ISOLATION AND CHARACTERISATION OF A GENE FOR THE T- LYMPHOCYTE TRIGGERING FACTOR FROM *Trypanosoma vivax.*

A thesis submitted in partial fulfilment for the degree of Master of Science in Zoology.

UNIVERSITY OF NAIROBI

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2000.

DECLARATION

I, Samoel Ashimosi Khamadi do hereby declare that this thesis is my original work and has not been presented for the award of a degree in any other university.

Signed ..... Date 16/11/2000

This thesis has been submitted for examination with my approval as the Internal University Supervisor:

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DEDICATION:

To my beloved parents for showing me the way of life.

To my fiancé Rehema Batti for her support.

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May God richly bless you all.

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ABBREVIATIONS

Abbreviation	Full Name
BSA	Bovine serum albumin
bp	base pair
kb	kilobase (s)
DEAE	Diethylaminoethyl
DMSO	Dimethyl sulphoxide
EDTA	Ethylene disamine tetra-acetic acid
ng	Nanogram (s)
nm	Nanometer(s)
O.D.	Optical density
PBS	Phosphate buffered saline
rpm	Revolutions per minute
UTP	Uridine triphosphate
Tris	Tris(hydroxy-methyl)amino methane
IPTG	Isopropyl β-thiogalactoside
PCR	Polymerase chain reaction
dNTPs	Deoxynucleotide triphosphates

ABSTRACT

Early during infection of mice with *T. brucei* parasites, the number of spleen mononuclear cells (MNCs) that produce interferon gamma (IFN-γ) is increased and the parasite produces a molecule, a T-lymphocyte-triggering factor (TLTF), which *in vivo* in a rapid antigen-non-specific way, triggers CD8+ T-lymphocyte cells to produce IFN-γ. From such activated lymphoid MNCs, the parasite receives a growth stimulus (Olsson *et al.*, 1991). Antibodies against IFN-γ abrogate this growth stimulus and recombinant rat IFN-γ directly supports parasite growth indicating that the synthesised IFN-γ may be a growth-stimulating factor for the parasite (Olsson *et al.*, 1991; Bakhiet *et al.*, 1993). The TLTF has been shown to bind directly to the CD8+ molecules on the T cells (Olsson *et al.*, 1991). IFN-γ in turn induces the production of a mitogen-activated protein (MAP) kinase in African trypanosomes (Hua & Wang, 1997), which might be a factor contributing to the proliferation of trypanosomes in the bloodstream. Consistent with this possibility, a monoclonal antibody directed against TLTF reduces parasite levels and increases the survival of mice infected with *T. brucei*.

The TLTF has been identified and characterised in *T. brucei*. It has been shown by studies with the TLTF fused to the green fluorescent protein that TLTF is localised to the small vesicles that are found at or near the flagella pocket, the site of secretion in trypanosomes (Vaidya *et al.*, 1997).

It has also been shown that *T. evansi*, *T. gambiense* and *T. rhodesiense* also contain factor (s) with similar activity. Homogenates from these parasite taxa triggered mouse or rat MNCs of the lymphoid tissue to proliferate. These responses were dependent on

CD8+ cells since the activity was (a) blocked by anti-CD8 antibodies and, (b) occurred in CD8+ enriched peripheral blood MNCs (Bakhiet *et al.*, 1996).

It was therefore important to determine whether livestock trypanosomes also have the gene for the TLTF and if so, whether it elicits the same immune response as seen in *T. brucei.* In the study, *T. vivax* genomic and cDNA expression libraries were screened using a *T. brucei rhodesiense* TLTF probe. The TLTF gene in *T. vivax* parasites was isolated by a novel technique referred to as Random PCR–assisted cloning of gene homologues.

Studies on the expression of the gene in different life cycles of the parasite were also done. Northern hybridisation was done on the bloodstream and insect forms. The gene was found to be expressed in both life cycle stages. Southern hybridisation studies showed that the *T. vivax* TLTF is a single copy gene.

CHAPTER 1: INTRODUCTION AND LITERATURE REVIEW

1.1 Trypanosomosis in Africa

African Trypanosomosis[1] is a spectrum of diseases in man and his domestic animals. It is caused by the species *Trypanosoma (Trypanozoon) brucei*, *Trypanosoma (Nannomonas) congolense* and *Trypanosoma (Duttonella) vivax* in animals. Biological races ("sub-species") of *T. brucei* designated *T. b. rhodesiense* and *T. b. gambiense* cause human sleeping sickness. The former causes the more acute East African illness, the latter the more chronic West African disease. *Trypanosoma b. brucei* is morphologically identical with these two subspecies in all phases of its life cycle, but does not infect man and is confined to wild and domestic ungulates and carnivores.

Trypanosomes are eukaryotic haemoflagellates belonging to the Phylum Protozoa, Order Kinetoplastida and Family Trypanosomatidae (Figure 1.1). Trypanosomes are long spindle-shaped cells (approximately 2x20 μm, though some can be as long as 100μm as evidenced in the stercorarian trypanosome *T. theileri* found in cattle, buffaloes and antelopes) that move by means of a single flagellum attached to the body of the cell. Morphological features such as length, shape and the location, within the parasite, of organelles such as the basal body, kinetoplast, nucleus and flagellum (Figure 1.2) are used to classify trypanosome species. *T. congolense* parasites are monomorphic, that is, they do not possess variable shapes, whereas the *T. brucei* and *T. vivax* parasites have marked pleomorphism (variable shapes) with blood stream

[1] The term trypanosomosis (instead of trypanosomiasis) will be used throughout this thesis according to the standardised nomenclature of animal parasitic diseases (SNOAPAD; Kassai *et al.*, 1988) to refer to the disease caused by African trypanosomes.

forms appearing as slender, intermediate and stumpy forms (Figure 1.4) (Kreier & Baker, 1992).

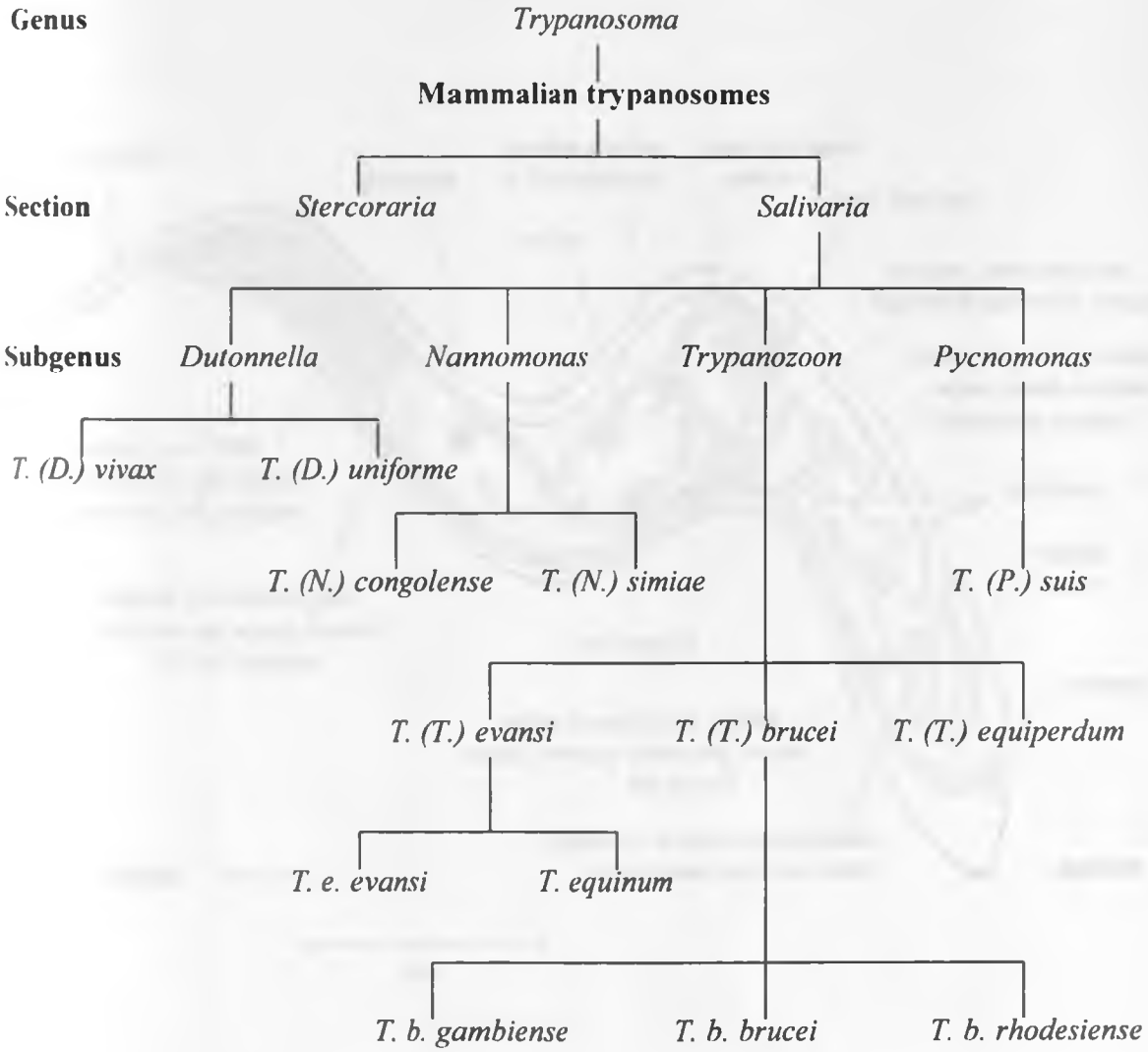

Figure 1.1: Classification of the Genus *Trypanosoma* [adapted from Hoare, 1972].

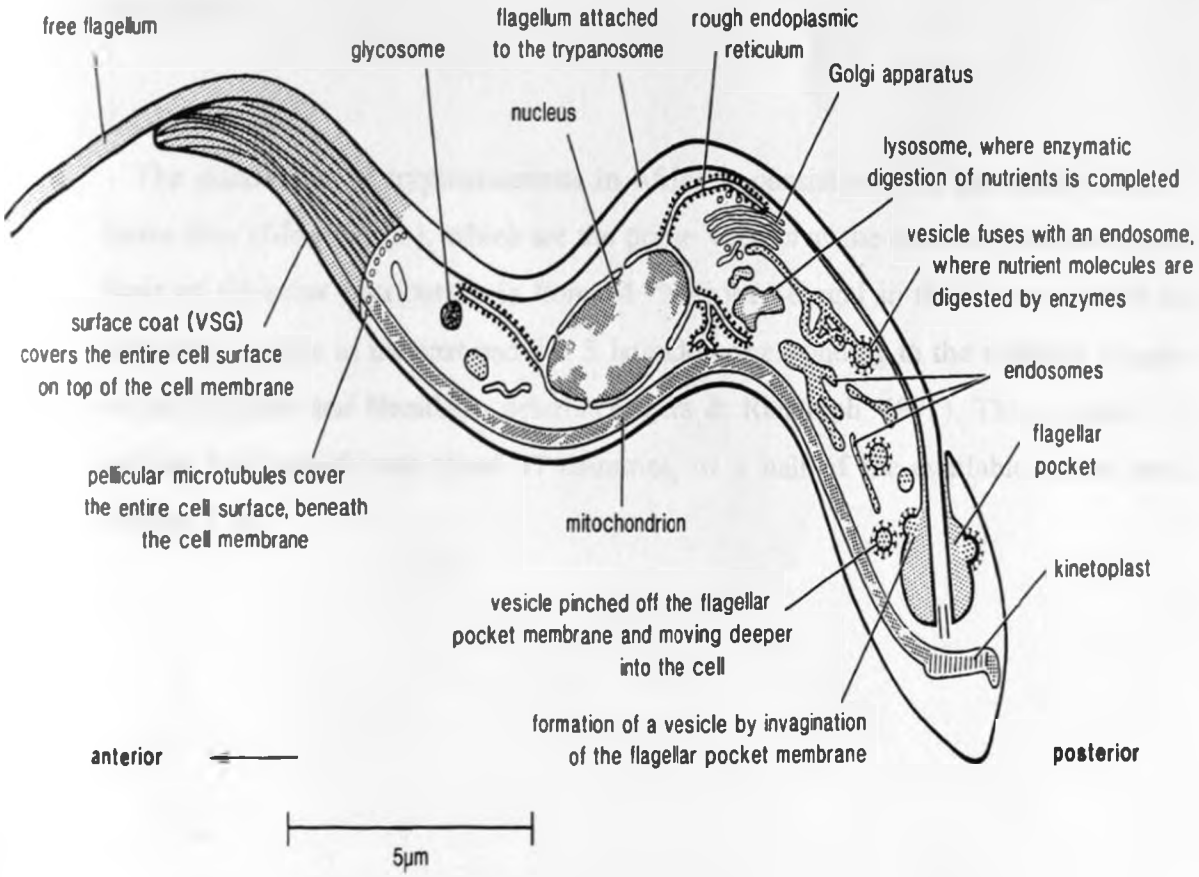

Figure 1.2: Schematic diagram of a bloodstream form trypanosome.

The diagram illustrates the major organelles of trypanosomes [adapted from Vickerman (1970) in: African Trypanosomiases (editor H.W. Mulligan)]

The species of the genus *Trypanosoma* affecting animals and humans in Africa have been assigned to the section Salivaria (Figure 1.1) where the developmental cycle of the parasite in the vector takes place in the anterior part of the alimentary tract and transmission is by inoculation. *T. evansi* is a less important trypanosome that affects livestock in Africa. It is transmitted only through mechanical means, that is, by direct, contaminative transmission between mammalian hosts and not cyclically. As a result of this, it has the widest geographical distribution world wide compared to the other pathogenic trypanosomes. It is slightly pathogenic in sheep, goats and pigs unlike in horses and camels where it causes a more severe disease generally referred to as Surra.

1.2 Distribution

The distribution of trypanosomosis in Africa is consistent with the distribution of tsetse flies (*Glossina sp.*), which are the prime vectors of the disease. The ecological limit of *Glossina* distribution is from 14 °N from Senegal in the West to 10°N in southern Somalia in the east and 20° S latitude corresponding to the northern fringes of the Kalahari and Namibian deserts (Rogers & Randolph, 1991). This is about 11 million km^2 spread over about 37 countries, or a half of the available arable land (Figure 1.3).

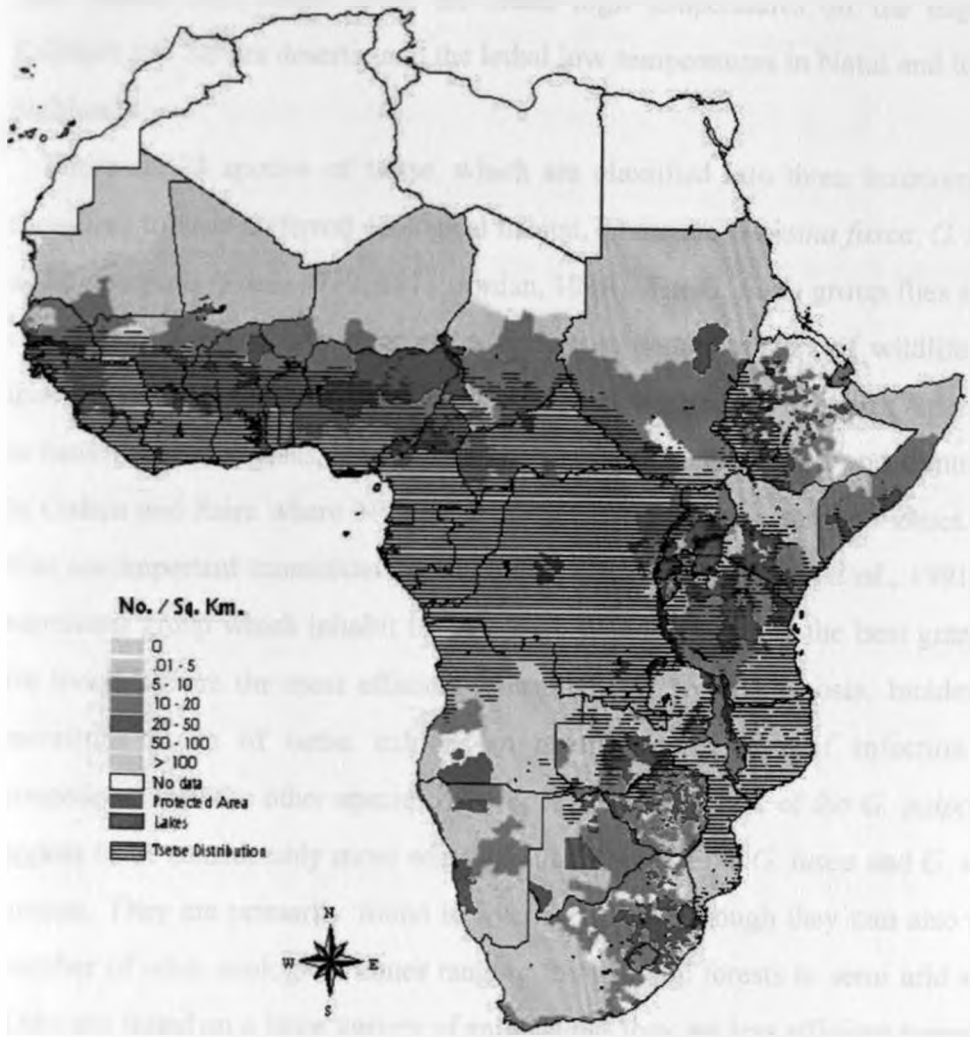

Figure 1.3: A map showing cattle rearing areas and tsetse fly distribution in Africa. The overlay of tsetse distribution with cattle density (coloured areas) is shown on the map. The abbreviation No/sq.km, which is the number of cattle per square kilometre, depicts the cattle density in various parts of Africa (Kruska *et al.*, 1995).

The limits of tsetse infestation are determined primarily by climate, and secondarily by vegetation. Temperature is a very important parameter as the distribution of these flies shows. This ranges from the lethal high temperatures on the edges of the Kalahari and Sahara deserts, and the lethal low temperatures in Natal and the tropical highlands.

There are 23 species of tsetse, which are classified into three taxonomic groups according to their preferred ecological habitat. These are *Glossina fusca*, *G. morsitans* and *G. palpalis* (Ford, 1970, 1971; Jordan, 1988). The *G. fusca* group flies are largely confined to humid forest areas and are more important vectors of wildlife infection than livestock trypanosomosis. This is because few breeds of livestock are maintained in heavily forested areas. These areas include large parts of West and Central Africa. In Gabon and Zaire where N'Dama cattle are maintained in forested zones, *G. fusca* flies are important transmitters of animal trypanosomosis (Leak *et al.*, 1991). The *G. morsitans* group which inhabit the savannah woodlands, often the best grazing lands for livestock, are the most efficient transmitters of trypanosomosis. Incidentally, *G. morsitans* group of tsetse exhibits in nature higher rates of infection with *T. congolense* than the other species (Hoare, 1972). Tsetse flies of the *G. palpalis* group appear to be considerably more adaptable than those of the *G. fusca* and *G. morsitans* groups. They are primarily found in riverine habitats though they can also occupy a number of other ecological zones ranging from humid forests to semi arid savannah. They are found on a large variety of animals but they are less efficient transmitters of trypanosomosis than *G. morsitans* flies.

1.3 Economic importance

Of all diseases affecting animals in Africa, trypanosomosis is considered one of the most important (Jawara, 1990). It was estimated that the annual loss to African farmers due to trypanosomosis is about U.S $ 1.9 billion (Jahnke *et al.*, 1988). Trypanosomosis seriously constrains development by the direct economic and social losses. This is in terms of mortalities and morbidity arising as a result of exposure of humans and livestock populations to disease. Nagana, the form of trypanosomosis

affecting cattle in Africa, is of more importance economically than sleeping sickness, which affects humans, and hence emphasis is mainly on this disease. Indirectly, economic and social losses are evidenced in rural communities and reducing productivity, ultimately this affects the national economy (Chadenga, 1994).

Losses in milk production, beef production, draught power and useful by-products such as hides and skins are enormous. Due to impaired fertility in sick animals, there is lower offtake:there are fewer animals for slaughter and sale. As a consequence of the lower birth rate, fewer animals lactate. Those that are milked produce less. These direct losses are difficult to quantify, but are only a part of the true cost of the disease. The cumulative effect of lower milk production, reduced work output, lower crop yields and impaired local transportation impede the development of the affected areas (Connor, 1994). The costs of control of the disease also contribute to the economic losses incurred. The different methods of control are expensive and the finances that could be used elsewhere are instead channelled into the control of the disease.

1.4 Life cycle

The life cycle stages of trypanosomes involves cyclical development in both a mammalian host and an insect vector (Woo, 1977). During development within their tsetse vectors, they shed antigenic coat typical of bloodstream forms and undergo division as epimastigotes (Figure 1.4). Some of these forms then differentiate to non-dividing metacyclic trypanosomes, which after resuming their antigenic coat are once again infective to the mammalian host. Cyclical development serves both to maintain the distribution of the parasite and to allow genetic re-assortment within species.

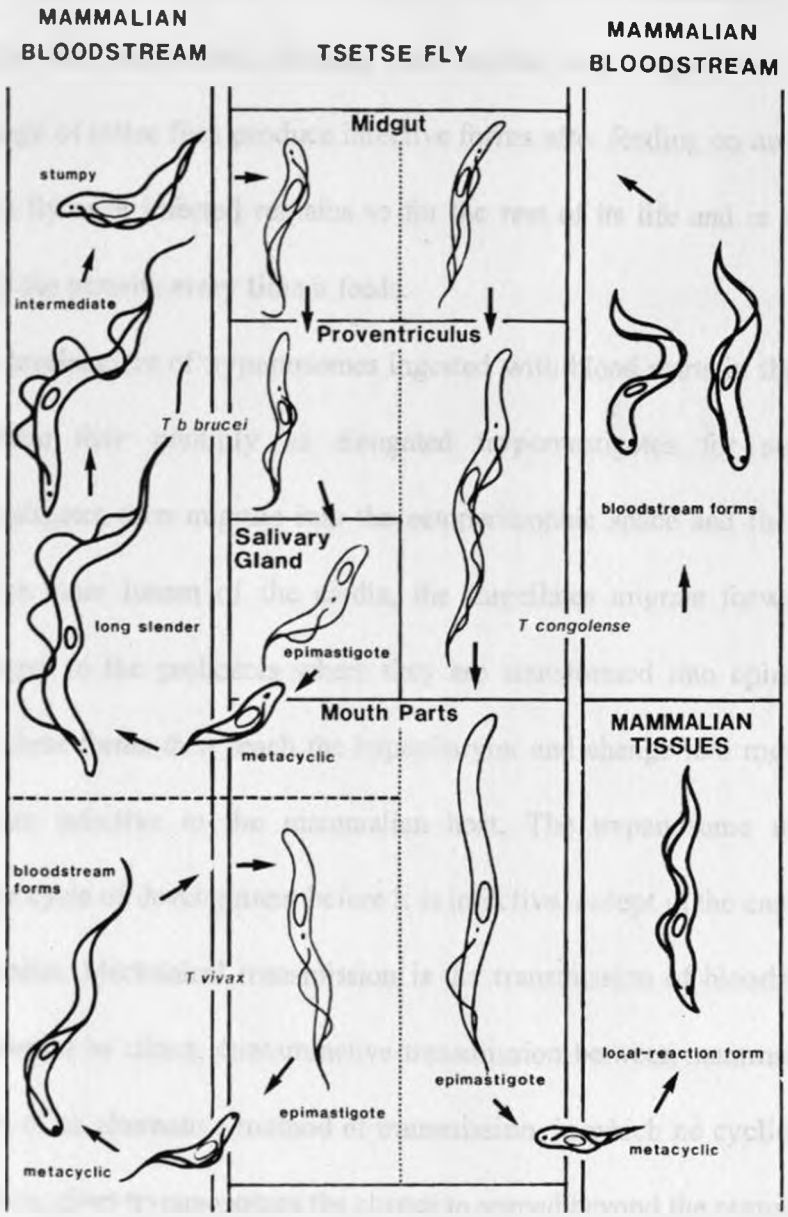

Figure 1.4: General outline of cyclical development of trypanosomes [Adapted from ILRAD 1991 Annual report].

