Evolutionary relationship between *Trypanosoma evansi* and *Trypanosoma brucei* with respect to specific mitochondrial antigen and phenotype knockout analysis

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A thesis submitted in partial fulfillment of the requirements for the Degree of Master of science in Microbiology of Kenyatta University.

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university or any other award.

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I thank thee, Father, Lord of heaven and earth. In appreciative memory of my parents Sephania and Jedida obanda. Dedicated to my wife Grace and my children, Jedida, Neema and Sifa.
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DEFINITION OF OPERATIONAL TERMS

**Heteroxenous:** involving an invertebrate and a vertebrate host.

**Monoxenous:** development only in a single species (usually an arthropod).

**Hemoflagellates:** development in the blood or fixed tissues of vertebrates.

**Kinetoplast:** a disk-shaped, DNA containing body within the large mitochondrion.

- kDNA: can make up to 25% of total DNA. Maxi circles (when edited, coding for mitochondrial proteins), Minicycles (coding for guide RNA for RNA editing).

**Kinetosome:** centriole from which an axoneme arises.

**Flagellar pocket:** A depression on the parasite surface, where a flagellum arises, undulating membrane, surface coat (glycocalyx).

**Salivaria:** trypanosomes that develop in the anterior parts of digestive tract of the vector and are transmitted from the vector to the vertebrate host by injection during blood feeding.

**Stercoraria:** Parasites develop in the hindgut of the vector and transmission of the parasites from the vector to the vertebrate host is through fecal contamination.

**Trypomastigote:** kinetoplast and kinetosome at the posterior end, flagellum runs along the surface. Typical of *Trypanosoma* in the blood stream and metacyclic form in the vector’s salivary gland.
Epimastigote: kinetoplast and kinetosome are between the nucleus and anterior end, a short undulating membrane lies near the base of the flagellum (Trypanosoma development in tsetse midgut).

Cryptogene: A gene whose transcript is edited.

Guide RNA or gRNA: A short 3'-uridylated RNA that can form a perfect duplex (except for the oligo [U] tail) with a stretch of mature edited mRNA.

Anchor duplex: the RNA duplex formed by hybridization of the 5' end of the gRNA and the mRNA sequence just downstream of the first editing site in an editing block.

Pre-edited region or sequence: Sequence that will be edited in the mature RNA.

Unedited region or sequence: Sequence that is never edited.

Editing block: Edited mRNA sequence mediated by a single gRNA.

Editing domain: Edited mRNA sequence mediated by 2 or more overlapping gRNAs.

Mature edited mRNA: A completely precisely edited mRNA.

Partially edited mRNA: An mRNA edited only in the 3' region.

Misedited sequence: Incorrectly edited sequence.

Pan-edited gene: extensively edited.

3' oligo [U] tail: The string of non-encoded U's at the 3' end of the gRNA.

gRNA-mRNA chimeric molecule: A molecule which consists of a gRNA 5' which is covalently linked at the 3' end to an mRNA, usually at an editing site.

RNA Polymerase: Transcribes RNA from DNA.
Post-transcriptional processing: Modification undergone by RNA during or after transcription.

Inducer: A substrate or chemical--related compound, which, along with the regulator protein, promotes the activity of a gene by aiding mRNA Polymerase.

Ligase: An enzyme that catalyses a condensation reaction that link two DNA molecules via the formation of a phosphodiester bond between the 3’hydroxyl and 5’ phosphate of adjacent nucleotides.
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<tr>
<td>Amp</td>
<td>Ampicillin</td>
</tr>
<tr>
<td>ATPase</td>
<td>ATPase Submit 9 Homolog mRNA, Nuclear Gene Encoding Mitochondria Protein.</td>
</tr>
<tr>
<td>C.P</td>
<td>Cysteine Protease</td>
</tr>
<tr>
<td>CSF</td>
<td>Cerebrospinal Fluid</td>
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<tr>
<td>DEET</td>
<td>Diethyltoluamide</td>
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<tr>
<td>DFMO</td>
<td>Difluoromethylornithine</td>
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<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid.</td>
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<tr>
<td>DNDi</td>
<td>Drugs for neglected diseases initiative.</td>
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<tr>
<td>ELISA</td>
<td>Enzyme-Linked Immunosorbent Assay</td>
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<tr>
<td>GFP</td>
<td>Green Fluorescent Protein.</td>
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<tr>
<td>Ggapdh</td>
<td>Glycosomal Glyceraldehyde-3-Phosphate Dehyrogenase</td>
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<td>gRNA</td>
<td>Guide Ribonucleic Acid.</td>
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<tr>
<td>HAT</td>
<td>Human African trypanosomiasis.</td>
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<td>K.E.</td>
<td>Glycerol Kinase</td>
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<tr>
<td>ktDNA</td>
<td>Kinetoplastid DNA.</td>
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<tr>
<td>LB</td>
<td>Luria-Bertani medium.</td>
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<tr>
<td>MP</td>
<td>Mitochondrial Protein.</td>
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<tr>
<td>mRNA</td>
<td>Messenger Ribonucleic acid.</td>
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<tr>
<td>NTTAT</td>
<td>non-tsetse transmitted animal trypanosomiasis.</td>
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<tr>
<td>PCR</td>
<td>Polymerase Chain Reaction.</td>
</tr>
<tr>
<td>RBCs</td>
<td>Red Blood Cells.</td>
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<tr>
<td>REL</td>
<td>RNA editing ligase.</td>
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<tr>
<td>RISC</td>
<td>RNA-Induced Silencing Complex.</td>
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<tr>
<td>RNA</td>
<td>Ribonucleic acid.</td>
</tr>
<tr>
<td>RNAi</td>
<td>RNA Interference</td>
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<tr>
<td>SDS</td>
<td>Sodium Dodecyl Sulfate.</td>
</tr>
<tr>
<td>TCA</td>
<td>Tricarboxylic Acid.</td>
</tr>
<tr>
<td>tRNA</td>
<td>Transfer Ribonucleic acid.</td>
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ABSTRACT

Trypanosomiasis is a disease caused by parasitic protozoans of the genus *Trypanosoma*. The agents of the disease are obligate extracellular parasites that occur in blood, cerebrospinal fluid and tissue fluids. *Trypanosoma brucei* causes sleeping sickness and Nagana in sub-Saharan Africa. *Trypanosoma evansi* causing surra is endemic in Asia, Middle East northern Africa including Northern Eastern Kenya. Salivarian trypanosomiasis is one of the most important and widespread diseases of domestic animals and man in the world. The causes of the re-emergence of this disease include widespread civil war, declining economies, reduced health financing and the dismantling of disease control programs. The current drugs in use are toxic and not effective because of drug resistance, hence, the need for developing new drugs. The study objective was to establish the evolutionary relationship between *T. brucei* and *T. evansi*, with respect to cell differentiation life cycle specific antigens and phenotype knockout analysis. PCR was used to compare genes encoding mitochondrial protein of *T. evansi* IL1695, *T. evansi* IL1934 and *Trypanosoma brucei rhodesiense* IL2343. Plasmid construction, preparation of plasmid DNA was done using alkaline lysis method. Extraction and purification of plasmid was by QIAGEN® plasmid protocol. Cell line of *T. evansi* and *T. brucei* for RNA interference experiments were established. Electroporation was by Gene-Pulse machine for generation of knockout phenotypes. Statistical analysis was by Student’s t-test. *T. evansi* IL1695, *T. evansi* IL1934 and *T. b. rhodesiense* IL2343 contain all the five genes for mitochondrial protein in their genomes. MP 48 and MP 52 RNA editing ligases genes were identified in *Trypanosoma evansi*. Specific RNA ligases MP 48 630 bp and MP 52 560bp primers were developed. These primers specifically identify *T. evansi*, *T. brucei* and *T equiperdum* from other organisms. Alignment of MP 48 and MP 52 gene sequences obtained in *T. evansi* and *T. brucei* show 100 % homology. Comparisons of MP 48 and MP 52 RNA ligase gene with data of closely related organisms available in Genbank® showed no significant homology with the RNA ligase sequences of *T. cruzi*REL and *L. major*REL2 sp. nor with the available sequences of Lt RNA ligase. Multiple alignment of *T. evansi* MP52 and MP 48 with related proteins show a perfect-match with *T. brucei* and near-perfect match of genes with data of closely related organisms available in Genbank. *T. evansi* was able to use T7 promoter gene, to recognize bacteriophage T7 RNA polymerase and produce RNA polymerase that synthesize mRNA encoding Green fluorescent protein, that was observed as Green fluorescent *T. evansi*. Approximately 40% of original populations of both the species were killed due to RNA interference. There was no significant difference in the effect of RNA interference in *T. brucei* Gutat 3.1 and *T. evansi* Tansui 13, using Student’s t-test two-tailed, p> 0.05 at 95% confidence interval. These new sub genus specific primers can be used as a diagnostic tool for monitoring pathogenic *Trypanozoon* parasites in humans, domestic animal. The primers can also be used in epidemiological survey. The RNA interference analysis identified MP48 and MP 52 RNA editing ligases as a drug target for the development of novel therapeutics in treatment of sleeping sickness, nagana and surra.
CHAPTER ONE: INTRODUCTION

1.1. Background

Trypanosomiasis is a disease caused by a parasitic protozoan of the genus *Trypanosoma*. Salivarian trypanosomiasis is one of the most important and widespread diseases of domestic animals and man in the world. The causative agents of the disease are obligate extra cellular parasites that occur in blood, cerebrospinal fluid and tissue fluids. The salivarian trypanosomiasis is further divided into tsetse – transmitted trypanosomiasis and non – tsetse transmitted animal trypanosomiasis (NTTAT).

Tsetse – transmitted trypanosomiasis, *Trypanosoma brucei rhodesiense* and *Trypanosoma brucei gambiense* cause sleeping sickness. Nagana in domestic animals is caused by *T. brucei brucei* and transmitted by tsetse fly. The disease occur in scattered foci throughout the sub-Saharan tsetse belts of Africa-an area of some 10 million sq km. Sleeping sickness is closely related to a cattle infection known as nagana, which restricts cattle rearing in many areas of the continent (WHO, 1979/1986).

Non – tsetse transmitted animal trypanosomiasis (NTTAT); *Trypanosoma evansi* causes a wasting disease of domestic livestock that is called surra. The parasite is transmitted mechanically by biting flies and it does not posses developmental stage in insect vector or a mitochondria (Aratama et al., 1992). Surra is prevalent in almost all tropical and subtropical areas of the world, such as sub Saharan Africa, Asia, Middle East and South America.

Human African trypanosomiasis (HAT) or sleeping sickness, a disease thought to have been largely conquered during the 1960’s, has re-emerged as a serious public health
problem over large swathes of sub-Saharan Africa (Welburn et al., 2001). It has been
estimated that 300,000–500,000 people are currently infected and 100,000 deaths are
caused each year by the disease (Cattand et al., 2001). However, less than 4 million
people are under surveillance and only about 40,000 are diagnosed and treated, due to
difficulty of diagnosis and remoteness of some affected areas (WHO, 1998). These
figures are relatively small compared to other tropical diseases, but African
trypanosomiasis, without intervention, has the propensity to develop into epidemics,
making it a major public health problem (WHO, 1998). Furthermore, the case fatality rate
in untreated patients is 100% (WHO, 1998). In many countries the tsetse and
trypanosomiasis problem has been exacerbated by population pressure, which has forced
pastoralists deeper into high-risk areas with increasing risk of infestation. Partly as a
result of this incursion of man and his animals into tsetse fly habitats there are indications
that fly populations are increasing and that some fly belts are currently expanding (WHO,
1998).

The chemotherapy of African trypanosomiasis relies on a few drugs which have
adverse side effects and are unsatisfactory: pentamidine, suramin and melarsoprol.
Eflornithine is the only alternative registered drug for the treatment of Trypanosoma
brucei gambiense in sleeping sickness patients who do not respond to melarsoprol.
However, apart from other drawbacks, it costs $US 300-500 per patient (WHO, 2000).
The risk of spreading sleeping sickness is closely related to the movement of cattle
populations, and the screening and treatment of livestock to prevent disease spread has
been advocated (Fève et al., 2001). New drugs that are safe, effective and affordable are
needed. New molecular diagnostic tools are extremely useful for elucidation of sleeping sickness and animal trypanosomiasis epidemiology.

MP 52 and 48 RNA Editing ligases were identified as drug targets in *T evansi*. Two new primers were designed from MP 48 and MP 52 RNA editing ligase DNA sequences for PCR diagnosis of infections caused by *T.brucei* and *T evansi*.

1.2. Justification.

Attempts have been made to distinguish *T.brucei* and *T evansi*, since analyses of isoenzyme electrophoresis pattern are similar (Gibson *et al.*, 1980). The sequence analyses of ribosomal RNA genes (rRNA) of these species have also been found to resemble (Urakawa, 1983). The PCR amplification patterns of procyclic acidic repetitive protein (PARP) genes that encode a major surface glycoprotein of procyclic forms of *T. brucei* (Mowatt *et al.*, 1987) were found to be the same with *T evansi*. The well-characterized difference between *T evansi* and *T brucei* was found in the Kinetoplast DNA (kDNA) (Borst *et al.*, 1979). PCR was performed on various mitochondrial (Kinetoplast DNA) protein genes to determine whether *T evansi* is different from *T brucei*. This scientific research was an attempt, to develop Polymerase chain reaction diagnosis, to differentiate *T evansi* from *T. brucei*. These organisms have not been differentiated by any other scientific method with exception of electron microscopy of Kinetoplast DNA (kDNA). RNA interference was used to identifying new chemotherapy target for chemotherapy of diseases caused by *T evansi* and *T brucei*.

1.3. Null hypothesis:

* T. brucei is not evolutionary different from *T evansi*. 
1.4. Main objectives

To establish the evolutionary relationship between *T. brucei* and *T. evansi*, with respect to cell differentiation life cycle specific antigens and phenotype knockout analysis.

1.4.1. Specific objectives

1) To find out the relationship of *T. evansi* and *T. brucei* at genome level, with respect to mitochondrial encoding genes.

2) To establish the presence of MP48 and MP52 RNA editing ligase in *T. evansi*.

3) To determine if T7 RNA polymerase drives the expression of Green Fluorescent Protein gene with the help of T7 promoter in *T. evansi*.

4) To determine the effect of introducing double stranded RNA (ds RNA) MP48 and MP52 ligase genes into *T. evansi* and *T. brucei*.
CHAPTER TWO: LITERATURE REVIEW

2.1. Classification of trypanosomes

The pathogens of the salivarian trypanosomiasis are members of the section salivaria and belong to the genus *Trypanosoma*, (Hoare *et al.*, 1972, Lun *et al.*, 1993, Riou *et al.*, 1979.) which are within the order Kinetoplastida and the class Mastigophora (Appendix 1). It is generally accepted that subgenus *Trypanozoon* is divided into 3 species: *T. brucei*, *T. evansi* and *T. equiperdum*, with *T. brucei* further subdivided into 3 subspecies defined by pathogenicity, distribution and host range (Hoare *et al.*, 1972).

Trypanosomes of subgenus *Nannomonas* are defined by their developmental cycle in the tsetse fly, which involves the midgut and proboscis. As bloodstream forms these trypanosomes are the smallest of the Salivaria, but there is considerable morphological variation, both in dimensions (length and maximum width), and in features such as body shape, prominence of the undulating membrane and presence of a free flagellum (Hoare *et al.*, 1972). Coupled with variation in host range and pathogenicity, Subgenus *Nannomonas* is split into 2 species: *Trypanosoma congolense*, which has a wide range of unregulated hosts, and *T. simiae*, for which pigs are regarded as the most important hosts (Hoare *et al.*, 1972). This simplifies clinical diagnosis: if you find a small trypanosome in the blood of a sick ox, goat or sheep, it will be *T. congolense*, while in pigs with acute trypanosomiasis it will be *T. simiae*. A number of hitherto cryptic subgroups have been discovered by molecular characterization during the past 20 years or so. Only one of these has been described in sufficient detail to warrant acceptance as a new species, *T. godfreyi* (McNamara *et al.*, 1994).
2.2. Distribution of trypanosome

The tsetse-transmitted trypanosomoses are prevalent in a vast region of Africa and cover approximately 10 million km², trypanosomoses distribution overlaps with that of the tsetse fly (WHO 1979/1986). Thirty six countries are locate in the tsetse infested areas (so-called tsetse belt) and approximately 50 million people, 50 million cattle, 30 million sheep and 40 million goats are at risk of infection (Figure 2.1) (WHO, 1979/1986). The non-tsetse transmitted trypanosomes are prevalent in almost all tropical and subtropical areas of the world, such as sub Saharan Africa, Asia, Middle East and South America. The area affected by non-tsetse transmitted trypanosomes NTTAT is approximately 3 times greater than that of the tsetse transmitted trypanosomes (WHO, 1979/1986, Williams et al., 1986).

