LIST OF ABBREVIATIONS

Abbreviation	Meaning
D.W.	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxyribonucleotide triphosphate
EDTA	Ethylene diamine tetra acetic acid
EtBr	Ethidium bromide
IPTG	Isopropyl – β- D- thiogalactoside
LB	Luria- Bretani
LMP	Low Melting Point
PBS	Phosphate buffer saline
PCR	Polymerase chain Reaction
PK Buffer	Proteinase K Buffer
PK Enzyme	Proteinase K Enzyme
R.T.	Room temperature
RE	Restriction enzyme
RoTat	Rode Trypanozoon Antigen Type
RNase	Ribonuclease
RPM	Revolution per minute

SDS	Sodium dodecyl sulphate
SOC	Super Optimal Broath with Catabolite Repression
TAE	Tris- Acetate EDTA
TbAT1	Trypanosoma brucei adenosine transporter 1
TevAT1	Trypanosoma evansi adenosine transporter 1
TE	Tris EDTA
UV	Ultra Violet
VSG	Variable Surface Glycoprotein
X- gal	5- bromo-4-chloro- 3 –indolylβDgalatase

Units of Measurement

%	Percentage
µg	Microgram
µl	Microlitre
^{0}C	Degree Celcius
A	Absorbance
bp	Base pair
cm	Centimeter
Da	Dalton
g	Gram (s)
h	Hour (s)

IU	International unit
Kbp	Kilo base pair
KDa	Kilo Dalton
Kg	Kilogram
M	Molar
ma	Milli ampere
mg	Milli gram
min	Minute (s)
Min	Minute
ml	Milli litre
mm	Mili meter
mM	Milli molar
ng	Nano gram
OD	Optical density
pmol	Picomole
sec	Second (s)
U	Unit
V	Volt
V / cm	Volt per centimeter
V / V	Volume / volume
W / V	Weight / volume

Identification and Molecular Characterization of Variable Surface Glycoprotein and Adenosine Transporter 1 Genes of *Trypanosoma evansi* of Camel Origin

ऊँट से पृथक *ट्रीपैनोसोमा इवान्सी* के वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर 1 जीन की पहचान और आन्विक अभिलक्षण

REHAB ALI IBRAHIM YAGI

THESIS

DOCTOR OF PHILOSOPHY

(Veterinary Parasitology)

2013

Department of Veterinary Parasitology
College of Veterinary and Animal Science
Rajasthan University of Veterinary and Animal Sciences,

Bikaner – 334 001 (Rajasthan)

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THESIS

Submitted to the

Rajasthan University of Veterinary and Animal Sciences, Bikaner

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DOCTOR OF PHILOSOPHY

(Veterinary Parasitology)

By

REHAB ALI IBRAHIM YAGI

2013

Ph.D (Veterinary Parasitology) **THESIS** **2013** **YAGI, R.A.I**

Rajasthan University of Veterinary and Animal Sciences, Bikaner
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This is to certify that this thesis entitled **"Identification and molecular characterization of Variable Surface Glycoprotein and Adenosine transporter 1 genes of *Trypanosoma evansi* of camel origin"** submitted for the degree of **DOCTOR OF PHILOSOPHY** in the subject of **Veterinary Parasitology** of the Rajasthan University of Veterinary and animal Sciences, Bikaner, embodies bona-fide research work carried out by **Rehab Ali Ibrahim Yagi,** under my guidance and supervision and that no part of this thesis has been submitted for any other degree. The assistance and help received during this work have been fully acknowledged. The draft of the thesis was also approved by the advisory committee on 28/10/2013.

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DEAN

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1. INTRODUCTION

Camel trypanosomosis is the most important parasitic disease that causes high morbidity and mortality among camel population. It has a wide distribution in Africa, Asia, South America and Middle East (Hoare, 1972). Camel trypanosomosis in

Sudan is usually encountered between latitudes 10^{o}–15^{o}N in area west of the Nile and 11-18^{o} N. East of it. In Africa most of the species are pathogenic to man and animals. *Trypanosoma evansi* was the first trypanosome to be described and identified as the causative agent of mammalian trypanosomosis. The earliest reports on trypanosomes were published by Evans (1880) who associated it with an endemic disease in equines and camels known as "Surra" at Dera Ismail Khan in the Punjab of India. The earliest records of camel trypanosomosis in Sudan dates back to 1905 (Oliver, 1907).

Camel is the most efficient domesticated animal for converting fodder into work, transport, milk and meat. Trypanosomosis is the most important single cause of economic losses in camel rearing areas, causing morbidity up to 30.0% and mortality of around 3.0% (Ngerenwa *et al.*, 1993; Pacholek *et al*., 2001). Studies have shown that the parasite can infect all species of domesticated livestock, although the principal host varies geographically (El-Sawalhy and Seed, 1999). Besides causing disease, trypanosomes are also responsible for producing a state of severe immune-suppression, which renders the infected host more susceptible to secondary infections and produce poor immune response to bacterial and viral vaccines (Holmes, 1980).

Camel trypanosomiasis caused by *T. evansi* is of great concern to countries like Sudan, which possesses the second largest camel population in the world, estimated at nearly 4,623,000 heads (Annual Report of Federal Ministry of Animal Resources and Fisheries, Sudan, 2010).

In Africa, beyond the northern-most limits of the tsetse fly belt, and in parts of East Africa, camels are the most important host (Dia *et al.*, 1997), whilst in Central and South America the horse is principally affected (Monzon *et al.*, 1990). In Asia, a much wider range of hosts is involved, including the Bactrian camel and dromedaries, cattle, buffalo, horses and pigs (Pathak *et al.,* 1993; Tuntasuvan *et al*., 1996; El-Sawalhy and Seed, 1999; Pacholek *et al*., 2001). This is contrary to observations in Africa and South America, where there is little evidence to suggest that domesticated livestock other than camels and horses, respectively, are clinically affected or infected with *Trypanosoma evansi*. Nevertheless, there are reports of serological evidence of infection in

goats and sheep from the Sudan and in cattle from Brazil (Boid *et al.*, 1981; Luckins, 1998) and both goats and cattle have been considered as potential reservoirs of infection (Luckins, 1998). In Nigeria, camels are found in the northern part of the country, most commonly in Borno, Kano, Katsina, Kebbi, Sokoto, Jigawa and Yobe states, where they are utilized considerably as sources of meat. Most of the camels found in these areas are traded from the neighbouring Niger and Chad Republics (Ochappa, 1988). Severe outbreaks, which occurred in different parts of the world where several thousand animals died in the 1970s and, of late, in 1994 and 1995, for instance, in Pantamal, Brazil, have also been well documented (Luckins, 1998).These epidemics pose a major constraint to camel productivity given their importance as a source of meat, milk production, transportation and draught power, as well as by-products (wool, hair, skin and hides). In addition, they also provide foreign currency to their owners from their export (Elamin *et al*., 1999).

Immunosuppression has been shown in *T. evansi*-infected mice (Juyal and Tiwari, 1982). Substantial immunosuppressive effect to Haemorrhagic Septicaemia (HS) vaccination was recorded in experimental *T. evansi* infection in buffalo-calves (Singla *et al.* 2001).

The high cost of regular drug and insecticidal treatment, the limited effectiveness of insecticide application in high rainfall areas, the possibility of environmental pollution by insecticides, increasing incidence of parasite resistance to available drugs, toxicity of existing drugs and absence of new drugs to replace them are some of problems that make vector and trypanosomosis control difficult and expensive.

The trypanosome surface is covered with a homogeneous and dense glycoprotein coat made of approximately five million dimmers of single antigen and termed as variable surface glycoprotein (VSG) that is repeatedly changed in a fraction of population. This allows the trypanosomes to escape antibody-mediated killing and repopulate the host thereby resulting in the development of long- lasting chronic infection. Development of a vaccine by conventional methods has been unsuccessful

due to antigenic variation by the parasite. The trypanosome parasite has at least 1000 different genes encoding antigenically distinct versions of the surface coat and has been a major obstacle toward the development of a vaccine.

One of the common mechanisms evolved is antigenic variation which has arisen independently in a number of single-cell eukaryotic (i.e. protozoan) parasites. The process allows the parasites to evade the immune responses of their mammalian hosts through the continuous appearance of antigenic variants. The new variants arise through a change in a small subset of the infecting population and proliferation of the variants produces a new parasite population unaffected by the immune response which kills the old variant but in turn subject to a new, specific immune response. Reiteration of the process secures an infection unless the parasite runs out of novel antigenic determinants. This is the basis of antigenic variation, which was discovered a century ago in African trypanosomes. Regardless of whether they are mainly tissue fluid dwelling, as is the case for *Trypanosoma brucei* , or blood dwelling, such as for *T. vivax* and *T. congolense*, the level of trypanosomes in the blood fluctuates with time, due to immune mediated killing of old variants and successive expansion of populations expressing new variants. This survival process shares a number of features with evasion systems in many other micropathogens, including viruses and bacteria. There are general logistic similarities among these systems, which collectively are known as contingency gene systems (Moxon *et al.,* 1994).

The basis of trypanosome antigenic variation is the protective coat on the parasite. The entire cell surface of trypanosomes in bloodstream and metacyclic form, including the flagellum, is covered with a coat that is thought to provide general protection against non-specific host resistance mechanisms. The coat is highly immunogenic and elicits high titres of lytic antibodies. Through antigenic variation, which operates simply by up to 0.01 individuals changing to another coat at each cell division; this rate is several orders of magnitude faster than fortuitous changes arising from background events. Each variant is termed a variable antigen type (VAT). The different VATs retain the general protectiveness of the coat, while providing the variation enabling avoidance of specific antibodies.

Rode Trypanozoon antigen type 1.2 (*RoTat* 1.2) is a variable antigen type 1.2 which was cloned from *T.evansi* isolated from an Indonesian buffalo in 1982 (Bajyana Songa and Hamers, 1988). It is one of the predominant VATs and has been found early in the infection in the majority of *T.evansi* strains. Rabbits infected with stocks and clones from different parts of the world developed antibodies to *RoTat 1.2* within 32 days of infection(Verloo *et al*.,2001, Claes and Verloo 2002). Test using *RoTat 1.2* donot cross react with antibodies to *T.theileri* or other pathogenic trypanosomes, although they can not distinguish between infections with trypanosomes currently classified as *T.evansi* and *T. equiperdum* (Claes and Verloo, 2002). The *RoTat 1.2* gene is a fairy specific marker for *T. evansi* type A strains, but is not present in *T. evansi* type B strains isolated from Kenya, which may limit its diagnostic utility (Claes, *et al*., 2004: Ngaira, *et al*., 2005).