When the metacyclic trypanosomes are inoculated into the animal by the tsetse fly, they change into normal blood forms and start dividing via binary fission. The cycle of development may take 19-53 days. This period depends on several factors including the subspecies infecting the animal and temperature. Only a small percentage of tsetse flies produce infective forms after feeding on an infected animal. A tsetse fly once infected remains so for the rest of its life and is therefore able to transmit the parasite every time it feeds.

The development of trypanosomes ingested with blood starts in the mid gut of the fly, where they multiply as elongated trypomastigotes for some days. The trypomastigotes then migrate into the ectoperitrophic space and then to the cardia. From the inner lumen of the cardia, the flagellates migrate forward through the oesophagus to the proboscis where they are transformed into epimastigote forms. Finally these forms then reach the hypopharynx and change into metatrypanosomes, which are infective to the mammalian host. The trypanosome must undergo a complete cycle of development before it is infective, except in the case of mechanical transmission. Mechanical transmission is the transmission of bloodstream forms of trypanosomes by direct, contaminative transmission between mammalian hosts. This adoption of an alternative method of transmission, in which no cyclical development takes place, gives trypanosomes the chance to spread beyond the normal boundaries of tsetse distribution. The principal agents for mechanical transmission of trypanosomes are numerous species of bloodsucking flies belonging to the family *Tabanidae*, which are exemplified by the widespread horse flies and stable flies (*Stomoxys* spp.). These insects have an interrupted pattern of feeding that carries the trypanosome-infected blood between members in herds of livestock.

The development cycles of *T. brucei, T. congolense* and *T. vivax* differ in locations in the fly in which they occur and in the lengths of time they require to complete their life cycles of development within the tsetse fly (Figure 1.5).The development cycle within the tsetse fly may be as short as five days in flies infected with *T. vivax,* two to three weeks in flies infected with *T. congolense* and as long as three to five weeks in flies infected with *T. brucei* (Vickerman, *et al.*, 1988).

The life cycle of *T. vivax* is restricted to the labrum and the hypopharynx of the proboscis (Hoare, 1972). Within the proboscis, epimastigotes of *T. vivax* attach to the chitinous wall of the food canal and divide by binary fission to form clusters of parasites (Vickerman, 1973). The epimastigotes then divide and mature to give premetacyclic trypomastigotes, which are found freely swimming in the hypopharynx. These forms then mature further and acquire a variable surface glycoprotein (VSG) coat and then become infective metacyclic trypomastigotes. In *T. congolense* and *T. brucei*, the development cycle is extended to the posterior midgut. Unlike *T. vivax,* bloodstream trypomastigotes of *T. congolense* and *T. brucei* survive and transform into procyclic trypomastigotes in the posterior midgut. They then replace their VSG coat with a coat of an invariant glycoprotein called procyclin. They divide and elongate rapidly in the gut. These parasites then pass into the ectoperitrophic space. The procyclic trypomastigotes then swim forward within this space into the proventriculus. They cease dividing and elongate further into mesocyclic trypomastigotes. These forms mature into metacyclics, which are infective to the host.

T. congolense and *T. brucei* life cycles differ at the point where the mesocyclic trypomastigotes develop. Those of *T. congolense* differentiate to epimastigotes and anchor themselves to the walls of the labrum and pharynx (Jefferies *et al.*, 1987).

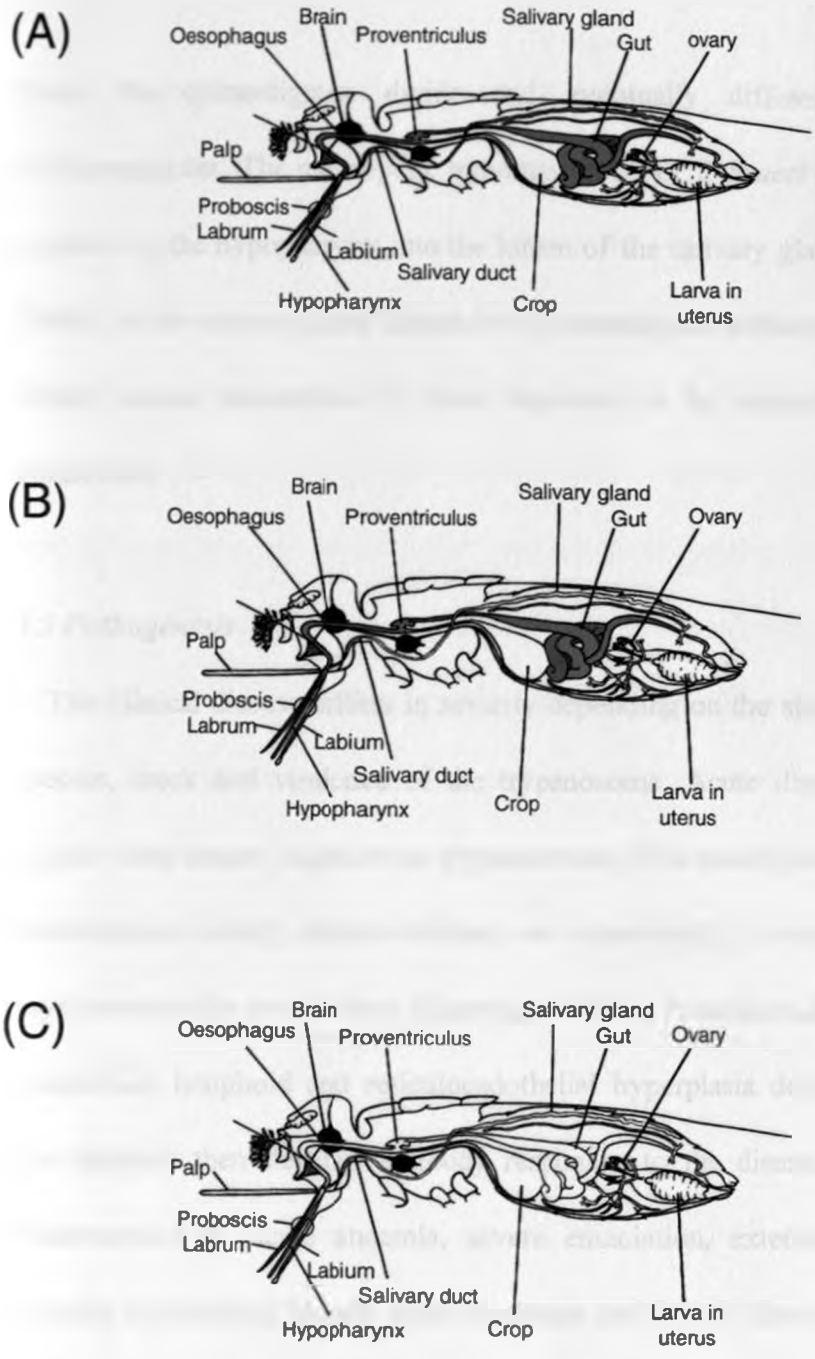

Figure 1.5: Schematic diagram of the development of *T. brucei* (A), *T. congolense* (B) and *T. vivax* (C) in the insect vector [Adapted from Vickerman *et al.*, 1988]. The red regions show the parts of the fly in which the development of the parasite occurs.

Here the epimastigotes divide and eventually differentiate to metacyclic trypomastigotes. The mesocyclic trypomastigotes of *T. brucei* in contrast continue to migrate up the hypopharynx into the lumen of the salivary glands (Vickerman *et al.*, 1988). In the salivary gland lumen the trypomastigotes differentiate to epimastigotes, which anchor themselves by their flagellum to the microvilli of the glandular epithelium.

1.5 ***Pathogenesis***

The clinical disease differs in severity depending on the susceptibility of the host species, stock and virulence of the trypanosome. Acute disease usually develops shortly after natural exposure to trypanosomes. This usually lasts several weeks and terminates in death, chronic disease, or occasionally, in recovery. Cattle remain asymptomatic for several days following infection. Parasitaemia, fever, anaemia and a generalised lymphoid and reticuloendothelial hyperplasia develop 1-2 weeks later. The animals then develop antibody responses to the disease. Trypanosomosis is characterised by acute anaemia, severe emaciation, extensive haemorrhages on mucous membranes, bloody nasal discharge and bloody faeces as seen in *T. vivax* (Schonefeld *et al.*, 1987). With onset of parasitemia, weakness, anorexia, weight loss and loss of condition result. As anaemia worsens, mucosal membranes become pale. After prolonged chronic infection, cachexia develops, infected animals lose muscle and fat from the axial and appendicular skeleton and their bony prominences are poorly covered. Trypanosome infected cattle usually die if not treated. Mortality from trypanosomosis occurs from either massive parasitosis or secondary infections due to immunosuppression, an important trait of the disease (Vaidya *et al.*, 1997).

Immunosuppression in trypanosome infections is mediated by several factors. Suppression of the host T cell responses is one of the hallmarks of infection with African trypanosomes. The cellular basis for immunosuppression includes the generation of suppressor macrophages that down regulate T cell proliferation. The suppressor macrophages induce the release of reactive nitrogen intermediates, specifically nitrogen oxide, and prostaglandins (Schleifer & Mansfield, 1993). The trypanosomes also release numerous pathogenic substances that cause alterations in cytokine/prostaglandin network. A 41-46 kDA molecule activates $CD8^+$ T cells to produce interferon-gamma which activates macrophages and promotes macrophage growth. The activated macrophages release tumour necrosis factor alpha and nitric oxide, which are trypanostatic and other cytokines and prostaglandins. These macrophage-released substances enhance immunosuppression (Dumas & Bouteille, 1996).

1.6 Control

The control of trypanosomosis currently relies upon the treatment of the infected host and on vector control. An alternative approach to trypanosomosis control depends on raising trypanotolerant livestock in tsetse-infested areas.

1.6.1 Chemotherapy

Chemotherapy is one of the most widespread and commonly used methods of controlling animal trypanosomosis (Leach & Roberts, 1981) This is inspite of the cost of the drugs and the problem of drug resistance. The trypanocidal drugs used are either for prophylactic or curative purposes. The main compounds used for treating

nagana include diminazine, isometamidium, suramin and quinapyramine. But resistance to these compounds has been reported in various sites in Africa (Rottcher & Schillinger, 1985; Schonefeld *et al.*, 1987). The cost of drugs is also very high. The ideal trypanocide should be safe and effective and must have a simple mode of administration to allow its use in rural conditions where health facilities are usually poor, and above all should be affordable.

1.6.2 Vector control

A variety of methods have been used in attempts to reduce the tsetse fly populations. These methods include the use of insecticides applied on odour-baited traps or screens or applied as aerosols by planes in the air onto the tsetse infested environment or ground spraying (Maclennan, 1981).

Cattle dipping or pour on is another target method used to kill tsetse. Insecticides like deltamethrin, which is used as a wash or pour on, are used. Up to 100% of alighting flies can be killed within two weeks. However, for the technique to succeed, domestic livestock must be present in the area in sufficient numbers, cattle must represent the overwhelming proportion of the host complex of the tsetse area and most of the cattle must be present for treatment on regular basis. Unfortunately, the cost of the insecticides is a serious constraint (Chadenga, 1994).

In recent years, use of insecticides has lost favour among tsetse control institutions. This is because of the environmental concerns regarding the use of DDT, dieldrin and other insecticides. Treated areas are easily reinvaded necessitating costly re-treatment programmes. Furthermore, these methods do not immediately protect cattle from being infected with trypanosomes.

Theoretically, the low reproductive capacity and infrequent mating of the female tsetse flies makes them ideal candidates for control by genetic techniques. One of these techniques is the sterile insect technique (SIT). This technique involves the release of male insects sterilised with gamma irradiation. This method has been used successfully in Zanzibar where *G. austeni* males sterilised by gamma irradiation were dispersed by air over the whole island in 1994. No wild tsetse have been caught since September 1996 and the eradication *of G. austeni* from Zanzibar was declared at the end of 1997 (Saleh *et al.*, 1998). But this method has hindrances which include the enormous cost of implementing a control program utilising SIT and, secondly, the density of the target population must be low for SIT to be effective (Politzar & Cuisane, 1982). Thirdly, this method works best in an isolated area like an Island, as evidenced in Zanzibar.

Other methods for the control of the tsetse vector include bush clearing, elimination of wild animal hosts and biological control. Bush clearing renders the habitat unsuitable for tsetse habitation. But this method has disadvantages, which include decrease in soil fertility, soil erosion and adverse effects on water supplies. Elimination of wild animal hosts is not a popular method as African economies rely on tourism as a source of foreign income. Wild animals are among the major attractions for tourists.

Biological control by use of natural enemies of the tsetse vector includes parasites and predators like fungi, bacteria and parasitic mites. To be successful, biological control organisms generally have to originate from a different geographical or ecological area from the potential pest to be controlled. Otherwise, it would be expected that the control and target organisms would be sufficiently adapted to one

another that no significant degree of control would easily be maintained over a long period. Most trials of parasites on tsetse flies have used insects that occur naturally in tsetse habitats, which may partly explain the lack of success.

Entomopathogenic fungi and bacteria have been used in experimental studies to show that they could be potential candidates for the control of trypanosomosis. These include *Pseudomonas aeruginosa*, *Serratia marcescens* and *Bacillus sphaericus* (Kaaya & Darji, 1989). The advantages of using biological control include the fact that they do not affect the environment negatively and it is a self-sustaining method. Once the entomopathogens are introduced in the environment, they will multiply and attack their intended targets naturally. However, these methods have shortfalls too. These include choosing a suitable method of formulation and dispersal of the pathogens and the chances of adaptation of the insect target to the pathogen so that they end up having a symbiotic relationship rather than a pathogen-host relationship (Kaaya, 1989).

1.6.3 Trypanotolerance

Raising of trypanotolerant animals is another method used to control trypanosomosis in livestock. Several breeds of cattle of western Africa origin have a significant degree of trypanotolerance and are able to thrive in tsetse- infested areas where susceptible breeds cannot survive. Although trypanotolerant cattle offer considerable potential as an alternative means of raising cattle in tsetse-infested areas, they currently represent only 5% of the cattle population of sub-Saharan Africa. The limited availability of animals of these breeds prevents their use in wide-scale programs to restock tsetse-infested areas. Nevertheless, N'Dama cattle have been

imported into countries such as Gabon and Zaire and are now being successfully raised on several large ranches (Shaw & Hoste, 1987). But the use of trypanotolerant livestock to combat the disease is limited as these animals are found mainly in parts of western Africa, where because of their small size are not always popular with farmers who aspire to owning the larger improved breeds.

The decreasing efficacy of available trypanocidal drugs and the difficulties of sustaining tsetse control increase the imperative need to enhance trypanotolerance through selective breeding, either within breeds or through cross-breeding. A major constraint on selection for trypanotolerance in cattle, both within breeds or through cross-breeding programmes, has been the absence of practical reliable markers of resistance or susceptibility (d' Ieteren *et al.*,1998).

1.6.4 Vaccination

It is evident that the control of African trypanosomosis is still a big problem. New control strategies are in the offing which include the use of vaccines. This is a desirable option. But an attempt to develop a vaccine against the disease has been dwarfed by the parasite's ability to change the composition of its exposed surface antigens. Since the conventional approaches to the control of the disease have largely met with failure, there has been a renewed interest in identifying novel aspects of the biology, biochemistry and molecular biology of trypanosomes that might be exploited to develop new targets for vaccines. If developed, then vaccination could form the best option for the control of this disease (Hajduk, *et al.*, 1992).

1.7 Immunology

The parasites are able to survive within an immunocompetent host through antigenic variation. These variable antigens change from one population of trypanosomes to another and are responsible for the differences between serological variants or variable antigenic types (VATs) of the trypanosome. The variable antigen composes the surface coat of the trypanosome. Bloodstream forms of all the African trypanosomes have a 12-15 nm thick coat overlying the plasma membrane. The coat is lost when the trypanosome embarks on cyclical development in the tsetse fly or undergoes transformation to the procyclic form *in vitro* (Vickerman, 1969). The coat is reacquired during differentiation to the metacyclic form so that trypanosomes already in possession of a surface variable antigen are inoculated into the mammalian host. Through antigenic variation the parasite is able to avoid mechanisms of the host immune system for eliminating it.

In addition, non-specific immune mechanisms are active during trypanosome infections. There is a polyclonal activation of T and B cells, and of macrophages. The activated macrophages play a key role in immunosuppression that evolves as the infection progresses (Askonas, 1985). This immunosuppression may partly be due to a macrophage-mediated induced suppression of both interleukin 2 (IL2) production and IL2 receptor expression in T cells (Sileghem *et al.*, 1987; Kierszenbaum *et al.*, 1991).

1.8 The T-lymphocyte triggering factor

A soluble factor derived from *T. brucei* has been shown to suppress IL2 receptor expression on lectin-stimulated human peripheral blood mononuclear cells (Sztein & Kierszenbaum, 1991). Another phenomenon related to the immune system is the homing-in and growth of parasites within the lymphoid organs such as the spleen and the lymph nodes (Askonas, 1985). It has been suggested that *T. brucei* growth promoting molecules may be produced in the lymphoid organs (Black *et al.*, 1985). It is evident that *T. brucei* depends on factor(s) from the host animal for its growth. The insect stage (procyclic) of *T. brucei* expresses lectin binding sites, and lectins secreted in the gut of the tsetse fly not only interfere with the establishment of the parasites in the mid gut, but also provides a signal for maturation of the parasites and induce hypopharyngeal infection (Maudlin & Welburn, 1988). *T. brucei* responds to molecules that stimulate proliferation and inhibit differentiation of the parasite (Black *et al.*, 1985).

Recently, a gene has been characterised encoding an immunomodulatory protein from *T.brucei,* which is related to pathogenic livestock species *T. congolense* and *T. vivax*. This protein, called the T-lymphocyte triggering factor (TLTF) is secreted by *T. brucei* during infection and triggers CD8+ T- lymphocytes to proliferate and to secrete interferon-γ (IFN-γ) (Olsson *et al.*, 1993). The IFN-γ contributes to immunosuppression and is used by the trypanosome as a growth factor. This is unlike other protozoal and viral infections where IFN-γ production is important in fighting these pathogens. Studies with the cloned *T. brucei* TLTF gene fused to the green fluorescent protein show that TLTF is localised to small vesicles located at or near the

flagellar pocket, the site of secretion and endocytosis in trypanosomes (Vaidya *et al.*, 1997).

Anti-TLTF monoclonal antibodies inhibit all IFN-γ inducing activities from *T. brucei*, suggesting that the activities result from a single molecule. The TLTF has been purified from *T. brucei*. This was accomplished by raising monoclonal antibodies (mAbs) against a fraction of *T. brucei* obtained by gel filtration, which contained high levels of materials inducing rat mononuclear cells to IFN-γ production. Monoclonal antibodies from four hybridomas strongly inhibited trypanosome-induced IFN-γ production. One of them (m Ab1) was used for the purification of TLTF by affinity chromatography. Sodium dodecyl sulphate (SDS) electrophoresis of the purified TLTF displayed a band of 42-45 kDa. A monoclonal antibody against TLTF inhibited mononuclear cell-supported growth of the parasites in mice. Treatments instituted at different time points after infection suppressed parasite growth, abrogated the IFN-γ production by splenocytes induced by the infection and prolonged survival of the animals (Bakhiet *et al.*, 1993).

The TLTF is released early during infection as evidenced in parasite-host interactions during experimental African trypanosomosis (Hamadien *et al.*, 1999). It was seen that in knockout mice with disrupted IFN-γ (IFN-γ $^{-/-}$), there were low levels of parasitaemia and they survived for a prolonged period as compared to wild type (WT) mice. This was thought to be caused by the absence of the IFN-γ effect on the growth of the parasites. Knockout mice without the IFN-γ receptor (IFN-γR $^{-/-}$) demonstrated very high levels of unbound IFN-γ. These mice demonstrated very high parasitaemia and survived for a very short time. The WT mice showed lower IFN-γ levels compared to IFN-γR $^{-/-}$ mice, and therefore exhibited intermediate parasitaemia

levels and survival time (Bakhiet *et al.*, 1996). There were increased levels of TLTF early during infection, which had declined markedly by late stages in all animals studied. The early induction of TLTF is a main tact used by the parasites to interact with its host (Bakhiet *et al.*, 1993). However, further induction may not be required at late stages because continuous induction of this molecule may lead to over-stimulation of the immune system of the host, which may not be beneficial to the parasite. The host may also generate antibodies that would neutralise the activity of the TLTF. In the experiments performed by Hamadien *et al.* (1999), it was shown in a biological assay that the host indeed produces neutralising antibodies against the TLTF. These antibodies are very specific. Low levels of anti-TLTF antibodies of Ig G isotype, detected in the study during infections in IFN-$\gamma^{-/-}$ and IFN-$\gamma R^{-/-}$ mice, propose a role for IFN-γ in the TLTF-specific Ig G antibody response. The mechanisms for the IFN-γ effect in this context are not clear. However, levels of anti-TLTF antibodies are probably dependent on the levels of both TLTF and IFN-γ. This was suggested because IFN-$\gamma^{-/-}$ mice which exhibited very low levels of TLTF did not produce anti-TLTF antibodies while IFN-$\gamma R^{-/-}$ mice, which showed increased levels of TLTF produced somewhat low levels of anti-TLTF antibodies (Hamadien *et al.*, 1999). It is not known whether IFN-γ affects B-cell functions directly or if a link between IFN-γ and required T-cell functions for the induction anti-TLTF antibodies is needed.

Detection of specific anti-TLTF antibodies by ELISA may represent a better approach of diagnosis of trypanosomosis because their levels are not dependent on both parasitaemia and stage of infection, but rather on the infection itself. This would mean that detection of anti-TLTF antibodies might indicate an intermediate or a late infection. In addition to measurement of TLTF and specific anti-TLTF neutralising

antibodies, IFN-γ was implicated as a key molecule in the generation of anti-TLTF neutralising antibodies (Hamadien *et al.*, 1999).

The depletion of CD8+ T- lymphocytes suppresses growth of *T. b. brucei* and IFN-γ production in infected Sprague-Dawley rats leading to increased survival of these animals. It is suggested that the CD8+ cells are involved directly or indirectly in growth regulation of the parasites and that IFN-γ induced by the parasite may be one of the factors that triggers immunosuppression (Bakhiet *et al.*, 1990). However, evidence has suggested IFN-γ to be a macrophage activating factor that mediates cellular defence to intracellular infectious agents (Nathan *et al.*, 1983). It can also inhibit macrophage-mediated antigen-specific T cell proliferation and the type of humoral or cellular immune responses may be dependent on the timing of IFN-γ induction (McKernan *et al.*, 1988).

The increase in IFN-γ production is largely abrogated by CD8+ T cell depletion, suggesting that this subset of T cells is mainly responsible for IFN-γ production. Differences in interplay between various strains of trypanosomes and factors derived from activated T cells during infection from different host animals may explain why the infection sometimes results in complete resistance, sometimes in tolerance with a low grade of parasite growth and sometimes in disease with a high level of parasite proliferation.

IFN-γ is a prominent cytokine product of activated CD 8+ T cells and has been identified as a growth promoting molecule released by the CD 8+ lymphoid cells after exposure to *T. brucei* (Olsson *et al.*, 1991) This growth-promoting role of IFN-γ for *T. brucei* contrasts with that for *T. cruzi* in which IFN-γ mediated macrophage activation inhibits intracellular parasite replication in the same cells (Munoz-

Fernandez *et al.*, 1992; Silva *et al.*, 1992). The role of IFN-γ in *T. brucei* infections also differs from that reported in leishmaniasis, in which recruitment of a predominant T cell response with production of IFN-γ is associated with a resolved infection, while a T cell response with interleukin 4 production is associated with disease progression (Heinzel *et al.*, 1989).

