Leu-Lys-AMC substrate (20 µM). The fluorescence was measured using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany) at Ex_{360nm} and $Em_{460nm.}$

2.8.5 Stop time inhibition assay

Stop time assays were carried out to assess the inhibitory activity of various protease catalytic class-specific inhibitors against purified *Tvi*CATL. Briefly, the enzyme (*Tvi*CATL, 6 µg; 14.8 µM active enzyme), in duplicates, was incubated with 100 µM of the different inhibitors in assay buffer for 15 min at 37°C. The H-D-Ala-Leu-Lys-AMC (20 µM) substrate was added and incubated at 37°C for 2 min. Thereafter, stopping reagent [100 mM monochloroacetate, 30 mM Na-acetate, 70 mM acetic acid, pH 4.3] was added and the fluorescence was measured (Ex_{360nm} and Em_{460nm}) using a FLUORStar Optima Spectrophotometer (BMG Labtech, Offenburg, Germany).

2.9 Enzyme-linked immunosorbent assay

In order to optimise an enzyme linked immunosorbent assay (ELISA) for determining appropriate concentrations of primary chicken anti-*Tvi*CATL antibodies that should be used in western blots and ELISA to detect *Tvi*CATL in mock-infected bovine serum, the optimal coating and primary antibody concentrations were evaluated. The wells of a 96-well Nunc-Immuno™ Maxisorp ELISA plate were coated with purified recombinant *Tvi*CATL (0.5 or 1 µg/mL, 100 µl per well) in PBS at 4°C for 16 h. The coating solution was discarded, and unoccupied sites blocked with blocking buffer [0.5% (w/v) BSA-PBS, 200 µl per well] for 1 h at 37°C. The wells were washed three times with [PBS-0.1% (v/v) Tween-20 (PBS-T)] using the BIOTEK® ELx50™ Microplate washer (BioTek® Instruments Inc., USA) and incubated at 37°C for 2 h with different dilutions of the appropriate primary antibody [diluted in blocking buffer, 100 µL per well]. The wells were washed again as before followed by the incubation with an optimised dilution of the appropriate

horseradish peroxidase (HRPO) labelled detection antibody [diluted in blocking buffer, 100 µl per well] for 1 h at 37°C.The wells were washed again and the ABTS substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in 0.15 M citrate-phosphate buffer, pH 5.0, 100 µL per well] was added. The plate was incubated in the dark for 15 minutes prior to reading the absorbance values at 405 nm with the VersaMax ELISA Microplate Reader (Molecular Devices, CA, USA), thereafter every 15 minutes until absorbance values above 1 were reached.

2.10 Evaluation of cross-reactivity between *Tvi*CATL and *Trypanosomal* spp. specific antibodies by western blot

Purified recombinant *Tvi*CATL was separated on a 12.5% reducing SDS-PAGE gel (Section 2.3) and a western blot was conducted as described in Section 2.4 for the evaluation of cross-reactivity between *Tvi*CATL antigen and chicken anti-*Tvi*CATL N-terminal peptide IgY, chicken anti-FL*Tvi*CATL$_{C25A}$ IgY, chicken anti-*Tco*CATL N-terminal peptide IgY and chicken anti-*Tbb*CATL IgY, which were the primary antibodies (5 µg/mL) and rabbit anti-chicken IgY HRPO (1:5 000) as the detection antibody. The membranes were incubated in substrate solution [0.06% (w/v) 4-chloro-1 naphthol, 0.1% (w/v) methanol, 0.0015% (v/v) H_2O_2] and allowed to develop in the dark until bands were visible. The image was captured using the G-box system (Syngene, Cambridge, UK). The antibodies chicken anti-*Tvi*CATL N-terminal peptide IgY, chicken anti-FL*Tvi*CATL$_{C25A}$ IgY, chicken anti-*Tco*CATL N-terminal peptide IgY and chicken anti-*Tbb*CATL IgY were produced against the following proteins: the N-terminal of *Tvi*CATL, the full length mutated protein of *Tvi*CATL (*Tvi*CATL$_{C25A}$), the N-terminal of *Tco*CATL and the full length protein of *Tbb*CATL, respectively (Table 2.2)

Table 2.2 Immunogenic sites of trypanosomal proteins and their respective antibodies

Antibody	Part of protein antibody was made against
chicken anti-*Tvi*CATL N-terminal peptide IgY	N-terminal peptide of *Tvi*CATL (APAAVDWRRKGAVTPVKD)
chicken anti-FL*Tvi*CATL$_{C25A}$ IgY	Full length protein of *Tvi*CATL with active site Cys25-to-Ala25 mutation to prevent processing to mature catalytic domain
chicken anti-*Tco*CATL N-terminal peptide IgY	N-terminal peptide of *Tco*CATL (APEAVDWRKKGAVTPVKD)
chicken anti-*Tbb*CATL IgY	Full length protein of *Tbb*CATL

2.11 ELISA for detection of *Tvi*CATL in *Tvi*CATL-spiked bovine serum samples

In order to develop an antigen detection ELISA diagnostic test, the interaction between antibody (chicken anti-FL*Tvi*CATL$_{C25A}$ IgY, 5 µg/mL) and antigen (*Tvi*CATL) in mock-infected bovine serum

samples was analysed, as illustrated in Figure 2.4. Mock infected bovine serum (bovine serum spiked with 1 µg/mL recombinant *Tvi*CATL) (1 µg/mL *Tvi*CATL diluted 1 in 1, 1 in 10, 1 in 20, 1 in 50, 1 in 100 and 1 in 200 with either PBS or PBS containing 0.1, 0.5 or 1% (v/v) PBS-Tween) was coated onto a 96-well Nunc-Immuno™ Maxisorp ELISA plate and incubated at 4°C for 16 h. The ELISA was completed as outlined in Section 2.9, using chicken anti-FL*Tvi*CATL$_{C25A}$ IgY (5 µg/mL) as the primary antibody and rabbit anti-chicken IgY HRPO secondary antibody (1:10 000 dilution).

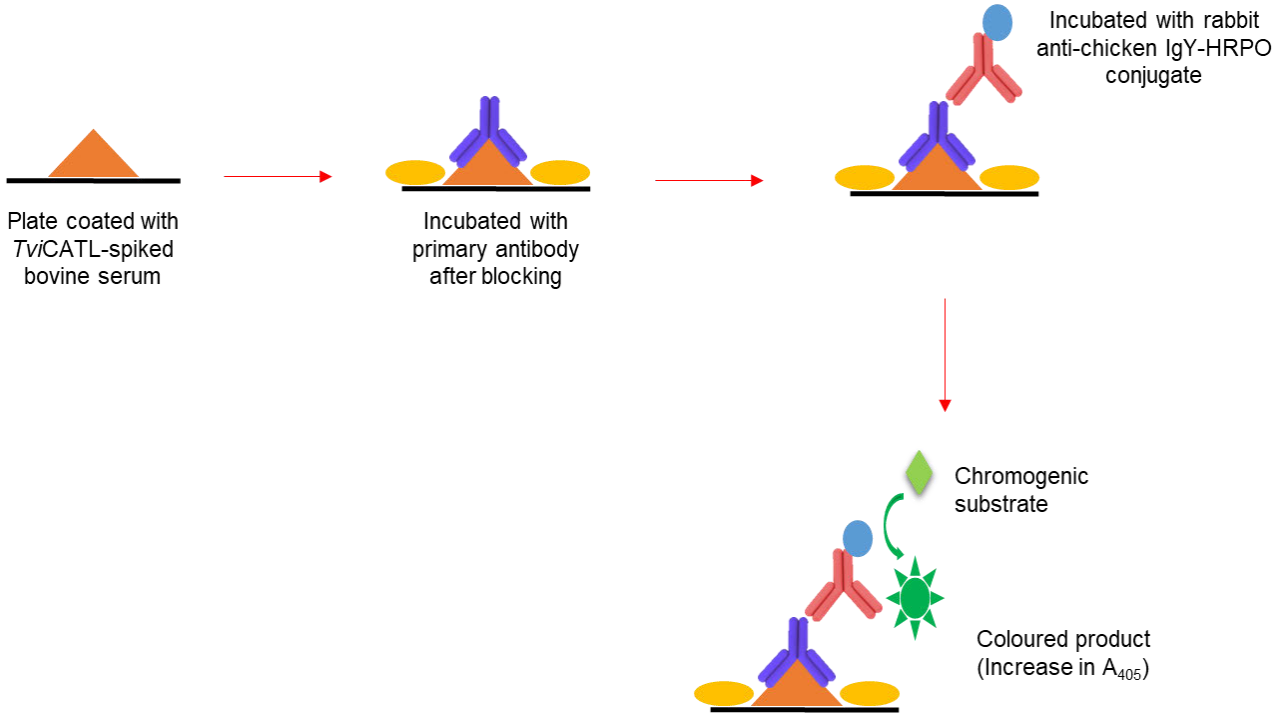

Figure 2.4: Indirect ELISA schematic representation of detection of *Tvi*CATL in *Tvi*CATL-spiked bovine serum samples

2.12 Production of scFvs antibodies

2.12.1 Culture of TG1 *E. coli* cells

A glycerol stock containing TG1 *E. coli* cells was streaked onto 2 x YT plates, without antibiotic, and grown for 16 h at 37°C. A single colony was inoculated in 2 x YT liquid medium [1.6% (w/v) tryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl] (5 mL), without antibiotic, and grown at 37°C for 16 h at 220 rpm. To 50 µl of the overnight culture, fresh 2 x YT medium (25 mL), without antibiotic, was added and incubated at 37°C until the log phase was reached (OD_{600} = 0.5) and stored at 4°C for a maximum of 7 days.

2.12.2 Titration and culture of M13KO7 helper phages

To determine the concentration (plaque forming units per mL) of the initial M13KO7 glycerol stock, serial dilutions of the M13KO7 helper phages were made in fresh 2 x YT medium (100 µl), without antibiotic, in order to obtain 30 to 300 colonies, which was incubated with TG1 *E. coli* cells that reached log phase growth (100 µl) for 5 min at RT. Pre-warmed 2 x YT [containing 0.7% (w/v) agar, 3 mL] to 44°C, without antibiotic, was added to each dilution and immediately plated onto pre-warmed 2 x YT plates, without antibiotic, and incubated for 16 h at 37°C.

A single M13KO7 plaque was punched out from the plated dilution using the reverse end of a sterile yellow pipette tip and added to 2 x YT medium (2 mL), without antibiotic, containing log phase TG1 *E. coli* cells (20 µl) and incubated at 37°C for 2 h at 100 rpm. Fresh 2 x YT medium (400 mL), without antibiotic, was added and incubated for 1 h at 37°C (100 rpm), followed by the addition of kanamycin (50 µg/mL) and left for further growth at 37°C for 16 h at 200 rpm. The cells were pelleted by centrifugation (10 800 x *g*, 15 min, 4°C) and ¼ volume of 20% (w/v) PEG 6000 in 2.5 M NaCl (100 mL) was added to the phage containing supernatant. The mixture was incubated on ice for 30 min, followed by centrifugation of the phages (10 800 x *g*, 15 min, 4°C). The pellet was resuspended in PBS (3 mL) before filtering through a 0.22 µM filter and the titre of the M13KO7 helper phages were determined by measuring the absorbance at 268 nm. The M13KO7 was stored at a concentration of $1x10^{12}$ pfu/mL in 15% (v/v) glycerol at -80°C.