![Map of trypanosome distribution in Africa](image)

**Figure 2.1. Distribution of sleeping sickness foci in Africa. WHO 1979/1986.**

A. *Trypanosoma brucei gambiense* area of the foci dotted line in west and central Africa.

*B. Trypanosoma brucei rhodesiense* to the right of the line.
B. Distribution of *Glossina morsitans* and *Glossina palpalis* in Africa.
C. Distribution of *Glossina palpalis* in Africa.
D. Distribution of *Glossina tachinodes* in Africa.

2.3. **Morphology of genus *Trypanosoma***

Bloodstream form trypanosomes of the 3 species are morphologically indistinguishable, save for the occurrence of short-stumpy forms in *T. brucei*.

Confusingly, the trait of pleomorphism can be lost in laboratory isolates of *T. brucei*, and they then become indistinguishable from the monomorphic species, *T. evansi* (Figure 2.3) and *T. equiperdum*. On the other hand, in the monomorphic trypanosomes such as *T. congolense*, *T. vivax* and *T. simiae*, there are no distinguishable forms during the infection in animals.

At the functional level pleomorphism reflects the ability of *T. brucei* to develop in its vector, the tsetse fly, and this is in turn dependent on possession of a complete and functional set of genes for mitochondrial operation. The mitochondrial genome is contained in the maxicircle DNA of the kinetoplast of *T. brucei*, together with the set of minicircle-encoded genes necessary for editing the maxicircle transcripts so they can be correctly translated. These features define *T. brucei*, and their absence defines *T. evansi* and *T. equiperdum*. *T. evansi* lacks a mitochondrial genome and its kinetoplast contains only a homogeneous set of mini circles. The few isolates of *T. equiperdum* examined also have missing kinetoplast DNA. One Chinese strain of *T. equiperdum* had maxi circles just over half the size of those of *T. brucei* and homogeneous mini circles like *T. evansi* (Lun et al., 1993).

Two other laboratory strains of *T. equiperdum* also had homogeneous mini circles; one had full-size and one reduced size maxi circles (Riou et al., 1979, Frasch et
al., 1979) *T. brucei* is a spindle shape, flagellated protozoan, which is about 8 to 39 mm in length. It has a centrally placed nucleus and a single unbranched mitochondrion, which extends along the outer margin of the undulating membrane. The flagellum emerges from the cell and there is a flagellar pocket. This is where most of the excretion and pinocytosis takes place. A Mitochondrial genome of the organism is known as the maxicircle kinetoplast, which is located beneath the flagellar pocket (Figure 2.2).

*Trypanosoma brucei* is a polymorphic trypanosome species. Two distinctly different forms can be distinguished, i.e. a long slender form and a short stumpy form. Often, intermediate forms, possessing characteristics of both the slender and stumpy forms, are observed. The cytoplasm often contains basophilic granules in stained specimens.

*Trypanosoma brucei* (long slender form): 17-30 μm long and about 2.8 μm wide, undulating membrane is prominent, free flagellum present at the anterior end, posterior end pointed, kinetoplast small and subterminal. *Trypanosoma brucei* (short stumpy form): 17-22 μm long and about 3.5 μm wide, undulating membrane is conspicuous, free flagellum absent, posterior end pointed, kinetoplast small and subterminal. *Trypanosoma vivax*: 20-27 μm long, undulating membrane is not obvious, free flagellum present at the anterior end, posterior end rounded, and kinetoplast large and terminal. *Trypanosoma congoense*: 8-25 μm (small species), undulating membrane not obvious, free flagellum absent, posterior end rounded, kinetoplast is medium sized and terminal, often laterally positioned. Although *T. congoense* is considered to be monomorphus, a degree of morphological variation is sometimes observed. *Trypanosoma theileri*: 60-70 μm (large species), undulating membrane is conspicuous, long free flagellum present, posterior end pointed, kinetoplast is large and positioned near the nucleus. *Trypanosoma theileri* is
normally nonpathogenic, but its presence can confuse the parasitological diagnosis (Frasch *et al.*, 1979).

Figure 2.2. Structures of *T. brucei*. Source (Adapted From Vickerman, (1969).
2.3.1. Kinetoplastid DNA

Kinetoplastid DNA (ktDNA) is relatively abundant and consists of mini circles and maxi circles. The two types of ktDNA occur in a concatenated mass within the mitochondria (mt) (Figure 2.4). Maxi-circles encode several mitochondrial genes and are more-or-less equivalent to the mtDNA (Borst et al., 1979). Mini circles are heterogeneous and rapidly evolving and their function is less clear. Both mini-circles and maxi circles encode guide RNA genes. Some genes on the maxi circles have ‘errors’
which need to be edited. The guide RNAs assist in RNA editing that takes place in the mitochondria of kinetoplastids. The editing of these ‘cryptogenes’ is believed to occur in a hypothetical ‘editsome’ particle. The extent of editing seems to correlate with different parasite life cycle stages and the corresponding changes in metabolism (i.e., aerobic vs. anaerobic) that is associated with the different life cycle stages. Mini circle DNA is also used for parasite detection and distinguishing different isolate. (Lun et al., 1992, Riou et al., 1979, Frasch et al., 1979).

Figure 2.4: (A.) *T. evansi* mini circle Kinetoplastid DNA. (B). *T.brucei* Maxi cycle and mini circle Kinetoplastid DNA. (Adapted from Borst 1979).
2.4. Transmission of trypanosomes

Non-tsetse-transmitted trypanosomes (NTTAT) are caused by *T. evansi* that is mechanically transmitted by haematophagous flies, but ingestion of meat from infected carcasses by carnivores can result in infections (Luckins *et al.*, 1977). The most important vectors in the field are *Tabanus spp.* *Stomoxys spp.* may be more important in spreading infection in stables due to the presence of large numbers of these flies, whereas in open conditions, tabanids are more important. Although the mode of mechanical transmission is well established, its dynamics are not understood and it is still necessary to define the relationship between the host species, the duration of infection and the level of parasitaemia, the period between feeds and the relative efficiency of different vector species. Other blood-sucking flies such as *Tabanus* and *Stomoxys* also transmit *Trypanosoma vivax* mechanically (Luckins *et al.*, 1977).

Dourine is a chronic trypanosomal disease of Equidae caused by *T. equiperdum*. The disease is transmitted almost exclusively by coitus and is characterized by edematous lesions of the genitalia, nervous system involvement, and progressive emaciation (Williamson *et al.*, 1986).

Both *T. brucei gambiense* and *T. brucei rhodesiense* are biologically transmitted by tsetse flies, and are the pathogens of chronic and acute sleeping sickness in man. *T. congolense*, *T. brucei brucei*, *T. vivax* and *T. simiae* are also tsetse-transmitted trypanosomes, which cause animal African trypanosomiasis called Nagana.
2.4.1. Vectors of trypanosomes

2.4.1.1. Tsetse-transmitted trypanosomes

The tsetse-transmitted trypanosomes are biologically transmitted by tsetse flies (Glossina sp). There are about 30 known species and subspecies of tsetse flies belonging to the genus Glossina. They can be divided into three distinct groups or subgenera: Austenia (G. fusca group), Nemorhina (G. palpalis group) and Glossina (G. morsitans group). Only nine species and subspecies, belonging to either the G. palpalis or the G. morsitans group known to transmit sleeping sickness. Most species fall into one of two major groups represented by G. palpalis and G. morsitans. The palpalis group is associated with riverine ecologies and is frequently found near streams, rivers, and lakes in west and central Africa (Ford et al., 1970). The morsitans group is most frequently associated with savannah woodlands and dry bush country in East Africa. The palpalis and morsitans groups are associated with the transmission of T. brucei gambiense and T. brucei rhodesiense, respectively. The differences in the ecologies and interactions with reservoirs of these two types of tsetse contribute the different manifestations of diseases caused by the two African trypanosome species (Minter et al., 1987).

2.4.1.2. Non-tsetse-transmitted trypanosomes

Tabanids are relatively large stout flies belonging to the sub-order Brachycera. They have acquired various colloquial names including horseflies mainly for Tabanus spp, clegs mainly for Haematopota spp, and deerflies mainly for Chrysops (Burger et al, 1981) Body length ranges from 5-25mm and the compound eyes are well developed. As with most haematophagous diptera it is only the females that take blood in addition to nectar with the males being solely nectar feeders. The principal mechanism for finding
their hosts is sight and so the large eyes serve this function well. There is also evidence that CO₂ acts as an Oduor source especially in some *Chrysops* spp (Burger et al., 1986). At present there are over 3,000 known species of Tabanids. The three main genera of economic importance are the *Chrysops*, *Tabanus*, and *Haematopota*. The three genera differ in their distributions although there is considerable overlap (Abbassian-Lintzen et al., 1964, Chainey et al., 1994). *Tabanus* has a cosmopolitan distribution with *Chrysops* being predominantly holoart and oriental. *Haematopota* is found in the palaeartic, the orient and afrotropical regions. *Tabanus* and *Haematopota* both show similar morphological features in having reduced ocelli and a proboscis, which is shorter than the head. *Chrysops* has a longer proboscis although not longer than the head and the ocelli are functional. Tabanids are major mechanical transmitters of *T. evansi*, causing “surra” in horses, camels, and dogs. It also affects cattle and other mammals but this is to a lesser extent.

Other trypanosomes transmitted include *T. vivax viennei*, in cattle and sheep, *T. simiae* in pigs, *T. theileri* of cattle (Krinsky et al., 1976).

2.5. Life cycle of *Trypanosoma brucei*

The trypanosome life cycle involves complex development of morphological distinct forms. These forms are present within the mammalian host and the insect vector. The major morphological difference involves changes in the cell structure and repositioning of the mitochondrial DNA that makes up the kinetoplast. During the progression of their life cycle, repositioning of the kinetoplast from the anterior to the posterior end of the trypanosome body occur, the flagellum begins anteriorly, passing to the posterior end and forming the end of the undulating membrane. These events were
discovered to be fundamental, after transfer of the parasite from the host blood to the tsetse midgut (Matthews et al., 1995).

The first prerequisite for successful transmission is that bloodstream forms must differentiate into procyclic form in the tsetse fly mid gut. The infection becomes established, proliferate and parasites migrate to the tsetse fly salivary glands. Parasite differentiates further into epimastigote forms and subsequently into mature metacyclic forms capable of initiating a fresh infection when transmitted to a new mammalian host through a tsetse fly bite. *T. brucei* life cycle is between mammals and the tsetse fly; they alternately express two types of surface coats. Bloodstream forms are covered by Variant Surface Glycoproteins VSG (Matthews et al., 1995), that shield underlying membrane proteins, preventing lysis of the parasites by serum components. The antigenic variation of the blood forms and their consequent evasion of the host immune responses, are caused by the consecutive expression of potentially as many as 1000 different VSG. When bloodstream forms differentiate to insect procyclic forms, the parasite surface is completely remodeled, VSG is repressed and the coat is shed, as it is progressively replaced by a new invariant coat composed of procyclins also known as procyclic acidic repetitive protein (Mowatt et al., 1987). The insect form has fully developed mitochondria (Figure 2.5). In most species of trypanosome multiplication is active in the vertebrate blood, by means of binary fission or multiple division (Noble et al., 1955).
Epimastigotes multiply in salivary gland. They transform into metacyclic trypanostigotes.

Trypomastigotes transform into bloodstream trypomastigotes. Trypomastigotes multiply by binary fission in various body fluids, e.g., blood, lymph, and spinal fluid.

Trypomastigotes multiply by binary fission in bloodstream trypomastigotes, which are carried to other sites.

Tsetse fly takes a blood meal (injects metacyclic trypomastigotes).

Trypomastigotes in blood

Bloodstream trypanostigotes transform into procyclic trypanostigotes in tsetse fly's midgut. Procyclic trypanostigotes multiply by binary fission.

Procyclic trypanostigotes leave the midgut and transform into epimastigotes.

Tsetse fly takes a blood meal (bloodstream trypanostigotes are ingested).

Figure 2.5. Life Cycle Of Trypanosoma brucei (Adapted From Vickerman)

2.6. Physiology of trypanosomes

The nutrition of most protozoa is holozoic; that is, they require organic materials, which may be particulate or in solution. Many trypanosomes have a permanent mouth, the cytosome or micropore, through which ingested food passes to become enclosed in food vacuoles. Pinocytosis is a method of ingesting nutrient materials where by fluid is drawn through small, temporary openings in the body wall. The ingested material becomes enclosed within a membrane to form a food vacuole (Englund et al., 1998).

Trypanosome has metabolic pathways similar to those of higher animals and requires the same types of organic and inorganic compounds. In recent years, significant
advances have been made in devising chemically defined media for the cultivation of parasitic trypanosomes. The resulting organisms are free of various substances that are present in organisms grown in complex media or isolated from a host and that can interfere with immunologic or biochemical studies. Competition for nutrients is not usually an important factor in pathogenesis because the amounts utilized by parasitic trypanosomes are relatively small. Extracellular or intracellular parasites that destroy cells while feeding can lead to organ dysfunction and serious or life-threatening consequences (Neva et al., 1994).

All living organisms make ATP as an energy carrier. This is produced mainly by the oxidation of carbohydrates using glycolysis and the Tricarboxylic Acid (TCA) cycle. Because free-living organisms do not have an abundance of food, they rely on the much more efficient TCA cycle for most of ATP production. Trypanosoma brucei meets very different environments at different stages of its life cycle. When this organism lives in the mammalian bloodstream, it depends completely on glycolysis for its supply of ATP. It possesses neither a functional Krebs cycle nor oxidative phosphorylation, nor does it store any carbohydrate. In the mammalian bloodstream forms there is an abundance of glucose. The opposite is true in the insect gut or hemolymph (Bakker et al.; Kacser et al., 1973; Heinrich et al., 1974; Westerhoff et al., 1987). The insect forms of T. brucei in the insect gut have mitochondria with full complement of TCA and glycolysis enzymes.

2.8. Clinical manifestations of Trypanosomiasis

2.8.1. Human trypanosomiasis

After an infected fly bites a person, a red painful swelling develops at the site of the fly bite, similar to that seen in Chagas disease. From this site, the parasite invades the blood
stream, causing episodes of fever, headaches, sweating, and generalized enlargement of
the lymph nodes. Parasites then invade the central nervous system (Early with
*rhodesiense* and later with *gambiense*) where they produce the symptoms typical of
sleeping sickness (Greenwood *et al.*, 1980). Ultimately the parasites invade the brain,
first causing behavioral changes such as fear and mood swings, followed by headache,
fever, and weakness. Simultaneously, the patient may develop myocarditis (Greenwood
*et al.*, 1980).

Without treatment, death may occur within six months from cardiac failure from
*T. brucei rhodesiense* infection. *T. brucei gambiense* infection may require up to two
years before symptoms of infection in the central nervous system appear. *Gambiense-
infected people develop drowsiness during the day, but insomnia at night. Sleep becomes
uncontrollable as the disease progresses until the patient becomes comatose (Dumas *et
al.*, 1999).

### 2.8.2. Animal trypanosomiasis

#### 2.8.2.1. Nagana

The clinical signs of the animal trypanosomiasis are parasitemia intermittent
fever, anemia, weight loss, progressive weakness and infertility in breeding animals. Host
susceptibility and virulence of parasite may vary, if left untreated hosts die of anemia
(Losos *et al.*, 1972).

Initial replication of trypanosomes is at the site of inoculation in the skin; this
causes a swelling and a sore (chancre). Trypanosomes then spread to the lymph nodes
and blood and continue to replicate. *Trypanosoma congoense* localizes in the endothelial
cells of small blood vessels and capillaries. *T. brucei brucei* and *T. vivax* localize in
tissues. Antibody developed to the glycoprotein coat of the trypanosome kills the trypanosome and results in the development of immune complexes. Antibody, however, does not clear the infection, for the trypanosome has genes that can code for many different surface-coat glycoproteins and change its surface glycoprotein to evade the antibody. Thus, there is a persistent infection that results in a continuing cycle of trypanosome replication, antibody production, immune complex development, and changing surface-coat glycoproteins. *T. brucei brucei* has a relatively short incubation period and causes severe to fatal infection in horses, camels, dogs, and cats (Losos *et al.*, 1972). It usually causes mild, chronic, or subclinical disease in cattle, sheep, goats, and pigs. A febrile response occurs in the horse 4-14 days after infection. This is followed by recurrent febrile reactions. The heartbeat and respiration may be accelerated and weakness are seen, whereas the appetite remains good (Losos *et al.*, 1972).

Progressive anemia and icterus, and edema of the ventral regions, especially the male genitalia, are characteristic. The organisms are not always easily perceived in blood smears and are best demonstrated in tissue smears or sections, (e.g., lymph nodes). Infected animals die in a few weeks or several months, depending on the virulence of the strain of *T. brucei brucei* (Losos *et al.*, 1972).