Drug resistance to trypanosomes is now a problem, but its underlying mechanisms are not fully understood. Cellular uptake of the major trypanocidal drugs is thought to occur through an adenosine transporter. The adenosine transporter-1 gene, *TbAT1*, encoding a P2-like nucleoside transporter has previously been cloned from *Trypanosoma brucei brucei*, and when expressed in yeast, it showed very similar substrate specificity to the P2-nucleoside transporter, but could not transport diamidines (pentamidine and diminazene). Witola *et al*. (2004) had cloned and sequenced a similar gene (*TevAT1)* from *Trypanosoma evansi* and found it to have 99.7% identity to the *TbAT1* gene. To elucidate the role of the *TevAT1* gene on diamidine trypanocidal effect, they genetically engineered *T. evansi* for conditional knock-out of the *TevAT1* gene by RNA interference (RNAi). Induction of the RNAi resulted in 10-fold depletion of *TevAT1* mRNA, with concomitantly significant resistance to diminazene aceturate (Berenil). The induced parasites propagated normally and attained peak cell density at an *in vitro* concentration of berenil, 5.5-fold higher than the inhibitory concentration 100% (IC_{100})of the wild-type. *TevAT1* knock-out had no effect on the trypanocidal activity of suramin and antrycide, but conferred some resistance to samorin. Their findings validate the significance of the *TevAT1* adenosine transporter-1 gene in mediating the trypanocidal effect of diamidines in *T. evansi*. Further, they showed for the first time that RNAi gene silencing in *T. evansi* can be induced using plasmids designed for *T. brucei* (Witola *et al*., 2004).

Treatment with trypanocidal drugs is the usual method of control of *T. evansi* and five compounds suramin, diminazine, isometamidium, quinapyromine and cymelarsan have been used (Luckins, 1999). Drug resistance is one of the big problems of the control of trypanosomosis in Sudan. It was noticed that camel trypanosomosis incidence has increasing and there was a wide increase of trypanocidals resistance for *T. evansi* reported from different part of Sudan (El Rayah *et al*.,1999). The number of anti-trypanosomal preparations available is limited and their value in disease control and eradication is reduced by the development of drug resistance in trypanosome populations (Leach and Roberts, 1981). There is so far no suitable methods for assessing drug resistance of trypanosomes. The problem of drug resistance in *T. brucei* appears to be increasing in the field. Sleeping sickness has recently become resurgent in sub-Saharan Africa, and the emergence of drug resistance is hindering efforts to control the disease. Melarsoprol treatment failures have reached alarming levels in several foci (Brun *et al*., 2001). In addition, resistance to the diamidine diminazene aceturate has also been reported from multiple foci (Geerts *et al., 2001*, Matovu *et al.,* 2001). Pentamidine resistance, in contrast, has so far not been reported from the field. The understanding of resistance mechanisms in bloodstream-form of trypanosomes is crucial to circumventing existing resistance problems and avoiding the emergence of resistance to the next generation of drugs.

Recently human infections with *T. evansi* have been reported in India making it a potential human pathogen (Joshi *et al*., 2005).

DNA cloning is a technique for isolating and reproducing a large numbers of identical DNA fragments. By introducing recombinant DNA into host cells the foreign DNA is reproduced along with the host cells.

Techniques for DNA cloning have opened incredible opportunities to identify or study the genes involved in almost every known biological process (Herfindal and Gourley, 2000). DNA cloning is the basis for other related technologies, such as gene therapy and genetic engineering of organisms. DNA cloning also made it possible to do genome sequencing.

Presently, there is an urgent need to improve the diagnostic tools and characterize resistant genes of *T.evansi* so as to formulate a proper disease control policy. In *T. evansi* infections *RoTat VSG* and *TevAT1* have a major role to play in persistant of disease. Indeed the parasite's surface antigen genes variation makes it clear that they can not serve as the basis of a recombinant vaccine. Nevertheless, it would clearly be very useful to have a means of control for this very important disease. Safe, inexpensive and effective recombinant protein based vaccine for *Trypanosoma evansi* based on variant antigen will be available for the prevention of Surra in camels.

Keeping in view the above facts, the proposed investigation has been undertaken with the following objectives:

1. To isolate Rode Trypanozoon Antigen Type (*RoTat VSG)* and *Trypanosoma evansi* Adenosine Transporter 1 *(TevAT1)* genes of *Trypanosoma evansi* from camel by polymerase chain reaction (PCR).

2. Cloning and characterization of above genes through sequencing**.**

2. REVIEW OF LITERATURE

Identification and Diagnosis

Trypanosomoses are important group of diseases affecting both man and animals caused by flagellated protozoan parasite of the genus *Trypanosoma* and transmitted by the bite of tsetse flies (*Glossina* spp.) and other heamatophagous flies or bugs. Trypanosomoses have a wide distribution in Africa, South America, Asia and the Middle East (Hoare, 1972). *Trypanosoma evansi*, a kinetoplastid haemoprotozoan with considerable economic importance, affects a wide range of hosts. The course of the disease and clinical picture varies from host to host thereby making the putative diagnosis more difficult.

Several techniques are available for diagnosis, depending on the type of diagnostic approach followed. For parasitological tests, a thick or thin wet blood smear can be prepared and examined under microscope. Positive results are achieved upon detection and observation of the parasite in the blood. The haematocrit centrifugation (HCT) technique involves filling heparinised capillary tubes with the blood sample, centrifuging the capillaries and then searching for viable parasites in the so-called buffy coat layer, found between the red blood cells and the plasma (Woo, 1970). A second concentration method, the mini-AECT (Anion Exchange Centrifugation Technique) involves the blood sample passing through a DEAE-cellulose column, which separates trypanosomes from red blood cells, based on charge. The highly negatively-charged red blood cells stick to the column, whilst the trypanosomes passing through the column are collected as elute at the other end. This elute can then be centrifuged and examined for the presence of trypanosomes (Lumsden *et al*., 1979). A further parasitological test includes animal inoculation, where the suspected blood sample is inoculated into small rodents (usually rats or mice) and in positive cases, a parasitaemia will establish

itself. The small rodents are checked regularly for the presence of *T. evansi* parasites via tail blood examination. Laboratory animals may be used to reveal subclinical infections in domesticated animals. *Trypanosoma evansi* has a broad spectrum of infectivity for small rodents, and so rats and mice are often used. In studies of *T. evansi* infections in camels, comparisons have been made between thick blood film examination and rat or mouse inoculation methods which gave more positive results than thick smears alone. However, even mouse inoculation is not 100% sensitive (Monzon *et al*., 1990).

Saarma (1979) in India identified *T. evansi* in the blood of camels, buffaloes and horses. He incriminated *Tabanus rubidus, Stomoxys calcitrans* and *Haematobia exigua* as mechanical vectors.

Camel trypanosomosis is an arthropod-borne disease transmitted by biting flies, Tabanus spp. being implicated as the main vectors, and other flies like Stomoxys and Hippoboscids (*Hippobosca camelina*) are also suspected (Losos,1980).

Improvement in the diagnosis of *T. evansi* infections has recently been introduced by Bajyana Songa and Hamers (1988) through substitution of the variant antigen type (VAT) used in the present test with a predominant VAT of *T. evansi.* The test is at present the simplest serological test for diagnosis of *T. evansi* infections. Its application to the diagnosis of the other species of pathogenic trypanosomes in livestock, however, is unlikely to succeed due to difficulties in stabilising their surface antigens by fixation.

Nantulya (1989) used CATT test to detect antibodies to the surface coat antigens of a commonly occurring trypanosome antigenic variant (VAT). The antigens were fixed and stabilised on the parasite using formaldehyde so that whole trypanosomes could be used in a direct agglutination test. The test was applied for the diagnosis of camel trypanosomiasis with some success because the VAT used in the test is found in the repertoire of VAT's expressed by *T. b. brucei, T. b. gambiense* and *T. evansi*. When applied for the diagnosis of *T. evansi* in water buffaloes, however, the test was found to give good results only after the inclusion of a specific anti-buffalo immunoglobulin.

In Haryana state and Nadia District in India outbreak of *T. evansi* in cattle and buffaloes were reported to increase with increase in numbers of *Tabanus* sp. and *Stomoxys* sp. (Batra *et al*., 1994). The cameline surra occurs in an acute or chronic debilitating form causing high morbidity and mortality (Pathak and Khanna, 1995). *Trypanosoma evansi* is mechanically transmitted between buffaloes and other host species by tabanid flies. Infection success is dependent on the fly intensity, susceptibility of the host, disease prevalence and level of parasitaemia in infected animals.

Some tests like antigen based ELISA and Suratex (Accu Pharma) were useful in detection of current infections (Pathak *et al.,* 1997) while antibody based ELISA and CATT (Card agglutination trypanosomal test) were useful for sero-epidemiology of cattle, buffalo and camels.

The life cycle of *T. evansi* is simple and direct. It does not undergo any development or multiplication in its insect vectors. The parasite multiplies by binary fission only in the animal host (Brun *et al.*, 1998). *Trypanosoma evansi* does not develop in its insect vectors because it lacks maxicircle DNA that is necessary for development in vectors (Ventura *et al.*, 2000).

The Haematocrit Centrifugation Technique (HCT) that has sensitivity equivalent to 85 trypanosomes/ml (Reid *et al.,* 2001). Mouse inoculation (MI) test is generally regarded and accepted as the most sensitive method to detect the animal trypanosomosis.

More recently PCR (Polymerase chain reaction) and DNA probes have been used for detection of trypanosomosis in animals and hold a ray of hope for the future. Wuyts *et al*. (1994) described a PCR targeted to repetitive nuclear sequence of *T. evansi* in mice and bovine calves from Thailand. The investigation attempted to detect the trypanosome DNA from clotted blood of camels, dogs and donkeys using this PCR and its comparison with blood smear examination after giemsa staining.

The PCR process allows a small amount or a specific region of DNA to be amplified, using precise temperature conditions and ingredients like primers, which read the specific region, a DNA polymerase, which can synthesise a copy of the DNA region and deoxynucleotide triphosphates (dNTPs), which build up the new DNA copy (Holland *et al*., 2001).

Reghu *et al.* (2008) compared two methods: PCR and blood smear examination for sensitive and specific detection of *Trypanosoma evansi* in camels, donkeys and dogs. Out of 131 blood samples tested, (61 camels, 44 donkeys and 26 dogs), 26 samples (21 camels, 3 donkeys and 2 dogs) were detected positive by PCR. Blood smear examination revealed the *T. evansi* organisms in only two camels.

Treatment and Drug Resistance

Leach (1961) reported the result of various drug regimes in treating camels involved in an outbreak of *T. evansi* infection in Sudan. He observed that the stock of *T. evansi* involved was resistant to dose of 5 g suramin but susceptible to 2 g quinapyramine sulfate.