2.12.3 Culture of *Nkuku*® phagemid library

The *Nkuku*® phagemid library, provided in *E. coli* cells as a glycerol stock, was added to 2 x YT medium [containing 100 µg/mL ampicillin, 2% (w/v) glucose, 200 mL]. The volume of the phagemid added was adjusted until the initial OD_{600} was as close as possible to 0.05. Incubation of this culture was carried out at 37°C at 240 rpm until an OD_{600} of 0.5 was reached.

Following incubation, 100 mL of the culture was inoculated with $8x10^9$ pfu/mL M13KO7 helper phages and incubated at 37°C for 30 min with no agitation, thereafter for an additional 30 min at 100 rpm. The cells were pelleted (3 300 x *g*, 15 min, 4°C), resuspended in 2 x YT medium [containing 100 µg/mL ampicillin, 25 µg/mL kanamycin, 200mL] and incubated at 30°C at 240 rpm for 16 h. The cells were pelleted (3 300 x *g*, 15 min, 4°C) and ¼ volume of 20% (w/v) PEG 6000 in 2.5 M NaCl (50 mL) was added to the phage containing supernatant. The mixture was incubated in ice for 1 h, followed by centrifugation (3 300 x *g*, 15 min, 4°C). After centrifugation, the phagemids were pelleted and resuspended in PBS (4 mL) followed by filter sterilisation (0.22 µM filter). The filtered phagemids were used for the first round of bio-panning.

2.12.4 Bio-panning of the *Nkuku*® phagemid library against *Tvi*CATL

The process of bio-panning is illustrated in Figure 2.5. The scFvs against recombinant *Tvi*CATL were selected from the *Nkuku*® phagemid library using a method called bio-panning or simply panning (Step A). Panning was performed as previously described by Van Wyngaardt *et al.* (2004). The ELISA plate was coated with 100, 50, 25 and 10 µg/mL of *Tvi*CATL for panning rounds 1, 2, 3 and 4, respectively, at 4°C for 16 h. The plate was washed three times with PBS to remove unbound antigen (Step B) and unoccupied sites blocked with either 2% (w/v) fat free milk powder-PBS (MP) or 3% (w/v) BSA-PBS (BP) (300 µl) at RT for 1 h. The plate was washed twice with 0.1% (v/v) Tween-20-PBS (PBS-T) and then twice with PBS. During the blocking step, the *Nkuku*® phagemid library was diluted to $1x10^{12}$ pfu/mL in either MP or BP [containing 0.1% (v/v) Tween- 20, 100 µl] and incubated at 37°C for 30 min. The pre-treated *Nkuku*® phagemid library was added to the plate after washing and incubated at RT for 30 min with gentle agitation and then for a further 90 min with no agitation. After washing 20 times with PBS-T and further 20 times with PBS, 100 mM triethylamine (100 µl) was added and incubated at RT for 10 min with gentle agitation to eluted bound phages (Step C). To neutralise, eluted phages were added to 1 M Tris-HCl buffer, pH 7.4 (50 µl), and log phase TG1 *E. coli* cells (1 mL) was transfected with the neutralised phages (150 µl) and incubated at 37°C for 30 min. The cells were pelleted (3 300 x *g*, 10 min, 4°C), resuspended in 2 x YT medium (100 µl), without antibiotic, and added to fresh 2 x YT medium [containing 100 µg/mL ampicillin, 2% (w/v) glucose, 5 mL] and grown at 30°C for 16 h at 200 rpm (Step D)

To fresh 2 x YT medium [containing 100 µg/mL ampicillin, 2% (w/v) glucose, 25 mL], overnight culture (~50 µl) was added to it until an initial OD_{600} of 0.05 was achieved, thereafter incubated at 37°C at 200 rpm until an OD_{600} of 0.5 was reached. Following incubation, 5 mL of the culture was inoculated with $8x10^9$ pfu/mL M13KO7 helper phages and incubated at 37°C for 30 min with no agitation. The cells were then pelleted (3 300 x *g*, 10 min, 4°C), resuspended in 2 x YT medium [containing 100 µg/mL ampicillin, 25 µg/mL kanamycin, 25 mL] and incubated at 30°C for 16 h at 240 rpm.

The cells were pelleted (3 300 x *g*, 20 min, 4°C) and 1/5 volume of 20% (w/v) PEG 6000 in 2.5 M NaCl (5 mL) was added to the phage containing supernatant. The mixture was incubated at 4°C for 1.5 h, followed by centrifugation (3 300 x *g*, 30 min, 4°C) and the pellet resuspended in PBS (1 mL). A second centrifugation step was performed (11 000 x *g*, 2 min, 4°C) and the phages were filtered through a 0.22 µM filter.

Instead of using the *Nkuku*® phagemid library, sterilised phages from each respective panning round were used for the next round of panning. The panning process was repeated for a total of four rounds with the plate being coated with decreasing concentrations of *Tvi*CATL, allowing for enrichment of specific binders.

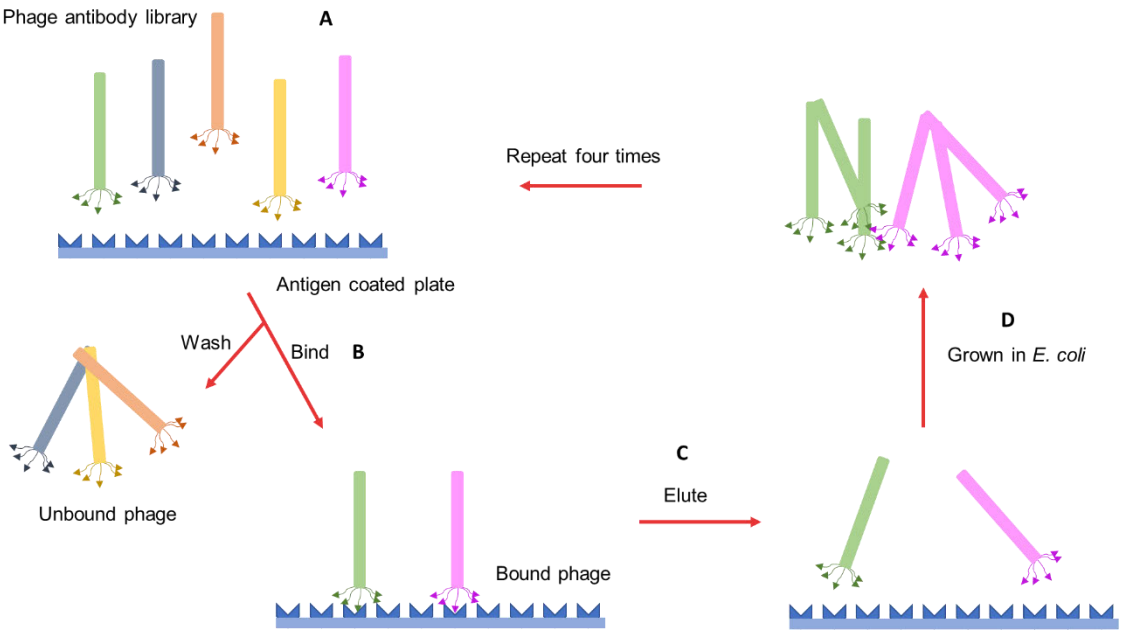

Figure 2.5: Schematic representation of the bio-panning process. The binding process of the scFv antibody to the coated antigen is represented in steps A to D.

2.12.5 Screening of enrichment of panned phages (polyspecific ELISA)

A 'polyspecific' ELISA was conducted in a similar way as described in Section 2.9 in order to enrich pooled phages after the four rounds of panning. Purified recombinant *Tvi*CATL (10 µg/mL, 100 µl per well) diluted in PBS, was used to coat a 96-well Nunc-Immuno™ Maxisorp ELISA plate for 16 h at 4°C. The coating solution was discarded, and wells were blocked with blocking buffer [MP or BP, 200 µl per well] at 37°C for 1 h. Phages from each panning round (rounds 1-4), including the *Nkuku*® phagemid library and M13KO7 [10^{12} phages diluted in MP or BP each containing 0.1% (v/v) Tween-20, 100 µl per well] was added and incubated for 2 h at 37°C. The wells were washed three times with PBS-T and thereafter incubated with mouse anti-M13 IgG [1:8 000 diluted in MP or BP, 100 µl per well] at 37°C for 1 h. The wells were washed as before, followed by the addition of goat anti-mouse HRPO conjugate [1: 1 000 diluted in MP or BP, 100 µl per well] and incubated at 37°C for 1 h. the wells were washed as before and the ABTS substrate solution [0.05% (w/v) ABTS, 0.0015% (v/v) H_2O_2 in 0.15 M citrate-phosphate buffer, pH

5.0, 100 µl per well] was added. The plate was incubated in the dark for 15 minutes prior to reading the absorbance values at 405 nm with the VersaMax ELISA Microplate Reader (Molecular Devices, CA, USA), thereafter every 15 minutes for 1 h.

2.12.6 Screening of phage-displayed binders (monospeficic ELISA)

The glycerol stock from the pan round where the most enrichment of phages occurred was serially diluted in fresh 2 x YT medium, without antibiotic, and this mixture (100 µl) was incubated with log phase TG1 *E. coli* cells (100 µl) for 5 min at RT. The dilutions were plated onto TYE plates [1% (w/v) tryptone, 0.5% (w/v) yeast extract, 0.8% (w/v) NaCl, 1.5% (w/v) bacteriological agar, containing 100 µg/mL ampicillin, 2% (w/v) glucose] and incubated at 37°C for 16 h, in order to obtain individual colonies (30 to 300). Forty-eight individual colonies were randomly picked and inoculated into a 96-well plate containing 2 x YT (100 µl), without antibiotic, and incubated at 30°C for 16 h at 220 rpm. After incubation, the plate was stored as a glycerol stock at -80°C containing 60% (v/v) glycerol (50 µl). This plate was called the master plate.

In a new 96-well plate, an aliquot from each of the wells from the master plate (5 µl) was used to inoculate 2 x YT plates [containing 100 µg/mL ampicillin, 2% (w/v) glucose, 150 µl per well] and incubated at 37°C for 2.5 h at 220 rpm. This serves as the clone plate. To each well of the clone plate, $2x10^9$ pfu/mL M13KO7 diluted in 2 x YT medium (50 µl per well), without antibiotic, was added and the plate incubated at 37°C for 30 min with no agitation. Following incubation, the plate was centrifuged (600 x *g*, 10 min, 4°C), supernatant discarded and to the wells containing pellets, 2 x YT medium [containing 100 µg/mL ampicillin, 25 µg/mL kanamycin, 150 µl per well] was added and incubated at 30°C for 16 h at 220 rpm. After centrifugation (600 x *g*, 10 min, 4°C), the pellet was resuspended in 2 x YT (containing 100 µg/mL ampicillin, 25 µg/mL kanamycin, 150 µl/well) and incubated at 220 rpm for 16 h at 30°C. The cells were pelleted and the phage containing supernatant (50 µl per well) was added with an equal volume of 6% (w/v) BSA-PBS-T to a pre-coated and pre-blocked ELISA plate and incubated at 37°C for 2 h. The ELISA was performed as described in Section 2.12.5, with only BP being used as the blocking and dilution buffer.