2.8.2.2 Surra.

An infected bite by *Tabanus* and *Stomoxys*, leads to *T.evansi* entering the blood stream of the host. Thereafter it multiplies to a sizeable population and causes the appearance of a number of symptoms like intermittent fever, progressive anemia, weakness, loss of condition and terminal nervous signs, including paralysis and convulsions. Surra invariably proves fatal in horses and camels within a few months,
though in camels it may run for a few years the working capacity is severely impaired (Lun et al., 1993).

2.9. Diagnosis of Trypanosomiasis

2.9.1. Microscopic examination of trypanosome

Several diagnostic techniques are available for the disease. The most primitive but reliable technique is direct detection of trypanosomes by microscopic examination of blood and/or Cerebrospinal Fluid (CSF).

The simplest techniques are examination of wet, thick or thin films of fresh blood, usually obtained from the ear vein, jugular vein or the tail and stained by Giemsa staining technique.

These are made by placing a drop of blood on a clean microscope slide and covering with a cover-slip (22 x 22 mm). The blood is examined microscopically or by phase-contrast. Approximately 50-100 fields are examined. Trypanosomes are recognized by their movement in red blood cells (RBCs). The method is simple, inexpensive and gives immediate results. Depending on the trypanosome size and movements a presumptive diagnosis can be made of the trypanosome species. Final confirmation of the species is made by the examination of the stained preparation. The diagnostic sensitivity of the method is generally low but depends on the examiner’s experience and the level of parasitaemia. Sensitivity can be improved significantly by lysing the RBCs before examination using a haemolytic agent such as sodium dodecyl sulfate (SDS) (Ndao et al., 1995).
2.9.2. Parasite concentration techniques

The probability of detecting trypanosomes in a sample from an infected animal depends largely on the amount of blood examined and the level of parasitaemia. The amount of blood examined with direct examination techniques is low and parasites are often very scanty in the blood of an infected animal. Both of these factors contribute to the low sensitivity of direct examination techniques. Sensitivity can be improved by increasing the volume of blood to be examined and by concentrating the trypanosomes (Woo et al., 1970).

2.9.3. Microhaematocrit centrifugation technique

The microhaematocrit centrifugation technique, or the Woo method (Woo et al., 1970), is widely used for the diagnosis of animal trypanosomiasis. It is based on the separation of the different components of the blood sample depending on their specific gravity. The microhaematocrit centrifugation technique is more sensitive than the direct examination techniques (Woo et al., 1970).

2.9.4. Serological tests

Several antibody detection techniques have been developed to detect trypanosomal antibodies for the diagnosis of animal trypanosomiasis, with variable sensitivity and specificity. The methods of choice are the indirect fluorescent antibody test (IFAT) (Katende et al., 1987) and the trypanosomal antibody-detection ELISA (Hopkins et al., 1998, Luckins et al., 1977). The development and testing of specific recombinant antigens could, in the near future, allow the detection of species-specific antibodies.
2.9.4.1. *Indirect fluorescent antibody test*

The original method for this test (Wilson *et al.*, 1969) has been replaced by a new technique for the preparation of trypanosomal antigens (Katende *et al.*, 1987), which involves fixation of live trypanosomes using a mixture of 80% cold acetone and 0.25% formalin in normal saline (Platt *et al.*, 1976, Well *et al.*, 1982).

2.9.4.2. *Card agglutination test*

This test was originally developed for the diagnosis of human sleeping sickness caused by *T. brucei gambiense* (Magnus *et al.*, 1978) and has been further adapted for the detection of antibodies against *T. evansi* (Pathak *et al.*, 1997).

2.9.4.3. *Enzyme-linked immunosorbent assay ELISA*

The original ELISA (Luckins *et al.*, 1977) has recently been further developed for use in large-scale surveys of bovine trypanosomiasis (Hopkins *et al.*, 1998). Both antibody-detection tests have high sensitivity and specificity. Their species specificity is generally low. They detect immune responses to current and past infections and can, therefore, only provide a presumptive diagnosis of active infection. This immunodiagnosis needs expensive, sophisticated equipment and expertise, which is not always available. It has to be performed in specialized laboratories and there is a substantial delay between the actual sampling and the availability of the results. Nevertheless, the ELISA diagnosis lends itself to a high degree of automation and standardization. Sample collection and storage is made easy through the use of filter papers. All of these factors make the antibody ELISA a very useful test for large-scale surveys to determine the distribution of tsetse-transmitted trypanosomiasis.
2.9.5. DNA amplification tests

A polymerase chain reaction (PCR) method has been developed as a tool for the diagnosis of infections with African trypanosomes in humans and animals, as well as tsetse flies. Specific repetitive nuclear DNA sequences can be amplified for *T. vivax* and each of the five *T. congolense* subgroups (Desquesnes *et al.*, 1997). A common primer set is available for detection of the three *T. brucei* subspecies. The primer sets available for different trypanosome species, subspecies and subgroups are referred to as follows:

- *T. brucei* subspecies: TBR1 and TBR2
- *T. congolense* (Savannah subgroup): TCN1 and TCN2
- *T. congolense* (Forest subgroup): TCF1 and TCF2
- *T. congolense* (Kenya Coast subgroup): TCK1 and TCK2
- *T. vivax*: TVW1 and TVW2.

Standard PCR amplifications are carried out in a reaction mixture containing Tris-HCl, MgCl₂, KCl, each of the four deoxyribonucleotide triphosphates, primers, DNA template and Taq DNA polymerase. Samples are incubated during several cycles at varying temperatures. The PCR products are electrophoresed through agarose. Gels are stained with ethidium bromide. However, at the moment, the cost of PCR analyses is prohibitive for the routine use of the test (Katakura *et al.*, 1997).

2.9.6. In-vitro cultivation of salivarian trypanosome bloodstream forms

Serum-free medium for the cultivation of *T. brucei* and *T. evansi* BSFs was achieved by Hirumi (Hirumi *et al.*, 1997). The serum-free culture is the ultimate in vitro system for studying growth-promoting factors, drug-sensitivity test, mode of trypanocidal activity, gene-selection, cloning, cell division cycle and gene expression that have been difficult earlier due to the serum supplementation in the culture medium.
2.10. RNA Editing

The RNA modification phenomenon of uridine (U) insertion/deletion RNA editing was discovered in mitochondria of trypanosomatid protists more than 15 years ago (Benne et al. 1986). These phenomena can be grouped into two basic classes—insertion/deletion editing and substitution editing. The term RNA editing (Benne et al. 1986) has been historically limited to certain RNA nucleotide modifications discovered after 1986, such as C to U editing of the mammalian apoB mRNA, plant mitochondria and chloroplast mRNAs, A to I editing of glutamate receptors mRNAs (Gott et al., 2000), C-insertion editing of Physarum mitochondrial mRNAs. Until very recently, however, little was known in detail about the proteins and enzymes involved in this process and their interactions, but this is rapidly changing due to the availability of Leishmania major and T. brucei genomic sequences and rapid gene identification techniques such as mass spectroscopy.

In kinetoplastid protozoa, RNA editing is a post-transcriptional RNA-processing event that occurs in the mitochondrion and results in the addition and deletion of uridine residues at specific sites in mRNA transcripts. Editing produces the sequence information necessary for functional mRNAs by correcting frame shifts, creating start codons, or in some cases, forming complete reading frames (Stuart et al., 1991). The genetic information necessary to direct the insertion or deletion of uridine residues in these mRNAs is present in small, 55-70-nucleotide primary transcripts called guide RNAs (gRNAs). The gRNAs are complementary to the edited mRNAs and contain a nonencoded poly (U) tail of 5-15 nucleotides (Blum et al., 1990). The uridine tail may donate or accept uridines during the editing process through the formation of a chimeric
gRNA/mRNA molecule consisting of the gRNA covalently linked via the poly (U) tail to the 3' segment of the mRNA (Blum et al., 1991). The gRNA also contains a short 7-10-nucleotide anchor region at its 5'-end, which is complementary to the pre-edited sequences immediately 3' to the editing site. The anchor region recognizes and base pairs with unedited mRNA and directs the initiation of the editing process. Uridine addition and deletion continues until the mRNA is fully complementary to the gRNA as in Figure 2.6.

Figure 2.6. The enzyme cascade model for RNA editing. (I–IV) U-insertion editing. (IIA, IVA) U-deletion editing. (Adapted from Simpson, 2003)

The second model proposes multiple rounds of cleavage-ligation and draws analogies from the mechanism of tRNA splicing. In this scheme, the gRNA-mRNA
chimera is formed by endonuclease and RNA ligase activities (Harris et al., 1992). The pre-edited mRNA is cleaved by an endonuclease at the editing site and RNA ligase joins the gRNA to the 3'-fragment of the mRNA producing the chimeric molecule. A second round of cleavage and ligation resolves the chimera.

Support for the cleavage-ligation mechanism comes from the identification of mitochondrial RNPs that are thought to be involved in RNA editing (Pollard et al., 1992). These were shown by glycerol gradient sedimentation of mitochondrial extract to consist of two ribonucleoprotein particles which sedimented as 19 S and 35-40 S complexes containing RNA ligase, chimera formation, and terminal uridylyl-transferase activities. Uridylyl-transferase presumably is required for addition of the nonencoded poly (U) tail to the gRNA. These results led to examine the role of the RNA ligase in chimera formation (Simpson et al., 2003).

The mechanism of RNA ligation has been studied for ligases from wheat germ (Konarska et al., 1982), yeast (Greer et al., 1983), and T4-infected Escherichia coli cells (Uhlenbeck et al., 1982). Even though the enzymes differ in the structures of their substrates and products, their overall mechanisms are quite similar. The T4 enzyme, for example, joins RNAs with a 5'-phosphate to 3'-hydroxyl termini in three distinct and reversible steps:

1. \( E + ATP \rightarrow E-AMP + PP \)
2. \( E-AMP + pN- \rightarrow E + AppN- \)
3. \( -N + AppN- \rightarrow -NpN- + AMP \)

The first step involves the adenylylation of the enzyme \( (E) \), by the transfer of AMP from ATP, via phosphoamide linkage with a lysine residue on the protein. In the
second step, AMP from the adenylylated enzyme is transferred to the 5'-phosphate of the RNA molecule forming an activated RNA molecule with a 5', 5'-phosphoanhydride bond. In the third and final step, the 3'-hydroxyl of the same or different RNA molecule attacks the activated RNA forming a phosphodiester (Sabatini et al., 1995, Panigrahi et al., 2001).

2.11. RNA interference

Regulation of gene expression, deciding how much of what proteins are produced in the cell, is controlled by a myriad of different molecules. One type of naturally occurring regulatory molecule is small interfering RNA (siRNA), which selectively disrupts the production of a protein it is programmed to recognize, a process called RNA interference (Hutvagner et al., 2004). These short stretches of nucleotides combine with other cellular proteins to form an RNA-induced silencing complex, called RISC, which locates and destroys a targeted messenger RNA, the molecule that carries a protein recipe from the nucleus to the site of production in the cytoplasm. RNA interference has been widely exploited tool to knock out gene expression and infer the function of missing proteins, very little is known about the mechanisms behind this regulatory process (Hutvagner et al., 2004). Trypanosoma brucei cells containing gene-specific double-stranded RNAs (dsRNAs) leads to specific degradation of the homologous messenger RNA leading to formation of a knockout phenotype (Ngo et al., 1998)

2.11. Control of Trypanosomiasis

The control of trypanosomiasis is by vector control, curative and prophylactic drugs. Until the development of synthetic organochlorine insecticides such as DDT and dieldrin, vegetation clearance was the major method for the vector control. Since the
insecticides were developed, the large-scale insecticides 'spraying was carried out in West Africa where over 200,000 km² of land was cleared of tsetse by the insecticides spraying. Thus the large-scale insecticides spraying were very effective method for the vector control. However, the insecticides spraying have become widely unacceptable in terms of the protection of environment.

The sterile insect technique was one of the method for the vector control, the method was not only expensive but also some of the sterile males of some species have been shown to be efficient vectors of pathogenic Trypanosoma species and would potentially increase the risk of trypanosomiasis (Moloo et al., 1982, 1988).

It is rare to change a small local environment to eliminate the breeding grounds of these flies, such as small ponds. This method however is not effective in most cases and rarely are ponds removed to at control a horsefly problem that mechanically transmits T.evansi. This is due mainly to the close locality of another site, which cannot be removed, and the flight and dispersal of the adult flies (WHO, 1989).

The main attempts at present are the use of traps, pour on and spot on insecticides, and for humans the use of repellents. These traps can be effective in localized areas and involve killing horseflies without the need for insecticides in traps, which can easily be home made(WHO, 1989).

The use of insecticides as spot and pour one is used mainly to treat against other livestock flies such as blowflies and warble flies. There is evidence that insecticides such as the pyrethroids are effective when first applied but the effects are short lived in relation to horsefly control. The use of self-application methods such as face bags or back scrubbers has been used but although extremely effective against other flies, such as
hornflies and faceflies, they are not very effective for horseflies. As far as humans are concerned the methods employed can be either repellents or traps or a combination of both. Traps are most effective when placed around the periphery of an area such as a garden to create a horsefly free zone. The positioning and amount of traps however need to be determined by trial and error as the population density and species will differ in different localities. The use of repellents involves either topical application onto the body with chemical such as diethyltoluamide (DEET), citronella oil or eucalyptus oil. DEET can cause certain plastics to degrade, spectacle frames, watch faces etc, and no repellents should contact the eyes or lips (WHO, 1989).

The use of these repellents is fine for the occasional trip out but become both expensive and laborious to apply when wanting to go out into the garden. In these situations it is probably better to use an impregnated patch or article of clothing. A light cotton jacket or a stick on patch can be effective at repelling horseflies. A garment can be soaked in DEET diluted in water to give effective protection. A cotton jacket (DEET affects some artificial fibers) weighing 120 grams can be impregnated by pouring 30ml of DEET into 250ml of water and immersing the garment into the solution. When not in use the jacket can be sealed in a plastic bag to retain the life of the DEET. A garment so soaked can be effective for several weeks (WHO, 1989).

While there are no drugs suitable for preventing sleeping sickness, there are drugs that can be used to treat it. Treatment of infections with African trypanosomes in humans is limited to chemotherapy, with diamidines (pentamidine), suramin, melaminophenylarsenicals (melarsoprol), and dl-ε-difluoromethylornithine (DFMO) (Fries et al., 2003). The latter two are effective against late-stage sleeping sickness (Pepin...
et al., 1994), with melarsoprol being the drug of choice against the acute form of the disease caused by *T. brucei rhodesiense* (Bacchi et al., 1990). Pentamidine isothionate and suramin are the drugs of choice to treat the early haemolymphatic stage of West and East African trypanosomiasis, respectively. Melarsoprol is the drug of choice for late-stage disease where there is central nervous system involvement (Atouguia et al., 1995). Recently, eflornithine has been licensed for use to treat late-stage sleeping sickness. Eflornithine provides an alternative drug for the treatment of *T. brucei gambiense*, the form of sleeping sickness that occurs in West and Central Africa. For the *T. brucei rhodesiense* form that occurs in East and southern Africa, there is no alternative treatment (Sunkara et al., 1987).

In severe cases of the disease where the trypanosomes appear in the cerebrospinal fluid, Mel B is the drug of choice for the reasons of its ability to penetrate into cerebrospinal fluid. However, the Mel B, which is organic arsenic, must be used with utmost care because of fatal side effects (WHO, 1989).

Trypanocidal drugs for use in cattle are limited to the salts of three-compounds:- Isometamidium chlorides, Homidium bromide and humdrum chloride and Diminazene aceturate. Diminazene aceturate is the most widely used chemotherapeutic drug. It has virtually no prophylactic activity (Leach et al., 1981). The homidium salts are used mainly for chemotherapy, but they do have some prophylactic activity. Isometamidium chloride is mainly used as a chemo prophylactic drug, but it too has some chemotherapeutic activity.

Strategies for control have relied heavily on the use of chemotherapy to treat infected animals. It is generally recognized that chemotherapy is most effective in the
early stages of infection. At late stage infections, where it is most likely that relapses will occur, or in animals that exhibit nervous signs cymelarsen may have a role to play (Singh et al., 1977).

Although these drugs have been used widely, there are often conflicting reports of their efficacy and curative dose, suggesting that there might well be differences in sensitivity amongst isolates of *T. evansi* from different regions. Suramin is almost universally effective at a dose rate of 10mg/kg. Diminazene aceturate has been used at dose rates from 3.5 mg/kg to cure *T. evansi* in cattle, buffalo and donkeys in India, Thailand and the Philippines to as high as 15 mg/kg in buffalo in India (Singh et al., 1977). Quinapyramine is used at 2 – 5mg/kg in cattle, camels and horses and is the preferred drug in India. Historically, drug combinations (e.g. suramin/tartar emetic) have been employed, requiring as many as seven treatments/animal. Although this strategy was considered effective for camels and horses, it did not achieve widespread application. Complexes of suramin/quinapyramine and suramin/diminazene aceturate have been developed and although found to be 80 – 100% curative experimentally, have not been tested under field conditions (Singh et al., 1977).