Apart from its direct growth- stimulating effects on *T. brucei*, IFN-γ may indirectly affect *T. brucei* survival in a host. High levels of endogenously produced IFN-γ are responsible for immunosuppression during graft versus host disease and IFN-γ may suppress T cell proliferation (Klimpel *et al.*, 1990; McKernan *et al.*, 1988). IFN-γ is also a potent activator of macrophages (Adams & Hamilton, 1987) Such activated macrophages produce other cytokines, such as interleukin 1, and tumour necrosis factor α (TNF- α) (Collart *et al.*, 1986). These cytokines are generated at high levels during *T. brucei* infections (Mathias *et al.*, 1990; Sileghem *et al.*, 1989).

Another study done by Hamadien *et al.*, (2000) showed that IFN-γ stimulation induced the secretion of TLTF in *T. b. brucei* cultures compared to control cultures without IFN-γ stimulation. This was seen in parasites cultured for 1 hour at 37 °C with or without 100u/ml IFN-γ. The ELISPOT assay was used to evaluate the bioactivity of the TLTF secreted in culture supernatants of *T. b. brucei*, spontaneously after IFN-γ stimulation. Recombinant TLTF which was previously reported to stimulate mononuclear cells to IFN-γ production (Vaidya *et al.*, 1997) induced the cells to IFN-γ secretion.

TNF-α has been shown to abolish the growth promoting effect of IFN-γ. This cytokine may exert a toxic effect on the parasites (Lucas *et al.*, 1994). It has been suggested that TNF-α together with IFN-γ plays a key role in the interaction between

the parasite and the host, whereby IFN-γ may have a stimulatory effect on the parasite, while TNF-α has a direct trypanocidal activity (Lucas *et al.*, 1993). The inhibition of the effect of rat IFN-γ on *T. brucei* growth by the anti- IFN-γ receptor antibodies suggests that the putative IFN-γ receptor on the trypanosomes has structures in common with mammalian IFN-γ receptor. The latter receptor is highly species specific, and IFN-γ from one species in general only affects that species (Bakhiet *et al.*, 1996).

African trypanosomes exploit the growth regulating mechanisms of the host immune system by selectively utilising IFN-γ as a factor to stimulate their proliferation and may thus increase the frequency of antigenic shifts. Secreted IFN-γ may influence growth or survival of the parasite, since it enhances [^{3}H] thymidine uptake in trypanosomes. Human pathogenic *T. brucei* strains are also stimulated by IFN-γ and the response is partially dependent on the species from which the IFN-γ is derived. The increased [^{3}H] thymidine uptake reflects an enhanced proliferation of trypanosomes in an optimised axenic culture system. The effects of IFN-γ can be inhibited by IFN-γ-receptor blocking antibodies. IFN-γ influences growth of *T. brucei* both *in vivo* and *in vitro (*Bakhiet *et al.,* 1996). This effect occurs only after exposure of the parasites to IFN-γ and not after exposure of the parasites to IFN-α or IFN-β. In addition to IFN-γ produced by lymphocytes, small sensory neurons may be a source of a molecule with activities of IFN-γ as observed in tissue culture studies (Eneroth *et al.*, 1992). These neurons contain a molecule that has a molecular weight distinct from lymphocyte-derived IFN-γ, but which cross-reacts immunologically with the latter. The molecule secreted from these sensory neurons though having a molecular weight differing from that of the IFN-γ secreted from the lymphocytes, has been shown to

potently stimulate *T. brucei* growth and can stimulate trypanosome growth. The molecule is also present in sensory axons in the tissues (Olsson *et al.*, 1994).

A soluble IFN-γ receptor has been found to be secreted from cells infected by a number of orthopoxviruses. This receptor interferes with anti-viral activity of IFN-γ and is encoded by the virus. In contrast to the restricted specificity of the host IFN-γ receptor, the virus-encoded soluble IFN-γ receptor has a broad species specificity, which would aid replication of the virus in different species (Alcami & Smith, 1995). The effects of rat and human IFN-γ on rodent and human pathogenic strains of trypanosomes were examined. It was seen that rat, but not human IFN-γ stimulated the rodent pathogenic *T. brucei*. Similarly, the human pathogenic *T. gambiense* was stimulated by human IFN-γ but not by rat IFN-γ. However, since human IFN-γ could inhibit the effect of rat IFN-γ and *vice versa*, the study also indicated that both IFN-γ can bind to the putative parasite receptor, but they may not necessarily elicit a response. During the isolation procedures, human pathogenic trypanosome strains had been passaged in rodents, and it is interesting to note that these strains could respond to both human and rat IFN-γ. The putative trypanosome IFN-γ receptor may therefore also have acquired unusual broadened species specificity (Bakhiet *et al.*, 1996).

It has been observed that mice infected with *T. brucei* show an increased survival time when treated with anti-IFN-γ antibody. Several lines of evidence point to a similar role of IFN-γ in human infections. First, human recombinant IFN-γ stimulated growth of strains of *T. gambiense* and *T. rhodesiense* isolated from human patients. Anti- IFN-γ antibodies have been reported to be present in humans infected with *T. gambiense*. This indicates that this cytokine is also produced in humans during trypanosome infection (Bonfanti *et al.*, 1995) and a preliminary analysis of cytokines

in patients affected by African trypanosomosis showed a selective and significant variation of only IFN-γ with the severity of the disease (Radomski *et al.*, 1994). In support of this, suramin, which has been in use for many decades for clinical treatment of early stages of trypanosome infections, has recently been shown to be a powerful suppresser of IFN-γ production (Czernin *et al.*, 1993). All this serves to show that IFN-γ production by T-lymphocytes, stimulated by trypanosomes, is important in parasite survival within an immunocompetent host.

The TLTF and any putative IFN-γ receptors on the parasite are obvious targets for attempts to use preventive immunotherapy against the disease. Thus these results with passive transfer antibody therapy encourage attempts for active vaccination with TLTF or fragments thereof after it has been cloned, sequenced and prepared in sufficient amounts. With such a strategy, not only may the growth of the parasites be substantially reduced, but also immunosuppression may be reduced. With this knowledge, a multicomponent vaccine can be developed to fight the parasite within the host. If successful, this control measure will be cost- effective and safer than other control methods (Bakhiet *et al.*, 1993). This will go a long way in alleviating the disease problem caused by trypanosomes.

1.9 Justification

Work done by Bakhiet *et al.* (1990) has shown that trypanosomes release a TLTF that induces CD8+ T cells to secrete IFN-γ. Subsequent studies by Bakhiet *et al.* (1993) indicated that TLTF binds to the CD8 molecule on T cells. When rodents are infected with *T. brucei,* IFN-γ production in the spleen increases markedly. Moreover, in rats depleted of CD8+ T cells by injection of anti-CD8 monoclonal antibodies or in

knockout mice carrying a deletion of the CD8 gene, a trypanosome infection does not induce as much IFN-γ production, the parasitaemia is decreased and the infected animals survive longer. In passive immunotherapy experiments, a mouse monoclonal antibody directed against TLTF greatly reduces parasite levels and increases survival of animals infected with *T. brucei* (Bakhiet *et al.*, 1993 & 1996). The gene for TLTF from *T. brucei* has been isolated and characterised by Vaidya *et al.*, (1997). This has not been done in *T. vivax*. It is therefore important to determine whether *T. vivax* also utilises this mechanism of modulating the immune system of its host during infection by TLTF production. To determine this it is essential to identify, clone and characterise the homologous genes from *T. vivax*.

1.10. Objectives

The aim of the project was to isolate and characterise a gene for the T lymphocyte triggering factor (TLTF) from *T. vivax*. Specific objectives were:

1. To screen *T. vivax* libraries for genes encoding TLTF using a *T. brucei* TLTF gene probe.
2. To characterise *T. vivax* genes encoding TLTF.

CHAPTER 2: MATERIALS AND METHODS

2.1 DNA libraries and T. brucei TLTF DNA clone.

i) *T. vivax* cDNA and genomic DNA expression libraries present at The International Livestock Research Institute (ILRI).

ii) *T. b. rhodesiense* TLTF cDNA clone from *Dr. Donelson's laboratory* (USA).

iii) Trypanosomes used in the study were *T. congolense* IL 3000 derived from stock C-49 (William *et al.*, 1981; Majiwa et *al.*, 1985), *T. brucei brucei* IL Tat 1.1, clone A4 which was derived from stock EATRO 795 and *T. vivax* IL 1392 derived from stock Y486 (Leeflang *et al.*, 1976).

2.2 Growth and isolation of trypanosomes

All trypanosome clones were grown in mice or rats. Cryopreserved glycerol stabilates of the trypanosomes were obtained from the ILRI stabilates bank. The stabilates were allowed to thaw on ice and diluted with cold phosphate-buffered saline containing glucose. The number of viable trypanosomes was estimated by counting them in a haemocytometer. Live parasites (1×10^4) were passaged intraperitoneally into mice or rats irradiated with cesium (550 rads/animal), using the IACUC SOP number 99026. The irradiation served to impair the immune system of the rodents making them unable to elicit immune responses against the parasites. This was important, as the parasites are able to multiply rapidly in an immunosuppressed host, hence giving a high number of parasites after a short while. The development of parasitaemia was monitored by examination of the tail blood and the trypanosomes harvested at the first peak of parasitaemia (10^8-10^{10} trypanosomes/ml of blood). Rats

or mice were anaesthetised and the parasitaemic blood harvested by cardiac puncture. The blood was collected in a container with heparin (10 units/ml) or Ammonium citrate as an anticoagulant. The trypanosomes were separated from the blood elements by chromatography in a column of DEAE-52 cellulose equilibrated with PSG pH 8.0 as described by Lanham and Godfrey (1970). The host blood cells being more negatively charged than the trypanosomes are adsorbed onto the DEAE, while the trypanosomes pass through, retaining their viability and infectivity.

The trypanosomes were collected in the eluate and pelleted by centrifugation at 2500 rpm at room temperature for 10 minutes in a Heraeus –Christ 11 minifuge RF. The pellets were used in the preparation and purification of DNA and RNA.

2.2.1 Genomic DNA isolation from trypanosomes

Genomic DNA was isolated from parasite cells at a concentration of 1 x 10^9/ml. The cell suspension was spun at 2500 rpm in a minifuge and the cell pellet resuspended in 1 ml of TNE solution. Fifty µl of 10 % SDS was added to the tube and mixed gently giving a clear and viscous solution. RNAse A was added to a final concentration of 100 µg/ml and the mixture was incubated at 37 °C for 90 minutes. Proteinase K was added to a final concentration of 100 µg/ml and mixed gently. This was incubated at 37 ° C for 4 hours.

An equal volume of phenol-chloroform (1:1) was added and mixed gently to achieve an emulsion. The samples were centrifuged at 14000g for 30 minutes and the top aqueous layer transferred to a fresh tube. Two and a half volumes of ice cold absolute ethanol were added and the high molecular weight DNA precipitated

immediately. The DNA pellet was washed in 70 % ethanol and then dried in a vacuum dessicator. The pellet was dissolved in Tris-EDTA buffer pH 7.6 and stored at 4 °C.

2.2.2 Total RNA isolation from trypanosomes

RNA was isolated from bloodstream and epimastigote insect form of the *T. vivax* parasite cells using the single-step method of Acid Guanidinium Thiocyanate-Phenol-Chloroform (AGPC) extraction described by Chomczynski and Sacchi (1987). The parasite pellet ($1x10^9$/ml) was resuspended thoroughly in 2 ml of RNA denaturing solution by vortexing and pippeting up and down. To the resulting homogenate was added 0.2 ml of 2M sodium acetate, pH 4, 2 ml water saturated phenol and 0.4 ml of chloroform-isoamyl alcohol (49:1) with thorough mixing after addition of each reagent. The final suspension was vortexed thoroughly and cooled on ice for 5-10 minutes. The samples were centrifuged at 14000 rpm at 4 °C for 10 minutes.

The top aqueous phase containing the RNA was transferred to a fresh tube and mixed with 1 volume of isopropanol. This was placed on dry ice for 15 minutes to precipitate the RNA. Spinning at 14000 rpm for 15 minutes sedimented the RNA. The pellet was dissolved in 0.3 ml of denaturing solution. One volume of isopropanol was added to precipitate the RNA on dry ice for 15 minutes. This was centrifuged and the pellet resuspended in 70 % ethanol. After centrifugation, the RNA pellet was vacuum-dried and dissolved in 50 µl of sterile distilled water.

2.3 Northern Transfer and hybridisation

Northern transfer of RNA was done using the rapid and simple electrophoretic conditions described by Pellé and Murphy (1993). A 1.4 % mini gel was prepared by

boiling agarose in 10 mM sodium phosphate buffer, pH 6.8 containing 1µl of 10 mg/ml ethidium bromide (EtBr) per 100 ml of buffer. This was cooled to 60 °C and poured in a gel form. One to 3 µg of Total RNA-poly (A^+) was alliquoted in 1.5 ml sterile eppendorf tubes. To this was added 2 µl of sterile 6x loading buffer and 3 µl of distilled water. The mixture was incubated at 75 °C for 5 minutes followed by immediate loading of the samples onto the 1.4 % agarose gel. The RNA was electrophoresed on the gel at 7 volts/cm until it was well fractionated. It was transferred immediately to a nylon membrane (Nytran, Schleicher and Schuell, Amersham, U.K) by vacuum. The RNA was fixed to the filter by UV light using a Stratalinker (Stratagene, USA). The filter was prehybridised using the prehybridisation solution and then hybridised with the TLTF gene probe at 65 °C overnight. This was followed by washing at a stringency of 1x SSC and 0.1 % SDS, 65 °C. The filter was exposed to an autoradiograph at –80 °C overnight and then developed.

2.4 DNA labelling for hybridisation

The DNA probes used in this study were labelled by random priming (Feinberg and Volgstein, 1983). The random priming radiolabelling of DNA was done using the Multiprime® DNA labelling system (Amersham Pharmacia Biotech, UK). Twenty eight µl (50 ng) of the DNA probe was placed in a 0.5 ml eppendorf tube and denatured at 94 ° C in a water bath for 5 minutes and then immediately chilled on ice. Ten µl of labelling buffer, 5 µl of random primer, 5µl of α-^{32}P dCTP, (3000 Ci/mM) and 2µl (5u/µl) of klenow fragment of DNA polymerase was added to the tube. The reaction was centrifuged briefly in an eppendorf minifuge and incubated at 37 °C for 1

hour. The unincorporated nucleotides were separated from labelled DNA by centrifugation through a 1 ml Sephadex G-50 column at 1,200 rpm for 2 minutes in a Heraeus-Christ minifuge II RF as described by Sambrook *et al.*, (1989). The purified probe was collected into a clean eppendorf tube. Before use, the probe was denatured by boiling at 95 °C for 5 minutes.

2.5 Screening the library by hybridisation

2.5.1 Propagation of E. coli strain Y1090 host cells

A single colony of *E. coli* Y1090 (see Appendix) was picked from a plate and inoculated into 50 ml of 2xYT medium supplemented with 0.5 ml of 20 % maltose, 0.5 ml of 1M $MgCl_2$ and 50 μg/ml ampicillin. The medium was incubated in a rotary shaker at 37 °C, 225 rpm overnight. An aliquot of the overnight culture was inoculated into fresh 50 ml of 2xYT medium supplemented as above and grown to mid-log phase. This was determined by measuring the OD at 600 nm on a spectrophotometer DU-50 (Beckman, UK). At mid-log phase, the OD was between 0.5-0.6. The bacterial cells were pelleted by centrifugation at 3000 rpm for 10 minutes in a Heraus Christ minifuge RF and then resuspended in 10 ml of 10 mM $MgSO_4$ and stored at 4 °C.

2.5.2 Optimisation of hybridisation conditions

T. brucei, *T. vivax* and *T.congolense* genomic DNA were electrophoresed on an agarose gel and a Southern transfer done (see section 2.11). *T. brucei* TLTF DNA probe was used for hybridisation under different hybridisation and washing

conditions. Hybridisation at 65 °C was found to be the optimum, while the optimum stringency for washing was 1X SSC & 0.1 % SDS at 65 °C.

2.5.3 Titration and plating of the library

One hundred µl of 1:00, 1:1000, 1:10000 dilutions of the stock λgt 11 (see Appendix) *T.vivax* genomic and cDNA libraries in SM buffer were used to infect 8 ml of Y1090 host cells. The cells were incubated at 37 °C for 15-20 minutes, then mixed with 8 ml of the molten top agar maintained at 45 °C and plated onto 135 mm NZYCM plates with ampicillin (50 µg/ml). The plates were incubated overnight at 37 °C and the following morning, the number of the plaques formed counted and used to estimate the titre of the libraries.

2.5.4 Screening the libraries by hybridisation

Screening of the libraries was done by hybridisation as described by Benton and Davis (1977). The library was plated at a density of 1 x 10^4 pfu/135 mm plate and the plates incubated overnight at 37 °C to grow the phage plaques. Dry nylon filters (Schleicher & Schuell, Germany) was overlaid onto the agar surface and orientation established by stabbing the agar through each filter with hypodermic needle (18G) dipped in Indian ink. The filters were lifted from the plates after 10 minutes using a sterile pair of forceps and placed plaque side up on Whatman 3MM paper soaked with denaturation solution for 5-10 minutes. The filters were transferred to a UV cross linker machine (UV Stratalinker™ 2400, Stratagene) to fix the DNA onto the filters (Khandjian, 1975). This was done at 1.2 x 10^5 µJoules.

The filters were transferred to a 3MM paper soaked with neutralisation solution and allowed to neutralise for 5-10 minutes. The filters were cross-linked again at 2.4 x 10^5 µjoules. The filters were then prehybridised in the prehybridisation solution for at least 3 hours. They were hybridised with the *T. b. rhodesiense* TLTF DNA probe overnight in a water bath with gentle shaking, at 65 °C. The filters were washed with 1X SSC, 0.1 % SDS at 65 °C and then exposed to an autoradiograph with an intensifying screen at –70 °C overnight. After developing the autoradiograph, the positive plaques were identified, picked and suspended in 500 µl of SM buffer. Aliquots of 20 µl of 1:100 dilution of each suspension were used to infect 200 µl of *E. coli* strain Y1090 and plated as described above for secondary screening of positive clones.

2.5.5 Preparation of DNA from the bacteriophage lambda

The positive clones isolated after several rounds of screening were used to extract the DNA clones of interest. The positive plaque was suspended in 500 µl of the SM in a 1.5 ml sterile Eppendorf tube and incubated at 37 °C in a water bath to allow the phage to diffuse into the SM. This was done for 2 hours with intermittent vortexing to hasten the diffusion. A drop of chloroform was added to kill *E. coli* cells and 200 µl of this suspension solution then mixed with an equal volume of log phase Y1090 bacterial cells and incubated at 37 °C for 15 minutes to allow the phage to infect the bacterial cells. Top agar was melted and cooled down to 45 °C in a water bath. Eight ml of the top agar was mixed with infected bacterial cells and plated on 135 mm NZYCM plates containing ampicillin at 50 µg/ml. The top agar was allowed to set at room temperature and the plates were incubated in an inverted position at 37 °C overnight.

To the confluent plaques on each plate, 10 ml of SM was added and the plate incubated at 37 °C for about 1 hour. The SM solution containing the phage DNA was recovered in a 15 ml corex tube (USA, No. 8441). A half volume of the retrieved SM solution of DEAE-sephacel slurry (Pharmacia Biotech, Sweden) was then added to the corex tube and mixed on a roller for 1 hour at room temperature. The tube was centrifuged at 8000 rpm, 4 °C for 15 minutes in a swinging bucket rotor (Beckman J-21 centrifuge) to remove bacterial debris.

The supernatant was recovered into a fresh corex tube. For every 1 ml of the supernatant recovered, 25 µl of 1M Tris.HCl (pH 7.5), 200 µl of 5 M NaCl and 200 µl of 50 % polyethylene glycol 6000 was added. The solution was mixed and incubated on ice for 15 minutes to precipitate the phage particles and then centrifuged as above. The supernatant was discarded and the tube drained completely. The phage pellet was resuspended in 400 µl of SM in a 1.5-ml Eppendorf tube. Ten µl of 10mg/ml of RNAse was added to the suspension and incubated at 37 °C for half an hour. To the suspension was added an equal volume of chloroform, vortexed, and spun at 14000 rpm for 5 minutes.

The aqueous phase was recovered in another tube and to it was added 10 µl of 10% SDS, 20 µl of 0.5 M EDTA and an equal volume of phenol /chloroform. This was spun again as before. The aqueous phase was recovered and re-extracted with an equal volume of chloroform to remove any residual phenol.

The aqueous phase was retrieved into a new tube and 2.5 volumes of absolute ice cold ethanol added to precipitate the DNA. To this was added 0.1 volumes of 3 M sodium acetate to increase the efficiency of the recovery of the DNA. The tube was incubated at –20 °C and then pelleted by centrifugation at 14000 rpm at 4 °C for 20

minutes. The resulting pellet was washed with 70 % ethanol and dried in a vacuum SpeedVac concentrator centrifuge (Savant Instruments. Inc, Farmingdale, NY). The pellet was resuspended in 50-100 µl of sterile deionised water or T.E buffer. The extracted phage DNA was electrophoresed on an agarose gel to determine its quality and then stored at 4 °C.

2.6 Plasmid DNA purification from bacterial cells (Minipreps)

All plasmid DNA used in the study were purified from bacteria cells using the Wizard (Plus) Minipreps DNA Purification System (Promega, USA). Five ml of the bacterial culture was put in a 10 ml sterilin tube (Bibby sterilin Ltd, England) and spun at 3000 xg for 10 minutes. The supernatant was poured off and the pellet resuspended in 300 µl of cell resuspension solution in a 1.5 ml Eppendorf tube. Three hundred µl of cell lysis solution was added to the tube and mixed gently by inverting the tube 4 times followed by incubation at room temperature for 5 minutes. Three hundred µl of the neutralisation solution was added and mixed by inverting the tube 4 times. The bacterial lysate was centrifuged at 14000 rpm for 5 minutes and the cleared lysate transferred into a fresh Eppendorf tube containing 1ml Wizard™ plus Miniprep DNA purification Resin.

A Wizard Miniprep Column was prepared by attaching a syringe barrel to the Luer-Lok extension of a Minicolumn. The tip of the Minicolumn/Syringe barrel assembly for each Minipreps was then inserted into the vacuum manifold. The resin mixed with the cleared lysate was pippeted into the barrel of the Minicolumn/Syringe assembly and a vacuum applied to pull the resin/lysate mix into the minicolumn. The vacuum was then stopped and 2 ml of the column wash solution added to the syringe barrel.

The vacuum was reapplied and the resin allowed to dry by continuing to draw the vacuum for 30 seconds. The syringe barrel was removed and the minicolumn transferred to a sterile 1.5 ml Eppendorf tube and centrifuged for 20 seconds at 14000 rpm to remove any residual column wash solution. Fifty µl of sterile deionised water pre warmed to 70 °C was added to the resin in the minicolumn and then spun at 14000 rpm for 20 seconds to elute the DNA. The eluted DNA was stored at 4 °C.

2.7 Electrophoresis

Conventional agarose electrophoresis was used to resolve various nucleic acid fragments such as restriction enzyme digests of genomic DNA, RNA, phage DNA, recombinant plasmid DNA and PCR products, as described by Sambrook *et al.*, (1989). The agarose gels were prepared with 1X TAE containing 0.5µg/ml ethidium bromide. The samples were mixed with gel loading buffer before loading into the wells on the gel. Electrophoresis was done at a constant voltage of 7 volts/cm using a Bio-Rad model 200/2-power supply source. The location of nucleic acid fragments on the gels was determined by direct examination of the gel and the size estimated by comparing with molecular weight size markers loaded alongside them (see Appendix), under UV light (Sharp *et al.*, 1973).

2.8 Restriction enzyme digest of nucleic acids

All restriction enzymes were used under optimal conditions as recommended by the manufacturer (New England Biolabs, MA, USA). Double digests of DNA were carried out with two enzymes simultaneously using a single appropriate buffer as indicated by the supplier. Purified PCR products, phage DNA, recombinant plasmid

DNA and genomic DNA prepared from trypanosomes and other organisms were digested in a total reaction volume of 20 µl. Some of the restriction enzymes that were used in the study are listed in Appendix.