A high incidence of trypanosomosis in conjunction with the use of therapeutic or prophylactic drugs often results in the appearance of drug resistant trypanosomes. Irregular treatment with prophylactic drugs or stopping their use altogether while animals are still under trypanosomosis risk can produce resistant strains of organism (Leach and Roberts, 1981). There is some evidence that the defense mechanism of the host plays a part in determining trypanocidal drug action, and this may also have a bearing on the development of drug resistance. Chemoresistance has been reported against most of trypanocidal drugs used (Williamson *et al*., 1982) and underdosing may be an important factor for development of resistance (Besier and Hopkins, 1988).

Relapses of *T. evansi* infections after treatment were reported soon after suramin was introduced for wide scale use in the field. Suramin resistant strains of *T. evansi* reported from Sudan, India, Russia and Indonesia (Boid *et al*., 1989).

Lun *et al*. (1991) used Cymelarsan in the treatment of buffaloes naturally infected with *Trypanosoma evansi* in south China. Forty buffaloes naturally infected with *T .evansi* were treated with a single dose of the new arsenical, Cymelarsan (Mel Cy) at 0.25 mg/kg to 3.0 mg/kg by intramuscular injection. All animals were cured, with the exception of two out of four animals treated with 0.25 mg/kg Mel Cy which relapsed two months after drug administration. Two out of eight buffaloes in control groups treated with a

single dose of diminazene aceturate (Berenil), 3.5 mg/kg, and relapsed two months after treatment. All cured animals showed no trypanosomes in their blood when tested within one to three years after administration.

Mohammed Ahmed *et al*. (1992) advised that Ethidium is to be withdrawn and replaced by Berenil in treating infected cattle in areas inside and outside the tsetse belt of the Sudan due to resistance. Cases of drugs resistance of different trypanosomes to nearly all the available drugs have been reported (Zhang *et al*., 1993,).

Kinabo (1993) reported that lack of much interest by the pharmaceutical industry to venture into development of new antitrypanosomal drugs has been a major stimulus to an intensification of research into the few existing drugs. Those indicated for animal trypanosomiasis include: isometamidium, homidium and diminazene, used primarily against *T. congolense*, *T. vivax* and *T. brucei*; and quinapyramine, mainly indicated for use against *T. evansi* infections.

Kaminsky and Brun (1993) used *in vitro* assays to determine drug sensitivity of trypanosomes. They advised the use of metacyclic or blood stream forms instead of procyclic forms in such assays.The advantage of this technique was that large number of isolates could be examined. Tests with metacyclic trypanosomes correlated well with field observations. However there were several disadvantages to this technique. *In vitro* cultivation of blood stream forms was only possible using pre-adapted lines and not using isolates directly from naturally infected animals (Hirumi *et al*., 1993). In vitro assays were also expensive to perform and require good laboratory facilities and well trained staff.

Peregrine (1994) stated that drug resistance was increasingly being recognized as a constraint of livestock production in many parts of Africa and he described the methods to identify drug resistance in trypanosomes which included test in mice. After expansion of an isolate in a doner mouse, groups of 5 or 6 mice were inoculated with trypanosomes. Twenty four hours later, or at the first peak of parasitaemia, each group except the control group was treated with a range of drug doses. Thereafter, the mice were monitored three times a week for 60 days. The ED_{50} or ED_{95} (effective dose which gives temporary clearance of the parasites

in 50% or 95% of the animals) could be calculated as the CD_{50} or ED_{95} (curative dose which gives complete cure in 50% or 95% of the animals).

Drug resistance may develop following repetitive and widespread use of a particular drug at a sub-curative level, coupled with immunosuppression due to medication, stress or concurrent infections (Mutugi *et al.*, 1994).

Musa *et al*. (1994) also reported resistance of *T. evansi* infection in camel to quinapyramine at a dose of 4.0 mg/kg. El Rayah *et al*. (1999) reported drug resistance in *Trypanosoma evansi* isolates from different part of Sudan to antrycide, suramin and samorin. She used equal to or higher doses than those usually recommended.

Treatment with trypanocidal drugs is the usual method of control of *T. evansi* and five compound suramin, diminazine, isometamidium, quinapyromine and cymelarsan have been used (Luckins, 1999). Suramin has been the mainstay of treatment for all host species for over 70 years (Luckins, 1999). Suramin is almost universally effective at a dose rate of 10 mg/kg. Quinapyramine has been used in camels and horses, whilst diminazine and isometamidium have been used for treatment of cattle and buffalo. Diminazine has been used successfully to treat cattle and buffalo in India and Thailand, but there are some doubts about its efficacy at the recommended dose rate of 3.5mg/kg, some authorities suggest a higher dose. Although the drugs have been in use for many years the reports of drug resistance from the field are few (Luckins, 1999).

Luckins (2000) reported that drug resistance occured amongst *T. evansi* isolates in several countries in Africa and Asia. Relapse of *T. evansi* infection after treatment with Cymerlarsan was reported (Haroun *et al*., 2003) in experimentally infected rats at the recommended dose of 0.5mg/kg. The mechanism of drug resistance is thought to be correlated with reduced drug uptake by the trypanosomes which are probably associated with adaptation of the parasite by mutation (Mäser *et al.*, 2003).

The standardised *in vivo* screening test in mice offers a simple, cheap and convenient method to detect drug resistance (Mamoudou *et al.*, 2008). Recently, the use of molecular markers to determine resistance of trypanosomes to a particular drug has also been explored (Mamoudou *et al.*, 2008) but requires a well established molecular laboratory to run the assay.

Variable surface glycoprotein genes of Trypanosomes

Cross (1984) reported that pathogenic trypanosomes had a unique mechanism for antigenic variation. Each cell was covered by a surface coat consisting of about seven million essentially identical glycoprotein molecules drawn from a large repertoire of variants, each encoded by an individual gene. Amino acid sequence variation extended throughout the molecule but reduced from the amino terminus to the carboxy terminus, where certain features, especially the grouping of cysteine residues, were quite conserved. The range of diversity within the thousand or so variant glycoprotein genes that existed in each cell was large. New variants may arise instantaneously by segmental gene conversion. Variant surface glycoproteins were synthesized with amino terminal signal sequences and hydrophobic carboxy terminal tails. The tails were extraordinarily conserved. After synthesis, they are replaced by a complex glycolipid structure in which myristic (dodecanoic) acid served to anchor the polypeptide to the surface membrane. Enzymic cleavage of myristic acid released variant glycoproteins from the surface coat.

There are several studies trying to unravel the different systems involved in the antigenic variation reviewed by Pays (2005). The first mechanism for the parasite to perform a VSG switch is to change the expression site. By switching off the active expression site and activating a silent expression site, a VSG switch takes place. However, as the VSG genes are transcribed as polycistronic units, at the same time an expression site associated gene (ESAG) switch will occur. This process could be one of the mechanisms which makes it possible for the trypanosomes to survive in various conditions and as such extending the host range. A second system of the antigenic variation occurs possibly via the VSG gene rearrangements including a reciprocal recombination

and a gene conversion. In this system, the active site is not changed (and the ESAGs remain the same) and only the VSG gene undergoes a modification. During the reciprocal recombination, the whole transcribed VSG gene in one telomere is replaced by another VSG gene present on a silent telomere. This system occurs by crossing over in the 70bp repeat region flanking every VSG gene on the promoter site of the gene.

The trypanosome genome contains hundreds of VSG genes (*VSG*) of which very few (7%) are fully functional (encoding all recognizable features of known functional VSG), whereas the majority (66%) are full-length pseudogenes with frame shifts and/or in-frame stop codons. Most of these *VSG*s are clustered in the subtelomeric arrays. Transcription of the *VSG* occurs in one of the telomeres of the large chromosomes, which contain the VSG expression sites (*VSG* ESs) (Engstler *et al.,* 2007). These expression sites are polycistronic transcription units having expression site associated genes (*ESAG*s) upstream of the VSG. These polycistronic mRNAs are matured by polyadenylation and addition of a spliced leader sequence by a process called trans-splicing. Among the different expression sites only one is active at a given time. Thus only one of the VSG molecules is present within the trypanosome surface coat, resulting in the homogeneous display of an identical surface coat. Transcription starts simultaneously in all *VSG* ESs, but only in the “active” one there is complete transcription and all the others are aborted. In the rapidly dividing long slender form of trypanosomes, the active expression site was found to be present in a specialized region called expression site body (ESB) and no similar structure was detected for the silent expression sites.

Molecular Characterization of *RoTat* VSG gene of *Trypanosoma evansi*

Ploeg *et al.* (1982) cloned large fragments of genomic DNA of *Trypanosoma brucei* in cosmid vectors in *Escherlchia coli*. Cosmids containing the BCs of genes 117, 118 and 121 were readily obtained, but DNA containing the expression linked copy (ELCs) was strongly selected against in the cosmid and plasmid cloning systems used. They had analysed the distribution of VSG

genes in the genome using probes for the sequences at the edges of the transposed segment which are partially homologous among these genes. In genomic cosmid clone banks, about 9% of all colonies hybridize with probes from the 5′- and 3′-edges of the transposed segment, showing that these sequences were linked in the genome. Moreover, the 117 and 118 BC cosmids contain several additional putative VSG genes in tandem, as deduced from hybridization and sequence analyses.

Bajyana Songa and Hamers (1988) reported that antigen type *RoTat* 1.2 of *T.evansi* could be assayed in the CATT test and compared with the testryp CATT. It was found that a more sensitive CATT test can be developed based on another early expressed VAT *RoTat* 1.2 of *T.evansi* in the sero- diagnosis of animal trypanosomosis.

Emmanuel *et al.* (1990) examined the distribution of restriction site polymorphisms in the nuclear DNA of 9 of these stocks, using 7 different variable surface glycoprotein (VSG) and non-VSG probes. Restricted kinetoplast DNA (kDNA) fragments of some of these strains were cloned into M13 or PUC 18 vectors and sequenced. The restriction and sequence mapping showed that most of *T. evansi* isolates belonged to A1 and A2 types of Borst and to two new closely related types A3 and A4. A notable exception was RoTat 4/1 derived from a Sudanese stock which was found to display a characteristic *brucei*-like minicircle heterogeneity. The *T. evansi* minicircles analysed were not only homogeneous in sequence but also the region similar to the conserved region in *Trypanosoma brucei* and *Trypanosoma equiperdum* flanked on its 5′ end by a palindromic repeat of part of the conserved region. The highly conserved sequence GGGCGGT which appeared to correspond to the initiation of synthesis of one of the Okazaki fragments contained an additional G and is located as in *T. brucei* and *T. equiperdum* about 73 bp 5′ from the ORI. The nuclear DNA analysis confirms the kDNA study in that all the *T. evansi* stocks were members of a very homogeneous group in terms of sequence divergence. Moreover, their analysis also confirmed that *T. evansi* was more closely related to the West African *T. b. brucei* and *T. b. gambiense* than to other African trypanosomes.