Chemoprophylaxis is not used for control of *T. evansi* as extensively as with the tsetse-transmitted trypanosomiases, but there are a number of reports of its use. Suramin binds to serum proteins and can confer a limited protection of about one month to treated animals, although there are reports of protection for as long as three months (Tuntasuvan et al., 2003).

Isometamidium has little prophylactic activity, but quinapyramine and quinapyramine prosalt can protect for one to two months. Experimental complexes of
quinapyramine/suramin were found to give resistance to challenge for up to 24 months, whilst diminazene/suramin complexes protected for approximately five months (Tuntasuvan et al., 2003).
CHAPTER THREE: MATERIALS AND METHODS

3.1. PCR of *T. brucei* and *T. evansi* specific Mitochondrial protein gene

DNA extraction, Total DNA was extracted from parasites (A) *T. evansi* 111695, (B) *T. evansi* IL 934 and (C) *T. b rhodesiense* IL 22343 according to Sambrook (2001) (Appendix 3). Lysis buffer (10 mM Tris-HCl [pH 8.0], 100 mM EDTA, 0.5% sodium dodecyl sulfate, and 100 μg of proteinase K per ml) was added to the samples, followed by overnight incubation at 55°C. DNA was extracted with phenol-chloroform-isoamyl alcohol (25:24:1) and precipitated with isopropanol. The purified DNA was dissolved in 100 μl of sterilized distilled water.

Dr Inoue of Obihiro University, Japan supplied the primers (Table 3.1.) PCR was conducted on Mitochondrial protein gene (Table 3.2.) of the following strains of *T. evansi* IL 1695 cattle in Africa (A), *T. evansi* IL 934 capybara (*Hydrochaerus hyrochaeris*) in South America (B) and *T. b rhodesiense* IL 22343 isolated from a patient in Ivory coast (C).

Table 3.1. Primer sequences and amplification product sizes of tested mitochondrial related genes.

<table>
<thead>
<tr>
<th>DNA</th>
<th>Primer sequence</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. <em>Trypanosoma brucei</em> ATPase submit 9 homolog MRNA, nuclear gene encoding mitochondria protein.</td>
<td>Primers F5' ATGATGCGCGCGGCTTGC3', R 5' GAAACGCAAGCATGAGTGCG3'.</td>
<td>340 bp</td>
</tr>
</tbody>
</table>
2. *T. evansi* glycosomal glyceraldehyde-3-phosphate dehydrogenase (Ggapdh)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5')</th>
<th>Reverse Primer (3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>F5' ATGGTTTTTGCCGTATTGG3'</td>
<td>R5' CACTCGTTATCGTACCACG3'</td>
<td></td>
</tr>
<tr>
<td>988 bp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

3. *T. evansi* cysteine protease

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5')</th>
<th>Reverse Primer (3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>F5' TCAACTATCGGCAACATCGA 3'</td>
<td>R 5' ACCAACGAGGAGCACCACCAT 3'</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
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</tbody>
</table>

RNA ligase MP48 gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5')</th>
<th>Reverse Primer (3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>F 5' ATGTTTCGTCGCTCGGTGT3'</td>
<td>R 5' AATGACCGCCCTCGCGTACA 3'</td>
<td></td>
</tr>
<tr>
<td>630 bp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

RNA ligase MP 52 gene

<table>
<thead>
<tr>
<th>Gene</th>
<th>Forward Primer (5')</th>
<th>Reverse Primer (3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>F5'ATGCAAACCTCCAAAGGTTGGTGCT3'</td>
<td>R5'ATAGGGAACCTCCTCCGGTGGTA GCCTG 3'</td>
<td></td>
</tr>
<tr>
<td>560 bp</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### 3.2 PCR reactions, conditions and amplification of mitochondrial protein genes

Table 3.2. Species specific PCR conditions and programmes.

<table>
<thead>
<tr>
<th>Trypanosome species</th>
<th>Amplification conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Trypanosoma brucei</em> ATPase submit 9 homolog MRNA, nuclear gene encoding mitochondria protein.</td>
<td>PCR primer concentration 10pMol, 94°C for 10 Min. <strong>Hot starts, 1 cycle.</strong> 94°C for 30 sec, 58°C for 30 sec (Annealing), (25 cycles). 72°C for 30 sec. 72°C for 7 min sec. Further Extension, 1 cycles. 4°C for ∞</td>
</tr>
<tr>
<td><em>T. evansi</em> glycosomal glyceraldehyde-3-phosphate dehydrogenase (Ggapdh)</td>
<td>PCR primer concentration 10pMol, 94°C for 10 Min. <strong>Hot starts, 1 cycle.</strong> 94°C for 30 sec, 56°C for 30 sec (Annealing), (25 cycles). 72°C for 30 sec. 72°C for 7 min sec. Further Extension, 1 cycles. 4°C for ∞</td>
</tr>
<tr>
<td><em>T. evansi</em> cysteine protease</td>
<td>PCR primer concentration 10pMol, 94°C for 10 Min. <strong>Hot starts, 1 cycle.</strong> 94°C for 30 sec,</td>
</tr>
</tbody>
</table>
3.3 DNA sequencing

PCR products were purified by Gene Clean II (BIO 101, Inc., Vista, CA). DNA sequencing of MP 48 RNA editing ligase gene in *T. brucei* and *T. evansi* was accomplished by the dideoxynucleotide chain termination method according to Sanger (1977), by an automated cycle sequencing using the dye terminator method (ABI PRISM big dye terminator kit, Perkin-Elmer). Purified PCR fragments were cloned into pT7 Blue-T vector. Automated cycle sequencing was by dye terminator method (ABI PRISM big dye terminator kit, Perkin-Elmer) 1 pmol/μl DNA was added to 1 μl recombinant pT7 Blue-T vector plasmid (60-89 ng/ml), mixed with 1.2 μl of double distil water. The solution was mixed with 2 μl of Premix solution containing Dideoxynucleoside triphosphates, T7 DNA polymerase and deoxynucleoside solution. The same procedure was repeated using 1 pmol/μl of pmol M13 M4 for a complimentary strand, which assist to confirm the DNA sequence of the gene. The solution was transferred in a thermocycler for PCR. The polymerase chain reaction amplification was set at 96°C for 10 sec to
denature the plasmid. Annealing of double strand DNA was at 50° C for 5 sec and
extension at 60° C for 4 min for a total of 25 cycles. Polymerase chain reaction was
followed by ethanol precipitation of the DNA and rinsing with 70% ethanol. The
resulting pellet was dried in air and resuspended in template suppression reagent, that
stop any further reaction. The resuspended single strand DNAs were incubated at 95° C
for 2 min to linearize the amplified single strand DNA. Sequences were analyzed on a
PE Applied Biosystems 310 automatic DNA sequencer (PE Applied Biosystems). The
MP 48 RNA editing ligase gene sequences obtained with *T. brucei* and *T. evansi* were
compared between themselves and with the data available on Genbank, using the clustal
V software (Higgins *et al.*, 1991). The same procedure was repeated for MP 52 ligase
genes.

3.4. Cloning of MP 48 and MP 52 RNA editing ligase amplified gene of *T. evansi* and
*T. brucei* into pT7-Blue vector and p2T7Ti plasmid from LaCount and Donelson 2000

Mitochondrial protein 48 and 52 RNA editing ligase amplified gene of *T. evansi*
and *T. brucei* were cloned into pT7 Blue-T vector, leading to a recombinant plasmid pT7-
Blue vector /MP 48 gene and recombinant plasmid pT7-Blue vector /MP 52 gene
respectively according to LaCount and Donelson (2000). The resulting recombinant
plasmids were transformed into a competent αDH 5 *E. coli* cell, using High Efficiency
Electro-transformation of αDH 5 *E. coli* protocol (Appendix 4). αDH 5 *E. coli* containing
the recombinant plasmids were propagated in Luria-Bertani (LB) medium. To obtain a
large amount of plasmid, αDH 5 *E. coli* was cultivated in Luria-Bertani (LB) medium -
Ampicillin in a Semi-large scale preparation of plasmid DNA using alkaline lysis method
(Appendix 5) or by QIAGEN plasmid protocol (Appendix 6). Plasmids were separated by electrophoresis in a 1.5% (w/v) TAE agarose gel stained by 0.5 μg/ml ethidium bromide and visualised by ultraviolet light. Mitochondrial protein 48 and 52 RNA editing ligase amplified gene were further cloned into p2T7Ti plasmid to form p2T7Ti /MP 48 and p2T7Ti MP52 gene recombinant plasmid respectively. The plasmids were propagated using same method as in the case of pT7 Blue-T vector. pT7 Blue-T vector contain ampicillin genes for the selection of αDH 5 E. coli containing the desired plasmids which are further amplified. pT7-Blue vector /MP 48 /mp52 gene plasmid were cut using appropriate restriction enzyme to produce a MP 48 /mp52 gene fragment which were ligated with p2T7Ti vector. Mitochondrial protein 48 and MP 52 RNA editing ligase gene were inserted between two-T7 promoter genes, to recognize bacteriophage T7 RNA polymerase in pLEW13 plasmid. Tetracycline was added to induce the p2T7Ti plasmid, to generate sense and antisense DNA from the DNA sequences of MP 48 and MP 52 RNA ligase gene. MP 48 and MP 52 RNA ligase gene were placed between two-T7 promoter in a head to head orientation for a successfully RNA interference analysis. p2T7Ti vector plasmid contains a neomycin gene, which is used for selection of parasite containing the plasmid.
Figure 3.1. Map of pT7 Blue-T Vector (Adapted From Novogen, Inc, 1998).
Figure 3.2. Map of pT7Ti-Insert MP 48/ Mp 52 (Adapted from LaCount and Donelson, 2002).
3.5. Semi-large scale preparation of plasmid DNA using alkaline lysis method for extraction of all plasmids

Plasmids DNA were extracted according to Sambrook (2001). Luria-Bertani (LB) medium -Amp cultures (25ml) containing αDH 5 E. coli with plasmids, were centrifuged at 8000 rpm for 10 min at 4°C and supernatant discarded. The pellet was resuspended in a buffer solution 1 (50Mm glucose, 25 mM Tris-cl pH 8.0, 10mM EDTA pH 8.0). E.coli cell wall was ruptured by Solution II (1%SDS, 0.2 N NaOH) to release the cell contents, Solution III (5 M potassium acetate / glacial acetic acid buffer) was added, and incubate on ice for 10 min. Chloroform was added to remove long strands of DNA and insoluble protein. The solutions were spanned at 15000 rpm for 15 min at 4°C. The upper aqueous phase supernatant was added to phenol; chloroform –isomylalcohol solution for further extract of soluble proteins. The supernatant containing plasmid were precipitated by isopropanol after spanning at 15000 rmp, 4°C for 5min. Precipitated plasmid was dried in air and dissolved in 50 μl of TE containing Rnase to degrade contaminating RNA molecule at 37°C for 30 min. Electrophoresis were performed using 1 μl of the sample in a 1.5% (w/v) agarose gel then stained by 0.5 μg/ml ethidium bromide and visualised by ultraviolet light, to check for purity and DNA concentration of DNA μg/ul = ODX 0.05X dilution. (Appendix 5).

3.6 Extraction and purification of plasmid by QIAGEN plasmid maxi protocol commercial kit

QIAGEN™ plasmid maxi protocol commercial kit was used to extract plasmid in α DH 5 E.coli according to manufacturing company QIAGEN™. Alpha DH 5 E. coli was grown in large-scale culture 200 ml and spanned at 8000 rpm at 4°C to get a
bacterial cell pellet. Pellet from overnight bacterial culture were mixed in the 4 ml of sol PI at 4°C for resuspension of the pellet, Sol P1 contain Rnase to Degrade contaminating RNA. Four milliliter of Sol P2 were added to lyse the cell membrane and release the cell contents, reaction time was 5 minutes at room temperature, 4 ml of Sol P3 were added and spanned at 10000rpm for 30 at 4°C to enhance the precipitation of soluble protein in the organic phase. Buffer P3 were added to precipitate genomic DNA, protein, cell debris and SDS, then incubated on ice for 20 minutes. The aqueous phase were passed though an ion-exchange chromatography column that had been equilibrated by 4 ml of QBT buffer solution. 10 ml of the QC buffer were added twice to wash and remove all contaminant in the plasmid DNA preparation, the second wash was necessary when large volumes or bacterial strains producing large amounts of carbohydrates are used. The QC buffer removes genomic DNA and RNA. QF buffer 5ml Eluted ion-exchange chromatography column, elute contains plasmid DNA. Isopropanol 3.5ml was added and centrifuged immediately at 10000 rpm for 30 min at 4°C for the plasmid to precipitate. The plasmids were washed three times with 1 ml of 70% ethanol in 1.5 ml tube at 10000 rpm at 4°C for 5 min, to remove ethanol completely. DNA plasmids were dried in air for 10 minutes before dissolving in 50 µl sterile double distilled water. Electrophoresis on was performed using 1 µl of the sample in a 1.5% (w/v) agarose gel stained by 0.5 µg/ml ethidium bromide and visualised by ultraviolet light, to check for purity and DNA concentration of DNA ug/ul = ODx 0.05X dilution (Appendix 6).

3.7. DNA purified Gene Clean

DNA was purified using a kit, GENECLEAN II according to the manufacturing company, BIO 101 Co., Ltd. The agarose containing DNA fragment was solubilized in a
potassium iodide solution. The DNA was adsorbed onto glass powder and eluted with a buffer of low salt concentration.

3.8 Construction of T7 Blue-T plasmid vector and p2T7Ti plasmid vector

Construction of T7 Blue-T vector and p2T7Ti vector was done according to LaCount and Donelson (2000) (Figure 3.1 and Figure 3.2). pT7 Blue-T vector 4μl (3-5μg) were cut at EcoI site by 4μl Restriction enzyme EcoRI in the presence of 5μl of H buffer in a 36μl of Double distilled water, 1μl of Rnase was added to degrade contaminating RNA. The master mix was incubated at 37°C for 1 hour. PCR products of MP 48 were ligated into the pT7 Blue-T vector (Novagen), using a DNA ligation kit containing T4 DNA ligase (manufactured by TAKARA SHUZO Co., Ltd.). Ten microlitres of DNA ligation mix were added to 4μl of PCR amplified MP 48 RNA editing ligase gene, 1μl of pT7 Blue-T vector and 5 μl double distilled water. The result was a recombinant plasmid PT7-Blue vector /MP 48 gene. The same procedure was repeated for MP 52 ligase genes.

The mixtures were incubated at 16°C for between 30 minutes to 24 hours. The resulting recombinant plasmids were transformed into a competent αDH 5 E. coli cell, using High Efficiency Electro-transformation of αDH 5 E. coli (Appendix 4). Alpha-DH 5 E. coli took up the recombinant vector/plasmid. 300 μl Luria-Bertani (LB) medium with Ampicillin containing a final concentration of 50μg/ml was added and incubated at 37°C for 45 min for the selection of αDH 5 E. coli containing the plasmids. These were followed by spreading on LB ampicillin agar plate for selection of recombinant αDH 5 E. coli, incubation was at 37°C overnight, single colonies were picked and the insert size were checked by PCR using each set of the specific primers of MP48, using the same
conditions as in Table 3.1 and Table 3.2. The same procedure was repeated for MP 52 ligase genes.

Propagation of recombinant αDH5 *E. coli* cell was by picking single colonies for semi-large scale cultivation and large-scale cultivation. The extracted p<sup>T</sup>T7<sup>T</sup>i /MP 48 gene recombinant plasmids were separately mixed well with 10μl of X6 loading buffer each and were separated by electrophoreses in a 1.5% (w/v) TAE agarose gel containing 0.5 μg/ml ethidium bromide stain and visualised by ultraviolet light, p<sup>T</sup>T7<sup>T</sup>i /MP 48 gene recombinant plasmid bands was cut out of the gel and purified by Gene Clean II (BIO 101, Inc., Vista, CA) according to the supplier’s instructions. The same procedure was repeated for MP 52 ligase genes.

3.9. p<sup>T</sup>T7<sup>T</sup>i vector Plasmid construction

Construction of p<sup>T</sup>T7<sup>T</sup>i vector was done according to LaCount and Donelson (2000). p<sup>T</sup>7-Blue vector /MP 48 gene recombinant plasmid were digested at Bam HI and Hind III site by corresponding restriction enzyme and suitable buffer (DNA 5ul. X10B buffer, D.W 35ul R1 BamH1 and Hind 111 2.5ul each). The mixture was incubated at 37°C for 3 hrs. The resulting volume of 50 μl was mixed well with 10 μl of X6 loading buffer and electrophoresis performed on 1.5% agarose TAE. The same procedure was repeated for MP 52 RNA editing ligase gene.