Each reaction contained 0.5- 2 µg of DNA, 2-10 units of appropriate restriction enzyme, 2µl of 10 x BSA (where required) 2µl of the appropriate buffer and sterile water added to make the final volume. Digests were carried out at 37 °C for 1-2 hours or overnight and resolved on an agarose gel as described in section 2.7 above.

2.9 DNA purification from agarose gels by GENECLEAN

The GENECLEAN II kit (BIO 101 Inc, CA, USA) was used to purify DNA from agarose gels. The DNA was resolved on an agarose gel by electrophoresis and the band of interest sliced from the gel under UV light using a sterile scalpel blade and placed in a sterile 1.5 ml eppendorf tube. The weight of the excised band was determined and 3 volumes of sodium iodide added. The mixture was incubated at 55 °C to melt the gel. Five µl of GLASSMILK® were added to every solution containing 5µg of DNA or less. An additional 1µl was added for each 0.5µg of DNA above 5µg, followed by mixing and incubation at room temperature for 5 minutes to allow binding of the DNA onto the silica matrix. The mixture was vortexed every 1-2 minutes to ensure that the GLASSMILK® stayed suspended.

The mixture was centrifuged at 14000 rpm for 5 seconds and the supernatant discarded. The pellet was resuspended thoroughly in 700 µl of NEW™ Wash solution and then centrifuged for 5 seconds at 14000 rpm. This procedure was repeated 2 more times and any residual solution pippeted off after the third wash.

DNA was eluted from the GLASSMILK pellet by resuspending in a volume of sterile deionised water equal to the volume of GLASSMILK used. The tube was incubated in a water bath at 45-50 ° C for 2-3 minutes, and then centrifuged at 14000 rpm for 30 seconds to elute the DNA. The supernatant containing the eluted DNA was carefully removed and placed in a fresh tube. A second elution was done to recover any DNA still bound to the matrix. One µl of the eluted DNA was electrophoresed on an agarose gel to determine the quality and quantity of DNA eluted.

2.10 DNA purification from agarose gels by centrifugation

After electrophoresis on an agarose gel, the DNA of interest was sliced from the gel using a sterile scalpel blade. The agarose block was transferred into a sterile 1.5-ml eppendorf tube pierced at the bottom with a small needle then packed 1/5 with sterile glass wool. This tube was inserted into a similar tube for the recovery of eluted DNA. DNA was eluted from the gel by centrifugation at 1400 rpm in a minifuge for 20 seconds.

2.11 Southern transfer

Southern blotting of DNA from agarose gels (Southern, 1975) was carried out as described by Sambrook *et al.*, (1989). After electrophoresis, the gel was photographed under UV light and soaked in denaturing solution for 30 minutes at room temperature with gentle agitation, then rinsed with distilled water. A nitrocellulose membrane (Schleicher &Schuell, Germany) was cut, slightly larger than the gel and soaked in 20x SSC. The transfer was conducted by use of a vacuum transfer machine (Vacugene pump, Pharmacia). The nitrocellulose membrane was placed on the vacuum machine

and the gel aligned onto it. 20 x SSC was used to transfer the DNA to the filter for 1 hour at 85 mb of vacuum pressure. The DNA transferred onto the nitrocellulose was immobilised onto it using UV on a cross linker machine (UV Stratalinker™ 2400, Statagene, USA). The nitrocellulose membrane was then rinsed in Dulbecco's PBS and then pre hybridised at 65 °C for 3 hours using pre hybridisation solution. Hybridisation was done overnight using the TLTF probe. The membrane was washed at a high stringency (0.1 % SDS, 1 X SSC) at 65 °C 3 times at intervals of 30 minutes. The membrane was wrapped in a plastic bag and exposed to an autoradiograph at -70 °C overnight with an intensifying screen. The DNA of interest was identified after developing the autoradiograph.

2.12 PCR amplification

2.12.1 Standard PCR amplification

The PCR amplification reactions were performed in a final reaction volume of 20-100µl. This contained 1ng/µl of DNA template, 1x PCR buffer, 1.5mM $MgCl_2$, 0.2 mM dNTPS, 0.025 u/µl Taq polymerase and 1ng/µl final concentration of each primer. Primers that were used in the study are listed in Appendix.

The samples were placed in a programmable heating block (M.J. Research Inc) and incubated at 94 °C for 1 minute, 55 °C for 1 minute and 72 °C for 1 minute. At the end of 30-40 cycles, the synthesis reaction was cooled to 4 °C. The annealing temperature was varied depending on the degree of specificity desired for individual PCR amplification assays. Five to ten µl of the reaction volume of each amplified sample was resolved in a 1 % agarose gel stained with EtBr and photographed under UV illumination.

2.12.2 Random PCR

The conditions for Random PCR using arbitrary primers i.e. arbitrarily primed PCR (AP-PCR) (Welsh and McClelland, 1990; Williams *et al.*, 1990), were different. These primers are short oligodeoxyribonucleotides (10 mers) of arbitrary sequence. A single primer was used for each sample DNA to be amplified. The other reagents were as those used in standard PCR. The cycles for the AP-PCR were denaturing at 95 ° C for one minute, annealing at 40 ° C for 45 seconds, extension at 72 ° C for 1 minute, for 40 cycles followed by 10 minutes extension at 72 °C. Five to ten µl of the products of the amplification were electrophoresed on a 0.7 % EtBr stained agarose gel. A Southern transfer and hybridisation was done to identify the amplified DNA of interest. This was cloned in a pGEM-T sequencing vector and sequenced.

2.12.3 Purification of PCR products

The purification of PCR products using the Magic® PCR system (Promega Corp; Madison, WI, USA) was performed to remove any contaminants, which may impinge on their further manipulation.

For each PCR reaction, the sample was transferred to a 1.5-ml minifuge tube. To this, 100µl of direct purification buffer (50mM KCl, 10mM Tris-HCl, pH8.0, 1.5 mM $MgCl_2$, 0.1 % Triton x-100; Promega Corp; Madison, WI, U.S.A) was added. The content in each tube was vortexed briefly to mix. One ml of Magic® PCR preps resin (Promega Corp.) was added to the mix and vortexed three times over a period of 1 minute. The DNA/resin mix was then pippeted into a mini-column syringe assembly attached to a vacuum manifold and vacuum applied to drain the DNA/resin through the column. To wash the column, 2ml of 80 % iso-propanol was added to the syringe

barrel and vacuum applied to draw the solution through the column. The column was vacuum dried for a further 2-3 minutes. The syringe was removed and the mini-column transferred to a fresh 1.5-ml microfuge tube. To elute the DNA bound to the resin, 50 µl of sterile water or T.E buffer preheated to 50 °C was added into the mini-column and allowed to stand on the bench for about 1minute. The mini-column was then centrifuged at 14000g for 20 seconds in an eppendorf minifuge. The purified PCR products were stored at 4 ° C for later use.

2.13 Subcloning

The DNA inserts that hybridised with the TLTF probe were subcloned in appropriate plasmid vectors. The subcloning involved ligation of the DNA insert to the plasmid vector DNA, transformation of competent *Escherichia coli* cells (strain JM109) with the ligated DNA, selection of transformants and sequencing of positive clones.

2.13.1 Preparation of DNA inserts for cloning

DNA inserts of interest were cloned in a sequencing vector. PCR products were cloned directly into a pGEM-T vector without further manipulation. DNA inserts resulting from digests with restriction endonucleases contain sticky ends and required modification before cloning them directly in T-vector. These were first blunt ended by resuspending the purified DNA insert in T4 DNA polymerase 1x buffer, containing 100 µM of each dNTP and 0.1 mg/ml acetylated BSA. Five units of T4 DNA polymerase per microgram of DNA was then added and the reaction incubated at 37 °C for 5 minutes. The reaction was stopped by heating the mixture at 75 °C for 10 minutes. Deoxy-adenosine molecules (A's) were then added to the ends of the purified

blunt ended DNA fragments by a one cycle PCR reaction done at 70 °C for 2 hours. The resulting DNA was purified by PCR Magic preps and used in the ligation reaction as described in section 2.13.2 below.

2.13.2 Ligation reaction

Ligation reactions of PCR products and a T-vector were performed at a molar concentration of 2:1 respectively. This also applied to non-PCR products. The T-vector (Promega Corp. U.S.A) was used to ligate PCR products and DNA fragments resulting from restriction digests. The ligation reactions were set up in total volumes of 10µl containing 1µl of the T- vector (25 ng/µl), 2µl of appropriately diluted DNA (25ng/µl), 1µl of T4 ligase (3u/µl), 5µl of 2x rapid ligation buffer and 1µl of sterile de-ionised water. The ligation reaction tubes were incubated at 15 °C for at least 2 hours. Another cloning vector that was used in the study was pBluescript (see Appendix).

2.13.3 Preparation of JM109 Electrocompetent cells

For the preparation of electrocompetent bacterial cells, the procedure described by Dower *et al.*, (1988) was used. A single colony of JM109 cells (see Appendix) streaked on a plate was inoculated into 50 ml of 2xYT medium. This was grown overnight at 37 °C in a shaker with gentle agitation without antibiotic. Two ml of the overnight culture was inoculated into 1 litre of fresh 2 xYT medium. This was put in an incubator shaker at 37 °C at 225 rpm and the cells grown to an optical density OD_{600} of 0.5-0.6 when they reached the mid-log phase. At this stage of growth the cells are at the greatest period of competence. The cells were harvested by chilling the flask

on ice for 30 minutes. All the following stages were performed at 4 °C since the efficiency of transformation drops as much as 100 fold when carried out at room temperature (Sambrook *et al.*, 1989). The medium was subdivided into pre chilled 50-ml Falcon tubes (Propylene conical tubes, Becton Dickinson, USA).

The cells were centrifuged at 3000 rpm in a Heraeus Sepatech RF minifuge at 4 °C for 15 minutes. The supernatant was discarded and 50 ml of 10 % glycerol chilled to 4 °C was added to each tube and the pellets resuspended in it. The tubes were centrifuged again as before and after each spin two tubes were pooled together so that after each spin there were half the number of the original tubes. Each time the cell pellets were resuspended in chilled 10% glycerol. At the end the pellets were pooled into one tube and resuspended in 2 ml of 10 % glycerol. By so doing, the cells' concentration was increased. This increases the yield of transformants. Eighty µl of the cells were then aliquoted into pre-chilled 1.5 ml eppendorf tubes and then snap frozen immediately in liquid nitrogen and stored at – 80 °C until when needed for use.

2.13.4 Transformation by Electroporation

E. coli cells can be transformed to extremely high efficiencies by subjecting a mixture of cells and DNA to brief but intense electric fields of exponential decay waveform (electroporation). This method was used to transform cells in this study.

The concentrated cells were thawed on ice. To a 1.5 ml Eppendorf tube containing 80 µl of the electrocompetent cells, 2 µl of the ligation mix was added and the tube swirled gently to mix. The tube was left on ice for 5 minutes. The cell/DNA mixture was transferred to a pre-chilled cuvette and electroporation done at 1.5 volts on a BIO RAD Gene Pulser at 25 µFD and 200 ohms. Following the pulse, the cells were

immediately mixed with 1 ml of LB medium warmed to 37 ° C containing 10 µl of 20 % maltose solution and 10µl of 1 M $MgSO_4$ in a propylene tube. The cells were incubated, with shaking at 225 rpm, for 1 hour.

In the meantime, 50 µl of 2 % Isopropyl-thio-galactopyranoside (IPTG) and 50 µl of 2% 5-bromo-4-chloro-3-indolyl-B-D-galactoside (X-gal) were mixed and spread evenly onto 135 mm NZYCM + Ampicillin pre warmed at 37 °C, using sterile beads. X-gal and IPTG are important in the differentiation of recombinants from non-recombinants. In the presence of X-gal and IPTG, the recombinants appear white in colour while the non-recombinants are blue in colour.

After one hour of growth in the incubator shaker, the cells were resuspended and 500µl of the cells spread onto the plate containing the IPTG and X-gal using sterile beads. The plate was incubated in an inverted position overnight at 37 ° C for the growth of the transformed cells to take place.

2.13.5 Identification of recombinant clones

The plates were checked for positive colonies. White colonies were picked and subjected to PCR colony screening. The PCR was carried out as explained in section 2.10.1. The colonies were diluted 1:10 in distilled water and used as template in the PCR. After the PCR, the samples were electrophoresed on agarose gel to determine whether they contained inserts. Clones that were positive by PCR screening were grown overnight at 37 °C in 5 ml of 2xYT medium containing 50 µg/ml ampicillin. Plasmid DNA purification from the bacteria cells was done as described in section 2.6. Restriction enzyme digestion analysis was done on the purified plasmid DNA to confirm the actual presence of inserts.

2.14 DNA Sequencing

DNA plasmid clones of interest were sequenced using the fmol® DNA Sequencing System from Promega. The chain termination method described by Sanger *et al.*, (1977) was used.

2.14.1 Primer radiolabelling for sequencing

DNA sequencing was performed using end-labelled oligonucleotide primers prepared by incorporating γ^{33}P dATP at their 5′ ends. For each primer, a 10µl reaction was prepared in a sterile 0.5 ml microfuge tube. The reaction mix was made up of 1µl of primer (50 ng/µl), 5 µl of γ-^{33}P dATP, 1 µl of T4 polynucleotide kinase (PNK) enzyme (5 u/µl), 1µl of 10x PNK buffer and 2µl of sterile distilled water. The reaction was incubated in a 37 °C water bath for 30 minutes. The primers were stored at ⁻20 °C.

2.14.2 Sequencing reaction

Sequencing was carried out using the dedeoxynucleotide chain termination (Sanger *et al.*, 1977) using the fmol® DNA sequencing system (Promega, Madison, WI, USA). For each DNA sample to be sequenced, a master mix was prepared in a tube by mixing: 200 ng of template DNA, 5µl of 5x DNA sequencing buffer, 15 ng of ^{33}P end labelled primer, 1µl of Taq DNA polymerase (sequencing grade 5u/µl) and distilled water to a final volume of 16 µl. Four µl of the master mix (primer/template) was added to each of the four tubes labelled A, C, G, T containing 2 µl of ddATP, ddGTP, and ddTTP, respectively. Each tube was then overlaid with a drop of mineral oil and placed in a Minicycler™ (MJ Research Inc. Massachusetts, USA) for cycle

sequencing PCR. Thirty cycles were performed in three steps: 94 °C for 30 sec, 55 °C for 30 sec, 72 °C for 1 min, followed by a final extension of 72 °C for 10 min. Sequencing reactions were stopped by adding 3 µl of the fmol® stop solution and stored at -20 °C. Before electrophoresis, reactions were heated at 94 °C for 5 minutes to denature the samples, cooled on ice for at least 1 minute and then loaded into a pre warmed sequencing gel.

2.14.3 Sequencing gel electrophoresis

Sequencing reactions were resolved in 6 % acrylamide gels. A 6 % acrylamide mix was prepared from a 40 % acrylamide stock (2 g bis-acrylamide and 38 g acrylamide in 100 ml of sterile distilled water). 42 g of urea, in 15 ml of 40 % acrylamide, 10 ml of 10x TBE and sterile distilled water added to make 100 ml, were dissolved to make the 6 % acrylamide mix. 600 µl of 10 % APS (ammonium per sulphate) and 100 µl of TEMED (N, N, N, N tetramethylethylethylenediamine) were added to 100 ml 0f the 6 % acrylamide mix which was subsequently poured in between 2 glass plates clamped and separated with 2 spacers of 0.3 mm width.

The glass plates measured 42 x 33cm and 39.5 x 33cm. The inner side of the large plate was coated with 1 ml of gel glue (2 % Methacryloxy Propyltrimethyloxylsaline (sigma M-6514) in 100 % ethanol). Excess glue was washed off with water and air-dried. The inner side of the smaller plate was coated with 1 ml of siliconising solution (5 % dichlorodimethylsilane in carbon tetrachloride) and polished with 70 % methanol.

Sequencing combs were placed on the top surface of the gel with the flat side stuck deep in the gel to form an even platform. The gel was left to set for about 45 minutes.

1x TBE buffer was used for electrophoresis of the sequencing gel. The plates were assembled on the manual sequencer machine (BRL model S2, Life Technologies Inc) and the sequencing buffer added. The combs were put in an inverted position in the gel with the teeth making contact with the gel so as to form wells for loading the samples. The gel was pre warmed for 20 minutes after which the wells were flushed with the buffer to remove urea. Two to 4 µl of the sequencing reactions were loaded in adjacent lanes with the reactions arranged thus: A, C, G, T for each of the samples. The electrophoresis was performed at 65 watts with a constant power supply for 2 to 5 hours. At the completion of the sequencing, the plates were disassembled and the large plate with the gel stuck on it fixed in a solution of 10 % glacial acetic acid & 10 % methanol for 10 minutes. The gel was then soaked in distilled water for at 10 minutes and dried in an oven at 75-80°C for one hour. The plate was exposed to an x-ray film without an intensifier screen at room temperature overnight.

2.14.4 DNA sequences analysis

The films were developed and the sequences read using a digital sequence analyser (Graf/Bar Mark II model, Science Accessories Corporation) and an illuminator (model 5000 RR Beard Ltd) linked to a computer connected to an IBM printer analysed using the computer programs DNAGO and DNASIS. The sequences were edited to remove any vector sequence. A search was done using the BLAST program (Altschul *et al.*, 1997) to identify homology with the *T. b. rhodesiense* TLTF gene.

CHAPTER 3: RESULTS

3.1 Introduction

To be able to identify whether *T. vivax* parasites have a gene for the TLTF, it was imperative to use the TLTF open reading frame (ORF) gene from *T. b. rhodesiense* (Figure 3.1) as a probe to screen *T. vivax* cDNA and genomic phage libraries. On identification of positive clones, the DNA would be isolated from them, cloned in a plasmid vector and sequenced to determine the sequence of the isolated DNA and perform homology studies.

3.2 Screening of bacteriophage libraries

T. vivax genomic and cDNA bacteriophage libraries were screened under the optimised conditions and after several rounds of primary and secondary screening, the positive clones of interest could not be identified. This was because the probe was unspecific as it was hybridising to the vector DNA and bacteria DNA. Hence a different approach was used. This method involved the screening of trypanosome DNA directly This novel approach is referred to as Random PCR-assisted cloning of gene homologues.

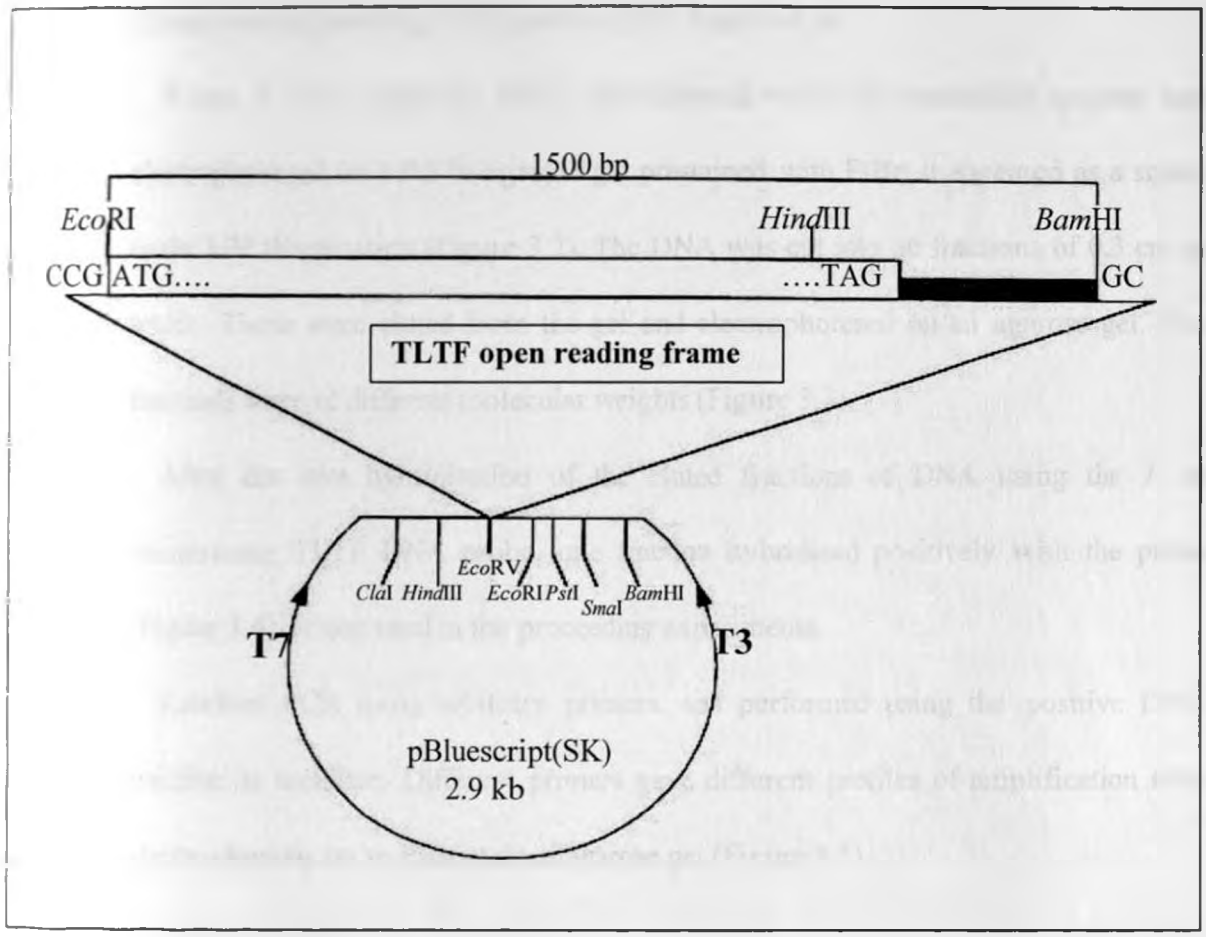

Figure 3.1: Map of *T. b. rhodesiense* TLTF cDNA cloned in pBSK plasmid vector. The 1.5 kb insert was released by an *Eco*RI and *Bam*HI double digest.

3.3 Random PCR- assisted cloning of homologue genes

This process involved digestion of genomic DNA into small fragments; dot blot hybridisation of the DNA fragments to identify DNA fragment (s) of interest; and cloning and sequencing of the positive DNA fragment (s).

When *T. vivax* genomic DNA was digested with *Pst*I restriction enzyme and electrophoresed on a 0.8 % agarose gel prestained with EtBr, it appeared as a smear under UV illumination (Figure 3.2). The DNA was cut into 36 fractions of 0.3 cm in width. These were eluted from the gel and electrophoresed on an agarose gel. The fractions were of different molecular weights (Figure 3.3).

After dot blot hybridisation of the eluted fractions of DNA using the *T. b. rhodesiense* TLTF DNA probe, one fraction hybridised positively with the probe (Figure 3.4). It was used in the proceeding experiments.

Random PCR using arbitrary primers was performed using the positive DNA fraction as template. Different primers gave different profiles of amplification after electrophoresis on an EtBr-stained agarose gel (Figure 3.5).