Urakawa *et al*. (2001) studied *Trypanosoma evansi* cloning and explained that a complementary DNA encoding the variant surface glycoprotein (VSG) of *Trypanosoma evansi* Rode Trypanozoon antigenic type *RoTat* 1.2, currently used for experimental serological diagnosis of *T. evansi* infection in livestock, was cloned as a recombinant plasmid and sequenced. A recombinant baculovirus containing the coding region of *RoTat* 1.2 VSG was constructed to express the protein in Spodoptera frugiperda [corrected] insect cells. From this, sufficient quantities of the recombinant protein were produced for empirical and wide-scale objective assessment of the diagnostic potential of this antigen. The gene encoding the *RoTat* 1.2 VSG was shown by PCR to be present in the genomes of many different cloned isolates of *T. evansi*, but not *T. brucei*, from geographically separate regions of Africa, Asia, and South America.

Claes *et al*. (2004) stated that a total of 39 different trypanosome stocks were tested using the *RoTat 1.2* based Polymerase Chain Reaction (PCR). The PCR yielded a 205 bp product in all *T. evansi* and in seven out of nine *T. equiperdum* strains tested. This product was not detected in the DNA from *T. b. brucei*, *T. b. gambiense*, *T. b. rhodesiense*, *T. congolense*, *T. vivax* and *T. theileri* parasites. The *Rotat* 1.2 PCR detected as few as 10 trypanosomes per reaction with purified DNA from blood samples, i.e. 50 trypanosomes/ml. Sequencing of the positive samples revealed that all amplicon were identical.

Marion *et al*. (2004) devised a strategy for isolating the repertoire of *T. brucei* 427 BES-containing telomeres in *Saccaromyces cerevisiae* by using transformation-associated recombination (TAR). They isolated 182 *T. brucei* 427 BES TAR clones, 167 of which could be subdivided into minimally 17 BES groups. This set gave the first view of the breadth and diversity of BESs from one *T. brucei* strain. Most BESs ranged between 40 and 70 kb (average, 57 ± 17 kb) and contained most identified *ESAG*s. Phylogenetic comparison of the cohort of BES promoter and *ESAG6* sequences did not show similar trees, indicating rapid evolution most likely mediated by sequence exchange between BESs.

Ngaira *et al.* (2004) determined the cause of the false negative results of CATT/*T. evansi*. A total of 20 field isolates comprised 16 stocks from camels that were negative by CATT/*T. evansi*, and 4 from CATT/*T. evansi*-positive camels. In addition, 15 known *T. evansi* and four *T. brucei* were used as reference. Purified DNA samples were tested using an established *RoTat* 1.2-based polymerase chain reaction (PCR) that yielded a 488 bp product for the specific detection of *T. evansi*. Antibodies to *RoTat 1.2* variant surface glycoprotein (VSG) were used in Western blotting to detect *RoTat* 1.2 VSG linear epitopes. Results of PCR and Western blot showed that the 16 stocks isolated from CATT/*T. evansi*-negative camels fell into three groups. In Group 1, both the *RoTat* 1.2 VSG gene and the VSG were absent in three stocks. In five trypanosome stocks in Group 2, the *RoTat* 1.2 VSG gene was detected, but Western blot was negative indicating absence of the expressed VSG. Five other stocks containing the RoTat 1.2 VSG gene were also in this group. The *RoTat* 1.2 VSG gene was detected and Western blot was positive in all four trypanosome stocks in Group 3. All four stocks from CATT/*T. evansi*-positive camels contained the *RoTat* 1.2 VSG gene and the expressed VSG. The reference *T. evansi* KETRI 2479 lacked the *RoTat* 1.2 VSG gene and there was no immune reactivity detected by Western blot. The rest of the reference *T. evansi* stocks examined contained the *RoTat* 1.2 VSG gene. All the four *T. brucei* samples examined were negative by PCR and Western blot. This study showed that the RoTat 1.2 VSG gene was absent from some *T. evansi* trypanosomes in Kenya.

Lejon *et al*. (2005) studied recombinant *RoTat* 1.2 VSG as antigen for diagnosis of *T*. *evansi* in dromedary camel and reported that transcript encoding a predominant *Trypanosoma evansi* variable surface glycoprotein *RoTat* 1.2 could be cloned and expressed as a recombinant protein in *Spodoptera frugiperda* and Trichoplusia ni (insect) cells. Its potential as an antigen for specific detection of antibody in serum of dromedary camels affected by surra, was evaluated. In ELISA, the reactivity of the recombinant RoTat 1.2 VSG was similar to that of native *RoTat* 1.2 VSG. An indirect agglutination reagent was therefore prepared by coupling the recombinant *RoTat* 1.2 VSG onto latex particles. The performance of the latex agglutination test was evaluated on camel sera, and compared with the performance of CATT/ T. evansi and LATEX/ T. evansi tests, using the immune trypanolysis

assay with *T. evansi RoTat 1.2* as a reference test. The relative sensitivity and specificity of the latex coated with recombinant *RoTat 1.2* VSG, using a 1:4 serum dilution, were respectively, 89.3 and 99.1%. No difference was observed between the performance of latex coated with recombinant *RoTat 1.2* VSG and LATEX/ *T. evansi* or CATT/ *T. evansi*.

Ngaira *et al*. (2005) reported that the majority of *T. evansi* could be detected using diagnostic tests based on the variant surface glycoprotein (VSG) of *T. evansi* Rode Trypanozoon antigen type (*RoTat*) 1.2. Exceptions are a number of *T. evansi* isolated in Kenya. To characterize *T. evansi* that are undetected by *RoTat 1.2*, they cloned and sequenced the VSG cDNA from *T. evansi* JN 2118Hu, an isolate devoid of the *RoTat 1.2* VSG gene. A 273 bp DNA segment of the VSG gene was targeted in PCR amplification for the detection of non-*RoTat 1.2 T. evansi*. Genomic DNA samples from different trypanosomes were tested including 32 *T. evansi*, 10 *T. brucei*, three *T. congolense*, and one *T. vivax*. Comparison was by PCR amplification of a 488 bp fragment of *RoTat 1.2* VSG gene. Results showed that the expected 273 bp amplification product was present in all five non-*RoTat 1.2 T*. *evansi* tested and was absent in all 27 *RoTat 1.2*-positive *T. evansi* tested. It was also absent in all other trypanosomes tested. The PCR test developed in this study was specific for non-*RoTat 1.2 T*. *evansi.*

Njiru *et al*. (2006) designed a specific PCR test to distinguish *T. evansi* type B from type A. Further a test based on maxicircles to distinguish *T. brucei brucei* from *T. evansi* was designed and evaluated.By using the designed PCR tests, they detected three type B isolates from camel blood samples collected in northern Kenya. Comparison of minicircle sequences from all four type B isolates showed >96% identity within the group, and 50–60% identity to type A minicircles. Phylogenetic analysis based on minicircle sequences revealed two clusters, one comprising isolates of type A and one of type B, while random amplification of polymorphic DNA showed slight polymorphic bands within type B. Most *T. evansi* isolates analysed were heterozygous at a repetitive coding locus (MORF2). All type B isolates had one genotype designated 3/5 based on the alleles present. Three camel isolates, which had homogenous type A minicircles, lacked the *RoTat 1.2* gene, while another five isolates were *T. b. brucei,* based

on the heterogeneity of their minicircles and presence of maxicircles as demonstrated by PCR amplification of the gene for cytochrome oxidase subunit 1. Their results confirmed the existence of *T. evansi* type B isolates, *T. b. brucei* and existence of *T. evansi* type A without *RoTat 1.2* gene in Kenyan isolates.

Oclyde *et al. (2007) observed* eighteen VSGs expressed from Ugandan isolates and compared with homologues (> 40 % sequence identity) in the two available *T. brucei* genome sequences. Fourteen homologues were present in the genome of *Trypanosoma brucei brucei* TREU927 from Kenya and fourteen in the genome of *T. b. gambiense* Dal972 from Cote d'Ivoire. The Ugandan VSGs averaged 71% and 73 % identity to homologues in *T. b. brucei* and *T. b. gambiense* respectively. The sequence divergence between homologous VSGs from the three different strains was not random but was more prevalent in the parts of the VSG believed to interact with the host immune system on the living trypanosome.

Konnai *et al*. (2009) applied a real-time PCR assay for the detection and quantification of parasites in water buffaloes using specific primers for the *T*. *evansi* Rode Trypanozoon antigen type (*RoTat) 1.2* Variable Surface Glycoprotein (VSG) gene, which is a known diverse DNA region in trypanosomes. The quantitative detection limit of the assay was 102 trypanosomes per mL of blood, and the identity of the amplicon was confirmed in all assays by melting curve analysis. To evaluate the clinical applicability of this procedure, detection and estimation of parasitemia in blood samples obtained from water buffaloes and horses were conducted. *T. evansi* was detected in 17/607 (2.8%) blood samples, with parasitemia levels ranging from >101 to 107 parasites per mL of blood. Interestingly, out of the 17 PCR positive animals, 3 had previously received trypanocidal treatment and 1 had abortion history. These data indicated that real-time PCR for the estimation of putative parasitemia levels was a quantitatively and objectively applicable technique for clinical diagnosis of Surra, and could help to understand disease stage and risk of transmission of *T. evansi*.

Fikru *et al*. (2010) studied comparative sensitivity of parasitological, serological, and molecular tests on 237 horses originating from two dourine-suspected districts of Arsi-Bale highlands of Ethiopia. The study was conducted to determine the prevalence of the disease and degree of agreement of the diagnostic tests. Accordingly, the prevalence of the disease was found to be 4.6%, 36.7%, and 47.6% by parasitological Woo test, *RoTat 1.2* and 18S PCR tests, respectively. The seroprevalence of the disease was 27.6% in CATT/ *Trypanosoma evansi* test. In Ethiopia, it was for the first time that trypanosomes from dourine suspected horses were demonstrated in 4.6% of the animals using Woo test.

Sengupta *et al*. (2010) suggested that the application of PCR over the parasitological methods for the detection of the early and chronic stage of surra in domestic and wild animals and they designed EXP3F/4R primer pair and amplified the 1.4 kb of VSG gene of *T. evansi* and studied the phylogenetic relationship by in silico analysis. The PCR method was standardised using another set of primer, DITRYF/R, and 400 bp was amplified from blood and tissue samples of experimentally infected animals. Applying the PCR method, they were able to detect as low as 0.15 trypanosome ml−1. Considering the number of parasite to DNA concentration, the PCR method has a sensitivity of 0.015 pg ml−1. The PCR could detect the presence of the parasite as early as 24 hours post-infection (p.i.) and 72 hours p.i., respectively, in experimentally infected rats and buffalo. No amplification was observed with DNA of *Babesia bigemina* and *Theileria annulata*, indicating the primers are specific for *T. evansi*. The PCR method could detect the dog, lion and leopard isolates of *T. evansi*. Similarly, amplifying the DNA from the experimentally infected tissues was also found to be sensitive.