The result in small band at 560bp for MP52 and 630 for MP48 RNA editing ligase gene respectively. The resulting small bands were cut out of the gel and purified by Gene Clean. 560bp for MP52 gene and 630 for MP48 gene fragments were ligated into the p<sup>T</sup>T7<sup>T</sup>i vector (from Dr Inoue), using a DNA ligation kit containing T4 DNA ligase (manufactured by TAKARA SHUZO Co., Ltd.) 10μl of DNA ligation mixture was
added to 4μl of PCR amplified MP 48 and MP 52 RNA editing ligase gene, 1μl of p2T7Ti vector and 5 μl double distilled water. The resulting recombinant plasmid was p2T7Ti /MP 48 gene recombinant plasmid. The same procedure was repeated for MP 52 ligase genes.

The mixture was incubated at 16°C for between 30 minutes to 24 hours. The resulting recombinant plasmids were transformed into a competent αDH 5 E. coli cell, using High Efficiency Electro-transformation of αDH 5 E. coli. Index 4 Alpha-DH 5 E. coli took up the recombinant vector/plasmid in a 300 μl Luria-Bertani (LB) medium containing Ampicillin in a final concentration of 50μg/ml was added and incubated at 37°C for 45 min. Incubation was followed by spreading on LB ampicillin agar plate and incubated at 37°C overnight. Single colonies were picked and the insert size was checked by PCR using the specific primers of MP48 RNA editing ligase gene. Using same PCR conditions as in Table 1 and Table 2. The same procedure was repeated for MP 52 RNA editing ligase gene.

Propagation of αDH5 E. coli cell was by picking single colonies for semi-large scale cultivation (Appendix 4) and large-scale cultivation (Appendix 5). The extracted plasmids p2T7Ti /MP 48 gene recombinant plasmid was mixed well with 10 μl of X6 loading buffer and were separated by electrophoreses in a 1.5% (w/v) TAE agarose gel was stained by 0.5 μg/ml ethidium bromide and visualised by ultraviolet light, p2T7Ti /MP 48 and p2T7Ti MP52 gene recombinant plasmid bands were cut out of the gel and purified by Gene Clean II (BIO 101, Inc., Vista, CA) according to the supplier’s instructions. The same procedure was repeated for MP 52 ligase genes.
3.10 Generation of transgenic trypanosome cells for expression and phenotype knockout analysis of MP 48 and MP 52 in *Trypanosoma evansi* and *T. brucei*

Generation of transgenic trypanosome cells for expression and phenotype knockout analysis of MP 48 and MP 52 RNA editing ligase gene in *Trypanosoma evansi* and *T. brucei* was according to LaCount and Achim (2000, 2001). pT7-Blue vector /MP 48 and pT7 – Blue vector/ MP52 gene recombinant plasmid were digested at Bam HI and Hind III site by corresponding restriction enzyme and suitable buffer (DNA 5ul. X10B buffer, D.W 35ul R1 BamH1 and Hind 111 2.5ul each). The mixtures were incubated at 37°C for 3 hrs for each gene. The resulting volume of 50 µl was mixed well with 10 µl of X6 loading buffer and electrophoresis performed on 1.5% agarose TAE. These resulted into two bands, a long band with over 1000bp, which was part of pT7- Blue T vector (Figure3.1), a small band at 560bp for MP52 RNA editing ligase gene and 630 for MP 48 RNA editing ligase gene respectively. The small bands were cut out of the gel and purified by Gene Clean II (BIO 101, Inc., Vista, CA) according to instructions.

To generate p2rRNAprom a 292-bp fragment containing the *T. brucei*, rRNA promoter was PCR-amplified with primers that added XhoI and BamHI sites to the ends and inserted into the SalI and BamHI sites of pH496 in the opposite orientation to the rRNA promoter already present according to Biebinger (1996).

Plasmid p2rRNAprom/MP 48 RNA editing ligase gene 630bp, 52 560bp RNA editing ligase, Green florescent protein GFP 758 bp were created by inserting 630bp, 560bp and 758 bp respectfully into the HindIII and BamHI sites of p2rRNAprom. A second T7 promoter in the opposite orientation to the T7 promoter already present was added to pBluescriptII SK (-) by annealing oligos 5’-
CGTAATACGACTCACTATAGGGCAGCT-3' and 5'-GCCCATATAGTGAGTCGTATTACGAGCT-3' and ligating into the Sacl site of pBluescriptII SK (-) to give p2T7 Ti. MP 48, p2T7 Ti 52 RNA editing ligase and p2T7 Ti Green florescent protein GFP plasmids.

The MP 48,52 RNA editing ligase, Green florescent protein GFP fragment was excised with BamHI and HindIII, then blunted with T4 DNA Polymerase gene and ligated into the EcoRV site of pBluescriptII SK (-) to generate a complete p2T7/ MP 48,52 RNA editing ligase, Green florescent protein GFP plasmid vector. Figure 9 (Hill et al., 1999)

3.11. Construction pLEW82: GFP plasmid

To create pLEW82 according to Wang and Wirtz (2000,1999) (Figure 3.3): GFP, a HindIII/BamHI fragment from pHD: HX-GFP that included the GFP coding sequence was substituted for the luciferase gene in pLEW82 (Figure 3.3) (Wirtz et al., 1999, Wirtz et al., 1998). pLEW 13 Plasmid (Figure 3.4) contain gene encoding T7 RNA polymerase, Neomycin resistance gene and Tetracycline repressor gene.

To evaluate the RNA interference in T.evansi, pLEW 13 Plasmid and pLEW 28 plasmid were simultaneously transfected into T.evansi by electroporation. T7 promoter in pLEW 28 recognize T7 RNA Polymerase in pLEW13 plasmid, this allowed the promoter to drive the bacteriophage T7 RNA polymerase gene. This lead to production of RNA polymerase, which synthesize mRNA from the open reading frame of pLEW82 to produce Green Fluorescent Protein. Green Fluorescent Protein fluorescence indicated a in a successful reaction the product was Green Fluorescent trypanosome. These were analyzed by Green fluorescent trypanosome images obtained using a Zeiss Axioplan
2 microscope equipped with a ×100 oil immersion objective. Images were captured with an RT Spot Camera. Green fluorescent trypanosome images were obtained using a Zeiss Axioplan 2 microscope equipped with a ×100 oil immersion objective. Images were captured with an RT Spot Camera.

Figure 3.3. Structure of plasmid pLEW82 (Adapted from Dr Noburo Inoue. National Research Center for Protozoan Diseases, Obihiro University, Obihiro, Japan. 2000).
Figure 3.4. Structure of plasmid pLEW13 (Adapted from Dr Noburo Inoue. National Research Center for Protozoan Diseases, Obihiro University, Obihiro, Japan. 2000)
3.13. Cell-line establishment of bloodstream form *T. brucei* Gutat 3.1 strain and *T. evansi* Tansui – 13 for RNA interference analysis

Cell-line establishment of bloodstream form *T. brucei* Gutat 3.1 strain and *T. evansi* Tansui – 13 was done according to LaCount (2000). Bloodstream form *T. brucei* Gutat 3.1 strain and *T. evansi* Tansui – 13 were used for RNA interference analysis. Bloodstream form *T. brucei* Gutat 3.1 strain and *T. evansi* Tansui – 13 were divided in three culture medium respectively. The cultures were treated as indicated in Appendix 6. Cells were maintained in Iscove’s modified Dulbecco’s MEM (Flow laboratories, Irvine, Scotland) media supplemented with 10% fetal bovine serum (FCS). The strains were transfected with *NotI*-linearized plasmids (5-10 μg) p2T71 MP 48,52 RNA editing ligase, Green fluorescent protein GFP (Figure 3.2) and pLEW13 plasmids (Figure 3.4) respectively. Log phase cells (3-6×10^7 ml) were collected by centrifugation, washed with EM (a 3:1 mixture of cytomix (120mM KCl, 0.15mM CaCl2, 10mM KiHPO4, 25mM HEPES, 2mM EDTA, 5mM MgCl2, pH 7.6) and phosphate-sucrose buffer (277mM sucrose, 1mM MgCl2, 7mM KiHPO4, pH 7.4)) (Appendix 7) and resuspended in EM at a concentration of 1×10^7 ml. 0.45ml of cells were mixed with 0.1ml of linearized p2T7/ MP 48,52 RNA editing ligase, Green fluorescent protein GFP (Figure 3.2) and pLEW13 plasmids (Figure 3.4) respectively in a 0.4-cm electroporation cuvette and subjected to two pulses from a Bio-Rad Gene Pulser electroporator set at 1500V and 25microfarads. After electroporation, cells (0.5 ml) were transferred to 4 ml of fresh HMI 93 medium +10% FCS (Appendix 7) and allowed to recover overnight. Stable transformants were selected by 2.5μg /ml; Induction of RNAi expression was by 1μg /ml tetracycline. The G418 Neomycin was added after 12 hours after transfection then 20 min
later tetracycline was added. Cells were counted every 6-hour using hemocytometer.

Drug-resistant cells typically grew out within 7 days (Li et al., 1996, Hirumi et al., 1989).

3.14. PCR analysis

The amount of template cDNA and the number of PCR cycles were optimized and standardized according to the protocol. Positive reaction was considered as PCR product bands analysed as DNA fragments stained with ethidium bromide and visualised by ultraviolet light. The photographs were taken by UVP inc camera then processed using Sony digital graphic printer. The results of the PCR analysis were confirmed in at least three independent experiments.

3.15. DNA Sequences analysis

DNA Sequences of MP 48 and MP 52 RNA ligase genes in *T. evansi* Tansui 13 and *T. brucei* Gutat 3.1 were analyzed on a PE Applied Biosystems 310 automatic DNA sequencer (PE Applied Biosystems). *Trypanosoma brucei* and *Trypanosoma evansi* were compared between themselves and with the data available on Genbank, using the clustal V software.

3.16. *T. evansi* Green Fluorescent Protein analysis

Evaluation of *T. evansi*, ability to use pLEW82 plasmid and pLEW13 plasmid was analyzed as Green fluorescent trypanosome images obtained using a Zeiss Axioplan 2 microscope equipped with a ×100 oil immersion objective. Images were captured with an RT Spot Camera.

3.17. Statistical analysis of RNA Interference

Results are representative of at least five independent experiments. Analysis was to compare the effect of introduction of double stranded MP 48 and MP 52 RNA editing
ligase into *T. evansi* Tansui 13 and *T. brucei* Gutat 3.1. Significance of differences was determined by Student’s t test. P value of <0.05 was considered significant.
CHAPTER FOUR: RESULTS.

4.0. PCR products from mitochondrial related genes.

Primer sets for mitochondrial proteins gene were evaluated, for their ability to diagnose *T.evansi* and *T.brucel* in blood. Several mitochondrial protein gene fragments were amplified by PCR, PCR products were obtained from genomic DNA of the following strains: A) *T.evansi* IL1695 cattle in Africa B) *T.evansi* IL1934 capybara (*Hydrochaerus hydrochaeris*) in South America C) *T.rhodesiense* IL2343 isolated from a patient in Ivory Coast. The following were positive mitochondrial protein gene-amplified products as shown in Figure 4.1:

![Figure 4.1. PCR products for mitochondrial gene](image)

1. *T.evansi* cysteine protease 420bp C.P.
2. *Evansi* glycerol kinase gene, 530 bp K.E.
3. MP52 RNA editing ligase RNA ligase MP52 gene 560bp
4. MP48 RNA editing ligase gene 630bp
5. *Trypanosoma brucei* ATPase submit 9 homolog MRNA, nuclear gene encoding mitochondria rotein.340bp
All the genes in these species were found to be present by PCR. The PCR products could not differentiate *T. evansi* from *T. brucei*.

The specific primers of MP48 RNA editing ligase and MP52 RNA editing ligase PCR products detect and identify *Trypanosoma evansi* causing surra in livestock, *Trypanosoma brucei brucei* causing nagana in livestock, *Trypanosoma brucei gambiense* and *T. b. rhodesiense* causing human African trypanosomiasis. Specific primers of RNA ligase MP48 gene and RNA ligase MP52 were specific for subgenus *Trypanozoon* (Figure 4.2).

![Image of gel electrophoresis](image)

**Figure 4.2.** PCR Specific primers of RNA ligase MP48 gene and RNA ligase MP52 were specific for subgenus *Trypanozoon*. The primers can detect and identify 1) *T. evansi*, 2) *T. brucei* and 3) *T. equiperdum* in clinical specimen.
4.2 DNA Sequencing

The DNA sequence of MP48 RNA editing ligase 630bp and MP52 RNA editing ligase 560bp gene in *T. evansi* and *T. brucei* obtained are presented in Figure 4.3 and Figure 4.4. DNA sequence of MP 48 and MP 52 RNA editing ligase indicate 100% homology in *T.evansi* and *T.brucei* (Figure 4.3 and Figure 4.4). Predicted amino acid sequences of MP48 and MP 52 RNA ligase were 416 and 469 amino acids respectfully as presented in Figure 4.5 and Figure 4.6.
<table>
<thead>
<tr>
<th>Gene</th>
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<th>Gene</th>
<th>Sequence</th>
<th>Gene</th>
<th>Sequence</th>
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<td>IL2343/mp48</td>
<td>ATGTTGCTGCCTCTCTTGGTAGTACGCTACCTCCGCCAACACGGCGGACTCTTTGGTCGTGGGACGGCAAGCA</td>
<td>IL1695/mp48</td>
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<td>IL1934/mp48</td>
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<td>IL2343/mp48</td>
<td>TTTTTGGCTACACAGAAGATCAGATACGCAAAGCGCAGTGGCATTATGCCTCCGAACGAGCACTTCTTTGGCTATCATA</td>
<td>IL1695/mp48</td>
<td>TTTTTGGCTACACAGAAGATCAGATACGCAAAGCGCAGTGGCATTATGCCTCCGAACGAGCACTTCTTTGGCTATCATA</td>
</tr>
<tr>
<td>IL1934/mp48</td>
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<td>IL2343/mp48</td>
<td>GGAAGAAAATGATCAGATACGCAAAGCGCAGTGGCATTATGCCTCCGAACGAGCACTTCTTTGGCTATCATA</td>
<td>IL1695/mp48</td>
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</tr>
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<td>IL2343/mp48</td>
<td>ATTGATCCTCGAGAAGTTGCAGCGATACGTAACTTCTATTCGTGAGATGTTGTGCGAGAAGCATGAAGAAGAA</td>
<td>IL1695/mp48</td>
<td>ATTGATCCTCGAGAAGTTGCAGCGATACGTAACTTCTATTCGTGAGATGTTGTGCGAGAAGCATGAAGAAGAA</td>
</tr>
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<td>IL2343/mp48</td>
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<td>IL1695/mp48</td>
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</tr>
<tr>
<td>IL1934/mp48</td>
<td>CGGAAAACGGTCATGGTGGCTGGTAAGCCGCGAACAATAAGCGCCGTGCAGACCGACTCTTCCCCCAAT</td>
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<td>CGGAAAACGGTCATGGTGGCTGGTAAGCCGCGAACAATAAGCGCCGTGCAGACCGACTCTTCCCCCAAT</td>
<td>IL1695/mp48</td>
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</tr>
<tr>
<td>IL1934/mp48</td>
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<tr>
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<td>IL1695/mp48</td>
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Figure 4.3. Aligned sequences MP 48 RNA ligase Gene, multiple alignments of *T.evansi* IL1934 (A), *T.b.rhodesiense* IL2343(B) and *T.evansi* IL1695 (C)
Figure 4.4. Aligned Sequences Mp52 RNA Ligase Gene, Multiple alignments of *T.evansi* IL1934 (A), *T.b.rhodesiense* IL2343 (B) and *T.evansi* IL1695 (C)
Figure 4.5. Predicted amino acid sequence of MP 48 RNA editing ligase in *T. brucei* and *T. evansi*.

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<th>11</th>
<th>21</th>
<th>31</th>
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<td>ANFGIYSIEG EKMRYAKRS GIMPPNEHFF GYHILELQ RVTVSIREML CEKQKKKLHV 120</td>
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<td></td>
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<tr>
<td>121</td>
<td>VLINGELFGG KYDHPSVPKT RKTYMVAGKP RTISAVQTDTS FPOYSPLHIF YAFDKKTYKET 180</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
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<td></td>
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Number of amino acids: 416

Figure 4.6. Predicted amino acid sequence of MP 52 RNA editing ligase gene in *T. brucei* and *T. evansi*.