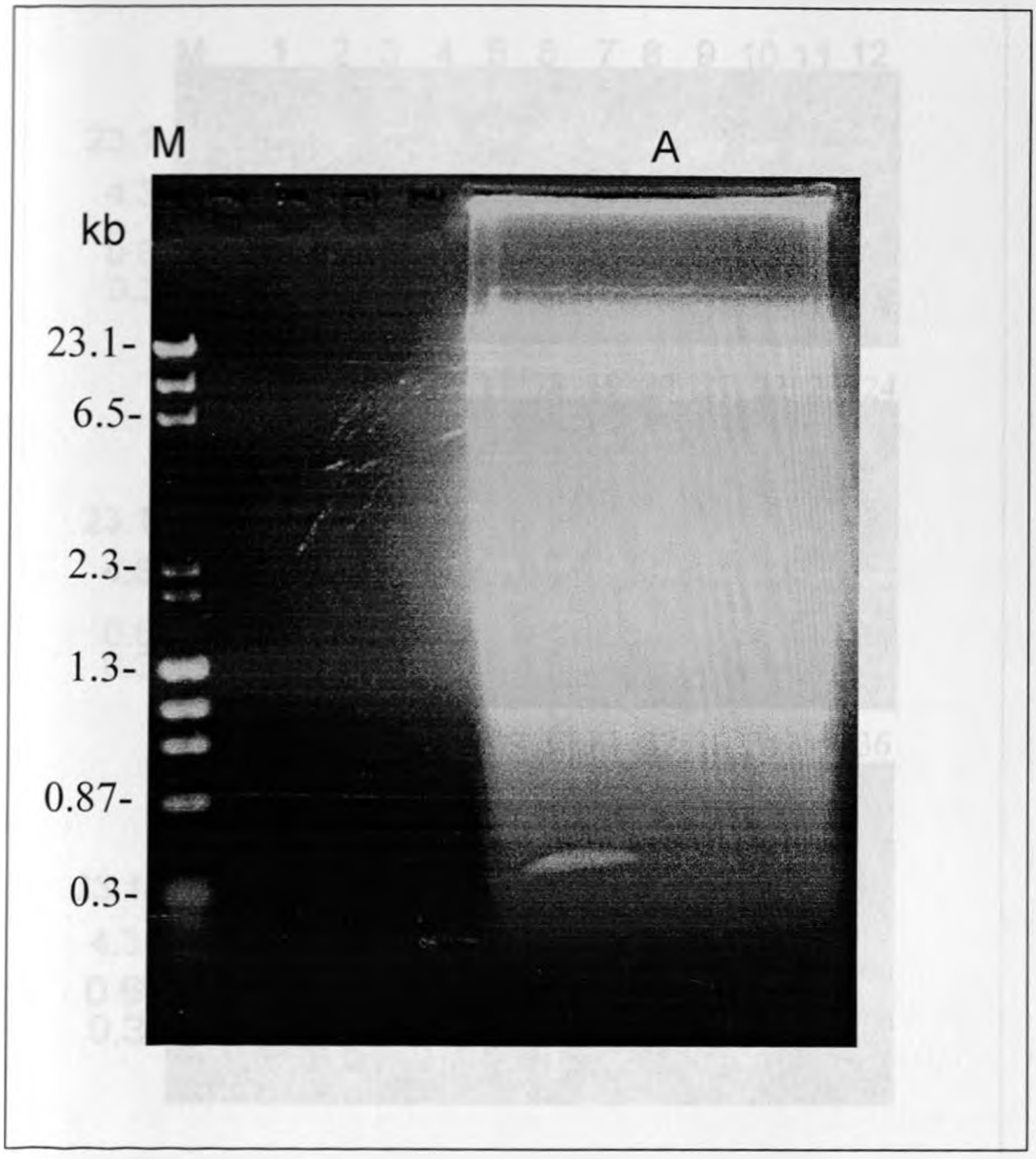

Figure 3.2: *T. vivax* genomic DNA digested with *Pst*I restriction enzyme.

Lane M represents a mixture of *Hae*III digested ϕx 174 DNA and *Hind*III digested λDNA size markers (GIBCO-BRL, Uxbridge, UK), while lane A represents the digested DNA.

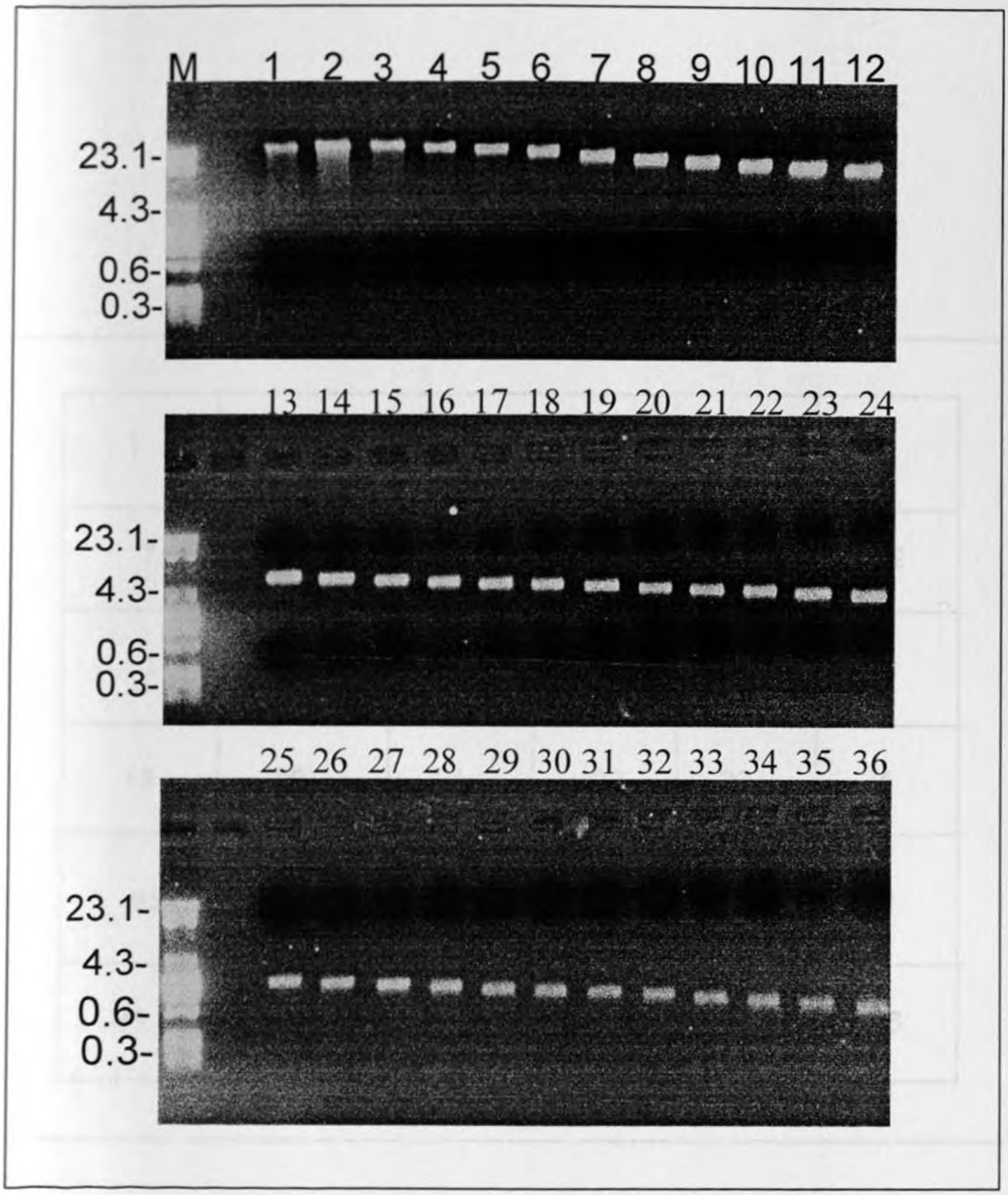

Figure 3.3: Et-Br stained agarose gel of 36 eluted DNA fractions after digestion with *Pst*I restriction enzyme. They were of different molecular weights as indicated.

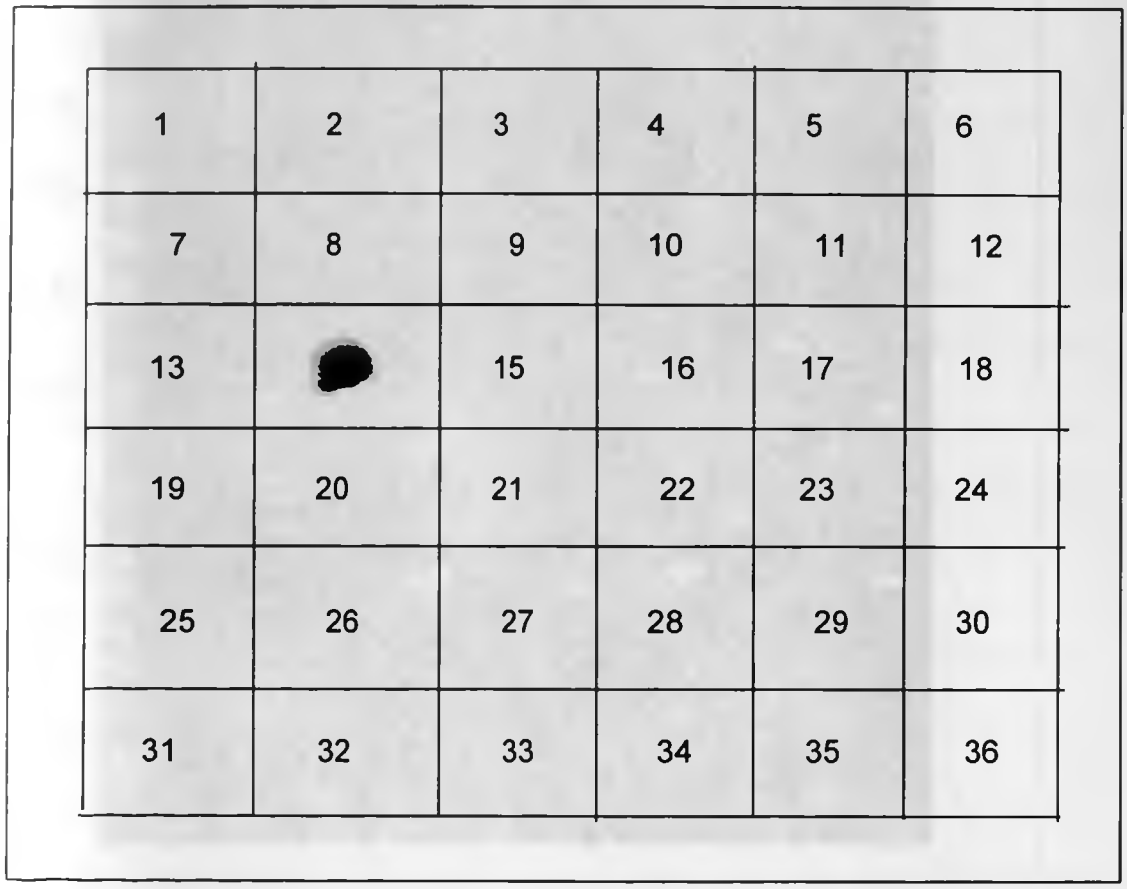

Figure 3.4: Dot blot hybridisation of the eluted DNA fractions.

One DNA fraction (No 14) hybridised positively to the probe. This fraction was used in performing a random PCR.

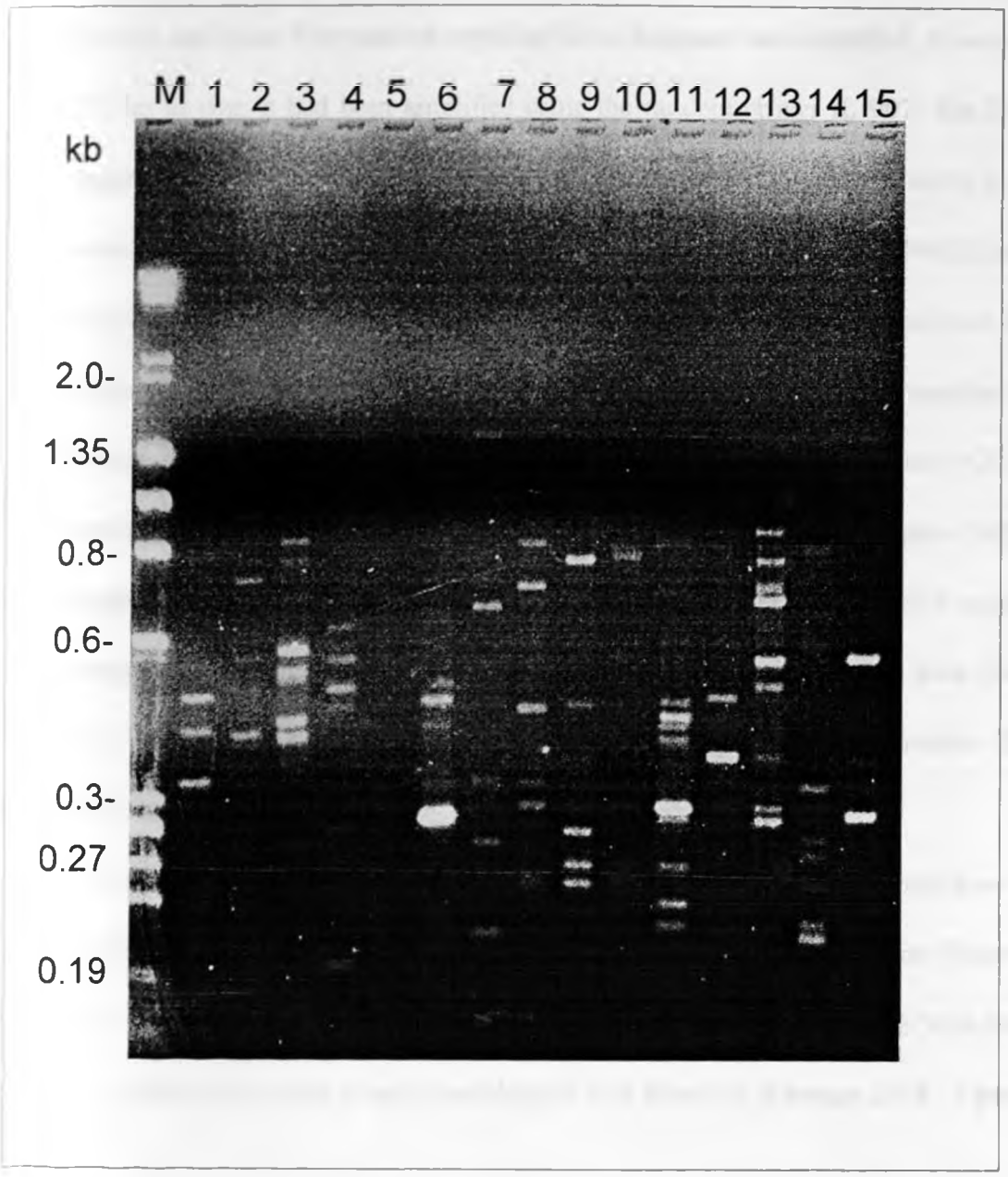

Figure 3.5: Random PCR of the positive DNA fraction with arbitrary primers.

The products of the PCR were electrophoresed on an EtBr- stained agarose gel. M represents a mixture of λ DNA digested with *Hind*III & ϕX 174 DNA digested with *Hae*III restriction enzyme. Lanes 1-15 represent the electrophoresed samples from amplification with different primers.

Southern blotting and hybridisation of the amplified products using arbitrary primers was done. The positive amplified DNA fragment was identified. It was about 250 bp in size. It had been amplified using the random primer IL 907. The 250-bp fragment of DNA resulting from the PCR was purified and cloned directly in a T-vector and sequenced. A DNA homology search was done using the DNASIS search program. It was found to be having a 67 % homology with the *T. b. rhodesiense* TLTF sequence. From this sequence, a specific primer was designed and together with miniexon and *T. vivax* sscDNA as template, was used to perform another PCR. The resulting amplified DNA was purified and cloned in a T-vector (Figure 3.6) and sequenced. The resulting sequence was 361bp long (Figure 3.7). A DNA sequence comparison between *T. vivax* TLTF and *T. b. rhodesiense* TLTF was done (Figure 3.8). The homology of the *T. vivax* TLTF DNA to that of *T. b. rhodesiense* TLTF DNA was 67.4 %.

An amino acid sequence comparison between *T. vivax* TLTF, *T. b. rhodesiense* TLTF and the Human growth arrest specific gene (GAS 11) was also done (Figure 3.9). The *T. vivax* amino acid sequence for TLTF has a homology of 70.8 % to that of *T. b. rhodesiense* while it has a homology of 51.5 % to that of human GAS 11 gene.

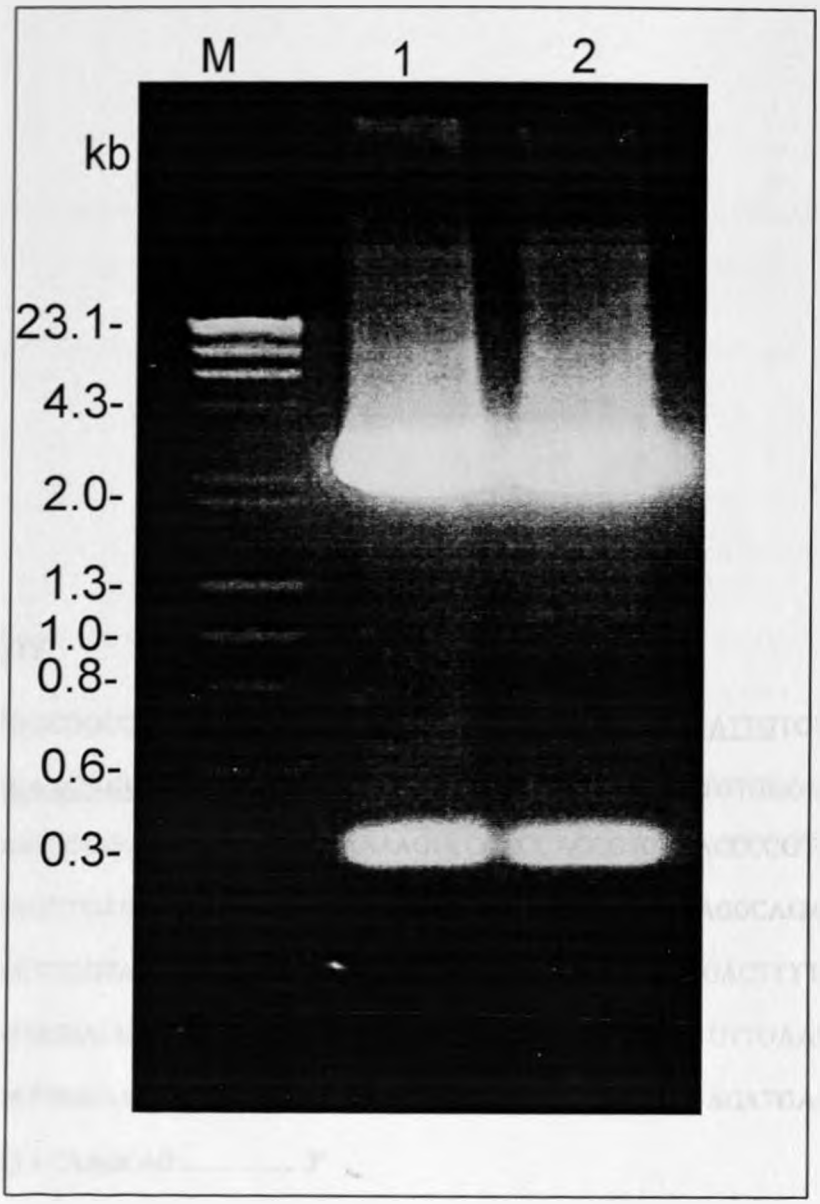

Figure 3.6: *T. vivax* 5' TLTF cDNA cloned in pGEM-T vector.

The clone was digested with *Sal*I and *Sac*II restriction enzymes to verify the presence of the insert, which was 361 bp.

5'Tv TLTF

5' GTGGCGGCCACAACGCTATTATTAGAACAGTTTCTGTACTATATTGTGTA 50

TAGAACAGCTGTTGACGTTACACTAGATGCCACCTAAGACTGTGGGGGCG.....100

IL7980

CGGGGCGGCAAAAAGGCTGAAAAAGCCCCACCACCGTGTAACCCCGTCAC.....150

AGAGCTGAGTTCCCTAGAGTCTTTGCACGAAAACCTCGATAAGGCAGAAG.....200

AACTTCGTAATTACTTTCAAGTTGAGCGCGATAAAGTGAACGACTTTTGG.....250

AATGCGACGAAGAGTGAAGTGGAAACATATCGCGCCCGACTGTTGAATGC.....300

GGATTCGGAGATTGAGGAGCTCGAGCGTGCCCACCAGGTAGAGATGAAGG.....350

TATACAAGCAG ..... 3'

Figure 3.7: DNA sequence of the 5 *T. vivax* TLTF cDNA.

The gene was 361 base pairs long. The Miniexon is underlined. The primer IL 7980 was designed from it later on.

Figure 3.8: The DNA sequence comparison between the 5′ *T. vivax* TLTF and that of *T. b.* rhodesiense. The dotted regions indicate regions of homology. The coding regions of the *T. vivax* TLTF and *T. brucei* TLTF have 67.4 % homology.

(Source: DNASIS homology search tool).

(A)

```
                10         20         30         40         50
5'TVTLTF.AMI  MPPKTVGARGG-KKAEKAPPPCNPVTELSSLESLHENLDKAEELRNYFQVERDKVNDFWN
              :::.:...::: .:. X:::: .:..:::.:::.:..:.::: :::::::::::::::.
TBTLTF.AMI    MPPRTAAERGGRRKSVKAPPPVDPLVELTTLESVHDALAKAERLRNYFQVERDKVNDFWT
                10         20         30         40         50         60
             60         70         80         90
5'TVTLTF.AMI  ATKSEVETYRARLLNADSEIEELERAHQVEMKVYKQ
               ::.::::::.::.::..::::::::::::::X
TBTLTF.AMI    ITKGEVETYRNRLFNAEASIEELERSHQVEMKVYKQ
                70         80         90
```

(B)

```
                10         20         30         40         50         60
5'TVTLTF.AMI  MPPKTVGARGGKKAEKAPPPCNPVTELSSLESLHENLDKAEELRNYFQVERDKVNDFWNA
                                                .....:.X:.. : :::::.:::::..::.
GASGENE.AMI   KGKKGKAKGTPIVDGLAPEDMSKEQVEEHVSRIREELDREREERNYFQLERDKIHTFWEI
                 10         20         30         40         50         60
                      70         80         90
5'TVTLTF.AMI  TKSEVETYRARLLNADSEIEELERAHQVEMKVYKQ
              :....:. .: : : :.:.:: :  ::::.::::X
GASGENE.AMI   TRRQLEEKKAELRNKDREMEEAEERHQVEIKVYKQ
                 70         80         90        100
```

Figure 3.9: (A): The *T. vivax* and *T. b. rhodesiense* TLTF amino acid sequence comparison. The homology was 70.8 %.

(B): The *T. vivax* TLTF and the Human Growth Arrested specific gene 11 (GAS) amino acid sequence comparison (B). The homology was 51.5 %.

(Source: Blastx homology search tool).

3.4 Screening of T. vivax genomic and cDNA libraries with the 5′ T. vivax TLTF cDNA probe

The 361 bp *T. vivax* TLTF DNA fragment was the 5′ end of the TLTF cDNA and it contained miniexon. This fragment was used to make a probe that was specific for the *T. vivax* TLTF gene. It was used to screen *T. vivax* genomic and cDNA libraries in λ gt 11 phage. After primary and secondary screening, positive plaques were identified (Figure 3.10). These plaques were used for phage DNA extraction.

3.5 Bacteriophage DNA extraction, cloning and sequencing

Bacteriophage DNA was extracted from the positive plaques. This DNA was digested to completion with *Eco*RI restriction enzyme to determine whether inserts were present. The inserts were about 5 kb in size (Figure 3.11, panel A). Southern blotting and hybridisation was done using the *T. vivax* TLTF probe and the filter exposed to autoradiography. The inserts hybridised to the probe indicating that they contained the gene of interest (Figure 3.11, panel B).

A double digest was done using *Eco*RI and *Hind*III restriction enzymes and electrophoresed on an EtBr-stained agarose gel (Figure 3.12, panel A). Six fragments resulted ranging from 2.3 kb to more than 23 kb. After Southern blotting and hybridisation with the TLTF probe, only the 2.3 kb insert hybridised to it (Figure 3.12, panel B).