Bashir *et al*. (2011) carried out study in Sudan in the period from September to November 2009 on samples collected from 687 camels from geographically distinct zones to detect all possible African trypanosomes, which could infect camels. They performed the ITS1-PCR to screen camels from four regions in the Sudan. The result showed higher prevalence in Halfa "Butana region" of 57.1% (117/205) with lower prevalence of 6.0% (4/67) recorded in Umshadeeda. Samples from Kassala showed prevalence of 24.0% (12/50) and those from South Darfur showed prevalence of 35.6% (26/73) taken into account that the actual

number samples from Darfur was 356 samples. Samples were first tested using agarose electrophoresis for visualizing positive samples. The presence or absence of fragment size differences in the positive *T. evansi* samples was then tested by using 2% agarose Zebra (BioTools Inc, Japan) with high resolution capability of differentiating small base pairs differences. No size difference was observed in all samples. In another experiment, the *RoTat* 1.2 gene encoding the variable surface glycoprotein (VSG) of *T. evansi* was analyzed for its presence or absence by a polymerase chain reaction (PCR) using *T. evansi* species-specific primers. The study showed that the *RoTat* 1.2 VSG gene was absent in thirteen out of thirty *T. evansi*-positive samples.

Amer *et al.* (2011) assessed prevalence of *T. evansi* in the blood of dromedary camels (*Camelus dromedarius*) brought to Al Bassatein abattoir, Cairo, Egypt, by mouse inoculation test ad found that out of 84 tested camels, 4 animals (4.7%) were infected. Molecular analysis was achieved by PCR amplification and sequence analysis of part of ribosomal RNA gene including 18S, ITS1, 5.8S and ITS2 regions. Despite the conserved nature of 18S region, ITS region showed obvious heterogeneity compared to analogous sequences in database. Analysis of transferrin receptor encoding gene (ESAG6) showed variable repertoire in the studied isolates, which may indicate to a novel structure of *T. evansi* population from Egypt and/or a difference in host range. Furthermore, analysis of variable surface glycoprotein RoTat 1.2 gene marker revealed some heterogeneity at this gene locus.

Sengupta *et al*. (2012) reported that the 5′ end of the partial VSG gene sequences (681 bp) encoding N-terminal protein of *RoTat* 1.2 VSG (227 amino acid) could be amplified, cloned into pET32a vector, and expressed in prokaryotic system. The fused His-tagged expressed VSG protein (43 kDa) of the *T. evansi* was characterized in SDS-PAGE and immunoblotting using hyperimmune/immune sera raised against buffalo, dog, lion and leopard isolates of *T. evansi*. The expressed protein remained immunoreactive with all the sera combinations. The animals immunized with whole cell lysate or recombinant protein showed similar antibody reactions in ELISA and CATT (Card Agglutination Test for Trypanosomiasis). This study suggested the expressed recombinant truncated VSG was having its importance for its possible use in sero-diagnosis of surra.

Tarek *et al*. (2012) conducted study to evaluate the use of polymerase chain reaction (PCR) using (Rode Trypanozoon antigen type) *RoTat 1.2* primers for the genomic DNA of eight Egyptian strains of *T. evansi* from camels, goats, sheep, cattle and donkeys from different areas of Egypt. In addition, six previously identified isolates of *T. evansi,* from Africa, Asia and South-America, isolated from different hosts. Besides, one *T. equiperdum*, one *T.b.brucei* and two *T. brucei* subspp strains from Africa were used as reference strains. The results supported previous suggestions that PCR amplification of the *RoTat* 1.2 (Variable surface glycoprotein) VSG gene was a specific marker for reference *T. evansi* strains, but it failed to differentiate some Egyptian strains that have been previously identified as *T. evansi*. However, it identified natural populations of *T. evansi* in Egypt with degree of heterogeneity, describing for the first time that those natural populations revealed higher levels of intra-specific genetic variability of *T. evansi.* In addition, the results revealed that mixed genotyping occurred between some *T. evansi* isolates with *T. brucei* and *T. equiperdum* in the border areas and produced evidence that strain difference might occur between Egyptian sub-populations and reference strains of *T. evansi*.

Marjo *et al*. (2013) assessed the prevalence and intraspecific diversity of *T. evansi* in the Philippine, blood samples from water buffaloes in different geographical regions were collected during an outbreak. *T. evansi* was detected in all 79 animals tested using PCR targeting the *RoTat 1.2 VSG* gene. Sequencing of the rDNA complete internal transcribed spacer (ITS) region including the 5.8S subunit showed high similarity (99–100%) between Philippine isolates and known *T. evansi* isolates in Genbank. Tree construction based on the same region confirmed the close relationship between Philippine and reported Thai isolates as compared to Egyptian isolates separated by relatively small genetic distances, 47 polymorphisms, despite the clustering in four branches. Overall, the results of this study proved genetic diversity within *T. evansi* species despite previous reports on limited heterogeneity among isolates worldwide.

Molecular Characterization of *TevAT1* gene of *Trypanosoma evansi*

Carter and Fairlamb (1993) were the first to perform a study that showed that a *T. b. brucei* melarsen-resistant clone derived from a wild-type parental clone lacked the P2 transporter and was 67-fold less sensitive to melarsoprol *in vitro* when compared to the parental clone. Further more, it was demonstrated, using melarsen resistant *T. brucei*
bloodstream forms, that the P2 purine transport system was implicated in the reduced import of the diamidine, pentamidine, leading to the suggestion of its involvement in pentamidine resistance.

Barrett *et al*., (1995) reported that P2 adenosine transport system was also found to be present in *T. equiperdum* whereby the P2 transporter of a berenil resistant clone also cross resistant to melarsoprol, had reduced activity and possibly decreased affinity for adenosine (the main P2 substrate) in comparison to the drug sensitive clone.

Scott *et al*. (1996)in an *in vitro study* revealed that alterations in P2 transport activity are associated with diminished drug uptake giving rise to drug resistance in *T. brucei* group organisms.

Wilkes *et al*. (1997) reported linking P2 changes in isometamidium resistant *T. congolense* although it was likely that isometamidium entered through other routes associated with changes in the mitochondrial membrane potential.

Mäser *et al*. (1999c) stated that molecular characterisation of the gene encoding the *T. brucei* adenosine transporter (*TbAT1*), played a major role in further understanding the mechanisms underlying drug resistance in *T. brucei*. Identification of the *TbAT1/P2* gene was undertaken by expression in the yeast *Saccharomyces cerevisiae*. The gene was found to encode an adenine sensitive adenosine transporter P2, whose alteration due to mutations resulted in melaminophenyl arsenical resistance *in vitro* and *in vivo.*

Mäser *et al*. (1999c) also demonstrated that the *TbAT1* gene cloned from a melarsen-resistant clone (STIB 777R) had ten nucleotide differences in its cloned *TbAT1* Open Reading Frame (ORF) when compared to the *TbAT1* of the melarsen-sensitive

clone (STIB 777S). Sequencing suggested the possibility of STIB 777S being homozygous for the sensitive allele. The *TbAT1* gene cloned from the melarsen-resistant clone (STIB 777R) was reintroduced into yeast and was shown to be unable to stimulate the uptake of exogenous adenosine and did not confer susceptibility to melarsen-oxide, confirming its lack of P2 activity. In the same study a PCR/ RFLP (Restriction fragment based polymorphism) method using the *Sfa NI* enzyme was shown to differentiate between the *T. brucei* melarsen sensitive (STIB 777S) and melarsen resistant clone (STIB 777R) by displaying different allele patterns, 566 bp & 111 bp for the wild type *TbAT1* and 435 bp and 242 bp for the mutated *TbAT1.*

Moore and Richer (2001) reported that in South Sudan, Ibba centre, melarsoprol was withdrawn in 2001, because of high treatment failure rates, analysis of isolates from *T. b. gambiense* sleeping sickness patients collected in 2003 was carried out using the same PCR/RFLP method and sequencing. The results indicated the presence of *TbAT1* wild type alleles only and no evidence for the resistant mutations. The isolates were tested *in vitro* and they were found to be sensitive to melarsoprol leading to the suggestion that

melarsoprol resistant alleles were never there or melarsoprol resistant mutations could not survive without drug pressure.

Matovu *et al.* (2001) studied *TbAT1* gene which codes for the P2 adenosine transporter, from *Trypanosoma brucei* field isolates to investigate a possible link between the presence of mutations in this gene and melarsoprol treatment failure. About 65 isolates of *T. b. gambiense* were analyzed from north-western Uganda with high treatment failure rates following melarsoprol therapy, 38 had a mutated *TbAT1*. All individual isolates contained the same set of nine mutations in their *TbAT1* genes. Of these, five point mutations resulted in amino acid substitutions, one resulted in the deletion of an entire codon, and three were silent point mutations. Identical sets of mutations were also found in a drug-resistant *T.b.rhodesiense* isolate from south-eastern Uganda and in a *T.b.gambiense* isolate from a relapsing patient from northern Angola. A deletion of the *TbAT1* gene was found in a single *T. b. gambiense* isolate from a relapsing patient from northern Angola. The data presented demonstrate the surprising finding that trypanosomes from individual relapse patients of one area, as well as from geographically distant localities, contain an identical set

of point mutations in the transporter gene *TbAT1.* They further demonstrated that many isolates from relapse patients contained the wild-type *TbAT1* genes, suggesting that melarsoprol refractoriness was not solely due to a mutational inactivation of *TbAT1.*

Matovu *et al.* (2003) described the role of *TbAT1* in drug uptake and drug resistance in *T. brucei* by genetic knockout of *TbAT1. Tbat1*-null trypanosomes were deficient in P2-type adenosine transport and lacked adenosine-sensitive transport of pentamidine and melaminophenyl arsenicals. However, the null mutants were only slightly resistant to melaminophenyl arsenicals and pentamidine, while resistance to other diamidines such as diminazene was more pronounced. Nevertheless, the reduction in drug sensitivity might be of clinical significance, since mice infected with *tbat1*-null trypanosomes could not be cured with 2 mg of melarsoprol/kg of body weight for four consecutive days, whereas mice infected with the parental line were all cured by using this protocol. Two additional pentamidine transporters, HAPT1 and LAPT1, were still present in the null mutant, and evidence is presented that HAPT1 may be responsible for the residual uptake of melaminophenyl arsenicals.