Chapter 3

Results

3.1 Expression of recombinant *Tvi*CATL

The full-length gene of *Tvi*CATL from *T. vivax* encodes a 376 amino acid protein with an expected molecular size of 41.36 kDa and a p*I* of 6.42 as predicted by the Compute p*I*/Mw tool on the ExPASy server (Gasteiger *et al.*, 2005)

Recombinant *Tvi*CATL expression was carried in the *E. coli* BL21 (DE3) system using auto induction (terrific broth) at 37°C. Expression was analysed on a 12.5% reducing SDS-PAGE gel. Following staining with Coomassie blue R-250, a prominent protein band was observed at approximately 47 kDa representing expression within the inclusion bodies of the insoluble pellet (Figure 3.1, A). Confirmation of the identity of the protein band as *Tvi*CATL was achieved using a mouse anti-His monoclonal antibody (Figure 3.1, B) in a western blot, which further validated the SDS-PAGE results. Large scale expression was conducted using terrific broth.

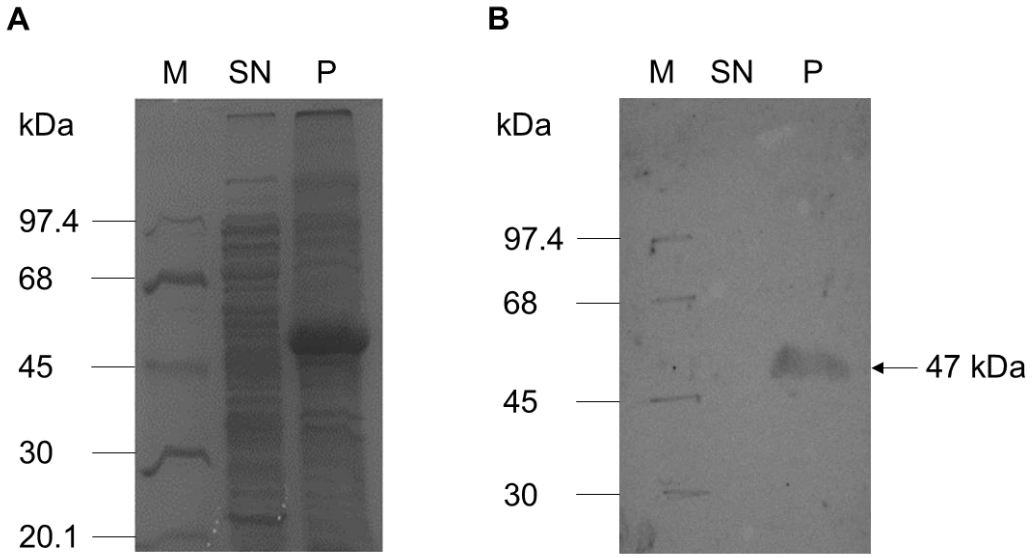

Figure 3.1: Analysis of *Tvi*CATL expression from a pET-28a vector. Samples from the recombinant expression of *Tvi*CATL containing the soluble (SN) and insoluble (P) fractions were electrophoresed on two 12.5% reducing SDS-PAGE gels with one **(A)** stained with Coomassie blue R-250 and the proteins separated on the other **(B)** transferred onto nitrocellulose and probed with primary mouse anti-His monoclonal antibody (1:1 500). Goat anti-mouse IgG HRPO secondary antibody (1:5 000) and 4-chloro-1-naphthol·H_2O_2 were used as the detection system. M: homemade molecular weight marker.

3.2 Solubilisation, refolding and purification of recombinantly expressed *Tvi*CATL

Inclusion bodies containing the recombinantly expressed *Tvi*CATL protein were solubilised using SDS and the sarkosyl method (Burgess, 2009) followed by refolding and purification on a nickel chelate column. Following extensive washing of the nickel chelate resin after the unbound fraction had been collected, a protein of ~47 kDa was eluted from the column showing as a prominent band on the gel (Figure 3.2, A). The 47 kDa protein band represented the expected size due to the addition of 6 kDa from the His-tag to the protein size of 41.36 kDa. This purified protein was used for subsequent experiments. The 47 kDa *Tvi*CATL protein was detected in a western blot probed with the mouse anti-His monoclonal antibody (Figure 3.2, B).

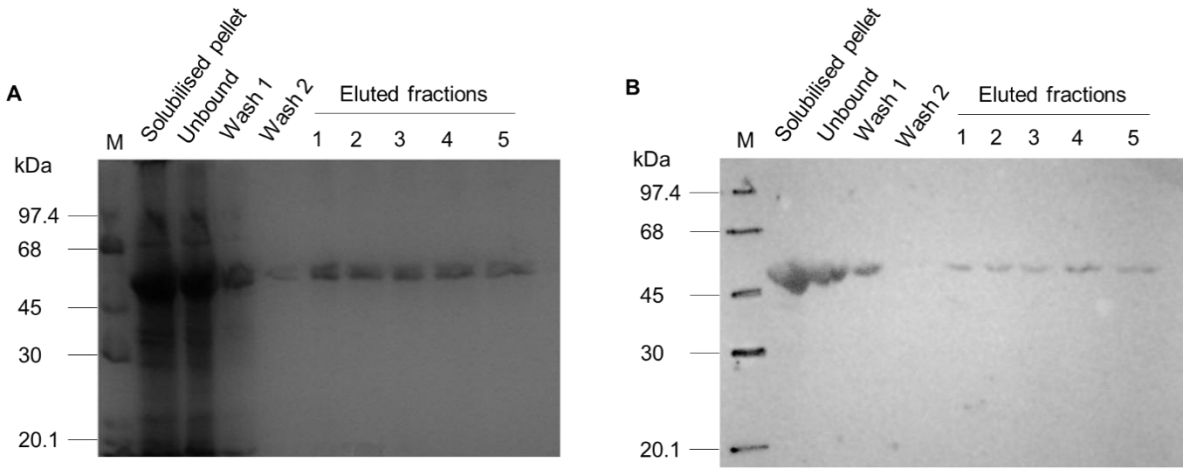

Figure 3.2: Solubilised, refolded and nickel purified recombinant *Tvi*CATL. Samples of the eluted fractions from the purification of the solubilised and refolded recombinant *Tvi*CATL was analysed on two 12.5% reducing SDS-PAGE gels with one **(A)** stained with Coomassie blue R-250 and the separated proteins other **(B)** transferred onto nitrocellulose and probed with mouse anti-His IgG primary antibody (1:1 500). Goat anti-mouse IgG HRPO secondary antibody (1:5 000) and 4-chloro-1-naphthol·H_2O_2 were used as the detection system. M: homemade molecular weight marker, Wash 1: first wash, Wash 2: last wash.

3.3 Enzymatic characterisation of *Tvi*CATL

The enzymatic characterisation of *Tvi*CATL included: substrate P_2 site specificity, the effect of catalytic class-specific inhibitors and optimal pH of hydrolysis. The peptide substrate, H-D-Ala-Leu-Lys-AMC, used in previous studies for characterising cathepsin L-like cysteine proteases from *Trypanosoma* spp. was the peptide substrate of choice in determining optimal enzyme concentration.

Hydrolysis of the H-D-Ala-Leu-Lys-AMC substrate (20 µM) was detected across the concentration range of *Tvi*CATL over time (Figure 3.3). Increasing concentrations of *Tvi*CATL resulted in higher

fluorescence values due to the hydrolysis of H-D-Ala-Leu-Lys-AMC. Enzyme amounts from 8 to 10 µg per assay resulted in complete hydrolysis of the substrate. *Tvi*CATL at a concentration of 6 µg was the optimal concentration used for subsequent assays in order to preserve enzyme.

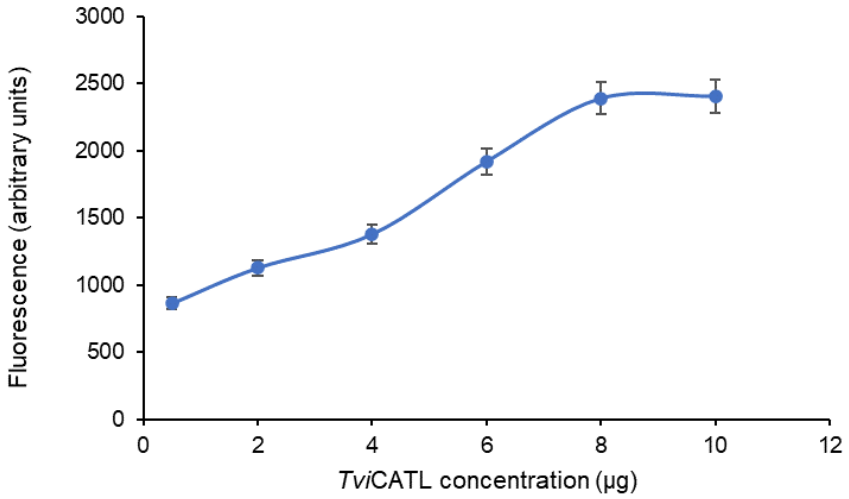

Figure 3.3: *Tvi*CATL activity measured at different enzyme concentrations. Amounts of recombinant *Tvi*CATL (0.5-10 µg) incubated in assay buffer, pH 6.5 were used to hydrolyse H-D-Ala-Leu-Lys-AMC substrate (20 µM) over a 15 minute time period. Each data point represents the mean activity of two replicates.

3.3.1 Active site titration

Active site titration of recombinant purified *Tvi*CATL was carried out using E-64 as the active site titrant (Barrett and Kirschke, 1981) and the H-D-Ala-Leu-Lys-AMC peptide substrate. This was done to obtain the concentration of enzyme that was active, which was shown to be ~20% (Figure 3.4) which is equivalent to 14.8 µM active enzyme.

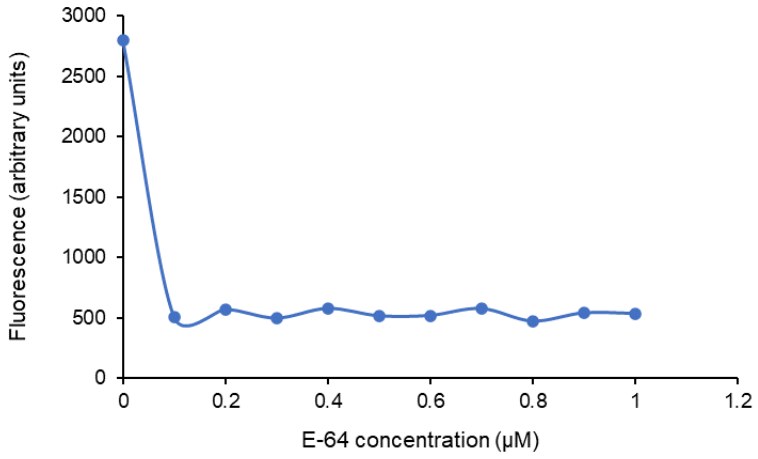

Figure 3.4: Active site titration of recombinant *Tvi*CATL. A constant amount of *Tvi*CATL (1 µM) was incubated with different concentrations of E-64 (0-1 µM) and the hydrolysis of H-D-Ala-Leu-Lys-AMC (20 µM) was measured. Each data point represents the mean activity of two replicates.