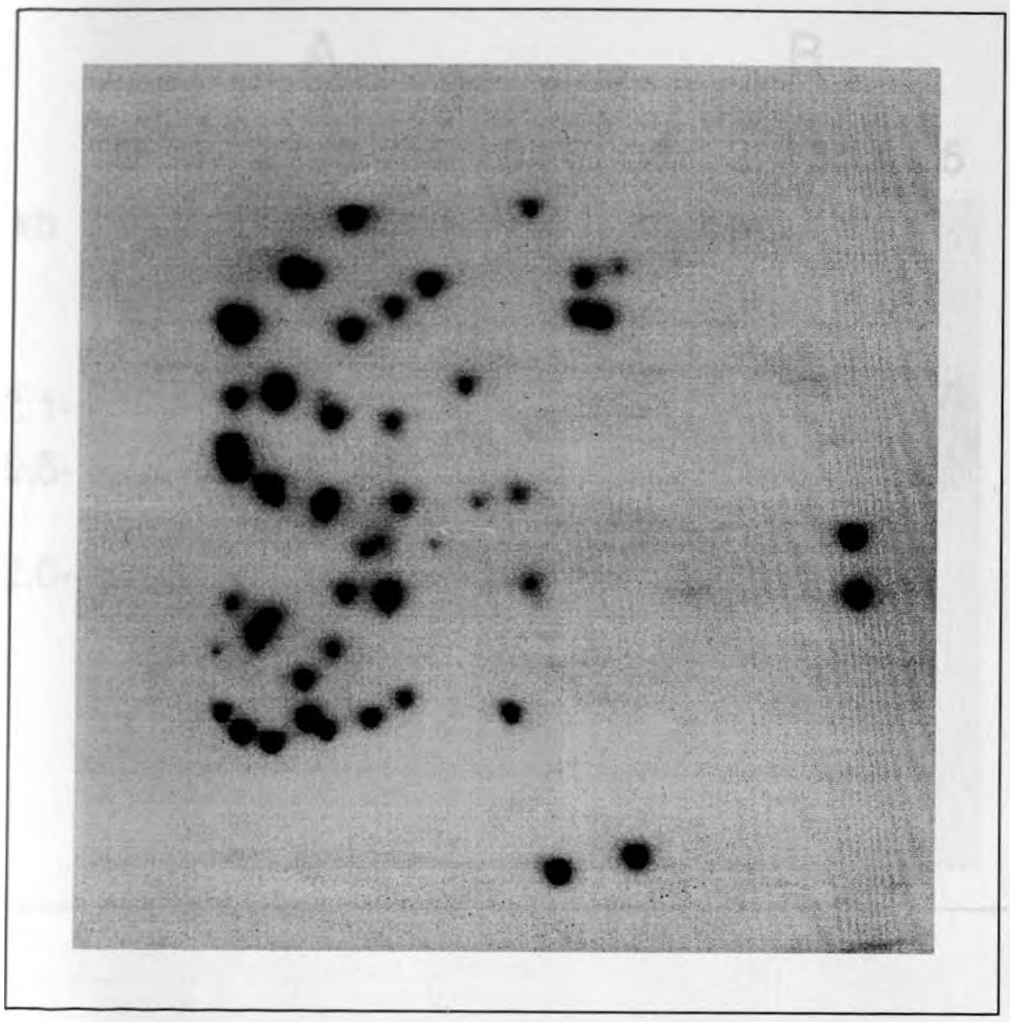

Figure 3.10: Identification of phage clones containing the TLTF gene in *T. vivax* genomic DNA library. The positive clones appeared as dark spots on the developed autoradiograph.

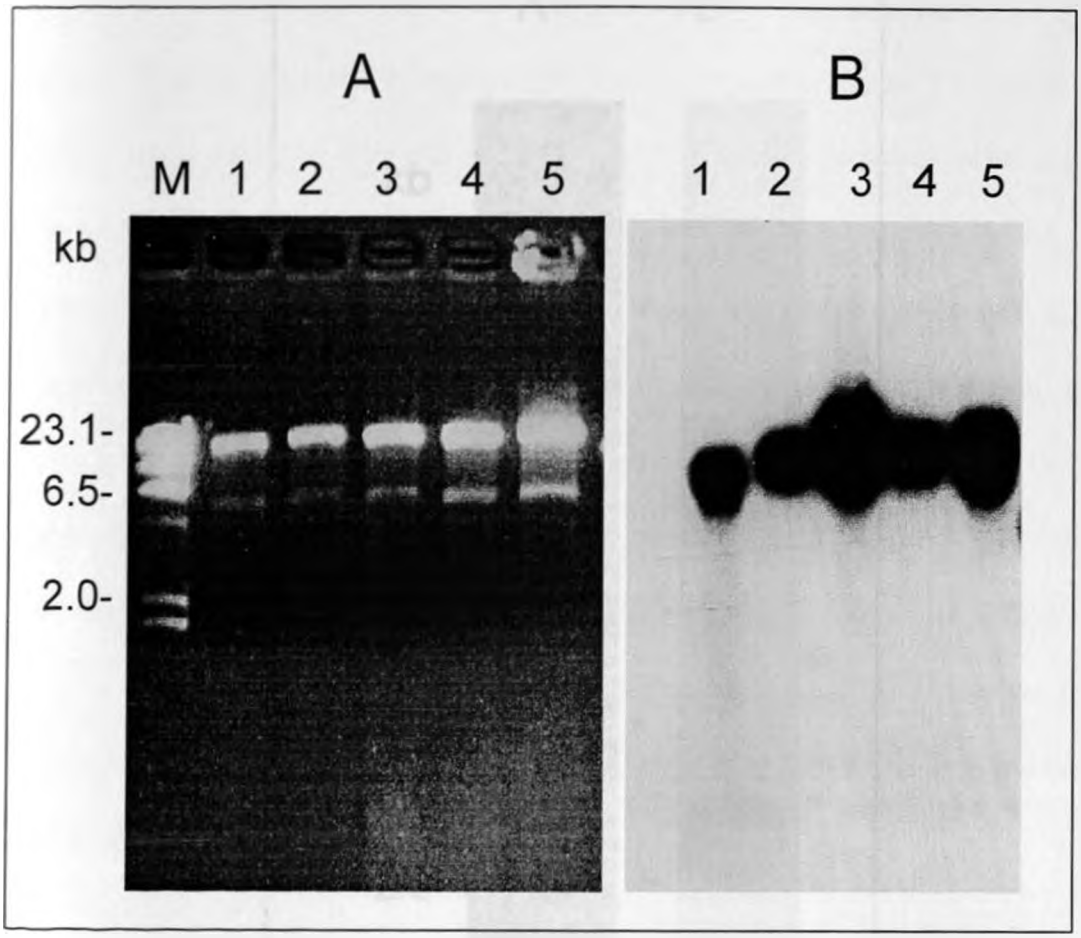

Figure 3.11: Phage DNA restriction and Southern blotting. Panel A indicates the phage DNA digested with *Eco*RI restriction endonuclease and electrophoresed on an Et-Br stained agarose gel. The inserts were about 5 kb. Panel B indicates the autoradiograph developed after Southern blotting and hybridisation. The inserts hybridised strongly to the TLTF probe.

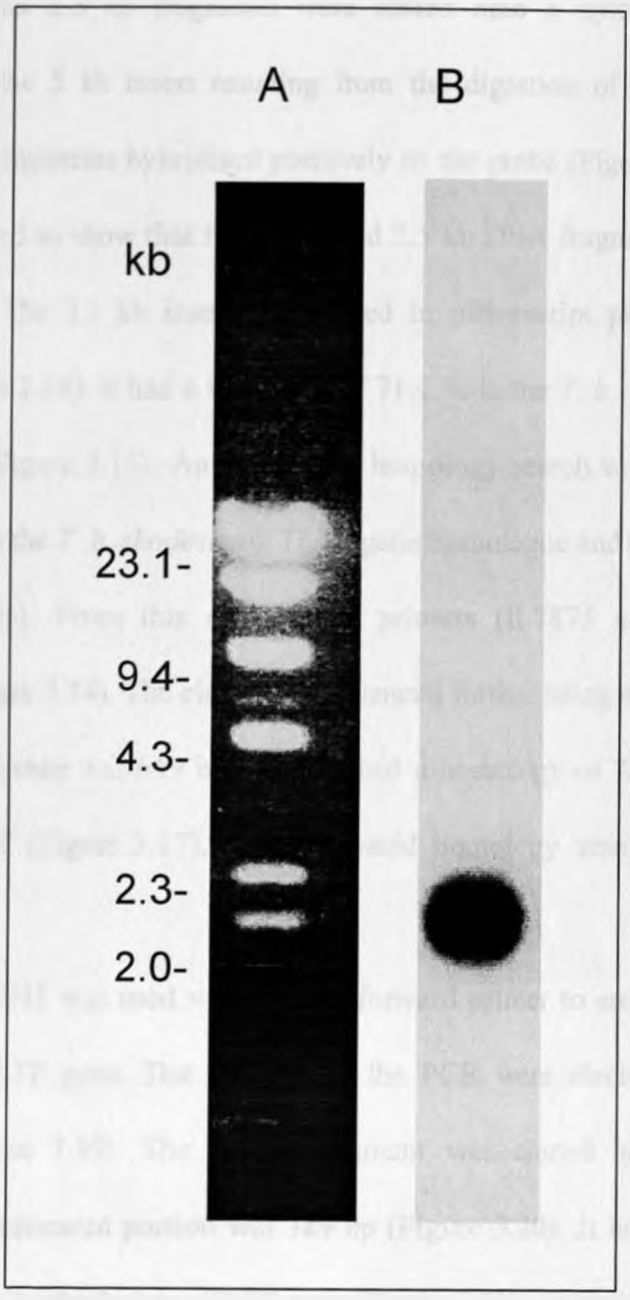

Figure 3.12: Phage DNA digested with *Eco*RI and *Hind*III restriction enzymes

Panel A shows the digested DNA after electrophoresis on an EtBr-stained agarose gel and photographed under UV light. Panel B indicates the autoradiograph that was developed after Southern blotting and hybridisation. Only the 2.3 kb insert hybridised to the 5' *T. vivax* TLTF probe.

The 2.3 kb and 2.5 kb fragments were dotted onto a nylon membrane and hybridised with the 5 kb insert resulting from the digestion of phage DNA with *Eco*RI. The two fragments hybridised positively to the probe (Figure 3.13, panel A). This process served to show that the 2.3 kb and 2.5 kb DNA fragments were parts of the 5 kb insert. The 2.3 kb insert was cloned in pBluescript plasmid vector and sequenced (Figure 3.14). It had a homology of 71.1 % to the *T. b. rhodesiense* TLTF DNA sequence (Figure 3.15). An amino acid homology search was also done. This was done both for the *T .b. rhodesiense* TLTF gene homologue and the human Gas 11 gene (Figure 3.16). From this sequence, 2 primers (IL7875 and IL7845) were designed (see Figure 3.14). The clone was sequenced further using the IL7875 primer. The resulting sequence was 649 bp long and had a homology of 72.3 % to the *T. b. rhodesiense* TLTF (Figure 3.17). An amino acid homology search was also done (Figure 3.18).

The primer IL7845 was used with λ gt 11 forward primer to amplify phage DNA containing the TLTF gene. The products of the PCR were electrophoresed on an agarose gel (Figure 3.19). The 2.5 kb fragment was cloned in a T-vector and sequenced. The sequenced portion was 389 bp (Figure 3.20). It had a homology of 66.2 % with the *T. b. rhodesiense* TLTF gene (Figure 3.21). An amino acid homology search was also done for this portion of the TLTF gene (Figure3.22). A primer IL7948 was designed from the DNA sequence (see Figure 3.20) and used to sequence the clone further. The resulting sequence was the 3′ end of the *T. vivax* TLTF gene and had a homology of 58.3 % with the *T. b. rhodesiense* TLTF (Figure 3.23). Figure 3.24 shows the amino acid homology of this portion of the gene with the *T. b. rhodesiense* TLTF and the GAS 11 gene. Hence the entire *T. vivax* TLTF gene was sequenced.

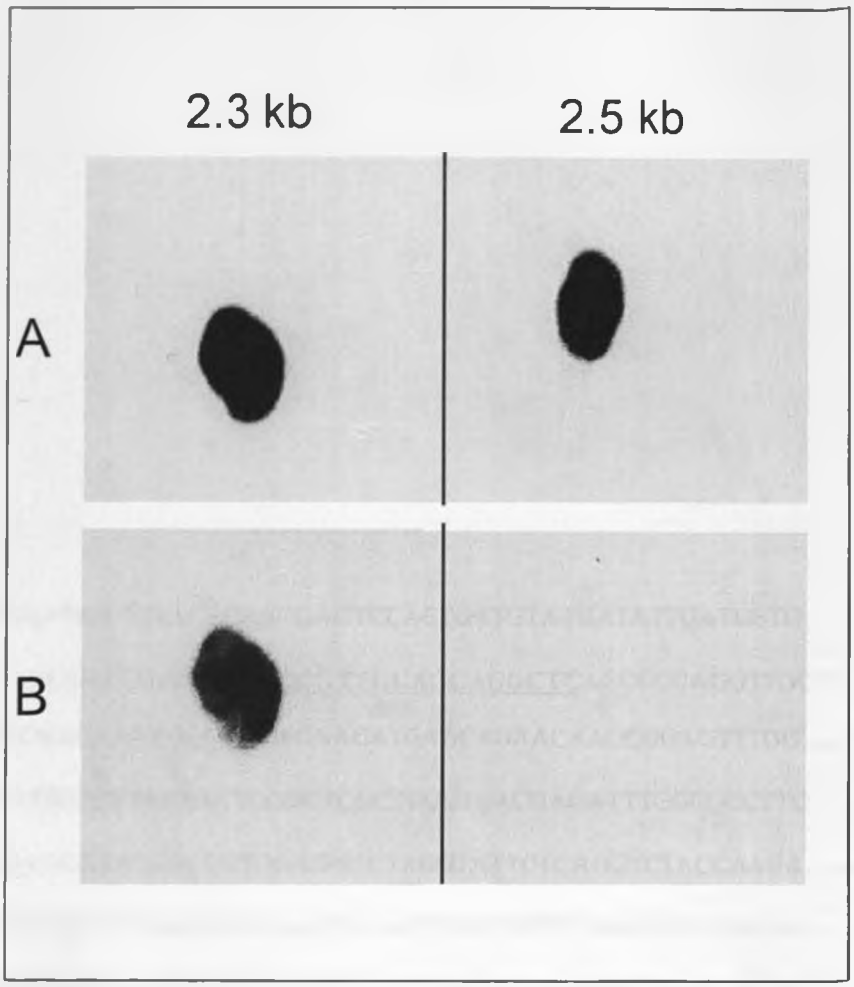

Figure 3.13: Dot blot hybridisation of insert DNA fragments.

Panel A shows an autoradiograph developed after dot blot hybridisation of the 2.3 kb and 2.5 kb DNA fragments that resulted from the *Eco*RI and *Hind*III double digest. The blot was hybridised with the 5kb insert that resulted from the digestion of phage DNA with *Eco*RI restriction enzyme alone. Both fragments hybridised with the probe. Panel B shows the same blot hybridised with the 5' *T. vivax* TLTLF DNA probe. Only the 2.3 kb fragment hybridised to the probe.

AAACAGAATGATGAACACAACGAGTCCACTGATGTATGATATTGATCGTG.....50

AGAACCAAAATTTAGTA**GCGCCGTTGGAGCAGGCTC**AGCGCGAGGTTGCT..... 100
7875

GAATTACAACAAAAGCGAAAACAACATGAGCAGAACAAGCGGAGTTTGG.....150

AGCTCACGCGCTTAAAACTGCGCTCGCTGCGTGACGAGATTTGGCGCCTTC.....200

GGGAGGAGCATAGCGCCCTGGAGGCCTAGGTATTCTCAGGTCTACCAAGA.....250

AAGGGAGGAGTTTAAAGGAAAATTCGAGTCCGCTCTTCGGCATGCGGCAG.....300

GG**GTGGTAGAGGAGCGCAATGAG**ATGCTGCAACAGAAGCTAATCACTAGT.....350
7845

GCGGCC

Figure 3.14: *T. vivax* TLTF sequence of the DNA insert (2.3 kb) derived from digestion of phage DNA with *Eco*RI and *Hind*III restriction enzymes. The sequence was 355 bp long. The underlined portions show the primers that were designed from it.

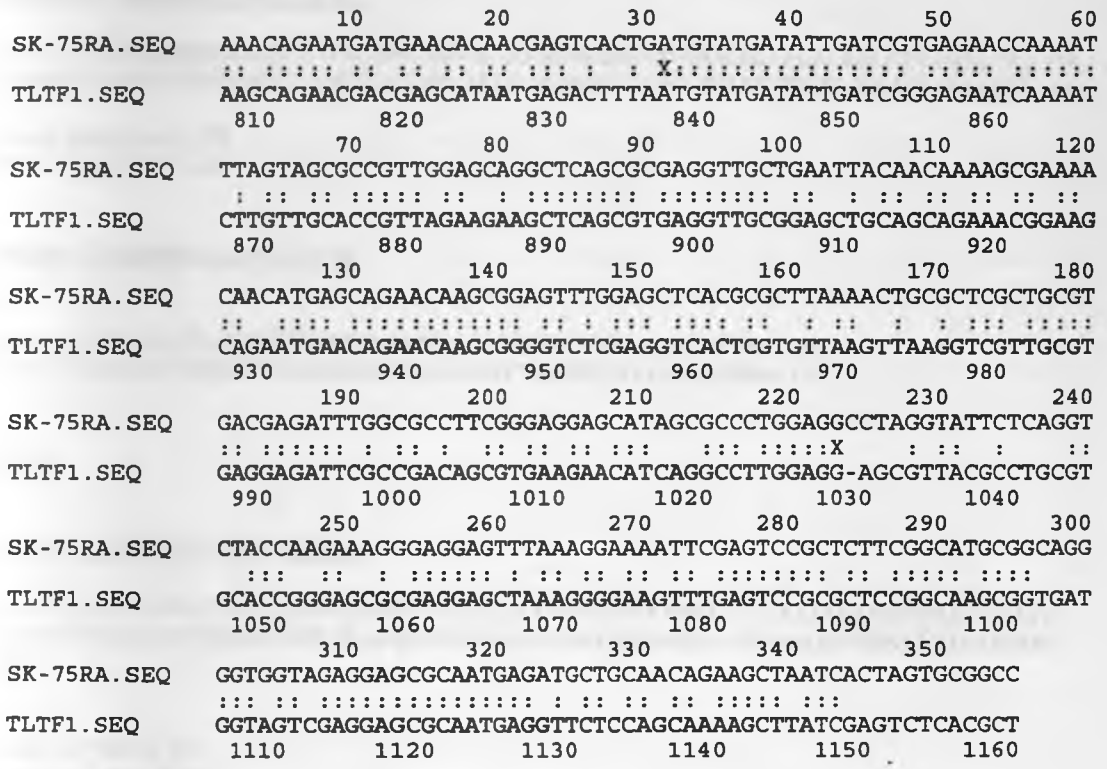

Figure 3.15: Sequence comparison between the *T. b. rhodesiense* TLTF (TLTF1) and the 355 bp reverse sequence (sk-75RA. SEQ) derived from *T. vivax* genomic DNA library. The homology was 71.1%.

(Source: DNASIS homology search tool).

(A)

Frame +1 :Identities 8/9 (88 %)

Query: 1 KQNDEHNES 27
Sbjct: 256 KQNDEHNET 264

Frame +2: Identities 55/65 (86 %)

Query: 29 LMYDIDRENQNLVAPLEQAQREVAELQQKRKQHEQNKRSLELTRLKLRSLRDEIWRLREE 208
Sbjct:265 LMYDIDRENQNLVAPLEEAQREVAELQQKRKQN EQNKRGLEVTRVKLRSLREEIRRQREE 324

Query: 209 HSALE 223
Sbjct: 325 HQALE 329

Frame +3: Identities 32/43 (74 %)

Query: 228 RYSQVYQEREEFKGKFESALRHAAGVVEERNEMLQQKLITSAA 356
Sbjct: 331 RYACVHREREELKGKFESALRQAVMVVEERNE VLQQKLIESHA 373

(B)

Frame +2 : Identities 20/64 (31 %)

Query:32 MYDIDRENQNLVAPLEQAQREVAELQQKRKQHEQNKRSLELTRLKLRSLRDEIWRLREEH 211
Sbjct:269 MAEVSGQNKRLADPLQKAREEMSEMQKQLANYERDKQILLCTKARLKVREKELKDLQWEH 328

Query: 212 SALE 223
Sbjct: 329 EVLE 332

Frame +3: Identities 16/43 (37 %)

Query: 228 RYSQVYQEREEFKGKFESALRHAAGVVEERNEMLQQKL-ITSAA 356
Sbjct: 334 RFTK VQQERDELYRKFTAAIQEVQQKTGFKNLVLERKLQALSAA 377

Figure 3.16 A: Amino acid sequence comparison between *T. vivax* TLTF (Query) and *T. b. rhodesiense* TLTF (subject).

B: Amino acid sequence comparison between the *T. vivax* TLTF (Query) and the Human Gas 11 gene (subject).

(Source: Blastx homology search tool).

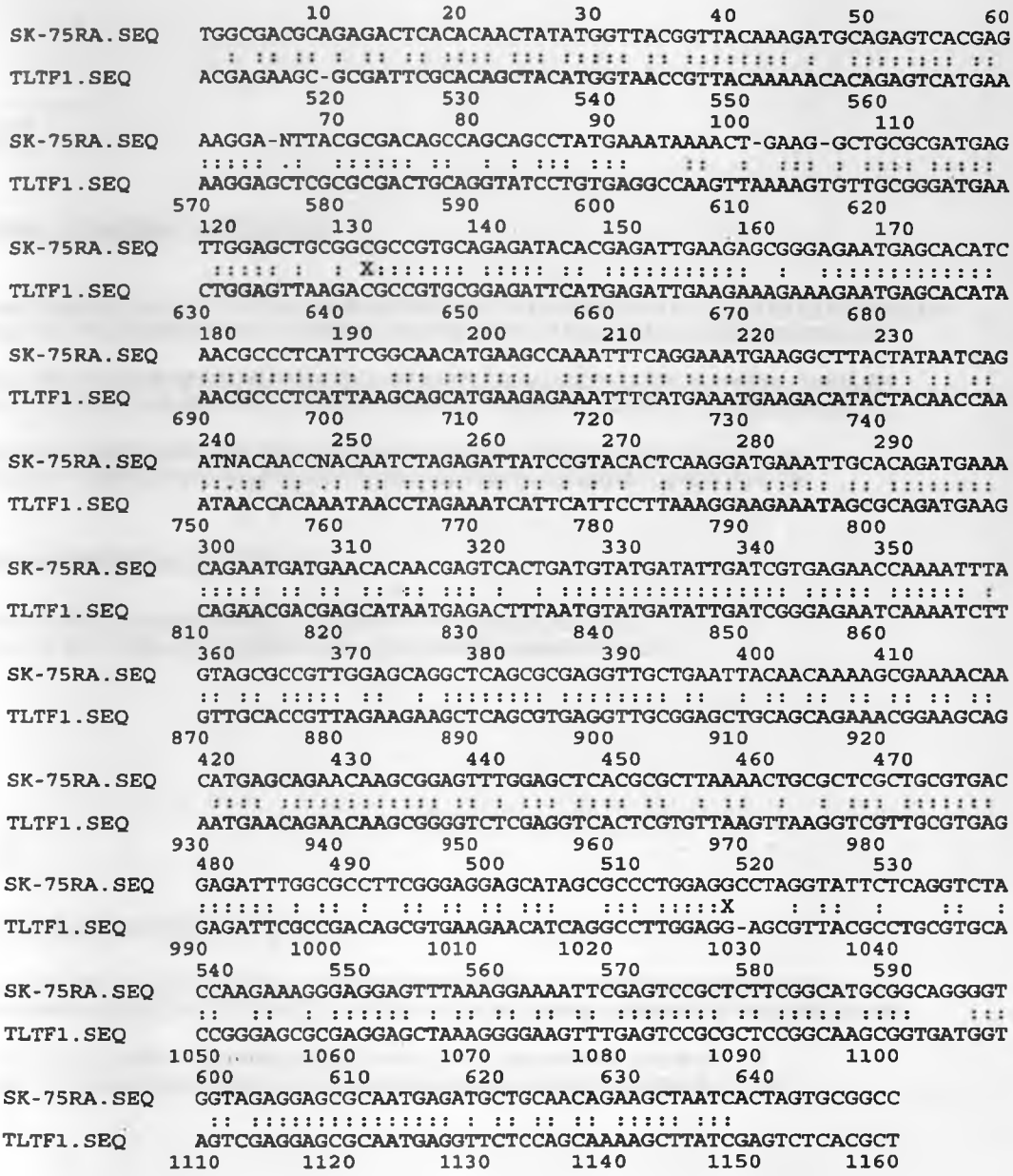

Figure 3.17: *T. vivax* TLTF sequence derived using IL 7874 and IL 7875 internal primers. This portion of the TLTF gene was toward the 3′ end of the gene and had a homology of 72.3 % to the *T. b. rhodesiense* TLTF.