Witola *et al*. (2004) cloned and sequenced *TevAT1* gene from *Trypanosoma evansi* and found it to have 99.7% identity to the *TbAT1* gene. They investigated the of the *TevAT1* gene on diamidine trypanocidal effect, they genetically engineered *T. evansi* for conditional knock-out of the *TevAT1* gene by RNA interference (RNAi). Induction of the RNAi resulted in 10-fold depletion of *TevAT1* mRNA, with resistance to diminazene aceturate (berenil). The induced parasites propagated normally and attained peak cell density at an *in vitro* concentration of berenil, 5.5-fold higher than the IC(100) of the wild-type. They found that *TevAT1* knock-out had no effect on the trypanocidal activity of suramin and antrycide, but conferred some resistance to samorin. Their findings validate the significance of the *TevAT1* adenosine transporter-1 gene in mediating the trypanocidal effect of diamidines in *T. evansi*. Further, they showed for the first time that RNAi gene silencing in *T. evansi* could be induced using plasmids designed for *T. brucei.*

De Koning *et al*. (2004) described the mechanisms by which [^{3}H]diminazene is transported by *Trypanosoma brucei brucei* bloodstream forms. Diminazene was rapidly accumulated through a single transporter, with a K_m of 0.45 ± 0.11 µM, which was dose dependently inhibited by pentamidine and adenosine. The K_i values for these inhibitors were consistent with this transporter

being the P2/*TbAT1* adenosine transporter. Yeast expressing *TbAT1* acquired the ability to take up [^{3}H]diminazene and [^{3}H]pentamidine. *TbAT1*-null mutants had lost almost all capacity for [^{3}H]diminazene transport. However, this cell line still displayed a small but detectable rate of [^{3}H]diminazene accumulation, in a nonsaturable manner. They concluded that *TbAT1* mediates [^{3}H]diminazene transport almost exclusively and that this explains the observed diminazene resistance phenotypes of *TbAT1*-null mutants and field isolates.

Federico *et al*. (2005) showed stronger phenotypes of the *tbat1* null mutant with respect to its sensitivity toward toxic adenosine analogs. Compared with parental *TbAT1*$^{+/+}$ trypanosomes, the *tbat1*$^{-/-}$ mutant was 77-fold less sensitive to tubercidin and 14-fold less sensitive to cordycepin. Resistance was further increased by the addition of inosine but was reverted by adenine. It was surprising that the *tbat1*$^{-/-}$ mutant grew faster than *TbAT1*$^{+/+}$ trypanosomes and that it overexpressed genes of the *trypanosoma brucei* nucleoside transporter (*TbNT)* cluster encoding P1-type transporters. These unexpected phenotypes showed that there were conditions other than drug pressure under which loss of P2 may confer a selective advantage to bloodstream-form trypanosomes. They stated that overexpression of P1 by trypanosomes after loss of P2 indicates that combinatorial chemotherapy with trypanocidal P1 and P2 substrates may be a promising strategy to prevent drug resistance in sleeping sickness.

Afework *et al*. (2006) investigated a possible link between the presence of mutations in *TbAT1* gene and isometamidium resistance. They analysed the gene from 11 isometamidium-sensitive field stocks isolated from *T.b. brucei* of cattle in Uganda, two sensitive reference clones and two resistant reference clones. A sequence alignment showed that the isometamidium-sensitive *T. b. brucei* contained the wildtype sequence patterns. In contrast, the isometamidium- resistant *T. b. brucei* stocks showed the mutant-type sequence patterns with six point mutations. They used the digestion with the restriction endonuclease Sfa NI to distinguish between isometamidium-sensitive and isometamidium-resistant *T. b. brucei.*

Delespaux *et al*. (2006) reported that screening by the single strand conformation polymorphism (SSCP) method was found to be more sensitive for detection of mutations in a *T. congolense* adenosine transporter (*TcoAT1*) gene with high similarity to *TbAT1/P2* when comparing diminazene resistant and sensitive *T. congolense* isolates. They investigated a possible link between

point mutations and diminazene aceturate (DA) resistance in mice, the TcoAT1 putative genes of 26 *T. congolense* strains, characterised for DA sensitivity in the single dose mouse test, were screened by (SSCP). Results showed that the SSCP profiles of 23 out of 26 (88.5%) *T. congolense* strains were confirmed by the sensitivity test in mice with the commonly accepted criterion for sensitivity to diminazene being a CD80 of 20 mg/kg in the mouse test. The remaining *T. congolense* strains showed a resistant SSCP profile and relapsed in mice after treatment at doses lower than 20 mg/kg indicating that the SSCP was more sensitive than the single dose mouse test for the detection of resistance to diminazene. However, none of the strains used in this study showed a sensitive SSCP profile while they were resistant in the single dose mouse test. The sequencing of the *TcoAT1* gene of two sensitive, two intermediate and two resistant strains allowed the set up of a PCR-RFLP test for the discrimination between sensitive and resistant strains confirming the SSCP results.

Daniel *et al*. (2007) investigated the basis for cross-resistance between the diamidine and melaminophenyl arsenical classes of drugs in African trypanosomes. They induced high levels of pentamidine resistance in a line without the *tbat1* gene that encodes the P2 transporter previously implicated in drug uptake. They isolated independent clones that displayed very considerable cross-resistance

with melarsen oxide but not phenylarsine oxide and reduced uptake of [3H]pentamidine. In particular, the high affinity pentamidine transport (HAPT1) activity was absent in the pentamidine-adapted lines, whereas the low affinity pentamidine transport (LAPT1) activity was unchanged. The parental *tbat1* line was sensitive to lysis by melarsen oxide, and this process was inhibited by low concentrations of pentamidine, indicating the involvement of HAPT1. This pentamidine-inhibitable lysis was absent in the adapted line KO-B48. Likewise, uptake of the fluorescent diamidine 4,6-diamidino-2-phenylindole dihydrochloride was much delayed in live KO-B48 cells and insensitive to competition with up to 10 µM pentamidine. They also showed that a laboratory line of *Trypanosoma brucei gambiense*, adapted to high levels of resistance for the melaminophenyl arsenical drug melarsamine hydrochloride (Cymelarsan), had similarly lost TbAT1 and HAPT1 activity while retaining LAPT1 activity. Therefore that selection for resistance to either pentamidine or arsenical drugs could result in

a similar phenotype of reduced drug accumulation, explaining the occurrence of cross-resistance.

Nerima *et al*. (2007) performed study in NW Uganda that showed the use of an allele specific PCR approach based on primers targeting the *TbAT1* PCR/*Sfa NI* 677 bp fragment and were found specific only for the wild type *TbAT1* and mutant *TbAT1* . This new method involved using a PCR universal reverse primer (Sfa-mut) in the same PCR assay with two forward primers one specific for the *TbAT1* wild type allele (Sfa-s) and another specific for the *TbAT1* mutant allele (Sfa-as) for detection of melarsoprol *TbAT1* mutants. However, the results correlated with those of the PCR/*Sfa NI* approach although the new method was faster and cheaper in terms of cost.

Anne *et al*. (2009) investigated whether collection of *T. brucei* infected blood from sleeping sickness patients spotted on to FTAÒ cards (Whatman) using the FTA method would enable the successful amplification by PCR and analysis of *T. brucei* microsatellite markers, the *T. brucei* drug resistance gene *TbAT1/P2* and the presence of *T. brucei* in blood. The study revealed that the method was very sensitive with the *T. brucei* multicopy locus (TBR) primers and allowed detection of a *T. brucei* infection from a DNA disc punched from an FTA card spotted with the lowest parasite dilution of 1 trypanosome/ml. However, it was observed that the high parasite density required for amplification of the single copy *TbAT1* gene, would be a limitation of the method if *T. b. gambiense* patient samples, which are known to have very low parasitaemia were to be spotted on FTAÒ cards. Otherwise the method was found to be very useful.

Anne *et al*. (2009) carried out study on *TbAT1* alleles in *T. b gambiense* isolates collected from sleeping sickness patients from areas within Arua and Moyo districts of NW Uganda. They used PCR/RFLP based method to determine if it could be useful as a predictive tool for the early detection of treatment failure or developing drug resistance. They collected 133 isolates either blood spots on FTA cards, whole blood or CSF, analysed, 91 (68 %) were successfully amplified by PCR using *TbAT1* primers and all possessed the *TbAT1*

wild type alleles after RFLP analysis with *Sfa NI* endonuclease, implying that trypanosomes in circulation have *TbAT1* alleles which showed normal melarsoprol transport as far as could be assessed with the *Sfa NI* analysis. Their finding indicates a shift from

previous results (58.5%) of the presence of *tbat1* mutant alleles or melarsoprol resistant alleles in Omugo, NW Uganda. The *TbAT1* RFLP fragments (677 bp) of 5 randomly selected samples from NW Uganda were sub-cloned and the sequencing was performed by MWG Biotech AG (Germany).

Mhairi *et al.* (2010) identified the genomic location of Tb*AT1* to be in the subtelomeric region of chromosome 5 and determined the status of the *TbAT1* gene in two trypanosome lines selected for resistance to the melaminophenyl arsenical, melarsamine hydrochloride (Cymelarsan), and in a *Trypanosoma equiperdum* clone selected for resistance to the diamidine, diminazene aceturate. In the *Trypanosoma brucei gambiense* STIB 386 melarsamine hydrochloride-resistant line, *TbAT1* was deleted, while in the *Trypanosoma brucei* brucei STIB 247 melarsamine hydrochloride-resistant and T. equiperdum diminazene-resistant lines, *TbAT1* was present, but expression at the RNA level was no longer detectable. Further characterization of *TbAT1* in *T. equiperdum* revealed that a loss of heterozygosity at the *TbAT1* locus accompanied loss of expression and that P2-mediated uptake of [^{3}H]diminazene was lost in drug-resistant *T. equiperdum*. Adenine-inhibitable adenosine uptake was detectable in a *Tbat1 T. b. brucei* mutant, although at a greatly reduced capacity compared to that of the wild type, indicating that an additional adenine-inhibitable adenosine permease, distinct from P2, was present in these cells.

Liao and Shen (2010) studied the correlative assays of effective dosage of quinapyramine on *T. b. evansi* disease between in vivo and in vitro methods which showed that their relationship was parabolic with positive correlation. On the other hand, the IC_{50} and CD_{100} values of 12 *T. b. evansi* isolates, AHB, GDB1, GDB2, HNB, JSB1, JSB2, YNB, ZJB, GDH, GXM, HBM and XJCA, collected from buffaloes, horses, mules and camels across nine provinces of China were examined using the two methods, respectively. Among them, the nine isolates, AHB, GDB1, GDB2, HNB, JSB1, JSB2, YNB, ZJB and GDH, became quinapyramine-sensitive *T. b. evansi*. Secondly, *T. evansi* populations could rapidly obtain antrycide-resistance when they were passed through immunosuppressed mice treated with low doses of the drug. But, the replication rate of trypanosomes with antrycide-resistance decreased as the level of drug-resistance increased. Thirdly, the analysis of the HK, G6PDH, ALAT and ASAT isoenzymes showed that they were not involved in the quinapyramine-resistance of *T. b. evansi*. But the protein bands of 15.79 kDa and 19.76kDa

might be involved in the antrycide-resistance of *T. b. evansi* population. At genetic level, the gene*, TbTA1*, could be amplified from the *T. b. evansi* isolate sensitive to quinapyramine-sensitivity but the *T. b. evansi* isolate with quinapyramine-resistance using not only the RT-PCR technique, but also PCR technique. They used the SSH (Suppression Subtractive Hybridization) to clone highly or low expressed cDNA fragments caused by production of antrycide-resistance in *T. b. evansi*. The 5 low and 9 high expressed new cDNA fragments were amplified.