3.3.2 Substrate specificity

High arbitrary florescence units were obtained for the hydrolysis both H-D-Ala-Leu-Lys-AMC and H-D-Val-Leu-Lys-AMC and these would therefore be good substrates for *Tvi*CATL (Figure 3.5). Results also show that Z-Phe-Arg-AMC is a good substrate. Previous studies have shown that H-D-Val-Leu-Lys-AMC is best suited for hydrolysis by *Tvi*CATL (Eyssen *et al.*, 2018) but in this study, favourable results were obtained using H-D-Ala-Leu-Lys-AMC.

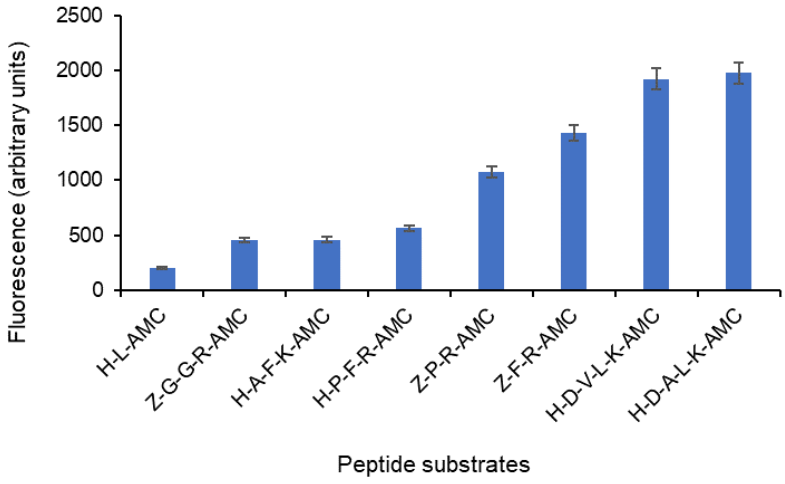

Figure 3.5: Substrate specificity of recombinant *Tvi*CATL. Recombinant *Tvi*CATL (6 µg) was incubated in assay buffer, pH 6.5 before the addition of various peptide substrates (20 µM). Each data point represents the mean activity of two replicates.

3.3.3 pH optimum

To establish the optimal pH for maximal activity of *Tvi*CATL, constant ionic strength buffers at different pH values were used. The hydrolysis of H-D-Ala-Leu-Lys-AMC was used to determine *Tvi*CATL enzyme activity. Between pH 6.5 and 7.5 *Tvi*CATL displayed maximal activity (Figure 3.6). At acidic pH values, *Tvi*CATL exhibited some activity; however, at basic pH values (pH 8-9) there was a significant drop in activity.

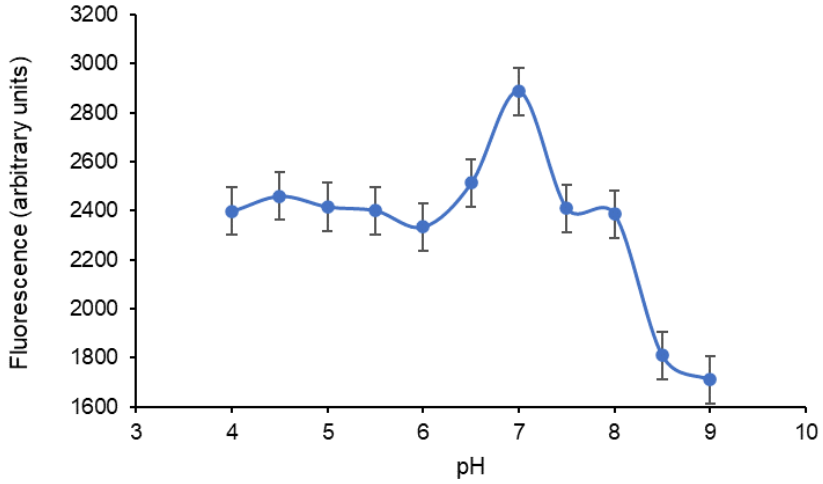

Figure 3.6: pH optimum profile of *Tvi*CATL. *Tvi*CATL (6 µg) in the presence of constant ionic strength buffers, pH 4-9, was used to determine optimum pH for maximal hydrolysis of H-D-Ala-Leu-Lys-AMC (20 µM). Each data point represents the mean activity of two replicates.

3.3.4 Stop time inhibition assay

The interaction of *Tvi*CATL with a variety of specific cysteine protease inhibitors (E-64), cysteine/serine protease inhibitors (antipain, leupeptin and iodoacetate), irreversible cysteine protease inhibitors (chymostatin and Z-Gly-Leu-Phe-CH_2Cl) and a metalloprotease inhibitor (bestatin) was tested (Figure 3.7). Activity of *Tvi*CATL when treated with bestatin was decreased to approximately 80%, where almost no inhibition was observed. Partial inhibition was shown when chymostatin or Z-Gly-Leu-Phe-CH_2Cl was added (approximately 60% and 58% activity, respectively), while antipain, leupeptin and iodoacetate diminished almost all activity (approximately 65-75% reduced activity; Figure 3.7, A)

The treatment with various concentrations (0.25, 0.5, 1, 1.5 and 2 µM) of the specific cysteine inhibitor, E-64, resulted in nearly complete inhibition of *Tvi*CATL activity (Figure 3.7, B). The highest E-64 concentration (2 µM) inhibited approximately 90% of *Tvi*CATL activity.

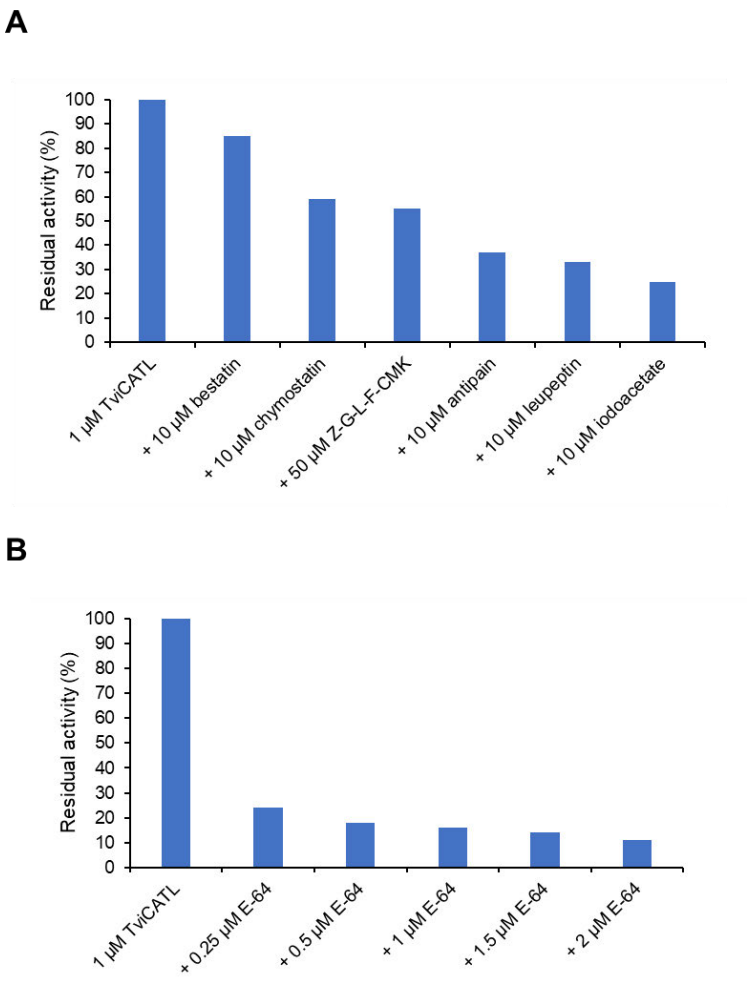

Figure 3.7: Activity profile of *Tvi*CATL in the presence of inhibitors. **(A)** Various catalytic class-specific inhibitors and **(B)** E-64 were incubated with *Tvi*CATL (1 µM) containing assay buffer, pH 6.5. Substrate (H-D-Ala-Leu-Lys-AMC) was added followed by the addition of stopping reagent. Each data point represents the mean activity of two replicates.

3.4 Evalution of *Tvi*CATL-specific antibodies

A checkerboard ELISA was performed to determine the antigen (*Tvi*CATL) coating and chicken anti-*Tvi*CATL N-terminal peptide IgY and chicken anti-FL*Tvi*CATL$_{C25A}$ IgY antibody concentrations yielding the highest ELISA signals. From Figure 3.8, it is evident that the highest absorbance value at 405 nm was obtained at a coating concentration of 1 µg/mL *Tvi*CATL in PBS for both, chicken anti-*Tvi*CATL N-terminal peptide IgY and chicken anti-FL*Tvi*CATL$_{C25A}$ IgY antibodies, while 5 µg/mL of both antibodies gave the highest absorbance values.

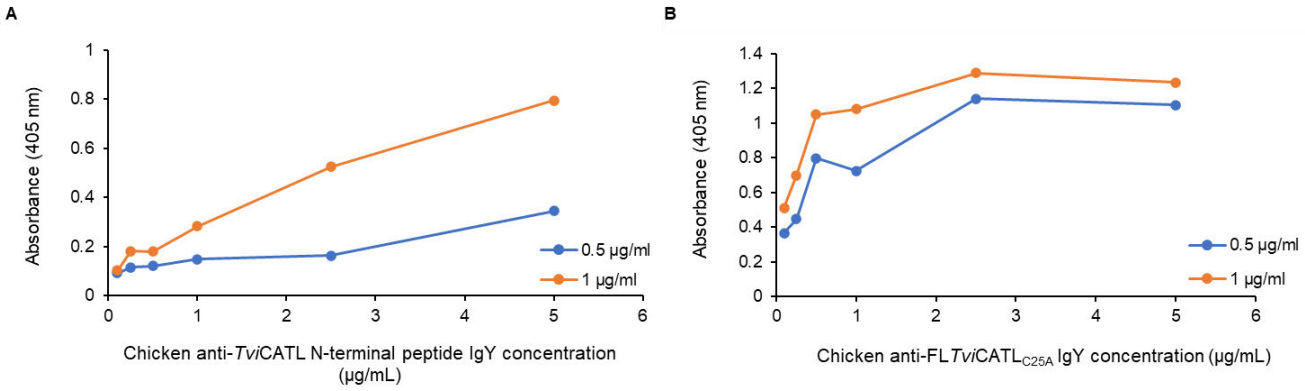

Figure 3.8: Checkerboard ELISA of *Tvi*CATL coating and chicken anti-*Tvi*CATL N-terminal peptide IgY and chicken anti-FL*Tvi*CATL$_{C25A}$ IgY antibody concentrations. ELISA plates were coated with *Tvi*CATL (1 and 0.5 µg/mL) and incubated with either (A) chicken anti-*Tvi*CATL N-terminal peptide IgY (5, 2.5, 1, 0.5, 0.25 and 0.1 µg/mL) or (B) chicken anti-FL*Tvi*CATL$_{C25A}$ IgY (5, 2.5, 1, 0.5, 0.25 and 0.1 µg/mL). Rabbit anti-chicken IgY HRPO secondary antibody (1:10 000) and ABTS·H_2O_2 were used as the detection system. The absorbance readings at 405 nm represent the average of duplicate experiments after 45 min development.