(Source: DNASIS homology search tool).

(A)

Frame –1: Identities 111/173 (64 %)

Query: 649WRRRDSHNYMVTVTKMQSHEKXLRDSQQPMK*NXXXXXXXXXXXXXXXXXXXXXXXXXXX 470
Sbjct: 157 YEKRDSHSYMVTVTKTQSHEKELARLQVSCEAKLKVLRDELELRRRAEIHEIEERKNEHI 216

Query: 469 -ALIRQHEAKFQEMKAYYNQXTTXNLEIIRTLKDEIAQMKQNDEHNESLMYDIDRENQNL 293
Sbjct: 217 NALIKQHEEKFHEMKTYYNQITTNNLEIIHSLKEEIAQMKQNDEHNETLMYDIDRENQNL 276

Query: 292 VAPLEQAQREVAELQQKRKQHEQNKRSLELTRLKLRSLRDEIWRLREEHSALE 134
Sbjct: 277 VAPLEEAQREVAELQQKRKQNEQNKRGLEVTRVKLRSLREEIRRQREEHQALE 329

Frame –2: Identities 32/43 (83 %)

Query: 129 RYSQVYQEREEFKGKFESALRHAAGVVEERNEMLQQKLITSAA 1
Sbjct: 331 RYACVHREREELKGKFESALRQAVMVVEERNEVLQQKLIESHA 373

(B)

Frame –1: Identities 37/111 (33 %)

Query: 466 LIRQHEAKFQEMKAYYNQXTTXNLEIIRTLKDEIAQMKQNDEHNESLMYDIDRENQNLVA 287
Sbjct: 222 LMQRHEEAFTDIKNYYNDITLNNLALINSLKEQMEDMRKKEDHLEREMAEVSGQNKRLAD 281

Query: 286 PLEQAQREVAELQQKRKQHEQNKRSLELTRLKLRSLRDEIWRLREEHSALE 134
Sbjct: 282 PLQKAREEMSEMQKQLANYERDKQILLCTKARLKVREKELKDLQWEHEVLE 332

Figure 3.18: A: Amino acid sequence comparison between *T. vivax TLTF* (Query) and *T. b. rhodesiense* TLTF (subject).

B: Amino acid sequence comparison between *T. vivax TLTF* (Query) and Human GAS 11 gene (subject).

(Source: Blastx homology search tool).

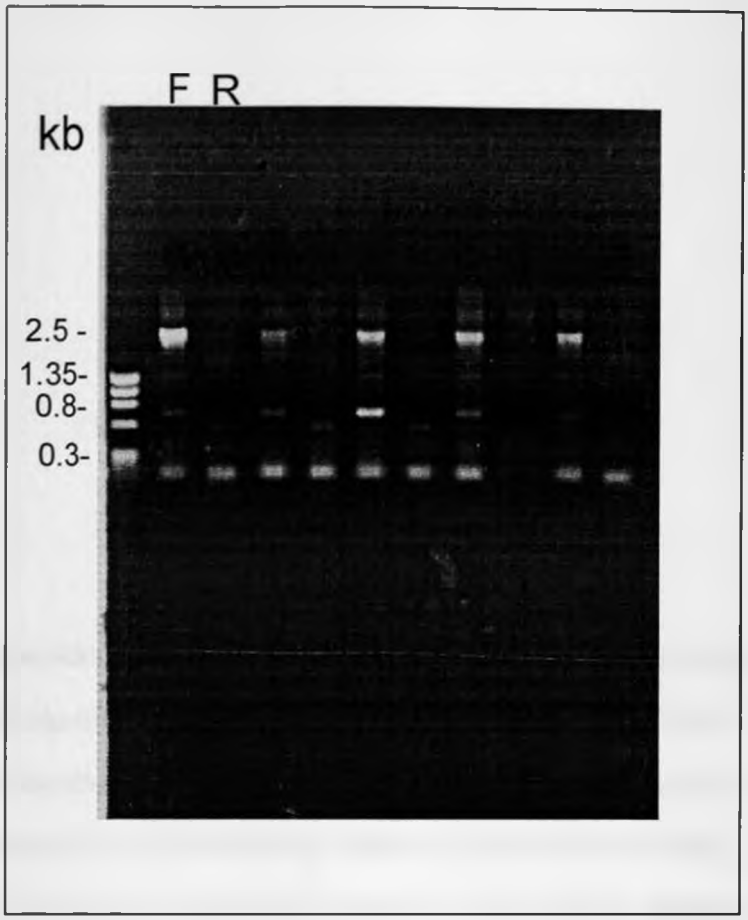

Figure 3.19: PCR of phage DNA extracted from *T. vivax* genomic library with λ gt11 forward and reverse primers with IL7845 primer.

The products of the PCR were electrophoresed on an EtBr-stained agarose gel. F represents the amplification with λ gt11 forward primer and primer IL7845 while R represents amplification with λ gt11 reverse primer and primer IL7845. Primer IL7845 had been designed from the sequence shown in figure 3.14. This was done for 5 clones; all giving similar results as seen on the diagram.

GTAGTCGAGGAGCGCAATGAGATGCTGCAACAGAAGCTTATCGAGTCCCA.....50

TGCACTTGTGGAAGAGCGGGATGAACAGCTGGAGGGAGTAATGCGTGCGA.....100

TGAATCTCGAACCCGGCGACGCTTGAGGCTATCTCAGCCGAGGTGGACCA.....150

GACATTGCAGCGTAAGAATCAGCTAATAAAGGATTTGCATTTTGAGCTGA.....200

AGAAGGCGGAAAAGTTGTTCAACGCCACATTGACTGAGATGGAAA**GGCGG**.....250

TGTCAGCGATTGAATATTCCTCCACTCCCGCGTANAATTTCACATAAGGG.....300
IL7948

GATTATTAATCCCGCGTGCGCGCTCTGTCTTTGATACTTCGCCAGCTTCA.....350

CATTTCTGTCCATTCGGTCCTGTG-GTTGTGAGCGTGATT

Figure 3.20: The *T. vivax* TLTF sequence resulting from PCR using IL7845 primer and λ gt 11 primer on phage DNA containing the TLTF gene. The sequence was 389 bp long. The underlined portion shows the primer that was designed from it.

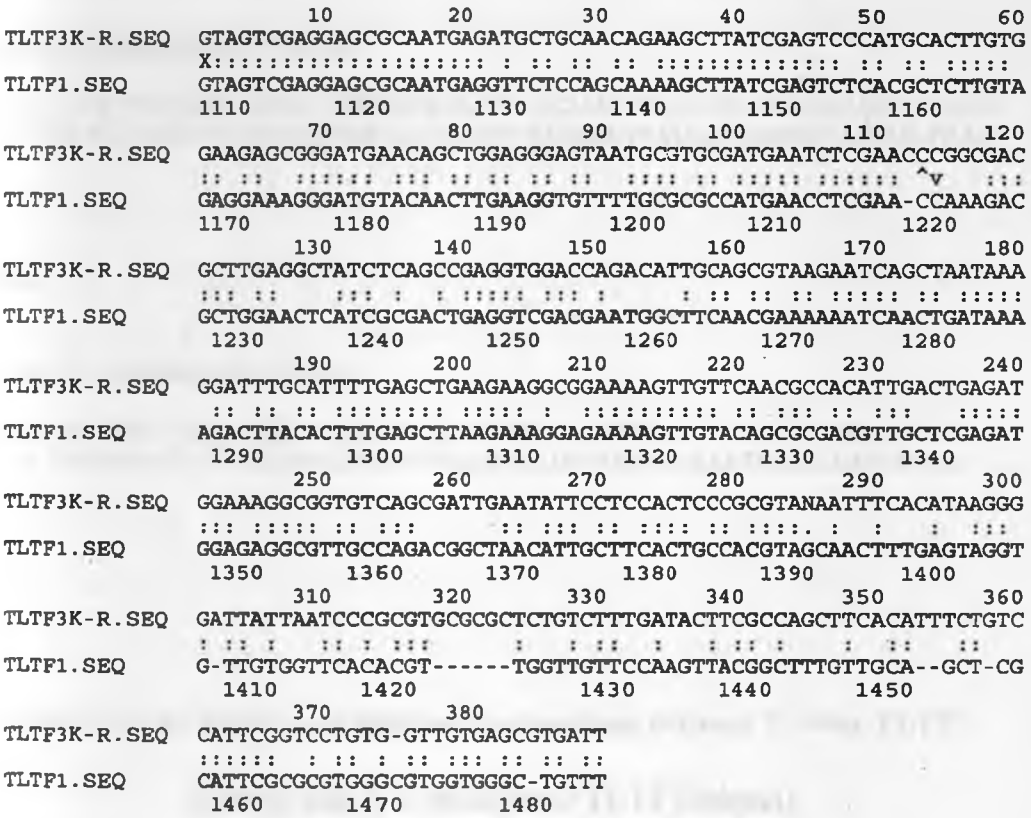

Figure 3.21: The sequence homology between the *T. b. rhodesiense* TLTF and the sequence of the DNA resulting from amplification of phage DNA with λ gt11 forward primer and IL7845 primer. The homology was 66.2 %.

(Source: DNASIS homology search tool).

(A)

Frame +1: Identities 35/38 (92 %)

Query: 1 VVEERNEMLQQKLIESHALVEERDEQLEGVMRAMNLEP 114
Sbjct: 356 VVEERNEVLQQKLIESHALVEERDVQLEGVLRAMNLEP 393

Frame +2: Identities 43/57 (75 %)

Query: 113 PATLEAISAEVDQTLQRKNQLIKDLHFELKKAEKLFNATLTEMERRCQRLNIPPLPR 283
Sbjct: 393 PKTLELIATEVDEWLQRKNQLIKDLHFELKKGEKLYSATLLEMERRCQTANIASLPR 449

(B)

Frame +2: Identities 19/54 (35 %)

Query: 110 NPATLEAISAEVDQTLQRKNQLIKDLHFELKKAEKLFNATLTEMERRCQRLNIP 271
Sbjct: 395 DPAALTLVSRKLEDVLESKNSTIKDLQYELAQVCKAHNDLLRTYEAKLLAFGIP 448

Figure 3.22: A: Amino acid sequence comparison between *T. vivax* TLTF (Query) and *T. b. rhodesiense* TLTF (Subject).

B: Amino acid sequence comparison between *T. vivax* TLTF (Query) and Human Gas 11 gene (Subject).

(Source: Blastx homology search tool).

TGACACTATAGAATACTCAAGCTATGCATCCAACGCGTTGGGAGCTCTCC.....50

CATATGGTCGACCTGCAGGCGGCCGCACTAGTGATTGTAGTCGAGGAGC.....100

GCAATGAGATGCTGCAACAGAAGCTTATCGAGTCCCATGCACTTGTGGAA.....150

GAGCGGGATGAACAGCTGGAGGGAGTAATGCGTGCGATGAATCTCGAAC.....200

CCGGCGACGCTTGAGGCTATCTCAGCCGAGGTGGACCAGACATTGCAGCG.....250

TAAGAATCAGCTAATAAAGGATTTGCATTTTGAGCTGAAGAAGGCGGAAA.....300

AGTTGTTCAACGCCACATTGACTGAGATGGAAAGGCGGTGTCAGCGATTG.....350

AATATTCCTCCACTCCCGCGTANAATTTCACATAAGGGGATTATTAATCCC.....400

GCGTGCGCGGCTCTGTCTTTGATACTTCGCCAGCNTCAC**ATTTCTGTCCAT**.....450
IL7979

TCGGTCCTGTGGTTGTGAGCGTGATTGTGGTTGGCGCATGGATGCCACCTA.....500

CTTTAAGGGAGCGGAAATTATCTGTAGGCAGGTAAGGGGAATGCACTTCA.....550

CCGGGGAGTATTATTGTAGAATAAGCATCAGGTACCCACTACGCTCTGTT.....600

TGTTTATATGCGCCTCTTTCACGCTATCCCCTCTCTACGGTTACGTGCGTT.....650

GTGTCGGTGTTACTTTACCGGGTGCTACCCCGTGTCGCGGCCGTAGTTAC.....700

GAAGTGACACTGGTGTGACCCGCTCACTGCCATTGTCACA

Figure 3.23: The 3′ end of the *T. vivax* TLTF sequence. The underlined portion shows the primer that was designed from it. It was used to generate the open reading frame of the TLTF gene.

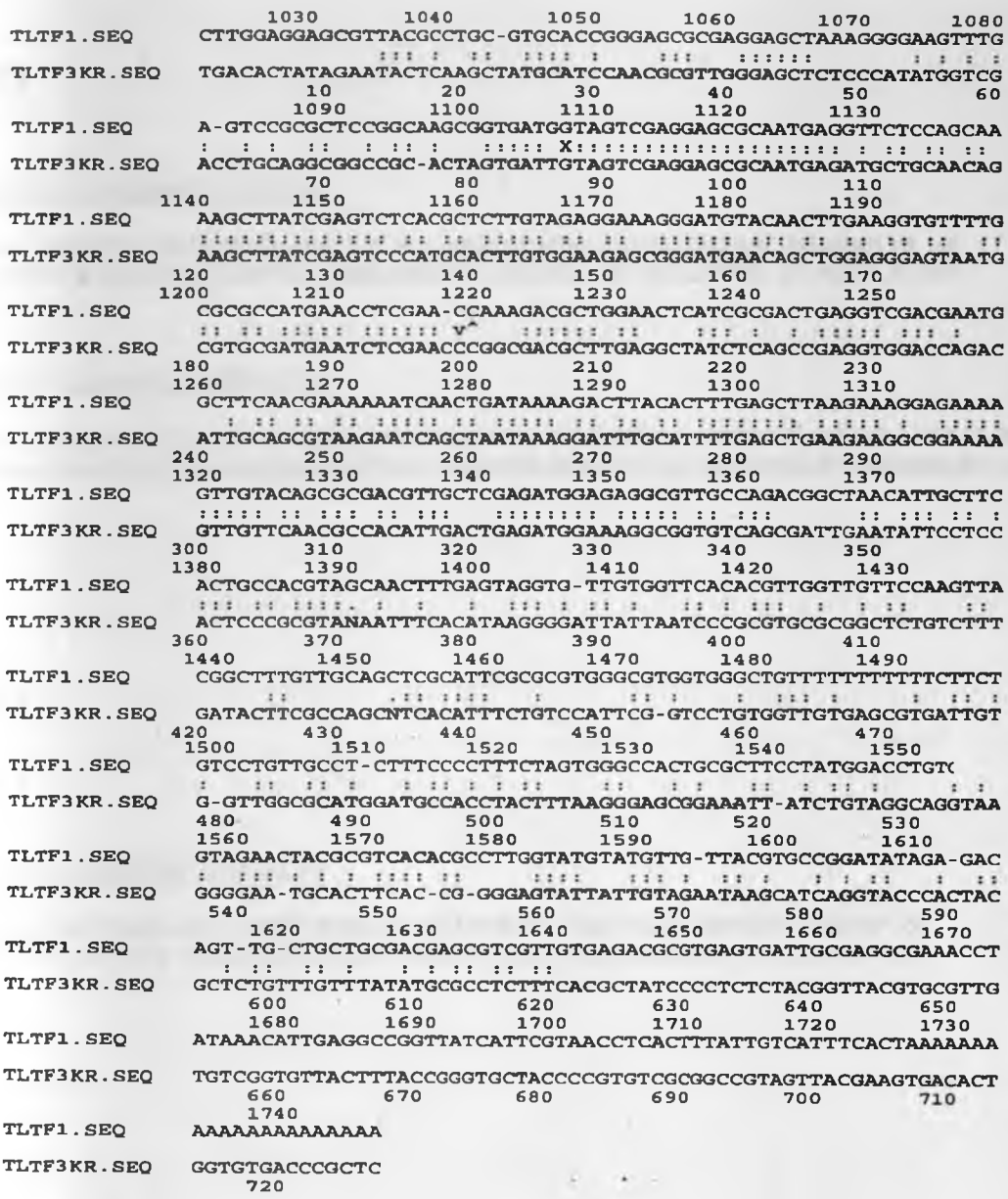

Figure 3.24: Sequence comparison between the 3′ end of the *T. vivax* TLTF gene (TLTF3KR.SEQ) and the *T. brucei rhodesiense* TLTF gene. The homology was 58.3%.

(Source: DNASIS homology search tool).

(A)

Frame +1: Identities 43/57 (75 %)

Query:199 PATLEAISAEVDQTLQRKNQLIKDLHFELKKAEKLFNATLTEMERRCQRLNIPPLPR 369
Sbjct:393 PKTLELIATEVDEWLQRKNQLIKDLHFELKKGEKLYSATLLEMERRCQTANIASLPR 449

Frame +3: Identities 40/59 (67 %)

Query: 24 MHPTRWELSHMVDLQAAALVIVVEERNEMLQQKLIESHALVEERDEQLEGVMRAMNLEP 200
Sbjct: 335 VHREREELKGKFESALRQAVMVVEERNEVLQQKLIESHALVEERDVQLEGVLRAMNLEP 393

(B)

Frame +1: Identities 19/54 (35 %)

Query: 196 NPATLEAISAEVDQTLQRKNQLIKDLHFELKKAEKLFNATLTEMERRCQRLNIP 357
Sbjct: 395 DPAALTLVSRKLEDVLESKNSTIKDLQYELAQVCKAHNDLLRTYEAKLLAFGIP 448

Figure 3.25: (A) Amino acid sequence comparison between *T. vivax* TLTF Query) and *T. b. rhodesiense* TLTF (Subject).

(B) Amino acid sequence comparison between *T. vivax* TLTF Query) and Human GAS 11 gene (Subject).

(Source: Blastx homology search tool).

3.6: Generation of the T. vivax TLTF Open Reading Frame (ORF)

From the *T. vivax* TLTF sequence, two primers were designed, one from the 5′ end of the gene and the other from the 3′ end of the gene. These primers were IL 7979 and IL 7980 (see Figures 3.7 and 3.23). These primers were used to amplify 2 clones of phage DNA containing the TLTF gene. The amplified DNA fragments were about 1.5 kb (see Figure 3.26 panel A). This was the open reading frame of the gene. This was of a similar size to the open reading frame of *T. brucei rhodesiense* TLTF (see Figure 3.1). Southern blotting and hybridisation of the gel using the TLTF gene probe was done to verify that the amplified portion was the open reading frame of the gel (Figure 3.26 panel B). The 1.5 kb fragment hybridised strongly to the probe indicating that it was the open reading frame of the gene.

3.7: Amino acid sequence comparison

An amino acid sequence comparison of the TLTF of *T. vivax*, *T. brucei* and *T. congolense* was done (Figure 3.27). The identity of the *T. vivax* TLTF amino acid sequence had over 70 % identity to that of *T. bucei*.

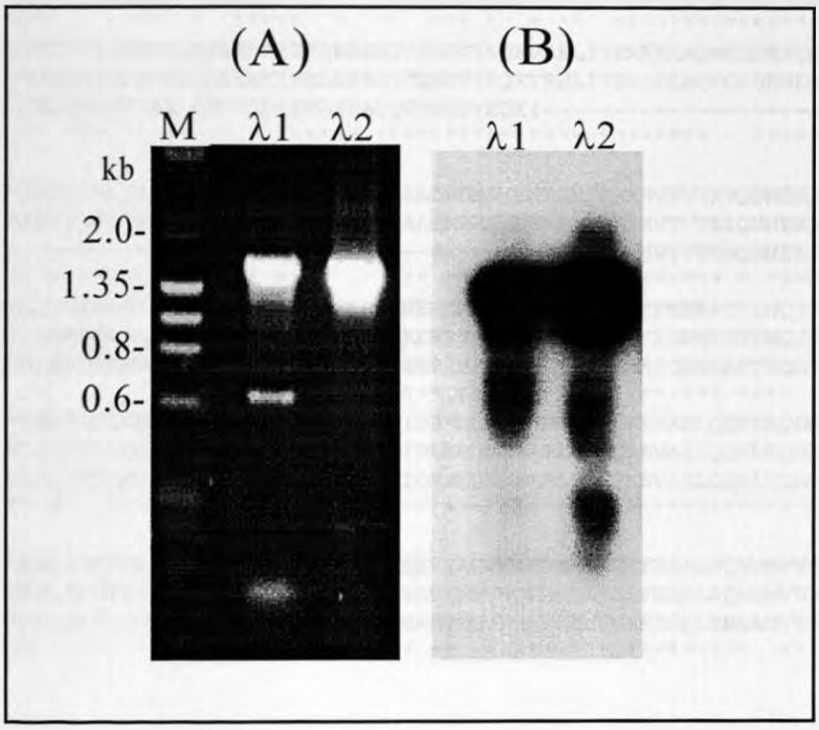

Figure 3.26: Phage DNA (2 clones λ1 and λ2) from *T. vivax* genomic DNA library containing the TLTF gene amplified with IL 7979 and IL 7980 primers.

The amplified DNA which was 1.5 kb in size was the open reading frame of the *T. vivax* TLTF gene (panel A). Southern blotting and hybridisation of the gel (panel B) with the *T. vivax* TLTF gene probe was done.

Figure 3.27: TLTF amino acid sequence comparison between *T. brucei*, *T. congolense* and *T. vivax*. The sequence is well conserved between the different trypanosome species: over 70 % amino acid identity with *T. brucei* TLTF.The *T. congolense* TLTF amino acid sequence was generated by Roger Pelle from ILRI.

3.8: Southern blot analysis: Genomic organisation of T. vivax TLTF gene

Total genomic DNA prepared from *T. vivax* parasites was digested with various restriction enzymes and the digests resolved on a 1 % agarose gel (Figure 3.28, panel A). The enzymes used were *Bam*HI, *Eco*RI, *Hind*III, *PstI* and *Xba*I respectively. Southern blotting and hybridisation was done using the *T. vivax* TLTF probe. After autoradiography it was seen that the probe hybridised to DNA fragments of different sizes with different restriction enzymes (Figure 3.28, panel B).

DNA digested with restriction enzymes, which did not have recognition sites within the gene coding of the TLTF, gave single hybridisation signals of large size. In the *Bam*HI and XbaI digests (Figure 3.28 panel B, lane 1 & 5), for example, the fragments detected were about 23 kb and 15 kb respectively. Similarly, a single major fragment of about 6 kb was detected in *Eco*RI digest (Figure 3.28, panel B, lane 2).

In the DNA Digest with an enzyme that has an internal site within the gene, at least 2 major fragments were detected. In the *Hind*III digest (Figure 3.28, panel B, lane 3), for example, two fragments of about 9.4 kb and 2.3 kb were detected. This information suggests that *T. vivax* has a single copy of the TLTF gene in its genome.