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<thead>
<tr>
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<th>21</th>
<th>31</th>
<th>41</th>
<th>51</th>
</tr>
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<td>MQLQRLGAFLKRLVGGCIR QSTAPIMPCV VVSFSGVFLT PVRTYMPLPN DQSDFSPYIE 60</td>
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<td>61</td>
<td>IDLPSRSHQQ SHHSGLAQ EVWACEKVHG TNFGYLINQ GDHEVVRFAK RSGMPDEN 120</td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>121</td>
<td>FFGHYHILDE FTAQIRIILND LLKQKYGLSR VGRLVLNGEL FGAKYKHPLV PKSEKWCTLP 180</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>181</td>
<td>NGKFPFIAQV IQQREPPPOQ SPELHFAFD IKYVSAGEE DFVLLGQDFE VEFSKVPNL 240</td>
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<td>421</td>
<td>AFRKLLTNTV YFESKRLVQ KWKELMQEAA AQQEAIPLP SPAAPTKGE</td>
<td></td>
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</tr>
</tbody>
</table>

Number of amino acids: 469
The MP 48 and MP 52 RNA editing ligase gene sequences obtained in *T. evansi* and *T. brucei* were identical. Comparisons of MP 48 and MP 52 RNA ligase gene with data of closely related organisms available in Genbank® show no significant homology with the RNA ligase sequences of *Trypanosoma cruzi* REL and *Leishmania major* REL2 sp. nor with the available sequences of Lt RNAligase as illustrated in Figure 4.7 and Figure 4.8 respectively. Multiple alignment of *T. evansi* MP52 and MP 48 with related proteins (Figure 4.9 and Figure 4.10), show a perfect-match with *T. brucei* and near-perfect match of proteins with data of closely related organisms available in Genbank
Figure 4.7. Multiple alignment of 630bp of T. evansi Mp48 ORF with the related genes

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<tr>
<th>TevansMP48</th>
<th>TbruceiMP48</th>
<th>TenuRel2</th>
<th>MinorRel2</th>
<th>LirNAligase2</th>
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</thead>
<tbody>
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<td>1</td>
<td>1</td>
<td>1</td>
</tr>
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<td>658</td>
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</tbody>
</table>

Note: The alignment shows the comparison of different genes with the T. evansi Mp48 ORF.
Figure 4.8.A. Multiple alignment of \( T. evansi \) MP52 ORF with those of related genes continues on page 63.
Figure 4.8.B. Multiple alignment of *T. evansi* MP52 ORF with those of related genes from page 62.
Figure 4.9. Multiple alignment of *Trypanosoma evansi* MP 52 with related proteins.
<table>
<thead>
<tr>
<th>Trypanosoma evansi MP48</th>
<th>T. evansi MP48</th>
<th>T. cruzi REL2</th>
<th>L. major REL2</th>
</tr>
</thead>
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<tr>
<td></td>
<td>Tbruce</td>
<td>Tevans</td>
<td>LtRNAilgasel</td>
</tr>
<tr>
<td></td>
<td>Tbruce</td>
<td>Tevans</td>
<td>Lm'ajor</td>
</tr>
<tr>
<td></td>
<td></td>
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</tr>
</tbody>
</table>

**Figure 4.10. Multiple alignment of Trypanosoma evansi MP48 with related proteins.**
4.3 Expression of Green Fluorescence Protein (GFP) in *T.evansi*

Molecular components of the RNA interference (RNAi) machinery and control of gene expression in *T.evansi* was evaluated. Since, different organisms are variably susceptible to RNAi (Wang *et al.*, 2000, LaCount *et al.*, 2000). Green Fluorescence Protein has been shown to be an effective target of RNAi strategies in both trypanosomes and other organisms. The expression of GFP in *T.evansi* was tested. In order to investigate RNAi functions in *T.evansi*, first, Neomycin resistance gene, T7 RNA polymerase and Tetracycline repressor genes were cloned into pLEW 13 Plasmid. Tetracycline operator, T7 promoter and green fluorescence protein genes were cloned into pLEW 28 Plasmid. pLEW 13 Plasmid and pLEW 28 Plasmid were simultaneously electroporated into *T.evansi*. Twenty four to forty eight hours of post-transfection, the expression of GFP was successfully as revealed by Green Fluorescent Protein expression, as a phenotype, including the appearance of the cell morphology Green Fluorescent *T.evansi*. (Figure 4.11 and Figure 4.12)

The result show the ability of *T.evansi* with the help of Bacteriophage T7 RNA polymerase gene in pLEW 13 plasmid to drive the expression of Green Fluorescent Protein gene in pLEW 28 Plasmid and appear as Green Fluorescent trypanosome under Fluorescent and differential inference contrast microscope. This determines that *T.evansi* has molecular components for RNAi experiment machinery functions as appears in Figure 4.11 and Figure 4.12.
Figure 4.11. Green Fluorescent *T.evansi*.

Figure 4.12. Plasmids are successful electropoarted by GFP expression in *T.evansi Tansui 13*. 
4.4. RNA interference of 48 and 52 RNA Editing ligase in *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1

Two opposing bacteriophage T7 promoters can be used to generate RNAi in procyclic *T.brucei* expressing the T7 RNA polymerase (LaCount et al., 2000). This experiment developed an integratable version of the two-T7 promoter vector (p2T7Ti) that utilizes tetracycline-inducible T7 promoters to generate sense and antisense RNA from the DNA sequences placed between them (MP48 and MP 52 ligase gene (Figure 3.2). *T.evansi* IL1695 MP 48 and MP 52 RNA editing ligase PCR products were cloned into p T7-Blue (Figure 3.1) to form recombinant plasmid that was transformed into α DH 5 *E. coli*.

The purified plasmid was cut at BamHI and HindIII site by corresponding restriction enzyme and suitable buffer. These resulted into two bands, a long band with over 1000bp that is part of into p T7-Blue and small band at 560bp for MP52 RNA editing ligase gene and 630bp for MP 48 RNA editing ligase gene (Figure 4.13).

MP 48 and MP 52 RNA editing ligase (Figure 4.14) fragment were ligated with p2T7Ti plasmid (Figure 4.15) to form plasmid p2T7Ti-48 and p2T7Ti-52 respectfully (Figure 4.16). The plasmid were simultaneously transfected with pLEW13 plasmids (Figure 4.12) into *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1.

These result indicate that *T.evansi* IL1695 contain both genes used for plasmids construction of double stranded MP48 and MP 52 RNA editing ligase gene (Figure 4.16) in p2T7Ti vector. The picture (Figure 4.16) shows that p2T7Ti MP48 and MP 52 RNA editing ligase gene respectively were successfully constructed for RNA interference experiment to generate phenotypic knockout parasites.
Figure 4.13. *T. evansi* IL1695 MP 48 and MP 52 RNA ligase gene PCR products cloned in p T7-Blue.

Figure 4.14. pT7-Blue vector/MP48 and MP52 gene recombinant plasmid cut at BamHI and Hind III site resulted into two bands over 1000bp pT7-Blue and small band at 560bp for MP52 gene and 630bp for MP 48 gene.

Figure 4.15. p2T7Ti was cut at BamHI and Hind III site resulting in two bands, a large size DNA band 6000bp that is part of p2T7Ti plasmids fragment to be ligated with 560bp for MP52 gene and 630bp for MP 48 gene form Figure 4.14.
Complete p2T7Ti containing of 48 RNA Editing ligase 630bp , ready for transfection.

Complete p2T7Ti containing of 48 RNA Editing ligase 560 bp , ready for transfection.

Figure 4.16. The bands as a result of ligation of large size plasmid DNA fragment with 6000bp Figure 4.15, which is part of p2T7Ti, and small DNA fragment band at 560bp Figure 4.14 for MP52 gene and 630 respectfully.

4.4.1 Effect of double stranded RNA interference of 48 RNA Editing ligase in T.evansi Tansui 13 and T.brucet Gutzat 3.1. Within 36 hours.

4.4.1.1. P value and statistical significance of 48 RNA Editing ligase interference in T.evansi Tansui 13 and T.brucet Gutzat 3.1.

The two-tailed P value of T.evansi Tansui 13 and T.brucet Gutzat 3.1. 48 RNA Editing ligase interference was 0.6617. This difference was considered not to be statistically significant. The mean of T.evansi 48 and T.brucet 48 was 0.3960 at 95% confidence.

t = 0.4543 df = 8 standard error of difference = 0.872

Table 4.3. Statistic analysis for RNAi using MP48 RNA editing ligase.

<table>
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<th>Group</th>
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<th>T.brucet 48</th>
</tr>
</thead>
<tbody>
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<td>5.5840</td>
</tr>
<tr>
<td>SD</td>
<td>1.1862</td>
<td>1.3715</td>
</tr>
<tr>
<td>SEM</td>
<td>0.5305</td>
<td>0.6917</td>
</tr>
<tr>
<td>N</td>
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</tbody>
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Table 4.4. Total % of dead *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1 caused by RNAi MP48 RNA ligase.

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<tr>
<th>Time in hours at an interval of 6 hours</th>
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<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>Total % of dead parasite</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T.evansi</em> MP 48 No of surviving parasites in 10^3</td>
<td>7.5</td>
<td>6.7</td>
<td>6.0</td>
<td>5.2</td>
<td>4.5</td>
<td>40 %</td>
</tr>
<tr>
<td><em>T.brucei</em> MP 48 No of surviving parasites in 10^3</td>
<td>7.5</td>
<td>6.6</td>
<td>5.6</td>
<td>4.62</td>
<td>3.6</td>
<td>52%</td>
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</tbody>
</table>

4.2.4 Effect of double stranded RNA interference of 52 RNA Editing ligase in *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1. Within 36 hours.

4.2.4.1 P value and statistical significance of 52 RNA Editing ligase interference in *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1:

The two-tailed P value value of *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1. 52 RNA Editing ligase interference was 0.5954 by conventional criteria, this difference is considered to be not statistically significant. The mean of *T.evansi* 52 and *T.brucei* 52 was 0.4280 at 95% confidence interval of this difference:

\[ t = 0.5529 \text{ df } = 8 \text{ standard error of difference } = 0.774 \]

Table 4.5. Statistic analysis for RNAi using MP 52 RNA editing ligase.

<table>
<thead>
<tr>
<th>Group</th>
<th>T.evansi 52</th>
<th>T.brucei 52</th>
</tr>
</thead>
<tbody>
<tr>
<td>Mean</td>
<td>6.1820</td>
<td>5.7540</td>
</tr>
<tr>
<td>SD</td>
<td>1.0560</td>
<td>1.3715</td>
</tr>
<tr>
<td>SEM</td>
<td>0.4723</td>
<td>0.6134</td>
</tr>
<tr>
<td>N</td>
<td>5</td>
<td>5</td>
</tr>
</tbody>
</table>
Table 4.6: Total % of dead *T. evansi* Tansui 13 and *T. brucei* Gutat 3.1 caused by RNAi MP 52 RNA editing ligase.

<table>
<thead>
<tr>
<th>Time in hours at an interval of 6 hours</th>
<th>12</th>
<th>18</th>
<th>24</th>
<th>30</th>
<th>36</th>
<th>Total % of dead parasite</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>T. evansi</em> MP 52 No of surviving parasites in $10^3$</td>
<td>7.5</td>
<td>6.8</td>
<td>6.1</td>
<td>5.81</td>
<td>4.7</td>
<td>38 %</td>
</tr>
<tr>
<td><em>T. brucei</em> MP 52 No of surviving parasites in $10^3$</td>
<td>7.5</td>
<td>6.62</td>
<td>5.7</td>
<td>4.95</td>
<td>4.0</td>
<td>46. %</td>
</tr>
</tbody>
</table>

Using an inducible RNAi system the experiment was set out to determine MP 52 and 48 RNA Editing ligases importance in *T. evansi* viability.

To examine whether RNAi-inhibition of MP 52 and 48 RNA Editing ligases affected the bloodstream form *T. brucei* and *T. evansi*, transfection with the plasmids were performed. The effects were compared 12 hours after transfection. In the absence of tetracycline, all four recombinant cell lines were found to grow at approximately the same rate as the parental cells.

When tetracycline was added, there was major decline in the population of the $7.5 \times 10^3$ parasites that had been transfected with p2T7Ti-48 10µg ml / pLEW13µg ml, p2T7Ti-52µg ml / pLEW13µg ml and p2T7Ti-GFPµg ml / pLEW13µg ml control constructs (Figure 4.12). Introduction of double stranded RNA had a dramatic effect on the survival of the parasites population (Figure 4.17). Within 24 h after transfection and induction by tetracycline, there were rapid declines in population of these parasites. In the next 18 hrs, 40% and 52% of *T. evansi* and *T. brucei* respectively died due to lack of MP 48 RNA editing ligase caused by RNA interference (Table 4.3 and 4.4). Thirty eight percent and forty six percent of *T. evansi* and *T. brucei* respectively died due to lack of MP 52 RNA editing ligase caused due RNA interference (Table 4.5 and 4.6). The parasites,
which survived, contain only pLEW13µg ml plasmids that contain a neomycin resistant gene. This wild type parasite has been observed in previously studies, following induction of RNAi against essential genes in *T. brucei* (Furuya *et al.*, 2002). It can be inferred that *T. brucei* and *T. evansi* were equally susceptible to RNAi-mediated knockouts (Figure 4.17). The parasites displayed a significant reduction in the original *T. brucei* and *T. evansi* population (Figure 4.17). The MP 52 and 48 RNA Editing ligase is are essential in the mammalian stage of *T. brucei* life cycle essential for the survival of both bloodstream form *T. brucei* and *T. evansi*. Parasites are standardized at time 0 to 6 organism are allowed to grow. At time 12, the parasites were standardized at 7.5 x 10³ cells for each parasite. Tetracycline was added to induce RNAi in the parasites.
CHAPTER FIVE: DISCUSSION

5.1 Relationship of *T. evansi* and *T. brucei* at genome level, with respect to mitochondrial encoding genes

Polymerase chain reaction has broad applications; its sensitivity permits enzymatic amplification of gene fragments from minute quantities of nucleic acids derived from limited amounts from parasitic material. The main difficulties encountered in the study, were the search for primers with good amplification potential for differentiation of a suitable DNA region giving a distinct difference between *T. brucei* and *T. evansi*.

This work attempted to differentiate *T. brucei* from *T. evansi* using PCR on mitochondrial related genes. *Trypanosoma brucei* ATPase submit 9 homolog mRNA, nuclear gene encoding mitochondria protein 340bp(T.B.ATP), *T. evansi* glycosomal glyceraldehyde-3-phosphate dehydrogenase 988 bp (Ggapdh), *T. evansi* cysteine protease 420bp (C.P), Evansi glycerol kinase gene, get product 530 bp, MP48 RNA editing ligase gene 630bp and MP52 RNA editing ligase gene 560bp. Polymerase chain reaction results indicate that these genes are found in both species. Therefore, PCR performed using specific primers cannot differentiate *T. brucei* from *T. evansi*. The results indicate that the mitochondrial genes are located in the genomic DNA of *T. evansi*, while in *T. brucei* they are located in maxi circles Kinetoplast DNA.

The mitochondrial related genes, are essential for survival of *T. brucei*, since it has an insect vector stage where the parasite depends completely on a functional Krebs cycle.
and oxidative phosphorylation for its supply of ATP. At the functional level pleomorphism reflects the ability of *T. brucei* to develop in its vector, the tsetse fly, and this is in turn dependent on possession of a complete and functional set of genes for mitochondrial operation.

The mitochondrial genes are in the maxi circle DNA of the kinetoplast of *T. brucei*, together with the set of mini circle-encoded genes necessary for editing the maxi circle transcripts so they can be correctly translated. These features define *T. brucei*, and their absence defines *T. evansi* and *T. equiperdum*. Neither *T. evansi* nor *T. equiperdum* is cyclically transmitted by tsetse and indeed; neither species is capable of cyclical development. *T. evansi* lacks a mitochondrial genome and its kinetoplast contains only a homogeneous set of mini circles. The few isolates of *T. equiperdum* examined also have missing kinetoplast DNA. One Chinese strain of *T. equiperdum* had maxi circles just over half the size of those of *T. brucei* and homogeneous mini circles like *T. evansi* (Lun et al., 1993). Two other laboratory strains of *T. equiperdum* also had homogeneous mini circles; one had full-size and one reduced size maxi circles (Riou et al., 1979, Frasch et al., 1979).

The well-characterized difference between *T. evansi* and *T. brucei* was found in the kinetoplast DNA (kDNA) (Borst et al., 1979). *T. brucei* possesses a typical kDNA that consists of 50 copies of similar 23 kb maxi circles DNA that is equivalent to a mitochondria DNA of other organisms. Mitochondrial protein genes are encoded in the maxi circles DNA (Aratama et al., 1992) and the organism has thousands of the heterogeneous mini circle DNA fragments. *Trypanosoma evansi* contains only one type of 1kbp mini circle DNA and does not have a maxi circles DNA equivalent to mitochondrial DNA (Borst et al., 1979).
The mammalian bloodstream forms of both species depend completely on glycolysis for their supply of ATP. They possess neither a functional Krebs cycle nor oxidative phosphorylation, nor do they store any carbohydrate (Bakker et al., 1995, Kacser et al., 1973, Heinrich et al., 1974, Westerhoff et al., 1987). Several works have been reported on the maxi circle and mini circle kinetoplast DNA of *T. brucei* and mini circle kinetoplast DNA of *T. evansi* (Borst et al., 1979), but they did not end at a PCR differentiation of these parasite.