3.5 Cross-reactivity evaluation by western blot between *Tvi*CATL and *Trypanosoma* spp. specific antibodies

Purified recombinant *Tvi*CATL was separated on a 12.5% SDS-PAGE gel (Figure 3.9, A), transferred onto nitrocellulose and allowed to interact with the different anti-trypanosome CATL antibodies in a western blot to test the possibility of cross-reactivity. All the antibodies detected the *Tvi*CATL antigen protein band at 47 kDa: chicken anti-*Tvi*CATL N-terminal peptide IgY (Figure 3.9, B), chicken anti-FL*Tvi*CATL$_{C25A}$ IgY (Figure 3.9, C), chicken anti-*Tco*CATL N-terminal peptide IgY (Figure 3.9, D) and chicken anti-*Tbb*CATL IgY (Figure 3.9, E). Though detection was observed for all antibodies, the strongest signal was detected using the chicken anti-FL*Tvi*CATL$_{C25A}$ IgY antibody. This is expected as this particular antibody was made against the same antigen, with only one amino acid mutated in the entire sequence.

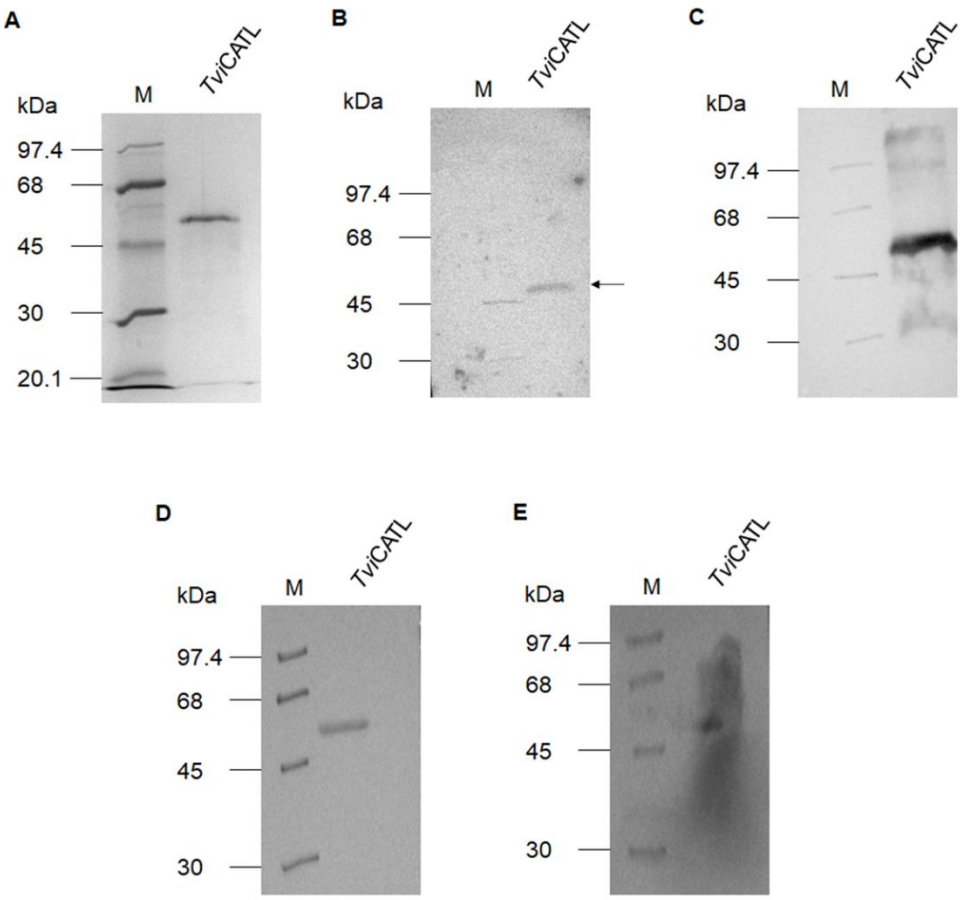

Figure 3.9: Western blot analysis of cross-reactivity between chicken anti-trypanosome CATL antibodies and *Tvi*CATL. *Tvi*CATL separated on a 12.5% SDS-PAGE gel and **(A)** stained with Coomassie blue R-250 or transferred onto nitrocellulose and probed with **(B)** anti-*Tvi*CATL N-terminal peptide IgY, **(C)** chicken anti-FL*Tvi*CATL$_{C25A}$ IgY, **(D)** chicken anti-*Tco*CATL N-terminal peptide IgY and **(E)** chicken anti-*Tbb*CATL IgY (all 5 µg/mL). Rabbit anti-chicken IgY HRPO secondary antibody (1:5 000) and 4-chloro-1-naphthol·H_2O_2 were used as the detection system. M: homemade molecular weight marker.

A sequence alignment of cathepsin L-like proteases from *T. cruzi*, *T. vivax*, *T. congolense* and *T. b. brucei* was done using Clustal Omega (Figure 1.10). *Tvi*CATL shares 62% identity with the homologue from *T. congolense* and 64% identity with that from *T. b. brucei* (Table 3.1). This explains the ability of the anti-*Tco*CATL and anti-*Tbb*CATL antibodies to detect the *Tvi*CATL antigen and hence explaining the cross-reactivity observed in the western blot.

Table 3.1 Sequence identities of cathepsin L-like proteases from *Trypanosoma* spp.

	CATL sequence identities			
Sequence	*Tcr*CATL	*Tvi*CATL	*Tco*CATL	*Tbb*CATL
*Tcr*CATL	100%	63%	55%	57%
*Tvi*CATL	63%	100%	62%	64%
*Tco*CATL	55%	62%	100%	70%
*Tbb*CATL	57%	64%	70%	100%

3.6 ELISA for detection of *Tvi*CATL in *Tvi*CATL-spiked bovine serum samples

The coating conditions were optimised for an ELISA to detect recombinant *Tvi*CATL spiked into bovine serum with chicken anti- FL*Tvi*CATL$_{C25A}$ IgY. A comparison between coating plates with PBS or serum spiked with recombinant *Tvi*CATL, showed a 'plasma matrix effect' for the serum samples evidenced by low absorbance values when compared to antigen in PBS (Figure 3.10, A). Therefore, to overcome this effect, spiked serum was diluted (1 in 1, 1 in 10, 1 in 20, 1 in 50, 1 in 100 and 1 in 200) with PBS (or PBS containing 0.1, 0.5 or 1% (v/v) PBS-Tween). A more diluted recombinant *Tvi*CATL-spiked serum sample (1 in 200) in PBS containing 1% (v/v) Tween-20 resulted in the best differentiating absorbance signal (Figure 3.10, B).

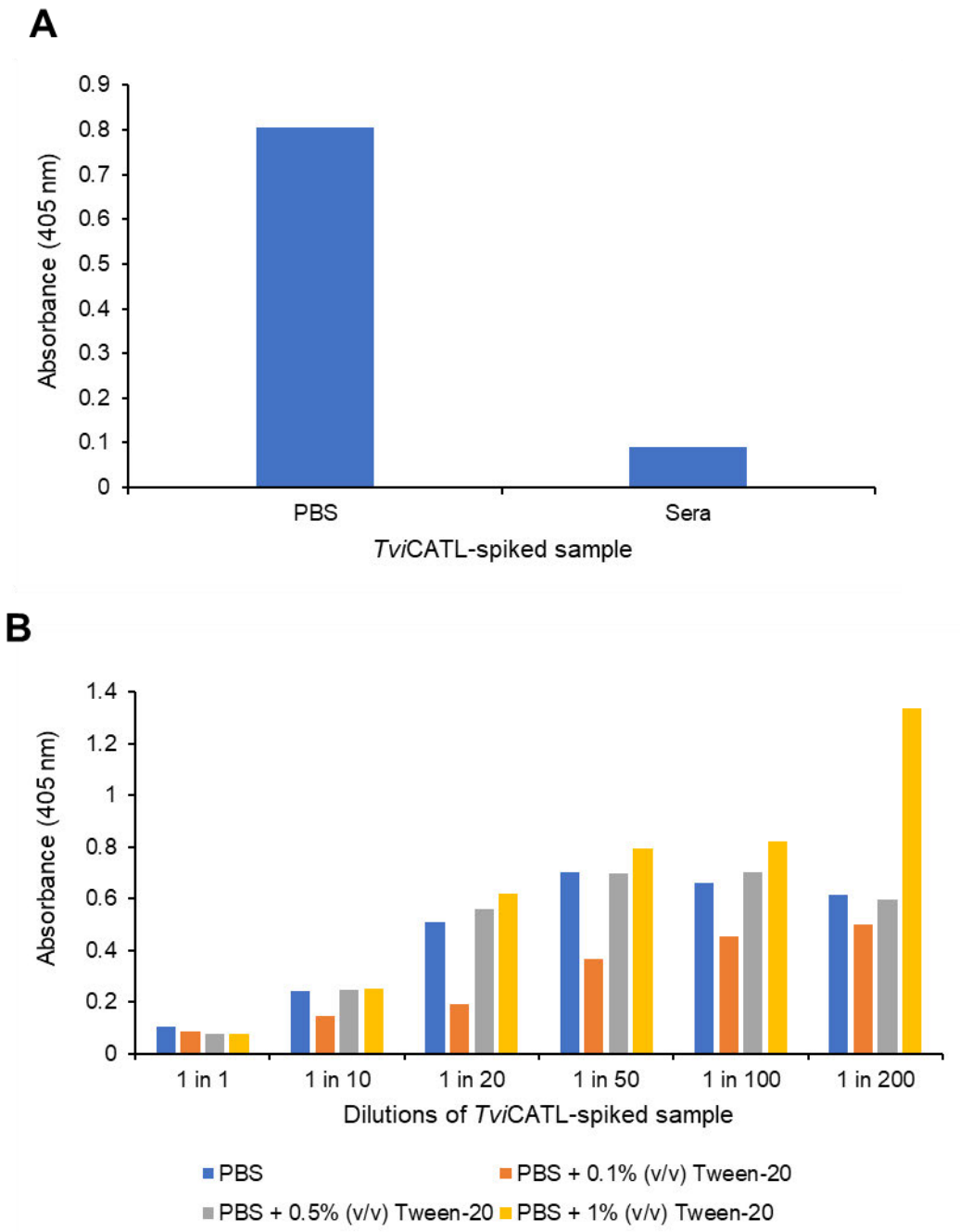

Figure 3.10: Optimisation of coating and buffer concentration in serum spiked with *Tvi*CATL antigen. ELISA plates were coated with **(A)** PBS or serum spiked with *Tvi*CATL (1 µg/mL) or **(B)** *Tvi*CATL-spiked serum dilutions (1 in 1, 1 in 10, 1 in 20, 1 in 50, 1 in 100 or 1 in 200) diluted in PBS (or PBS containing 0.1, 0.5 or 1% (v/v) PBS-Tween). Chicken anti-FL*Tvi*CATL$_{C25A}$ IgY (5 µg/mL) was used as the primary antibody; and rabbit anti-chicken IgY HRPO secondary antibody (1:10 000) followed by ABTS·H_2O_2 were used as the detection system. The absorbance reading at 405 nm represent the average of duplicate experiments.