Jane *et al*. (2013) reported the functional cloning and expression of *TcoAT1* and showed that the syntenic homologue of another *T. brucei* gene of the same Equilibrative Nucleoside Transporter (ENT) family: TbNT10. The *T. congolense* genome did not seem to contain a syntenic equivalent to *TbAT1*. Two *TcoAT1* alleles, differentiated by three independent SNPs, were expressed in the *T. brucei* clone B48, a *TbAT1*-null strain that further lacks the High Affinity Pentamidine Transporter (HAPT1); *TbAT1* was also expressed as a control. The *TbAT1* and *TcoAT1* transporters were functional and increased sensitivity to cytotoxic nucleoside analogues. However, only *TbAT1* increased sensitivity to diamidines and to cymelarsan. Uptake of [3H]-diminazene was detectable only in the B48 cells expressing *TbAT1* but not *TcoAT1*, whereas uptake of [3H]-inosine was increased by both *TcoAT1* alleles but not by *TbAT1*. Uptake of [3H]-adenosine was increased by all three ENT genes.

3. MATERIAL AND METHODS

3.1. Material

3.1.1. Experimental organism

For isolation of *RoTat* VSG and *TevAT1* genes, the experimental organism was *Trypanosoma evansi*, a blood protozoa included in Phylum *Sarcomastigophora*, Sub-phylum *Mastigophora*, Class *Zoomastigophorea*, Order *Kinetoplastida* and Family *Trypanosomatidae*. It is an important parasitic protozoon of camelids causing high morbidity and mortality.

3.1.2. Equipments

- Refrigerated micro centrifuge - Hettich, model- Mikro 22 R, Germany
- Refrigerated high-speed centrifuge - Biofuge Primo R, Heraeus, Germany
- Single Pan digital balance - Precisa, 125 A SCS, Switzerland
- Water Bath cum shaker - Aqua shake, Kuhner, Switzerland
- Incubator Shaker - Lab-Therm, Kuhner, Switzerland
- Trans illuminator - UVP, USA
- Micropipettes: 2.5, 10, 20, 100, 200 and 1000 µl capacity – Eppendorf, Germany and Nichipet, USA

- Horizontal Agarose Gel Electrophoresis apparatus with power supply - Genei, India
- Ultra low freezer (-80°C) - Model U410, NBS, USA
- Deep Freeze (-20°C) - Heto, Denmark
- Microwave oven - Kenstar Electronic Ltd., India
- Dry heating block - Thermocon, Genei, India
- Magnetic Stirrer– Remi, India
- pH meter– µ pH system 361, Systronics, India
- Incubator– Model BD-53, Binder, USA
- Horizontal laminar air flow – Telstar, AV- 30/70, USA
- Thermocycler – Mastercycler Gradient, Eppendorf, 5330, Germany
- Gel Documentation System – AlphaImager 2200, USA
- Water Purification System – Elix-Milli-Q, Synthesis Type, Millipore, USA
- UV- vis Spectrophotometer– UV mini 1240, Shimadzu, USA
- Trinocular Compound microscope, Nikon, USA

3.1.3. Chemicals

- Trizol (Invitrogen, USA)
- Ampicillin, Bovine serum fraction-V (BSA), Bromophenol blue, RNAse-A, Sodium dodecyl sulphate (SDS), Sucrose and Xylene cyanol F.F. were from Sigma Chemicals Co., St.Loius, USA
- DNAse (New England Biolabs, USA)
- DNA purification kit and Plasmid isolation kit (GE healthcare, USA)

- Proteinase K, Tris Base, Ethidium bromide, (Sigma Chemicals Co., St.Loius, USA)
- dNTPs (Larova GmbH, Germany and from Promega, USA)
- Tryptose Phosphate Broth (TPB) were from HiMedia (India)
- Glycerol and other biochemicals were from Sigma Chemicals Co., St.Loius, USA
- DNA molecular size markers were from Invitrogen, USA
- Agarose – Analytical and Preparative grades, Peptone and Yeast Extract were from Invitrogen, USA.
- Seakem Low Melting Point Agarose from Lonza, Switzerland
- Other chemicals of analytical grade were either from Sisco Research Laboratories India, Glaxo Laboratories India Ltd., or E.Merck (India) Ltd.

3.1.4. Glass and plastic ware

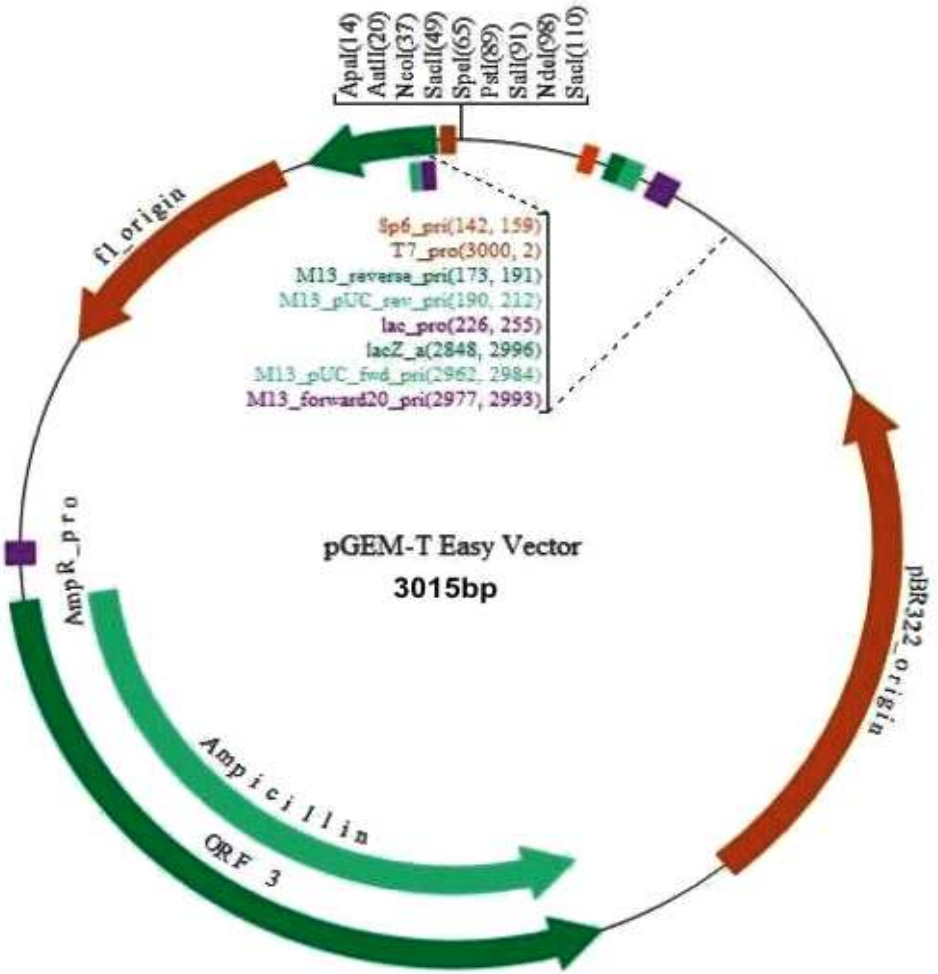
Glassware used in this study was procured either from Borosil India Ltd., or from Duran Schott, Germany. All the Glassware were thoroughly washed and sterilized as per standard protocols before use. Micro centrifuge tubes and micropipette tips were either from Axygen or Eppendorf, India Ltd.

3.1.5. Media and buffers

Composition of media, buffers and solutions used in this study is given in the Appendix No. I - III or at appropriate places.

3.1.6. Vector

pGEM- T Easy vector (Promega) available in the laboratory was employed. The vector was propagated in *Escherichia coli*, JM109 cells, extracted and used for cloning studies. The map of the pGEM- T Easy vector is shown in plate 1.


ApaI(14)
AatII(20)
NcoI(37)
SacII(49)
SpeI(65)
PstI(89)
SalI(91)
NdeI(98)
SacI(110)
f1_origin
Sp6_pri(142, 159)
T7_pro(3000, 2)
M13_reverse_pri(173, 191)
M13_pUC_rev_pri(190, 212)
lac_pro(226, 255)
lacZ_a(2848, 2996)
M13_pUC_fwd_pri(2962, 2984)
M13_forward20_pri(2977, 2993)
pGEM-T Easy Vector
3015bp
AmpR_pro
Ampicillin
ORF 3
pBR322_origin

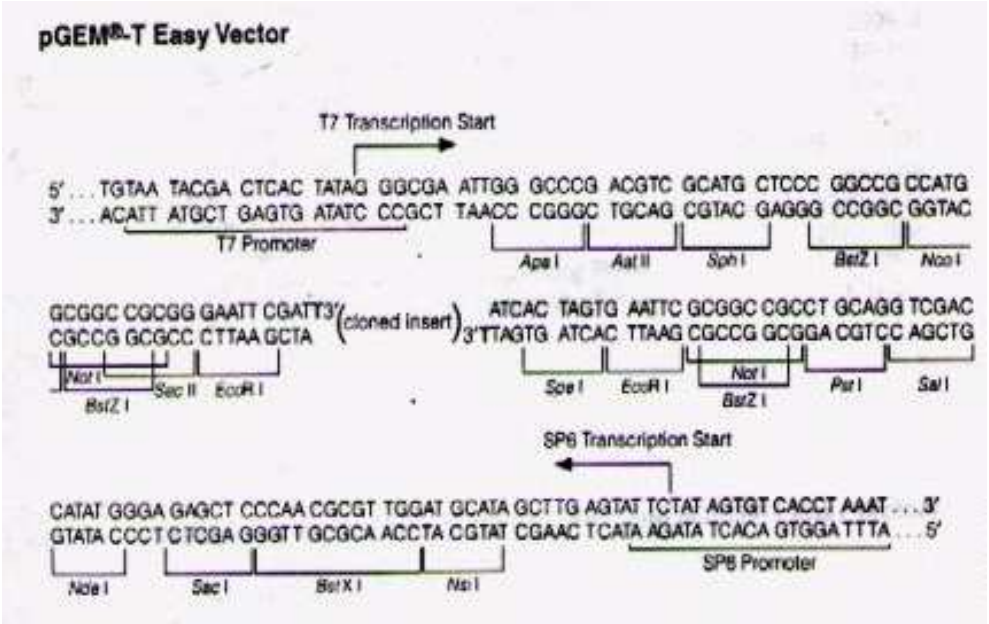


Plate 1. Map of the pGEM- T Easy vector

3.1.7. Host Systems

Escherichia coli strains JM109 (Promega) stored at –70^{0}C was used for propagation of plasmids.