Analyses of isoenzyme electrophoresis pattern are a useful tool for characterization of a variety of protozoan parasite, such as *Trypanosoma* (Godfrey et al., 1976), *Leishmania* (Al-Taqi et al., 1978), *Toxoplasma* (Barnet et al., 1988) and *Theileria* (Musisi et al, 1981). However Gibson demonstrated that *T. evansi* was indistinguishable from *T. brucei* especially from West Africa tsetse transmitted trypanosome, using isoenzyme analyses (Gibson et al., 1980). Stevens and Gibson (1999) demonstrated similarities of isoenzyme electrophoresis patterns between *T. evansi* and West Africa *T. brucei* (Stevens et al., 1992), (Gibson et al., 1983). The sequence analyses of ribosomal RNA genes (rRNA) are a useful tool for molecular phylogenetic analysis among related species. According to the results from the sequence analyses of rRNA, *T. evansi* was not so different as to be classified as an independent species from *T. brucei* (Urakawa Abstract, SURRA symposium, 1999), Furthermore. Artama demonstrated that the 177 bp nuclear RNA repeat was identical in the two species (Artama et al 1992). PCR amplification patterns of procyclic acidic repetitive protein (PARP) genes, which encoded a major surface glycoprotein of procyclic forms of *T. brucei* were the same (Mowatt et al., 1987). These results may suggest a striking resemblance between *T.
evansi and T. brucei at a genomic DNA level. The findings are that, T.evansi and T.brucei are evolutionary closely related and cannot currently be differentiated by PCR of tested mitochondrial genes.

5.2 Presence of MP48 and MP52 RNA editing ligase in T. evansi

Progress in diagnosis, treatment, and epidemiology of human African trypanosomiasis, Nagana and surra depends on the existence of specific and sensitive diagnostic tools. Inherent shortcomings of serologic and parasitologic diagnostic methods can be overcome by molecular biology techniques. A new polymerase chain reaction (PCR) test using specific primers was developed, from the recently identified sequence of the T.evansi and T.brucei MP48 and MP 52 RNA editing ligases. The specific primers are MP 48 RNA editing ligase gene F5' ATGTTGCGTCGCTGCTCGGGTCGTT 3', R5' ATGACCGCCCTCGCTGACTACA 3' 630 bp and MP 52 RNA editing ligase gene MP525'ATGCAACTCCAAAGGTTGGGTGCT3',R5'ATAGGGAATTCTCTTCCGGTT TGGTAGCCTG 3' 560 bp.

Presence of MP 48 and 52 ligases in total DNA extraction of T. evansi, T. brucei, T. equiperdum, T. congoense, T. vivax, T. simiae total bovine DNA, Plasmodium vivax and Theileria were tested and compared. DNA's of T. evansi, T. brucei and T. equiperdum indicated positive PCR products on agarose. Deoxyribonucleic acid sequence coding for MP48 RNA editing ligase gene, 630 bp and MP 52 RNA editing ligase gene 560 bp were also found to be present in the genome of T. evansi, T. brucei and T. equiperdum. Search of the above oligonucleotide sequence in the GenBankTM database show no homology to any sequence of other organisms. The DNA sequences of MP48 RNA editing ligase and MP 52 RNA editing ligase can be of great value for
distinguishing pathogenic subgenus *Trypanozoon* from pathogenic subgenus *Nannomonas*.

These specific primers are of major importance when using the test as an epidemiological tool to screen large numbers of field samples in geographical area where presence of this, these parasites overlap with pathogenic subgenus Trypanozoon. Finally, these primers are an ideal candidate for PCR diagnosis for infections caused by pathogenic parasites of the subgenus Trypanozoon in animals and human beings.

After purifying and sequencing of MP 48 and MP 52 ligase gene in *T. evansi* and *T. brucei*, alignment of the MP 48 and MP 52 RNA Editing ligase gene was possible, since the sizes and sequences were completely the same with a 100% homology. Comparisons of MP 48 and MP 52 RNA ligase gene with data of closely related organisms available in Genbank® showed no significant homology with the RNA editing ligase sequences of *T.cruziREL* and *L. majorREL2* nor with the available sequences of *Lt RNAligase* as illustrated in Figure 4.7, 4.8, 4.9 and 4.10. Multiple alignment of *T. evansi* MP52 and MP 48 with related proteins show a perfect-match with *T. brucei* and near-perfect match of genes with data of closely related organisms available in Genbank (Figure 4.5 and 4.6). This characteristic may serve as a basis for molecular differential diagnosis of *T. evansi* and *T. brucei* against other organism. The evidence further strengthens the close evolutionary relationship between the two organisms.

5.3 *Trypanosoma evansi* ability to drive T7 RNA polymerase lead to the expression of Green Fluorescent Protein gene with the help of T7 promoter.

To study the RNA interference in *T.evansi*, it was important to establish whether constructed plasmids can operate within this organism. Currently, there is no literature or
work concerning studies on RNAi in *T.evansi*. A method for RNAi interference experiment in *T.evansi* was developed. Promoter in pLEW13 plasmid was able to recognize the bacteriophage T7 RNA polymerase gene in pLEW82 plasmid to produce RNA polymerase to synthesize mRNA that was translated into Green Fluorescent Protein (GFP) and observed as Green Fluorescent trypanosome. The presence of GFP in *T.evansi* indicates that an RNA interference mechanism was possible in the organism. Therefore, it was possible to insert MP 48 and MP 52 RNA editing ligase gene between two-T7 promoters of the p2T7Ti plasmid in a head to head orientation to generate phenotype knockout. The promoters recognize bacteriophage T7 RNA polymerase to synthesize sense and antisense double stranded RNA. Further more T7 promoters- induction by tetracycline successfully generated sense and antisense double stranded RNA MP 48 and MP 52 RNA editing ligase leading to the death of *T.evansi* cells due to RNAi activity.

5.4 The generation of knockout phenotype due to the introduction of double stranded RNA (ds RNA) MP 48 and MP52 RNA editing ligase genes into *T. evansi* and *T. brucei*. (RNA interference)

Over 30 million people are infected with the parasites belonging to the family Trypanosomatidae, with a further 510 million at risk (WHO, 1998). The parasites cause a variety of diseases including African sleeping sickness (*T. brucei*), Chagas’ disease (*T. cruzi*), and cutaneous/visceral leishmaniasis (*Leishmania spp.*). These diseases are prevalent in parts of the world least able to afford the economic burden. With no immediate prospect of a vaccine and problems associated with current drug regimes, the requirement for new cost-effective treatments is a priority.
RNA silencing processes are widespread in almost all organisms. They have various functions including genome protection, the control of gene expression, development and heterochromatin formation. RNA interference (RNAi) is the post-transcriptional destruction of messenger RNA. Ribonucleic acid interference is functional in *T. brucei*, a protozoan parasite that separated very early from the main eukaryotic lineage and exhibits several intriguing features in terms of the control of gene expression.

Ribonucleic acid interference (RNAi) of MP 48 and MP 52 RNA editing ligase was investigated in *T. evansi*. MP 48 and MP 52 RNA editing ligase are known to be essential for survival of bloodstream forms of *T. brucei* (Achim *et al.*, 2001). RNA editing ligase enzymes are involved in a posttranscriptional specific insertion and deletion of uracil residues in mitochondrial transcript that involved in MP 52 and 48 RNA Editing ligase activity which are essential in the mammalian stage of *T. brucei* life cycle. *T. brucei* MP 52 and 48 RNA Editing ligases have been identified genetically and characterized at the biochemical level in *T. brucei*.

The introduction of double stranded RNA into *T. brucei* causes specific degradation of the corresponding homologues endogenous mRNA (LaCount *et al.*, 2002). Messenger RNA degradation will lead to inhibition of the translation of mRNA and stop protein synthesis of MP 48 and MP 52 RNA editing ligases, leading to the death of *T. brucei* cells. The dead cells are referred to as phenotypic knockouts.

It was hypothesized that, since *T. evansi* depends entirely on glycolysis for energy production and does not have mitochondria, therefore, *T. evansi* does not require posttranscriptional editing of maxi circle kinetoplast DNA transcripts by RNA editing.
complex that contain MP 48 and MP 52 RNA editing ligases for its survival. Therefore, inhibition of these ligases were not expected to be lethal in bloodstream form of *T.evansi*

*Trypanosoma brucei* has a maxi circle kinetoplast DNA equivalent to mitochondria DNA of other organisms (Borst *et al.*, 1979) and depends on functional glycolysis in bloodstream forms, krebs cycle and oxidative phosphorylation in the insect forms. RNA editing ligase enzymes are involved in posttranscriptional editing by specific insertion and deletion of uracil residues in mitochondrial transcript. These enzymes are essential in the mammalian stage of *T. brucei* life cycle (Achim *et al.*, 2001). The repression of MP 52 and 48 RNA Editing ligase genes are lethal in bloodstream form of *T.brucei* (Achim *et al.*, 2001).

The primary components of the experiment dealt with whether introduction of double stranded RNA (RNA interference) causes specific degradation of corresponding homologous mRNA in *T.brucei* and *T.evansi*.

The effect of introduction of double stranded MP 48 and MP 52 RNA ligase in *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1 was compared. Growth curve in Figure 4.17 indicated that both *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1 cell died as a result of RNAi (phenotypes), due to lack of 48 and MP 52 RNA editing ligase caused by the degradation of corresponding homologous mRNA within the first 42 hours of transfection. These confirm successful ds RNA interference activity in both strains. Approximately 40% of original populations of both the species were killed due to RNAi (Tables 4.3,4.4,4.5 and 4.6) The were no significantly differences in the effect of RNAi in *T.brucei* Gutat 3.1 and *T.evansi* Tansui 13 p> 0.05 for both ligases. A new drugs target in *T.evansi* was identified using RNA interference.
This was an interesting finding, since, *T.evansi* does not require post transcription editing of maxi circle kinetoplast DNA transcripts by RNA editing complex. The organism does not have maxicircle kinetoplast DNA or a mitochondrial DNA transcript to be edited. There is no scientific explanation about this strange behavior, therefore further research work need to be conducted, to find out whether these ligases have other essential activity not related to mitochondrial transcripts. The results deduce that these two species are very closely related, since they have 100% DNA sequence homology and perfect-match multiple alignment of MP52 and MP 48 proteins and are affected by lack of the ligase.

MP 48 and MP 52 RNA editing ligase are unique to *T.evansi* and *T.brucei* with DNA sequence and amino acid sequence different to the other known RNA editing ligase found in *L. major, T.cruzi*. There is no homology to any other protein or genes in the GenBankTM database, making MP 48 and MP 52 RNA editing ligases an ideal chemotherapy target for treatment of infection caused by these group of parasites.

Among different disease agents, parasites are the most similar to their human hosts. Glycosylphosphatidylinositol (GPI) transamidase has been identified as a good target for antitypanosome drugs but some components are similar to humans and animals.

Glycosylphosphatidylinositol (GPI) anchor is a membrane attachment mechanism for cell surface proteins widely used in eukaryotes. GPIs are added to proteins posttranslationally by a complex enzyme, GPI transamidase. Previous studies have shown that human and *Saccharomyces cerevisiae* GPI transamidases are similar and consist of five homologous components: GAA1, GPI8, PIG-S, PIG-T, and PIG-U in humans and Gaa1p, Gpi8p, Gpi17p, Gpi16p, and Cdc91p in *S. cerevisiae*. *Trypanosoma brucei* (*Tb*), a causative
agent of African sleeping sickness, shares only three components (TbGAA1, TbGPI8, and TbGPI16) with humans and S. cerevisiae but has two other specific components, trypanosomatid transamidase 1 (TTA1) and TTA2 (Nagamune et al., 2003).

MP 48 and MP 52 RNA editing ligases are unique to T.evansi and T.brucei and eliminate chances of side effects to drugs targeted against the parasites in humans and animals. Currently there are other essential enzymes already identified as drug targets, e.g. farnesyl pyrophosphate synthase (TbFPPS) of Trypanosoma brucei. The protein (TbFPPS) is an attractive target for drug development because the growth of T. brucei has been shown to be inhibited by analogs of its substrates, the nitrogen containing bisphosphonates currently in use in bone resorption therapy (Montalvetti et al., 2003). In addition, MP 48 and MP 52 RNA editing ligases are synergistic, that is, when one ligase is destroyed the other cannot perform its function. Therefore each of RNA editing ligases is an independent target for drug development.
CHAPTER SIX: CONCLUSION AND RECOMMENDATIONS.

6.1. Conclusions

Relationship of *T. evansi* and *T. brucei* at genome level, with respect to the mentioned mitochondrial encoding genes are similar. *T. evansi* cysteine protease, *Evansi* glycerol kinase gene, MP52 RNA editing ligase gene, MP48 RNA editing ligase gene, *Trypanosoma brucei* ATPase submit 9 homolog MRNA, nuclear gene encoding mitochondria protein and *T. evansi* glycosomal glyceraldehyde-3-phosphate dehydrogenase.

Presence of functional MP48 and MP52 RNA editing ligases in *T. evansi* were established.

It was determined that T7 RNA polymerase can drive the expression of Green Fluorescent Protein gene with the help of T7 promoter in *T. evansi*. Therefore, phenotypic knockouts in *T. evansi* are possible, when applying RNA interference with the help of electroporated pLEW13 and pLEW82 plasmids.

Introduction of double stranded RNA (ds RNA) MP 48 and MP52 ligase genes into *T. evansi* and *T. brucei* affected both organisms, leading to their death as a result of RNA interference.

6.2. Recommendations.

The new sub genus specific primers for MP 48 and MP52 RNA editing ligase genes should be tested in the field as diagnosis and epidemiological tool, to monitor pathogenic subgenus *Trypanozoon* in humans and domestic animals.

New and vulnerable protein targets within the cellular processes of the parasite are needed in the search for novel therapeutics. Research on the metabolism of parasites
is of immediate interest because function that are essential for the parasite but not the
host are potential targets for antiprotozoal compounds that would block that function but
be safe for humans for example MP 48 & MP 52 RNA editing ligase. New compound
that can inhibit MP 48 & MP 52 RNA editing ligase need to be identified, for future drug
development against trypanosomiasis.
References.


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Nagamune, K; Ohishi, K; Ashida, H; Hong, Y; Hino, J; Kangawa, K; Inoue, N; Maeda, Y and Kinoshita T. (2003). GPI transamidase of *Trypanosoma brucei* has two previously uncharacterized (trypanosomatid transamidase 1 and 2) and three common subunits. *Proc Natl Acad Sci USA.* **100; 19**: 10682-10687.


Appendix 1: Classification of phylum (Adapted from Cavalier-Smith, 1993)

**Taxonomic status:**

Class Mastigophora

Family Trypanosomatidae

Order Kinetoplastida Euglenozoa

Genus Trypanosoma and Leishmania

Species *T. b. gambiense*, *T. b. rhodesiense*

*T. b. brucei*, *T. congoense*, *T. vivax* and *T. evansi*.

*T. equiperdum* and *T. cruzi* —
Appendix 2: Genomic DNA isolation from blood. (Sambrook et al., 2001)

Protocol.

Blood samples will be taken in EDTA vacutainer tubes. Fresh samples can be used immediately or stored at -70°C. Thaw before use. Remove one ml of blood and add 0.8 ml 1X SSC buffer, and mix. Centrifuge for one minute at 12,000 rpm in a microcentrifuge.

Remove the supernatant and discard into a disinfectant.

Add 1 ml of 1X SSC buffer, vortex, and centrifuge as above for 1 minute, and remove all of the supernatant. Discard the supernatant into a disinfectant.

Add 375 µl of 0.2M NaOAc to each pellet and vortex briefly. Then add 25 µl of 10% SDS and 5 µl of proteinase K (20 mg/ml H2O) (Sigma P-0390), vortex briefly and incubate for 1 hour at 55°C.

Add 120 µl phenol/chloroform/isoamyl alcohol (25:24:1) and vortex for 30 seconds.

Centrifuge the sample for 2 minutes at 12,000 rpm in a microcentrifuge tube.

Carefully remove the aqueous layer to a new 1.5ml microcentrifuge tube, add 1ml of cold 100% ethanol, mix, and incubate for 15 minutes at -20°C.

Centrifuge for 2 minutes at 12,000 rpm in a microcentrifuge. Decant the supernatant and drain.

Add 180 µl TE buffer, vortex, and incubate at 55°C for 10 minutes.

Add 20 µl of 2M sodium acetate and mix. Add 500 µl of cold 100% ethanol, mix, and centrifuge for 1 minute at 12,000 rpm in a microcentrifuge.
Decant the supernatant and rinse the pellet with 1 ml of 80% ethanol. Centrifuge for 1 minute at 12,000 rpm in a microcentrifuge.

Decant the supernatant, and dry the pellet in a Speed-Vac for 10 minutes (or until dry).