3.7 Selection of scFvs specific to *Tvi*CATL

The selection of scFvs against recombinant *Tvi*CATL was achieved by using the *Nkuku*® phagemid library. Enrichment of phages occurred during each round of panning when phage antibody clones recognise the *Tvi*CATL antigen. Intrinsic washing allows unbound phages to be washed away and bound phages are amplified (Figure 1.13). Low levels of enrichment were obtained when blocked with fat free milk powder compared to that of BSA (Figure 3.11). Signals were low when using fat free milk powder for blocking even after four rounds of panning and stringent washing to remove non-specific binders. Enrichment of phages were, however, achieved when blocked with BSA. Increasing signals were observed from pans 1 to 4. *Tvi*CATL-specific scFvs occurred at pan 3 and even higher at pan 4.

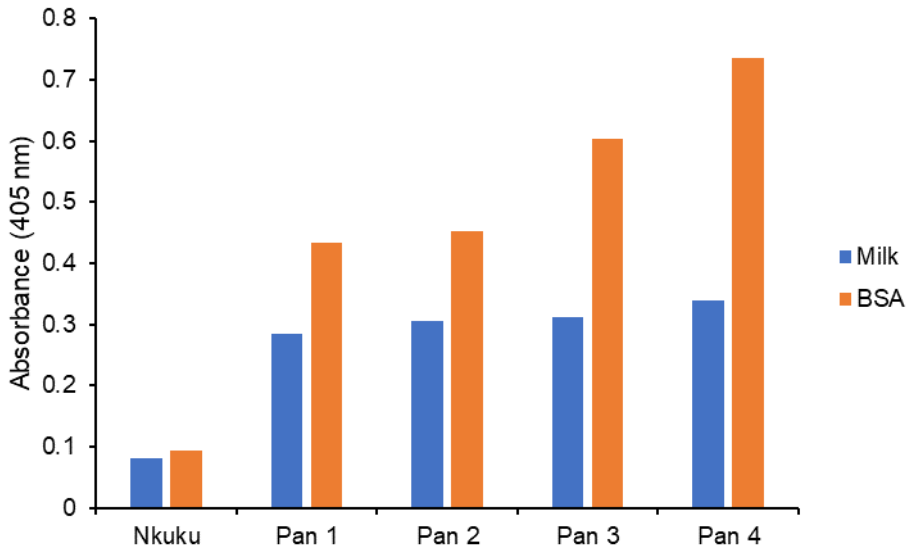

Figure 3.11: Enrichment of panned phages by polyclonal ELISA. The wells of an ELISA plate were coated with *Tvi*CATL (10 µg/mL) and blocked with either 2% (w/v) fat free milk powder-PBS or 3% (w/v) BSA-PBS. Phages isolated from each round of panning ($1x10^{12}$ pfu/mL) was incubated in the plate followed by incubation with mouse anti-M13 IgG (1:8 000). Goat anti-mouse HRPO conjugate (1: 1 000) and ABTS·H_2O_2 were used as the detection system. The absorbance reading at 405 nm represent the average of duplicate experiments after 60 min development.

Enrichment of phages was observed during rounds 3 and 4 of panning, using BSA as the blocking agent. These phages were used for the screening of monospecific phage antibodies by ELISA (Figure 3.12). Clones 8 and 14 (•) (Figure 3.12, A) had high signals when using colonies from pan 3. For colonies from panning done with pan 4 clones, clones 1, 24, 25 and 44 (•) had promising signals (Figure 3.12, B)

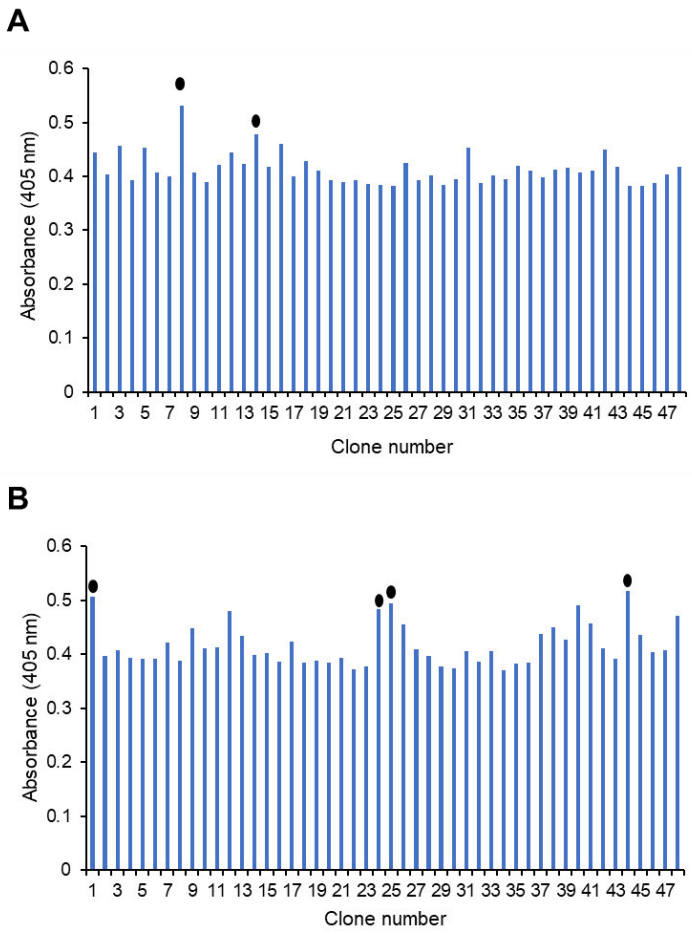

Figure 3.12: Monospecific ELISA for the screening of phage-displayed binders. Forty-eight colonies from **(A)** pan 3 and **(B)** pan 4 blocked with 3% (w/v) BSA-PBS were incubated in a coated ELISA plate with *Tvi*CATL (10 µg/mL) and probed with mouse anti-M13 IgG (1:8 000). Goat anti-mouse HRPO conjugate (1: 1 000) and ABTS·H_2O_2 were used as the detection system. The absorbance reading at 405 nm represent the average of duplicate experiments after 60 min development.

Chapter 4

General discussion

Human and animal African trypanosomiasis (HAT or sleeping sickness and AAT or nagana) are blood-feeding insect-borne parasitic diseases. The causative agents of HAT are *Trypanosoma gambiense* in west and central Africa and *T. rhodesiense* in east and southern Africa; while *T. b. brucei*, *T. congolense* and *T. vivax* are the animal infective species (Brun *et al.*, 2010). African trypanosomiasis places a major economic burden on the continent, with an estimated loss of US $5 billion a year and $30 million spent on treatment (Angara *et al.*, 2014). These parasites (with the exception of *T. vivax)* are transmitted by the tsetse fly (*Glossina* spp.) in sub-Saharan Africa. Since *T. vivax* develops in the insects' proboscis, it is spread mechanically by other haematophagous flies such as horse flies *(Tabanus* spp.) and stable flies (*Stomoxys* spp.) to other continents including South America (Vieira *et al.*, 2017)

Through antigenic variation and the deleterious effects trypanosomes have on the host's B-cell memory compartment, the parasite is able to escape the host's immune system which has made vaccine development unlikely (Magez *et al.*, 2021). Consequently, research has been aimed at treatment and control measures for trypanosomiasis. The control strategies for curbing the spread of AAT include vector control, using trypanotolerant cattle breeds and trypanocidal drugs. Vector control methods such as insecticide spraying, fly traps and the sterile insect technique had moderate success and insecticides have a harmful environmental impact (Vreysen *et al.*, 2013). Although trypanotolerant cattle breeds are more resistant to infection, their small size makes them less attractive to farmers. In contrast to the recently introduced fexinidazole and benzoxaborole SCYX-7158 for *gambiense*-HAT (Deeks, 2019), no new veterinary trypanocides have been introduced for more than 60 years and there are increasing reports of drug resistance (Giordani *et al.*, 2016). The identification of new drug targets for the control of AAT is therefore essential.

Effective disease control requires accurate diagnosis of trypanosomiasis because 1) many drugs have adverse side effects, and 2) increasing reports of drug resistance will allow the parasite to become resilient thus surviving in the host (Giordani *et al.*, 2016). Parasitological detection of trypanosomes in infected blood via microscopy has low sensitivity and is not species specific and therefore an accurate diagnosis cannot be made. Molecular diagnostic tests such as PCR and LAMP are highly sensitive, however these techniques require sophisticated equipment that depend on a constant electricity supply and highly skilled laboratory technicians. An appropriate

diagnostic test should be user-friendly, cost-effective and rapid and such test which meet these requirements, and more are rapid diagnostic tests. Currently used RDTs are SD Bioline HAT 1.0 and HAT Sero *K*-SeT (Büscher, 2014) however, both tests are only for the detection of *T. b. gambiense* infection and specific antibodies produced against this trypanosome species.

Serodiagnosis of AAT is also achieved by using ELISA-based antibody detection tests. However, since antibodies remain in circulation long after cure, these tests are not able to detect current infections which is species specific, sensitive, and cost-effective. Alternatively, a sandwich ELISA, or antigen detection ELISA, could detect circulating antigen in the bloodstream of the infected host and hence will be able to detect current infections. The currently used tests, however, have their limitations. Both ELISAs, the antibody detection and antigen detection ELISA, use whole trypanosomal lysate as the antigen, which is difficult to prepare and lacks standardisation (Greiner *et al.*, 1997; Rebeski *et al.*, 1999). Alternative antigens, such as trypanosomal cysteine proteases with potential diagnostic properties, have been identified and produced using recombinant technology (Eyssen *et al.*, 2018).

African trypanosomal cysteine proteases are expressed throughout the parasite life cycle and have essential roles in parasite survival (Mbawa *et al.*, 1992; Sajid and McKerrow, 2002). Cathepsin L-like proteases from *T. congolense* (*Tco*CATL) and *T. vivax* (*Tvi*CATL) are released into host bloodstream by dying parasites (Ximba, 2014). A study by Authié *et al.* (2001) showed that trypanotolerant cattle had a higher antibody response to *Tco*CATL compared to susceptible cattle; and host antibodies were detected in serum of cattle infected with *Tvi*CATL and not in in uninfected cattle (Ximba, 2014). These finding suggest that these proteases are potential candidates for the development of diagnostic tools.