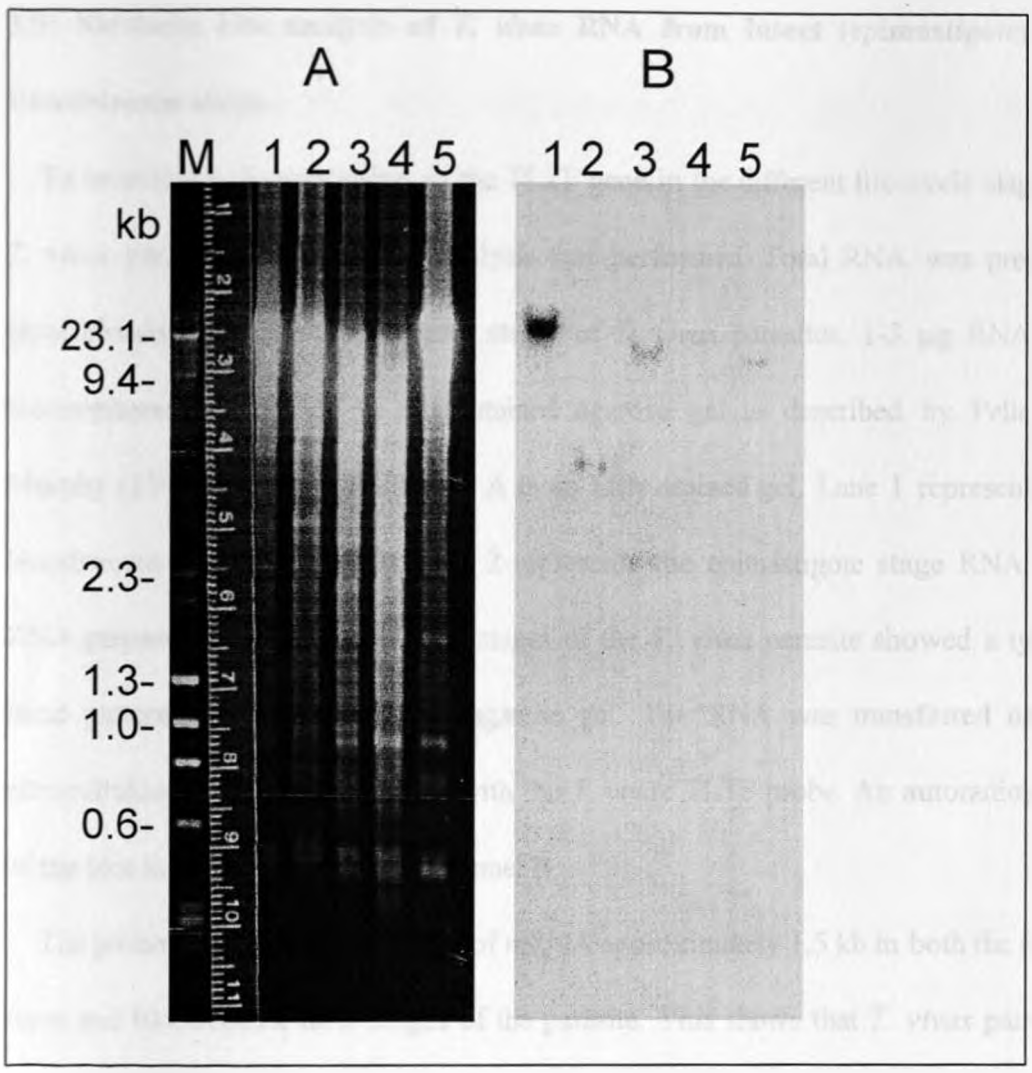

Figure 3.28: Southern blot analysis: Genomic organisation of *T.vivax* TLTLF gene. Panel A is the photograph of the gel stained with EtBr. DNA was digested with *Bam*HI (lane 1), *Eco*RI (lane 2), *Hind*III (lane 3), *PstI* (lane 4) and *Xba*I (lane 5). Lane M shows a mixture of molecular weight markers, the *Hind*III digest of λ DNA and the *Hae*III digest of ϕ x 174 DNA. Panel B shows the developed autoradiograph after Southern blotting and hybridisation with the *T. vivax* TLTF probe.

3.9: Northern blot analysis of *T. vivax* RNA from Insect (epimastigote) and Bloodstream stages

To investigate the expression of the TLTF gene in the different life cycle stages of *T. vivax* parasite, Northern blot analysis was performed. Total RNA was prepared from bloodstream and epimastigote stages of *T. vivax* parasites. 1-3 μg RNA was electrophoresed on a 1.4 % EtBr-stained agarose gel as described by Pelle and Murphy (1993) (Figure 3.29). Panel A is an EtBr-stained gel. Lane 1 represents the bloodstream stage RNA while lane 2 represents the epimastigote stage RNA. The RNA prepared from these different stages of the *T. vivax* parasite showed a typical band pattern on the EtBr-stained agarose gel. The RNA was transferred onto a nitrocellulose filter and hybridised with the *T. vivax* TLTF probe. An autoradiograph of the blot is shown in Figure 3.29, panel B.

The probe hybridised with a band of mRNA approximately 1.5 kb in both the insect form and bloodstream form stages of the parasite. This shows that *T. vivax* parasites have mRNA that transcribes the TLTF gene in both the bloodstream and insect form stages of its life cycle. The photograph of the EtBr-stained agarose gel of the total RNA under UV in Figure 3.29, panel A shows that the quantity of the fractionated RNA from the bloodstream form of the parasite (lane 1) was of a higher concentration than that of the insect form stage (lane 2). It also shows that RNA from the insect form of the parasite was degrading, as evidenced by the partial smearing seen in lane 2. The autoradiograph in panel B shows that the intensity of the signal in lane 1 representing the bloodstream mRNA was stronger than that of the insect form mRNA in lane 2, which was fainter.

These results serve to indicate two factors, one, that amount of total RNA of the bloodstream stage of the parasite electrophoresed on the agarose gel was more concentrated than that of the insect form stage. Secondly, not withstanding the concentration of the total RNA, it can be deduced that in the bloodstream stage of the parasite the mRNA responsible for the transcription of the TLTF gene is more pronounced than in the insect form stage of the parasite.

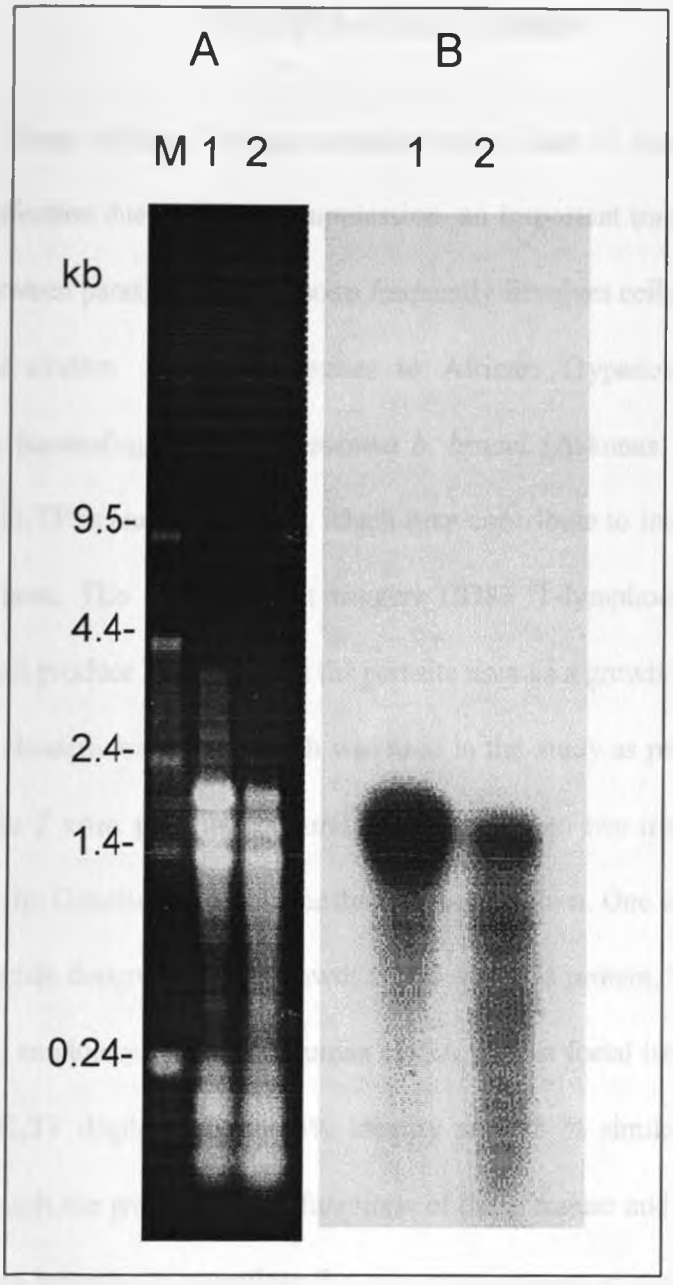

Figure 3.29: Northern blot analysis of *T. vivax* total RNA from epimastigote and bloodstream stages. Panel A represents a photograph of the RNA from the bloodstream form stage (lane 1) and epimastigote stage (lane 2) of the parasite electrophoresed on an agarose gel stained with EtBr. Panel B represents the developed autoradiograph after Northern blotting and hybridisation of the gel.

CHAPTER 4: DISCUSSION

Mortality from African Trypanosomosis occurs due to massive parasitosis or secondary infection due to immunosuppression- an important trait of the disease. The interplay between parasites and the hosts frequently involves cells and the products of the immune system. This also applies to African Trypanosomosis, caused by extracellular haemoflagellate *Trypanosoma b. brucei* (Askonas, 1984; Black *et al.*, 1985). The TLTF is one such factor, which may contribute to immunosuppression in the animal host. This is because it triggers CD8+ T-lymphocytes of the host to proliferate and produce IFN-γ, which the parasite uses as a growth stimulus.

The *T. b. rhodesiense* TLTF which was used in the study as probe to search for its homologue in *T vivax* parasites is similar in sequence to two mammalian sequences deposited in the GeneBank without further characterisation. One is a mouse protein of 489 amino acids designated as a growth arrest- specific protein. The other protein is encoded by a randomly sequenced human cDNA from a foetal lung cDNA library. In each case, TLTF displays about 35% identity and 58 % similarity (Vaidya *et al.*, 1997). Although the properties and functions of these mouse and human proteins are unknown, it is tempting to speculate that they may possess an activity towards CD8+ cells that resemble that of TLTF. If so, then TLTF, a trypanosome-encoded protein mimics the activity of an immunomodulatory protein of its host.

Apart from being homologous to the *T. brucei rhodesiense* TLTF, the *T. vivax* TLTF which was isolated in the study had a homology with the growth arrest – specific 11(Gas 11) gene which is a tumour suppresser gene isolated in humans (Whitmore, *et al.*, 1998). This gene is highly homologous to a mouse Gas cDNA (Accession number U19859, unpublished reference in the GenBank). It represents the

identification of the human homologue of this gene. The Gas genes are associated with the negative control of mammalian cell growth. The steady-state of their mRNAs are increased by three to fivefold when exponentially multiplying cells are exposed to a variety of stresses including inadequate nutrition or the removal of serum (Fleming *et al.*, 1997).

Gas genes were first isolated from a mouse NIH 3T3 subtracted cDNA library enriched for RNA sequences preferentially expressed during serum deprivation (Schneider *et al.*, 1988). At least 11 separate mouse Gas genes have been isolated to date with evidence implicating them in a variety of functions. These include negative regulation of cell proliferation in transformed cells (Del Sal *et al.*, 1995). The Gas genes are down regulated after induction of growth. Expression of Gas genes reflects requirements to sustain growth arrest or is related to differentiation rather than to growth control (Schneider *et al.*, 1988). But not all products of Gas genes have been shown to have negative effect on the growth of cells. Lih *et al.*, (1996) reported that Gas 9 encodes the platelet-derived growth factor α receptor, which acts in conjunction with other growth factors to promote cell cycling.

While parasitaemia is feasible in the absence of TLTF (Bakhiet *et al.*, 1993), the action of this molecule in stimulating IFN-γ production by CD8+ lymphocytes, potentiates parasite proliferation, and this is critical in determining the course of infection. While trypanosomes appear to constitutively secrete TLTF, this process is greatly enhanced after stimulation with IFN-γ, indicating that secretion can be intrinsically regulated by the parasites. Such regulation is a result of signal transduction pathways following presumed IFN-γ ligation with a parasite receptor (s) (Hamadien *et al.*, 2000). Complete understanding of the molecular interplay of this

protein-ligand interaction will be critical in the development of therapeutic strategies. Such strategies may be directed either at regulation of parasite gene expression or at blocking signalling events initiated by exposure to host molecules.

Since trypanosomes, by the release of TLTF, trigger CD8+ T cells to produce IFN-γ early during infection, the present observation demonstrates the presence of a bi-directional parasite-host interaction, in which the parasite sends signals the host to produce a specific growth stimulus. By using a key cytokine like IFN-γ as a growth stimulus, this extracellular parasite exploits the growth regulating molecules of the white blood cells of the host (Kamijo *et al.*, 1993) for its own benefit. Further studies on this aspect of infection may elucidate how the parasite survives in the reservoir animals and open new therapeutic strategies of treatment.

It was therefore important to determine whether other trypanosomes of economic importance like *T. vivax* and *T. congolense* have the TLTF gene and whether it elicits the same effect in cattle as does the *T. b. brucei* TLTF in rats. If it so does, then it can, along with other factors, be used as a candidate for vaccine development.

The TLTF is important in determining the course of parasitaemia in infected animals. Therefore, it was important to determine whether *T. vivax* parasites have the gene. This study was able to determine this through the aid of a novel technique for identifying homologue genes referred to as Random PCR-assisted cloning of homologue genes. Using this technique, the 5′ end of the gene was cloned and sequenced. It was 361 bp long. This DNA was used to screen *T. vivax* genomic and cDNA libraries in λgt 11. From the screening process, positive clones were identified from the *T. vivax* genomic DNA library. A fragment of size 2.3 kb which hybridised strongly with the 5′ end of the *T. vivax* TLTF gene was cloned in a pBluescript

sequencing vector and sequenced. From the resulting sequence internal primers were designed and used to sequence the remaining portion of the gene. The open reading frame of the *T. vivax* TLTF was of equal size to that of *T. brucei rhodesiense* i.e. 1.5 kb. The TLTF amino acid sequence comparison (Figure 3.27) of *T. congolense* and *T. vivax* had over 70 % amino acid identity to that of *T. brucei*, showing that the sequence is well conserved between the different trypanosome species. The TLTF may have similar functions for the different trypanosome species, regardless of the host (similar to cytokines in different higher eukaryotes).

From gene expression studies by Southern hybridisation of genomic DNA (section 3.8), the *T. vivax* TLTF gene was found to be a single copy gene. The gene is expressed in both the bloodstream and insect form stages of the parasite as shown by the Northern hybridisation done on RNA from both life cycle stages (section 3.9). The expression of the TLTF gene seems to be up regulated in the bloodstream form stages of the parasite as the Northern hybridisation signal in this stage was stronger than that of the insect form stage (Figure 3.29).

The TLTF is very important as it binds to CD8+ T-lymphocytes causing them to proliferate and produce IFN-γ which trypanosomes use as a growth stimulus. Whereas parasitaemia is feasible in the absence of TLTF (Bakhiet *et al.*, 1993), the action of this molecule in stimulating IFN-γ production by CD8+ cells potentates parasite proliferation, and this is critical in determining the course of the infection. Hence it follows that if anti-TLTF antibodies can be produced *in vivo*, then normal parasite proliferation can be hindered resulting in less parasitaemia or death of the parasites. Hence the TLTF is a potential target in the control of the disease, especially considering that the *T. vivax* TLTF has been identified, cloned and sequenced.

The initial screening process to identify the TLTF gene in *T. vivax* parasites was done using a novel technique referred to as Random PCR-assisted cloning of homologue genes. This technique incorporates the use of Southern hybridisation and Random PCR to identify DNA fragments of interest from gnomic DNA. It is reliable, easy and straightforward as it helps to avoid problems of contamination with foreign DNA during the initial screening process. This is important as it helps to eliminate the problem of false hybridisation with non-target DNA.

This study shows that *T. vivax* parasites have the gene for the TLTF. It is important to determine whether the parasite's TLTF elicits a similar immune response to the one elicited by *T. b. brucei* TLTF. If it does, then antibodies against the TLTF protein can be developed and used as a vaccine candidate in a multi--component vaccine against trypanosomosis. This will be done once the *T. vivax* TLTF open reading frame is cloned in a protein expression vector so as to express the protein.

Many questions remain about the TLTF, is there a unique secretory pathway for TLTF in trypanosomes? What is the sequence in the TLTF that targets it to the cytoplasmic vesicles? How do TLTF and CD8+ cells interact? What is the signalling pathway within CD8+ T cells that stimulates IFN-γ production? Does vaccination with recombinant TLTF reduce susceptibility to trypanosome infection? All these questions can be answered with more research on the gene.

CHAPTER 5: REFERENCES

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APPENDIX

1. BACTERIAL MEDIA AND PLATES

2 X YT (per litre)

16 g bacto-tryptone

10 g yeast extract

5 g Nacl

Ampicillin and tetracycline were dissolved at a concentration of 100 mg/ml each and stored at $^{-}$20 °C. Ampicillin was added to the media at a concentration of 50 µg/ml while tetracycline was used at a concentration of 12.5 µg/ml.

NZYCM medium for Plates (Per litre)

10 g NZ amine

5 g Nacl

5 g bacto-yeast extract

1 g casamino acids

1.71 g $MgSO_4.7H_2O$

12.06 g Agar

All plates contained 15 g/l of agar and were poured in 150 mm large petri dishes with about 60 ml of agar per plate. When required, ampicillin or tetracycline was added to the autoclaved agar after it cooled to 60 °C before pouring. All plates were dried by incubation at 37 °C, with their lids open overnight.

LB medium (per litre)

10 g bacto-tryptone

10 g NaCl

5 g bacto-yeast extract

2. BUFFERS AND SOLUTIONS

TE Buffer

10 mM Tris.Cl pH 7.4

1 mM EDTA, pH 8.0

10 x TBE Buffer

900 mM Tris

880 mM Boric acid

25 mM EDTA

TAE Buffer

40 mM Tris-acetate

1 mM EDTA, pH 8.0

TNE Solution

25 mM Tris-HCL

10 mM NaCl

5 mM EDTA, pH 8

Cell Resuspension Solution

50 mM Tris-HCL, pH 7.5

1 mM EDTA

100 μG/ML RNAse A

Cell lysis Solution

0.2 M NAOH

1 % SDS

Neutralisation Solution

2.55 M Potassium acetate pH 4.8

Column wash Solution

200 mM NaCl

Tris-HCL, pH 7.5

5 mM EDTA

Dilute 1:1 with absolute ethanol

Direct purification Buffer

10 mM Tri-HCL pH 8.8

50 mM KCL

1.5 mM $MgCl_2$

0.1 % Triton-X

DNA loading Buffer

0.25 % bromophenol blue

0.25 % xylene cyanol

15 % Ficoll

6X RNA Sample loading Buffer

98 % deionised formamide

30 % glycerol

1.2 % SDS

60 mM sodium phosphate

RNA gel Buffer

10 mM sodium phosphate (Na_2HPO_4/NaH_2PO_4)

Sequencing gel-loading Buffer

98 % deionised formamide

10 mM EDTA, pH 8.0

0.025 % xylene cyanol ff

0.025 % bromophenol blue

DNA Denaturing Solution

1.5 M NaCl

0.5 M NaOH

20 X SSC

0.3 M Sodium citrate

1 M NaCl

DNA Neutralising Buffer

1M Tri-HCL pH 8.0

1.5 M NaCl

Pre-hybridisation Solution

6 x SSC

0.5 % SDS

5 x Denhardt's solution

0.1 % Sodium pyrophosphate

50 X Denhardt's Solution

5 % Ficoll

5 % Polyvinyl pyrrolidone

5 % BSA

10 X Tag polymerase Buffer (magnesium-free)

50 mM KCL

100 mM Tris-HCl, pH 9.0

1 mg/ml gelatin

1 % Triton X-100

10 X Klenow Buffer

0.5 M Tris-HCL, pH 7.6

0.1 M MgCl

Frozen Storage Buffer (FSB) pH 6.2

100 mM KCL

45 mM $MnCl_2$

10 mM $CaCl_2$

3 mM hexamine cobalt chloride

10 Mm CH_3COOK

10 % glycerol

SM Buffer (per litre)

5.8 g NaCl

2 g MgSO4

50 mM Tris-HCl pH 7.5

5 ml 2 % gelatin

3. RESTRICTION ENZYMES USED IN THE STUDY

Enzyme	Restriction site
*Bam*HI	5′...G˘GATCC...3′ 3′...C CTAG▲G...5′
*Sac*II	5′...GAGCT˘C...3′ 3′...C▲TCGAG...5′
*Sal*I	5′...G˘GATCC...3′ 3′...C CTAG▲G...5′
*Hind*III	5′...A˘AGCTT...3′ 3′...T T CGA▲A...5′
*Eco*RI	5′...G˘AATTC...3′ 3′...C TTAA▲G...5′
*Pst*I	5′...CTGCA˘G...3′ 3′...G▲ACGTC...5′
*Hae*III	5′...GG˘CC...3′ 3′...C C▲GG...5′

4. NUCLEIC ACID MOLECULAR WEIGHT MARKERS USED IN THE STUDY

Marker	Marker sizes (kilobases)
Lambda DNA/*Hind* III	23.130, 9.416, 6.557, 4.36I, 2.322, 2.027, 0.564, 0.125
ϕX174 DNA/*Hae* III	1.353, 1.078, 0.872, 0.603, 0.310, 0.281, 0.271, 0.234, 0.194, 0.118, 0.072
RNA/BRL	9.5, 7.5, 4.4, 2.4, 1.4, 0.24

5. VECTORS USED IN THE STUDY

Vector	size (base pairs)	Uses
pGEM®-T	3003	- Cloning and sequencing
pBluescript(SK)	2900	- Cloning and sequencing
λgt 11	50000	- Construction of cDNA and genomic libraries

6. STRAINS OF *E. coli* AND BACTERIOPHAGE USED IN THE STUDY

E. coli strain	Genotype	Reference
JM 109	F$^+$, *Tra*D36, *pro*A$^+$, *pro*B$^+$, *lac*I^q, *lacZ*/*rec*A1, *end*a1, *gyr*A96, Nal^r, thi, *hsd*R17, *sup*E44, *rel* A1	Sambrook *et al,* 1989
Y1090	SupF, Δ*lac* U169, *pro*A$^+$, Δ*lon*, *ara*D139, *str*A, *hsd*R, *rps*L, *trp*C22 :: Tn10(tet^r) pMC9 amp^R	Young and Davis,1983

Bacteriophage strain	Genotype	Reference
λgt 11	λ*lac*5, Δ*shn*dIIIλ2-3, *sr*Iλ3°, *cI ts*857, *sr*Iλ4°, *nin*5, *sr*Iλ5°, Sam100	Young and Davis, 1983a

7. PRIMERS USED IN THE STUDY

Primer	ILO No	Nucleotide sequence
TLTF/FWD 1	6745	5′ CACCAGGTAGAGATGAAGGTATACAAGCAG 3′
TLTF/REV 1	6746	5′ CTGCTTGTATACCTTCATCTCTACCTGGTG 3′
TLTF/REV2	6748	5′ CAAACTTCCCCTTTAGCTCCTCGC 3′
T.vTLTF/FWD1	6794	5′ GAAGTGGAAACATATCG 3′
T.bTLTF /FWD A	6805	5′ GTAGAGATGAAGGTATACAAG 3′
T.bTLTF/REV A	6806	5′ CTTGTATACCTTCATCTCTAC 3′
TLTF/REV3	6836	5′ CGCTCAACTTGAAAGTAATTACGA 3′
λgt11 FWD	7705	5′ GGTGGCGACGACTCCTGGAGCCCG 3′
λgt11 REV	7706	5′ TTGACACCAGACCAACTGGTAATG 3′
TLTF/REV	7845	5′ GTGGTAGAGGAGCGCAATGAG 3′
TLTF/FWD	7874	5′ CCATGTCGGAGAACTTC 3′
TLTF/REV	7948	5′ GGCGGTGTCAGCGATTG. 3′
Random primer	907	5′ GGCCGAACCG 3′
TLTF/FWD	7980	5′ GAACAGCTGTTGACGTT 3′
TLTF/FWD	7875	5′ GCGCCGTTGGAGCAGGCTC 3′
TLTF/FWD	7979	5′ CGAATGGACAGAAAT 3′