Resuspend the pellet by adding 200µl of TE buffer. Incubate overnight at 55°C, vortexing periodically to dissolve the genomic DNA. Store the samples at -20°C.
Appendix 3: Growth of bacterial cultures (Sambrook et al., 2001).

From glycerol stocks:

Pipet 1-2ml of LB medium into a 17x150cm sterile snap cap culture tube (Falcon). Add an appropriate amount of antibiotic to the medium. Ampicillin should be at a final concentration of 50μg/ml of medium.

If the ampicillin stock concentration is 100mg/ml, add 1μl ampicillin stock to 2ml LB medium to obtain a final ampicillin concentration of 50μg/ml of medium.

If the ampicillin stock concentration is 50μg/ml, add 1μl ampicillin stock to each ml LB medium to obtain a final ampicillin concentration of 50μg/ml of medium.

Inoculate the LB/ampicillin medium with transformed cells stored at -80°C in glycerol.

Transfer the cells from the culture tube into 500ml LB containing an appropriate antibiotic. The amount of ampicillin added is again dependent on the stock concentration, see the above examples. Incubate this culture overnight at 37°C and 225rpm.

Prepare plasmid DNA using an appropriate protocol.

From an isolated colony of transformed cells

Prepare 1ml LB medium as described above. Inoculate the culture by picking a well-isolated colony from a LB plate containing an appropriate antibiotic. Incubate the cells overnight at 37°C and 225rpm.
Transfer the cells from the culture tube into 500ml LB containing an appropriate antibiotic. Incubate this culture overnight at 37°C and 225rpm.
Appendix 4. Transformation of *E. coli* by electroporation Adapted from (Eppendorf Electroporator USA 2000)

Preparation of Electrocompetent bacterial cells:

Start a fresh overnight culture with the *E. coli* strain of interest. Grow at 37°C with moderate shaking.

Inoculate a flask of LB medium 1:200 with the fresh overnight culture, being sure that the flask is large enough to allow adequate aeration. Grow at 37°C with shaking to an O.D.600 of 0.5 to 0.6.

Chill the cells in an ice-water bath 15 minutes and transfer to a pre-chilled centrifuge bottle. Centrifuge 20 minutes at 5000-x g, 2-4°C. Resuspend the pellet in 5ml ice-cold water. It is important to keep the cells cold during the entire procedure. Add the original culture of ice-cold water. Centrifuge as above. Repeat this wash once. Pour off the supernatant immediately and resuspend pellet by swirling in remaining liquid.

If using the cells immediately, place suspension in a pre-chilled, narrow-bottom, 50ml polypropylene tube and centrifuge 10 minutes at 5000 x g, 2-4°C. Resuspend in a volume of ice-cold water to yield approximately 2 x 10^11 cells/ml. Aliquot 40-300 μl cells into pre-chilled microcentrifuge tubes.

If freezing the cells for later use, add 40μl of ice-cold 10% glycerol, mix, and centrifuge 10 minutes at 5000 x g, 2-4°C. Estimate pellet volume, add an equal volume of ice-cold 10% glycerol to resuspend cells, and aliquot 40 to 300μl into pre-chilled microcentrifuge tubes. Quick-freeze on dry ice and store at -80°C.
Electroporation of bacterial cells:

Set the electroporator to desired voltage. Generally for *E. coli*, field strengths of 16-19kV/cm are required to obtain maximum transformation efficiency. Each strain of *E. coli* will have optimum field strength and this will need to be determined empirically.

Add 1μl plasmid DNA (5pg to 0.5μl) to tubes containing 40-50μl electrocompetent cells.

Mix. If using frozen cells, thaw the cells on ice immediately before use. Once thawed, the cells must be used or discarded. Any leftover cells may not be re-frozen for later use.

Transfer cell/DNA mixture to a pre-chilled cuvette, incubate on ice 5 minutes, dry the outside of the cuvette, insert into cuvette holder, and place into the electroporator.

Transfer cell/DNA mixture to a pre-chilled cuvette, incubate on ice 5 minutes, dry the outside of the cuvette, insert into cuvette holder, and place into the electroporator.

Apply the pulse. Remove cuvette and immediately add 1ml SOC medium (without antibiotics) and transfer to a sterile culture tube with a Pasteur pipette. Allow the cells to recover by incubating 30-60 minutes with moderate shaking at 37° C.

Plate various aliquots on LB plates containing the appropriate selection chemical
Table 7.1. Electroporation of *E. coli*

<table>
<thead>
<tr>
<th><em>E. coli</em> Strain</th>
<th>Field Strength (kV/cm)</th>
<th>Transformation efficiency (transformants/µg DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C600</td>
<td>19</td>
<td>2 x 10⁹</td>
</tr>
<tr>
<td>K12</td>
<td>17</td>
<td>3.5 x 10⁹</td>
</tr>
<tr>
<td>DH5</td>
<td>17</td>
<td>3 x 10⁹</td>
</tr>
<tr>
<td>DH10B</td>
<td>16.6</td>
<td>4 x 10⁹</td>
</tr>
</tbody>
</table>

Electroporation was performed using the Electroporator 2510 (Eppendorf), 1mm cuvettes, 40µl of cells, and 10pg of pUC19.
Appendix 5. Preparation of miniprep DNA (Adapted from Sambrook et al. 1989)

Protocol

Pick a well-isolated colony grown under selection (e.g., ampicillin) on an LB plate. Inoculate 3mL LB liquid culture containing the same selection antibiotic. Incubate the culture overnight (16 hours) at 37° C and 225rpm.

Pipet approximately 1.5ml bacterial culture into a microcentrifuge tube. Store the remaining culture at 4° C. Centrifuge to pellet the cells for 2-3 minutes at maximum speed in a microcentrifuge. Aspirate the culture medium and resuspend the cells in 100μl Solution I. Pipet the cells up and down several times to completely break up the pellet. Lyse the cells by the addition of 200μl of fresh Solution II. Mix gently by inverting the tube back and forth several times. Do not vortex.

Precipitate bacterial membranes and genomic DNA by the addition of 150μl Solution III. Mix gently (by inversion) as above. Allow the tube to stand undisturbed at room temperature for 3-5 minutes. This requirement is not an absolute.

Centrifuge for 5 minutes at maximum speed in a microcentrifuge to pellet the bacterial debris. Pipet the supernatant into a fresh microcentrifuge tube. Extract thaw supernatant with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Mix by vortexing and centrifuge for 3 minutes to separate the two phases. Pipet the upper aqueous phase into a fresh microcentrifuge tube (be careful not to include the phenol layer).

Precipitate the plasmid DNA with 2 volumes of ethanol (95-100%). Allow the tube to stand undisturbed at room temperature for 2-5 minutes. Centrifuge for 5 minutes at maximum speed in a microcentrifuge to pellet the plasmid DNA. Aspirate or decant
the supernatant and wash the white pellet with 70% cold ethanol. Decant the ethanol onto a paper towel and invert the tube to allow the remnants of ethanol to drain away from the pellet (Alternatively, while holding the tube inverted, use gentle aspiration to remove excess ethanol from the sides).

Redissolve the plasmid DNA in 25-50μl of suitable buffer (water or Low T.E).

To remove contaminating RNA, add 1-2μl of 0.5mg/ml DNase-free, RNase.

Solutions:

Solution I
50mM glucose, 25mM Tris-HCl (pH 8.0), 10mM EDTA (pH 8.0). Filters sterilize and store at 4°C.

Solution II (prepare fresh)
0.2N NaOH (1.0mM 10N NaOH), 1% SDS (2.5mL 20% SDS or 5.0mL 10% SDS). Deionized H2O to a final volume of 50mL.

Solution III
60.0mL of 5M potassium acetate, 11.5ml of glacial acetic acid, 28.5ml of deionized H2O. This solution is 3M with respect to potassium and 5M with respect to acetate. RNase, DNase free. Boehringer Mannheim catalog number 1119915, 500 g/ml.
Miniprep protocol (abbreviated)

a) Pellet bacteria from approximately 1.5ml culture from a well isolated colony

b) 100μl Solution I (4° C)

c) 200μl Solution II

d) 150μl Solution III

1) Incubate 3-5 minutes at RT, spin 5 minutes at maximum speed

2) Extract with an equal volume of phenol/chloroform/isoamyl alcohol, spin 2 minutes at maximum speed

3) Precipitate DNA with 2 volumes of 95% ethanol, incubate for 3-5 minutes at RT

4) Spin 5 minutes at maximum speed

5) Aspirate supernatant, wash with 70% cold ethanol

6) Redissolve in 25-50μl suitable buffer

7) Add 1-2μl of 0.5mg/ml RNase, DNase free to remove contaminating RNA

8) Store DNA at -20°C.

Alkaline lysis plasmid miniprep (Adapted from Sambrook Molecular Cloning: A Laboratory Manual 2001.)

Solutions:

1. TE buffer 50 mM glucose, 25 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0); filter sterilized and stored at 4°C.

2. 0.2 N NaOH (freshly diluted from 10 N stock), 1% SDS; This solution should be made up fresh on the day of use.

3. 3 M potassium and 5 M acetate. Store at 4°C.

   5 mM potassium acetate 60 ml
   Glacial acetic acid 11.5 ml
   H2O2 8.5 ml

Method:

Grow up a 5 ml culture overnight in the presence of the appropriate antibiotic.

Harvest 1.5 ml of culture by centrifugation in an Eppendorf tube.

Resuspend the pellet in 100 μl of ice-cold solution 1.

Store for 5 minutes at room temperature.

Add 200 μl of solution 2 and mix the contents by inverting the tube rapidly two or three times. Do not vortex.

Store for 5 minutes on ice.

Add 150 ul of ice-cold solution 3 and mix by vortexing in an inverted position.

Store for 5 minutes on ice.

Centrifuge for 5 minutes in an Eppendorf centrifuge at 4°C.
Transfer supernatant to a fresh tube and add an equal volume of phenol/chloroform. Mix by vortexing the tube and centrifuge for 2 minutes in an eppendorf centrifuge at room temperature.

Transfer the top layer to a fresh tube and add two volumes of ethanol. Vortex gently and let stand at room temperature for 2 minutes.

Centrifuge for 5 minutes in an Eppendorf centrifuge at 4°C.

Remove supernatant and add 1 ml of 70% ethanol. Vortex briefly and recentrifuge.

Remove supernatant and dry the pellet briefly in a vacuum desiccators.

Add 50 μl of TE (pH 8.0) containing DNase-free pancreatic RNase (20 μl/ml).

Qiafilter plasmid kits (QIAGEN hand book)

For fast purification of up to 10 mg ultra pure plasmid or cosmid DNA

Features and benefits

Up to 10 mg of ultra pure plasmid DNA
Cleared lysates from up to 2.5 liters of LB culture without centrifugation
Reduced plasmid purification time
Fast, easy, and convenient handling for small to large culture volumes

QIAfilter principle.

QIAfilter Cartridges, provided in QIAfilter, Hi hg Speed and EndoFree Plasmid Kits, are special filter units designed to replace centrifugation following alkaline lysis of bacterial cells. QIAfilter Cartridges completely remove SDS precipitates and clear bacterial lysates in a fraction of the time needed for centrifugation, reducing plasmid-purification time by up to 1 hour.

QIAfilter Midi and Maxi Cartridges have a syringe-format and lysates are cleared in a matter of seconds by pushing the liquid through the filter. QIAfilter Mega-Giga Cartridges operate with house vacuum to efficiently clear even large volumes of bacterial lysate with minimal effort

Procedure

Neutralized bacterial lysates are incubated directly in the QIAfilter Cartridge and cleared in seconds by filtration. The filtrate is then applied directly to a QIAGEN-tip for plasmid DNA purification.
Applications.

DNA obtained with QIAfilter Plasmid Kits provides excellent results in all applications, from cloning to transfection and automated fluorescent sequencing, including capillary

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Appendix 6. Transfection of *T.evansi* Tansui 13 and *T.brucei* Gutat 3.1 (according to LaCount 2000).

Materials: Plasmids p2T7 Ti 48 60μg, p2T7 Ti 52 60μg, pLEW 13 60μg, p2T7 Ti GFP 60μg. Medium HMI-93, 24 Micro titer well, Tetracycline 1 μg/ml, Neomycin G418 1.5-3 ug/ml, 3 sterile curvettes. 5μm TU, Cell counting chambers, coulters counter.

Cell-line is established in a maintained culture of *T.evansi* and *T. brucei*, growth to be attained at 2.3 X10^7 cells or more. Cell count is conducted using a cell counting chambers.

All medium containing cells are span at 3150 rpm for 15 min at 4°C so as to concentrate the cells.

Discard supernatant

Wash pellet with 350ul EM medium

Centrifuge at 3150 rpm for 15 min at 4°C

Discard supernatant and resuspend with 350ul EM

*T.evansi* and *T. brucei* is distributed into three different gene pulses curvettes electroporation chambers. Mix with a) pLEW 13 60μg and p2T7 Ti 48 60μg b) pLEW 13 60μg and p2T7 Ti 52 60μg c) pLEW 13 60μg and p2T7 Ti GFP 60μg

Electroporate with the plasmids using one pulse from BTX electroporator, set for the peak at 1.6kV and resistance timing mode R 2 (24Ω), Time constants typically about 0.3 m Sec.

After electroporation, cells are transferred to 9.5ml of HMI-9

Count the number of surviving trypanosomes typically 10% or less are counted

24 well micro titer plates are seeded with 3-10 x10^4 per well in a volume of 0.5-1ml.
Selection applied the following day by adding the same volume of HMI-9 containing 5 µg/ml G418 for a final concentration of 2.5µg/ml.

Transfection clones are grown for 1 week in the presence of Tet 1µg/ml to induce ds RNA expression followed by the addition, at zero time, of 5µm TU. Cells densities are determined using a coulter counter. Cells are counted every 12 hours in counting chambers.
Appendix 7. Trypanosome media (Adapted from Hirumi et al., 1997) (preparation protocols)

IMDM (Invitrogen/GIBCO Cat. No. 12440)
1 FBS (heat-inactivated at 55 °C for 1 h)
2 Serum Plus™ (JRH Biosciences Cat. No. 14001)
100 ml Hypoxanthine stock (dissolve 4.0 g of NaOH in 1 l water, add 13.6 g hypoxanthine and freeze in 100 ml aliquots).
0.28 g Bathocuproine disulfonic acid (final concentration is 50 μm)
1.82 g Cysteine (add after Bathocuproine) (final concentration is 1.5 mM)
1.1 g Pyruvic acid
0.39 g Thymidine
140 μl 2-mercaptoethanol
Filter-sterilize and store in 500 ml bottles at 4 °C.

SDM-79 minus NaHCO3 for 10 liters
Note: SDM is available from JRH Biosciences Inc, prepared to order, Cat. No. 57453
(revised formulation 12th November 1998)
Add 1 bottle (254.7 g) of JRH powder to 10 liters water.
pH to 7.4 with ~ 35 ml of 10 M NaOH.
Filter sterilize into 500 ml bottles and freeze.
Before using; add 1.5 ml hemin stock solution (final concentration will be 7.5 mg/l) and 50 ml heat-inactivated serum to 500 ml medium.
Hemin for 200 ml

Stock solutions are prepared at 2.5 mg/ml. Dissolve 400 mg of NaOH in 200 ml water (0.05 M) and add 500 mg hemin. Autoclave for 20 min and store as 20 ml aliquots at 4 °C.

DTM for 1 liter

One of the key features of this medium is the absence of glucose. Because MEM minus glucose is not commercially available, DTM is a bit more tedious to prepare. IMDM is available without glucose, but its amino acid composition differs significantly from MEM. DTM calls for adding BME vitamins solution, which GIBCO (Invitrogen) no longer supplies, for lack of demand. The formulation of MEM vitamins (100x, Invitrogen/GIBCO product number 11120) is identical to BME except for the absence of Biotin, which must now be added separately.

For 1 liter (nominal final volume), add the following (mg, unless otherwise stated) to 900 ml water adjust pH to 7.2 with 5 M NaOH and filter-sterilize.

6,800 NaCl, 400 KCl, 200 CaCl2, 140 NaH2PO4.H2O, 200 MgSO4.7H2O,
7,940 HEPES, NaHCO3 2,200,110 Sodium pyruvate, 10 Phenol Red, 14 Hypoxanthine,
1.0 Biotin, Glycerol (1.21 ml of a 50% solution of glycerol) 760, 640 Proline, 236 Glutamic acid, 1,340 Glutamine, 3.75 ml Hemin (3 stock solution at 2 mg/ml in 0.05 M NaOH), 20 ml50x MEM amino acids solution (Invitrogen/GIBCO Cat. No. 11130), 10 ml 100x MEM non-essential amino acids solution (Invitrogen/GIBCO Cat. No. 11140), 10 ml 100x MEM vitamin solution (Invitrogen/GIBCO Cat. No. 11120), 14 μl 2-mercaptoethanol (final concentration will be 0.2 mM), 150 ml Heat-inactivated FBS.