Due to antigenic variation and hence the unlikelihood of vaccine development; toxic drugs and drug resistance; and the current limitations and challenges of diagnosis, an alternate approach was considered to combat these shortcomings and the result was focussing on the cathepsin L-like cysteine protease expressed by *T. vivax*, *Tvi*CATL, as a drug and diagnostic target. Therefore, the overall aim of this study was to recombinantly express and enzymatically characterise *Tvi*CATL, and to produce phage-displayed single chain fragment variable (scFv) antibodies specific for *Tvi*CATL to be used in antigen-based serodiagnosis of AAT caused by *T. vivax.*

The use of recombinant technology for the production of protein antigens as supposed to isolation from native parasite lysate has many advantages: it is standardised, not hazardous and difficult to prepare, and recombinant protein can be produced in sufficient quantities using various

eukaryotic and prokaryotic expression systems (Büscher, 2014). In this study, *Tvi*CATL was expressed using the *E. coli* expression system. The *Tvi*CATL gene, ligated into the pET-28a expression vector, consisted of the full length coding region from parasitic DNA comprising of the propeptide, catalytic domain and the C-terminal extension that is unique to trypanosome cysteine proteases (Eyssen *et al.*, 2018). *Tvi*CATL was previously expressed using the *Pichia pastoris* yeast expression system resulting in the expression of an inactive pro-enzyme (Ximba, 2014). Purification with three phase partitioning and molecular exclusion chromatography were used in that study leading to two protein bands of 29 and 33 kDa. Due to the secreted protein being asparagine-glycosylated, two protein bands were observed, and this is expected when using the *P. pastoris* expression system that incorporates this post-transitional modification (Macauley-Patrick *et al.*, 2005), and acidification of the expression supernatant resulted in a mature active enzyme. Using the same recombinant expression system and purification method, Eyssen (2018) also obtained recombinant *Tvi*CATL that showed the 28 and 32 kDa protein bands on reducing SDS-PAGE. However, due to providing a quicker method to express proteins and higher levels of recombinant protein produced during small scale-expression due to the strong T7 promoter (Bill, 2014) associated with the *E. coli* expression system, this expression system was utilised in the present study. pET vectors such as the pET-28a vector is desirable for recombinant protein expression in *E. coli* due to the small His-tag which consequently allows for an easier purification method such as nickel affinity chromatography. *Tvi*CATL was therefore expressed using the pET-28a vector and in an *E. coli* host as an insoluble protein and purified using nickel chelate column under denaturing conditions.

Despite favourable protein expression levels, protein produced using the pET-28a vector are often expressed within inclusion bodies, which is a result of misfolded protein due to hydrophobic residue interaction and therefore resulting in protein aggregation (Wetzel, 1994; Fink, 1998). Although expression of recombinant protein in inclusion bodies is not ideal as the expressed protein will require solubilisation and refolding to be functional, collection of inclusion bodies by centrifugation represents a purification step in itself. In this study solubilisation was carried out using anionic SDS and sarkosyl detergents as conducted by Schlager *et al.* (2012), after inclusion bodies were obtained by centrifugation of the culture medium, followed by sonication. Solubilisation using urea could also have been used, however, it is a strong protein denaturant and requires many dialysis steps which also lead to protein loss (Singh *et al.*, 2012). Following solubilisation, on-column refolding and purification of *Tvi*CATL was performed on a nickel affinity resin using 250 mM imidazole, with sarkosyl, a mild detergent used to refold proteins.

Although solubilisation results in an unfolded, functional protein the use of SDS, sarkosyl and DTT, a reducing agent, causes significant amounts of *Tvi*CATL to be eluted in the unbound fraction during nickel affinity purification. This could be due to nickel ions being removed from the affinity matrix due to repeated use of the resin in the presence of detergents and reducing agents (Palmer and Wingfield, 2012). An alternate to nickel purification is Co^{2+}carboxylmethyl-aspartate affinity resin (Bornhorst and Falke, 2000), however this method is not favourable as the cobalt resin has a lower affinity for the polyhistidine affinity tag hence resulting in the elution of the tagged protein.

The *Tvi*CATL His-tagged fusion protein migrated as a 47.4 kDa protein on a reducing SDS-PAGE gel where protein was detected within the first five fractions eluted, using 250 mM imidazole, from the nickel affinity resin. This was confirmed by performing a western blot using mouse anti-His IgG primary antibody, which detected protein in the insoluble pellet as well as in the first five eluted fractions. The insoluble pellet may also contain other *E. coli* cellular proteins that are rich in histidine and are therefore co-eluted with the target protein due to their affinity for nickel ions (Schmitt *et al.*, 1993; Bornhorst and Falke, 2000).

Enzymatic characterisation of trypanosomal cysteine parasites is of utmost importance for the design of anti-trypanosomal therapeutic drugs and includes: determining substrate specificity, interaction with potential inhibitors and optimum pH for substrate hydrolysis. The substrate specificity of a protease depends on the characteristics of amino acid residues, described as occupying the P_1, P_2, P_3 etc. and/or $P_{1'}$, P_2' or P_3' positions on a peptide substrate relative to the sessile bond (Schechter and Berger, 1967). A number of substrate peptide sequences with different amino acid residues in P_1 to P_3 were subjected to hydrolysis by *Tvi*CATL to determine its substrate preference. Cysteine proteases have a S_2 pocket, which is complimentary to the P_2 substrate binding site (Figure 1.7), that determines substrate specificity and has a preference for bulky, hydrophobic residues (Barrett *et al.*, 1982). This preference was confirmed in the present study by the enzymatic activity shown by *Tvi*CATL hydrolysing substrates which have hydrophobic residues (preferably Phe or Leu) in P_2. The greatest activity was shown using H-D-Ala-Leu-Lys-AMC, with almost similar results shown using H-D-Val-Leu-Lys-AMC which indicates that *Tvi*CATL prefers substrates with Leu>Phe in P_2, further supported by Z-Phe-Arg-AMC showing the third best fluorescence signal. The inability of the enzyme to hydrolyse the aminopeptidase substrate, H-Leu-AMC, supports the fact that an amino acid residue is required in the P_2 position of the substrate. Furthermore, basic residues (Arg or Lys) in the P_1 position are also preferred and this was supported by H-D-Ala-Leu-Lys-AMC, D-Val-Leu-Lys-AMC, Z-Phe-

Arg-AMC and Z-Pro-Arg-AMC all displaying relatively high arbitrary fluorescence units. The ability of *Tvi*CATL to hydrolyse Z-Pro-Arg-AMC is notable as Z-Pro-Arg-AMC is the peptide substrate of choice for mammalian cathepsin-K, which refers Pro at P_2 (Choe *et al.*, 2006). A structurally constrained amino acid such as proline in the P_3 position of the substrate indicates a low preference and hydrolysing potential of *Tvi*CATL, even with Phe in the P_2 position as seen with the substrate, H-Pro-Phe-Arg-AMC. Also in agreement with the preference for a hydrophobic amino acid in the P_2 position are studies reported by Eyssen *et al.* (2018) for the native *Tvi*CATL, recombinant *Tco*CATL (Pillay *et al*, 2010), recombinant *Tcr*CATL (Eakin *et al*, 1992; Nery *et al*, 1997) and *Tbb*CATL (Troeberg *et al.*, 1996; Lonsdale-Eccles and Grab, 2002). Based on the S_2 pocket substrate specificity, high hydrolysis by *Tvi*CATL and availability in the laboratory, H-D-Ala-Leu-Lys-AMC was the substrate of choice and used for further experiments.

Several reversible and irreversible inhibitors decreased or inhibited the activity of *Tvi*CATL. Inhibition by reversible aldehyde inhibitors chymostatin, antipain and leupeptin decreased *Tvi*CATL activity by 40%, 65% and 70%, respectively. These reversible aldehyde inhibitors contain a basic arginine residue in P_1 which confirms that cysteine proteases prefer basic arginine residues in P_1. Similar results are that of *Tvi*CATL (LE PAPER 2018), *Tco*CATL (Pillay *et al*, 2010) and *Tcr*CATL (Garcia *et al*, 1998). The enzyme was insensitive towards inhibition by bestatin, with almost 85% activity retained, explained by the fact that bestatin contains Phe in P_1. Validation towards *Tvi*CATL being a cysteine protease is supported by the inhibition of *Tvi*CATL hydrolytic activity by thiol specific irreversible inhibitors, E-64 and iodoacetate, which diminished residual activity by 90% and 75%, respectively. Partial inhibition was achieved using Z-Gly-Leu-Phe-CMK, with the protease showing only 50% activity, and this furthers shows the importance of Leu in P_2 for effective binding in the S_2 pocket by the enzyme (Eyssen *et al.*, 2018).

The hydrolysis of H-D-Ala-Leu-Lys-AMC by *Tvi*CATL was tested to determine the pH optimum using constant ionic strength AMC buffers across a pH range. *Tvi*CATL showed optimal activity against the peptide substrate between pH 6.5 and pH 7.5 These results are similar to the study by Eyssen *et al.* (2018) where recombinant and native *Tvi*CATL functioned optimally around pH 7.0 and pH 7.5, respectively. The pH optima around physiological pH suggest that *Tvi*CATL is active in the host bloodstream following parasite lysis, and *Tvi*CATL could be a virulence factor. *Tvi*CATL exhibited approximately 70% activity at an acidic pH, which is comparable to the activity of *Tbr*CATL and *Tcr*CATL (Caffrey *et al.*, 2001) and indicates the lysosomal localisation of trypanosomal proteases within the parasite. Above pH 8.0, activity decreased considerably.

The second objective of the present study was to devise a test for serodiagnosis of trypanosomiasis based on *Tvi*CATL and specific anti-*Tvi*CATL antibodies. To this end optimal antibody and antigen concentrations and reactivity were determined in an indirect ELISA format. In addition, an alternate antibody production method was explored using the *Nkuku*® phage display library for use in an antigen-detection diagnostic test. Other ways of antibody production for trypanosomiasis diagnostics include producing nanobodies. Nanobodies are favourable because of their small size and low immunogenicity (Stijlemans *et al.*, 2017; Kariuki and Magez, 2021).

Currently, diagnosis of AAT is performed using ELISAs such as the sandwich, indirect and direct ELISA. Some of the technical difficulties faced is the use of whole trypanosomal lysate as the antigen which is difficult to prepare and needs to be stored frozen which is sometimes a challenge in countries which are affected by trypanosomiasis. Bossard *et al.* (2021) tested the effect of freeze-drying reagents and their results demonstrated that freeze-drying reagents had no impact. This will enable shipping antigens at a low-cost and enabling their safety.

Monospecific antibodies are important for antigen-detection diagnostic tests for animal African trypanosomiasis, and previously produced in-house produced antibodies were evaluated for their application in the serodiagnosis of AAT (Büscher and Lejon, 2004). Chickens are excellent high-yield antibody producers. Since the antibodies are isolated from the egg yolk compared to blood serum (when using mice, goats, or rabbits) this is also a more humane way to produce antibodies. Due to the evolutionary distance between birds and mammals (or parasites, in this case) the immunogenicity of protein antigens is also higher in chicken (Carlander *et al.*, 1999; Schade *et al.*, 2005).

In this study, an ELISA was optimised to determine the optimal concentration of primary antibody that should be used to detect *Tvi*CATL in western blots and in mock-infected bovine serum. A checkerboard ELISA was performed and two different concentrations (1 µg/mL and 0.5 µg/mL) of recombinant, purified *Tvi*CATL were incubated with various concentrations of two antibodies; chicken anti-*Tvi*CATL N-terminal peptide IgY and chicken anti-FL *Tvi*CATL$_{C25A}$. The optimal conditions arrived at 1 µg/mL antigen (*Tvi*CATL) with 5 µg/mL of either antibody was the baseline used for other ELISAs and western blots.

Antibodies against recombinantly expressed *Tvi*CATL, *Tco*CATL and *Tbb*CATL were evaluated by western blot to determine cross-reactivity with *Tvi*CATL. As treatment is not specific for infection by different trypanosome species, it is not critical if the test is parasite-specific. However,

these tests can be beneficial for the development of rapid tests, where any animal or human *Trypanosoma*-infective parasite can be used as the standard antigen for detecting circulating antibodies (Desquesnes *et al.*, 2022). This is seen by chicken anti-*Tvi*CATL N-terminal peptide IgY and chicken anti-FL*Tvi*CATL$_{C25A}$ IgY readily detecting the *Tvi*CATL antigen on western blots, as expected. This finding is also supported by the observed cross-reactivity of chicken anti-*Tco*CATL N-terminal peptide IgY and chicken anti-*Tbb*CATL IgY with *Tvi*CATL. This result was not unexpected as *Tco*CATL and *Tbb*CATL share 62% and 64% sequence identity respectively to *Tvi*CATL (Table 3.1). Although trypanosomal antibodies are not specific due to this cross-reactivity, sensitivity is however important and should differentiate trypanosome infection from those by other cattle infective organisms such as *Anaplasma*, *Babesia* and *Theileria* (Desquesnes *et al.*, 2022). In a study conducted by Bossard *et al.* (2010), anti-*T. congolense* antibodies cross-reacted with sera from *T. vivax* and *T. brucei*; and cross-reactivity of *T. vivax* GM6 antigen with *T. congolense*-infected sera was observed when performing diagnostic assays (Pillay *et al.*, 2013). Further studies can be conducted in this field where antibodies from different Kinetoplastida can be exploited.

An antigen detection ELISA was optimised to test if the *Tvi*CATL antigen could be detected in bovine sera spiked with *Tvi*CATL. Various other studies have used proteins from other trypanosomes in antigen detection ELISAs, or tests which use the concept of an ELISA. Boulangé *et al.* (2017) developed an RDT for the serodiagnosis of *T. vivax* and *T. congolense* infection using proteins expressed by both parasites. In a study reported by Sullivan *et al.* (2013) developed a test for the diagnosis of HAT using recombinant ISG65. Although these tests showed promising results, both detect antibodies and can therefore not distinguish between current and past infection since antibodies remain in circulation long after cure. Therefore, an objective of this study was to design an antigen detection ELISA to detect current infections. Thus, various coating (bovine serum spiked with recombinant *Tvi*CATL) and buffer concentrations were tested, and an optimal concentration was found to be a 1 in 200 dilution of 1 µg/mL *Tvi*CATL -spiked sera diluted in PBS containing 1% (v/v) Tween-20 that gave the highest absorbance reading at 405 nm. The positive control (PBS with *Tvi*CATL antigen) resulted in a higher reading than that of the test (serum spiked with *Tvi*CATL antigen). However, the antibodies did detect antigen in the spiked serum (test). These results indicate that *Tvi*CATL-specific antibodies are able to detect *Tvi*CATL antigen in blood in an indirect ELISA, and that this concept is promising in the field of serodiagnosis of trypanosomiasis. Although results were obtained, it should be noted that low absorbance values were obtained, and more studies should be conducted to validate these results.

To increase the sensitivity of an antigen-detection ELISA, an antigen capture (sandwich) format ELISA (Figure 4.1) which can detect circulating trypanosomal antigens in the blood of an infected animal would be desirable. This approach requires antibodies that could be coated on the plate to capture the trypanosome-derived antigen that is detected by a second enzyme-labelled antibody in an ELISA.

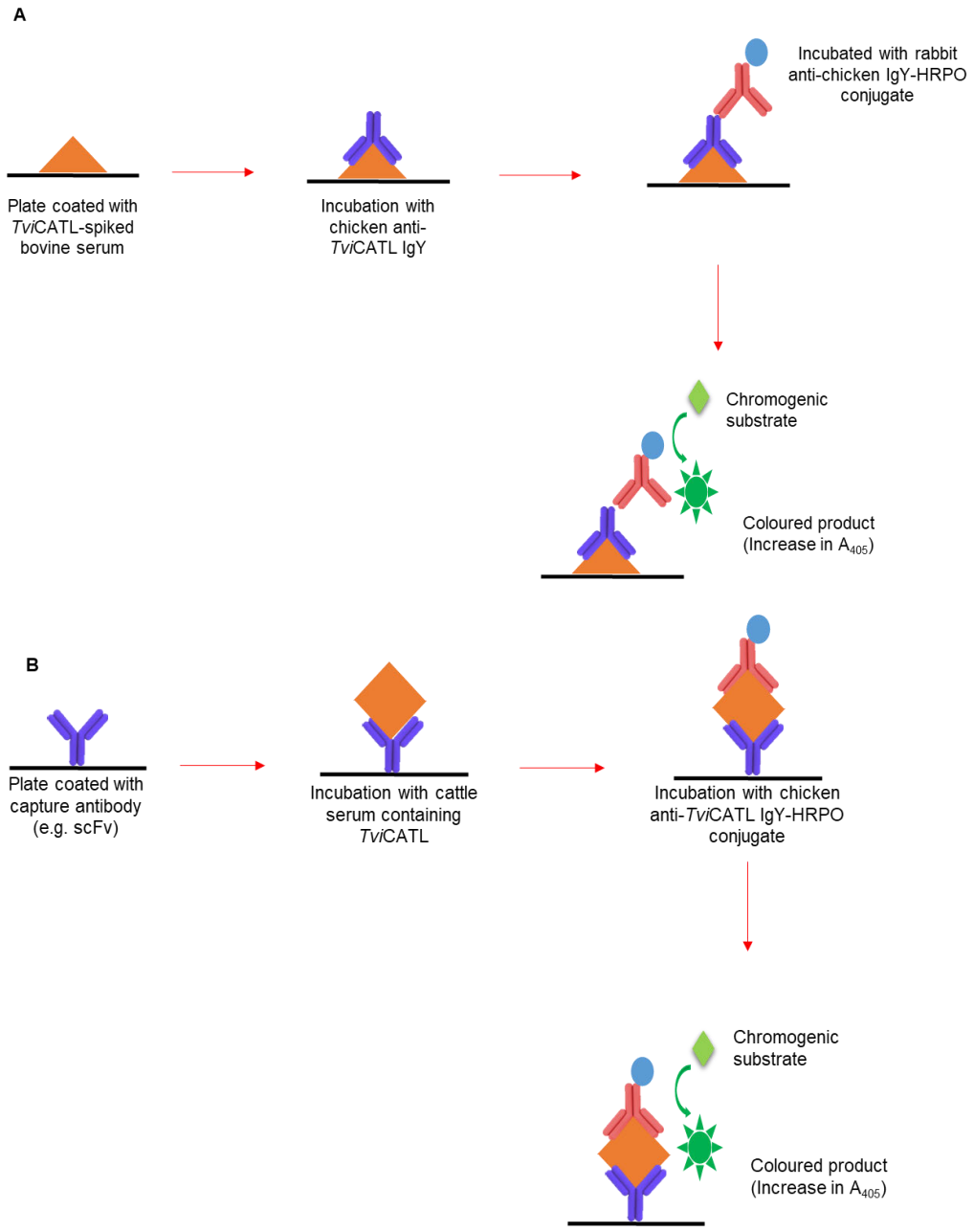

Figure 4.1: Schematic of (A) indirect ELISA and (B) sandwich ELISA for the detection of *Tvi*CATL in cattle serum

A different approach to using animal-produced antibodies is the use of phage display. The single chain variable fragment (scFv) antibodies have been produced and used in the development of ELISA diagnostic tests for bluetongue virus (Fehrsen *et al.*, 2005; Rakabe *et al.*, 2011) and African horse sickness (Van Wyngaardt *et al.*, 2004; Van Wyngaardt *et al.*, 2013).

In the present study, scFvs with a high affinity and specificity to *Tvi*CATL were selected from the *Nkuku*® phage display library (Van Wyngaardt *et al.*, 2004) through four rounds of panning. During a round of panning, proteins or antibody fragments are selected according to their binding affinities; and enrichment occurs when phage antibody clones recognise the antigen of interest. In this study, after four rounds of panning, enrichment of phages occurred as determined by polyclonal ELISA. Additionally, a comparison between milk and BSA as the preferred blocking agent showed greater enrichment using BSA as the blocking agent. Although enrichment of the polyclonal phages occurred during round four of panning, the signal was very low, but this was still higher than that of the unpanned *Nkuku*® phages which was used as a control.

Randomly picked bacterial clones, which were titered through exposure to bacterial TG1 cells and plated out, from pans three and four were evaluated using a monospecific ELISA. The specificity of these clones was tested in an ELISA due to the ability of a clone to successfully recognise the *Tvi*CATL antigen coated on the plate. Although several clones from pans three and four showed a signal, these results were very low.

Nanobodies, which are antibody constructs derived from the variable domains of camel antibody heavy chains, were successfully used as capture antibodies for the detection of *T. congolense* in lateral flow assays (Pinto Torres *et al.*, 2018). Similarly, when optimised, scFvs could be used as capture antibodies in lateral flow assays as well, for the detection of *T. vivax* infections.

In the present study, *Tvi*CATL was recombinantly expressed using the *E. coli* expression system where sufficient amounts of protein was achieved to conduct characterisation studies. Through purification and dialysis, the protease was purified to homogeneity for use in subsequent experiments. To test potential inhibitors for drug design as treatment for trypanosomiasis, enzyme characterisation was done. Secondly, *Tvi*CATL and chicken anti-trypanosome antibodies were evaluated in western blots and an antigen-detection ELISA for their potential as a species-specific diagnostic target. Rather than an antibody detection ELISA, an antigen detection ELISA is beneficial as current infections would be detected due to the circulating antigens in the bloodstream. This method can use chicken anti-*Tvi*CATL IgY, or alternately *Tvi*CATL-scFv specific antibodies. This study paved the way for *Tvi*CATL as a chemotherapeutic target; and the

application as antigen towards the development of an antigen detection ELISA for the serodiagnosis of AAT.

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