3.1.8. Primers

Gene specific primers for *RoTat* VSG and *TevAT1* genes were designed from published sequences (Accession No. HQ286335 for *RoTat VSG gene* and Accession No. AB124588 for *TevAT1* gene) using the primer designing tool at NCBI. All the

primer sequences were stretched 5’ to 3’ end. The primers obtained in lyophilized form from Clontech, USA, Eurofins, India and GCC Biotech., India were reconstituted as 100 mmol. stock in sterile TE buffer. Primers at working concentrations of 10 pmol in sterile TE buffer were used for PCR amplification.

Table 3.1: Primer sequences used to amplify *RoTat VSG* and *TevAT1* genes

Genes of *T. evansi*	**Forward and reverse Primers (5’ to 3’)**
RoTat VSG	**F** 5’ CACTGCTTTACGCCATCACTC 3’ **R** 5’ GCATTCTTTTCCATCCCATTTGC 3’
TevAT1	**F** 5’ CGGGTTTGACTCAGCCAATGA 3’ **R** 5’ CGTTTTACGTTTATGTCGTGACC 3’

3.1.9. Enzymes

Restriction enzyme *Eco*R1 and *Taq* DNA polymerase used were from Promega, USA.

3.2. Methods

During the present study attempts were made to identify the *RoTat* VSG and *TevAT1* genes of *T. evansi* from camel (*Camelus dromedarius*). The procedure followed has been described in details as under:

3.2.1. Identification of *Trypanosoma evansi* infected camel

Initially, camels suspected of trypanosomosis were identified in the National Research Centre on Camel, Bikaner (Rajasthan). Blood smear from suspected animals was prepared and stained with Giemsa stain after proper fixation with methanol (Appendix-II.1). Properly stained blood films were examined under compound microscope to confirm the infection of *T. evansi* in the camels. After confirmation of *T. evansi* isolates, blood from infected host was collected. For this 5 ml blood was collected from the jugular vein using 9 ml vaccutainer tube containing EDTA (ethyl diamine tetra acetic acid). 0.5 ml blood (with the help of insulin syringe) was inoculated intraperitoneally into each experimental animal which were Swiss albino mice (maintained at Small Animal Laboratory, NRC on Camel, Bikaner).

3.2.2. Propagation of trypanosomes

Based on the infectivity titration as given by Lumsden *et al*. (1973), a convenient passage dose/interval for mice for each of the isolates of *T. evansi* was arrived at and routinely used throughout the investigation. The method consisted of examination of wet blood films from peripheral blood of the laboratory hosts (mice/rats) and scoring the degree of parasitaemia. The following scheme described by Desowitz and Watson (1951) was followed for interpretation of degree of parasitaemia:

S : Scanty infection i.e. less than one trypanosome per microscopic fields

\+ : Average 1-5 trypanosomes per microscopic fields

++ : Average 6-10 trypanosomes per microscopic fields

+++ : Average 11-20 trypanosomes per microscopic fields

++++ : Average more than 20 trypanosomes per microscopic fields

M : Massive infection i.e. trypanosomes equal or exceeding the number of erythrocytes in the field

The estimation of the working/infectivity dose for routine passage in mice was then arrived by dilution of the suspension of trypanosomes collected through tail blood (++++ or M).

3.2.3. Collection of isolates

The blood of mice was collected from heart region in 5 ml disposable syringe containing 0.1 ml heparin solution after dissecting the mice which had massive infection.

3.2.4. Purification of trypanosomes

DEAE (Diethyl amino ethyl) cellulose column chromatography method was used for purification of trypanosomes (Lanham and Godfrey, 1970). This technique resulted in a suspension of trypanosomes that was free of any other figurative elements. This

technique was based on the electrostatic characterization of DEAE- cellulose at a given pH, to fix figurative blood elements (white and red cells, platelets) and not trypanosomes.

3.2.4.1. Preparation of column

Purification of trypanosomes was essentially needed to isolate the total cellular RNA/genomic DNA and further amplification, cloning and sequencing purposes.

10 grams diethyl amino ethyl (DEAE) cellulose powder was suspended in 0.1M NaOH for 1h with intermittent stirring. The cellulose was then washed repeatedly with distilled water until the pH of the supernatant was neutral. The sediment was then mixed with an equal volume of 0.1M HCl for 1h and thereafter, washed with distilled water as described previously until the pH of the supernatant was 6.8-7.0. The slurry was stored at 4^0C until used. The slurry was equilibrated with PSG (Phosphate saline glucose) buffer (pH 8.0, Appendix–I.3) supplemented with glucose at 1% level by repeated washings. The slurry was then packed carefully to a column height of 10 cm (2.5 cm diameter) fitted with a sintered glass disc of zero porosity. The flow of elute was controlled by Teflon screw clamp fitted to the column outlet. The column gel was further equilibrated by passing PSG buffer (pH 8.0) until the pH of the eluent was 8.0.

3.2.4.2. Charging of column

Collected blood was diluted with 1:3 chilled PSG (Phosphate saline glucose) buffer (pH 8.0, Appendix–I.3) before application to the column. The diluted blood was then carefully charged through the sides of the column of the gel surface and after the blood sample entered the bed, small quantities of PSG buffer (pH 8.0) were applied. Drops of eluted product from column were examined

time to time under the microscope to spot the separated trypanosomes. The trypanosomes were collected in a beaker and were pelleted by centrifugation at 1000 rpm for 10 min. The separated trypanosomes were pooled and were either resuspended in PBS (Appendix–I.2) for use in various applications or the pellet was kept at -20^{0}C for further processing.

3.2.5. Isolation of genomic DNA from pellets of *Trypanosoma evansi*

DNA isolation from collected pellet of *Trypanosoma evansi* was done as per the method utilized by Desquesnes and Davila (2002) for the preparation of animal Trypanosomes DNA from plane blood. The procedure for DNA isolation was same as the most commonly used procedure for DNA isolation from blood suggested by Sambrook and Russel (2001) and involving four major steps:

- Lysis of *Trypanosoma evansi* pellet by using the Proteinase K buffer (Appendix–I.4), Proteinase K Enzyme and RNase A.
- Digestion of proteins.
- Extraction of DNA with Phenol and Chloroform.
- Precipitation of DNA with Alcohol.

Collected pellet of *Trypanosoma evansi* was taken in an eppendorf tube and 500 µl PK Buffer, 50 µl PK Enzyme and 20 µl RNase A were added into it and mixed gently. After proper mixing it was incubated at 56ºC for 3 to 4 hours, during incubation tapping was done time to time. After incubation, 500 µl of phenol: chloroform: Isoamyl alcohol (25: 24: 1) was added to above eppendorf tube and mixed well. The mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature. The upper phase was transferred to a fresh eppendorf tube and treated with equal volume of phenol: chloroform: Isoamyl alcohol (25: 24: 1) and mixed well. The mixture was centrifuged at 10,000 rpm for 10 minutes at room temperature. Then the upper phase was transferred into a clean eppendorf tube and 500 µl chilled chloroform was added. After proper mixing, the mixture was again centrifuged at 10,000 rpm for 10 minutes at room temperature. Upper phase was transferred in to a clean eppendorf tube and 10µl sodium acetate (3M) and 250 µl chilled ethanol (0.1 volume 3M sodium acetate and 2.5 volume ethanol) were added in it and then kept at -20°C for overnight. Next day, above eppendorf tube was centrifuged at 13, 000 rpm for 10 minutes at room temperature. The upper phase was discarded and the DNA pellet washed by 500 µl of 70% chilled ethanol then centrifuged it at 13, 000 rpm for 10 minutes at room temperature. Supernatant was poured off and the pellet was dried by Dry Bath and dissolved this DNA pellet in 50 µl of TE Buffer. Then we determined the concentration and purity of DNA sample.

3.2.6. Quantitative and Qualitative assessment of DNA

3.2.6.1. Spectrophotometric Determination

For quantifying the amount of DNA, O.D. values were recorded at wavelengths of 260 and 280 nm (The reading at 260 nm allows calculation of the concentration of nucleic acid in the sample and an O.D. of 1 corresponds to approximately 50 µg/ml for the double stranded oligonucleotides).

The DNA was diluted with TE Buffer at the ratio of 1: 50 and mixed accordingly. TE buffer was taken as a blank and respective O.D. was recorded to calibrate at zero.

After setting spectrophotometer with blank the O.D. values at 260 nm and 280 nm were recorded for DNA and protein respectively. The concentration of unknown double stranded DNA sample was estimated, using the following formula:

DNA concentration (µg/ml) = Absorbance at 260nm x dilution factor x50 = A1 X 50 X 50

The ratio between the readings at 260 nm and 280 nm (O.D. 260/280) provides estimates of purity of nucleic acid. The ratio of O.D. values of high quality DNA ranged between 1.8 and 2.

A1 / A2 = Absorbance at 260/280 = DNA/protein = 1.85

3.2.6.2. Checking of quality of DNA

The genomic DNA isolated from the *T. evansi* was checked for quality, purity and concentration. Only the DNA samples of good quality were used for further analysis.

Horizontal submarine agarose gel electrophoresis was carried out to check the quality of genomic DNA using 0.8 % w/v agarose. At the start the gel casting tray was prepared by sealing it's both ends with adhesive tape and then the comb was set over it in away to keep a gap of at least 0.5 mm between the tips of comb teeth and floor of the casting tray, so that the wells got completely sealed by agarose.

Subsequently, 0.8% agarose (w/v) suspension in 1 X TAE buffer was made and heated on an electric heater or in microwave until the agarose was completely melted and dissolved to give a clear transparent solution. After cooling it to about 50^0C, ethidium bromide (10 mg/ml) @ 5 µl per 100 ml of agarose solution was added to a final concentration of 0.5 µg/ml and was mixed gently. The agarose solution was poured into the sealed casting tray. The gel was prepared to about 4 mm thicknesses. The agarose gel was allowed to set completely at 4^0C temperature before the comb was gently removed. The adhesive tape was also detached and gel casting platform was submerged in the electrophoresis tank containing 1 X TAE buffer.

For loading the samples, 10 µl of autoclaved triple distilled water was mixed with 5 µl DNA. One µl of 6 X gel loading dye (Appendix-II.2) was mixed with 5 µl diluted DNA for loading in each well of gel. Electrophoresis was performed at 4V/cm (60-70V) for hour after loading the DNA sample into the well. Once the electrophoresis was over, the gel was visualized under UV trans-illuminator and documented by photography. Only DNA sample showing intact bands was used for further analysis.

3.2.7. Amplification of *RoTat and TevAT1* genes by PCR

Principle of PCR

The purpose of PCR (polymerase chain reaction) is to make a huge number of copies of a gene. This is necessary to have enough starting template for sequencing.

3.2.7.1. The cycling reaction:

There are three major steps in a PCR, which were repeated for 30 to 40 cycles. This was done on an automated thermo cycler, which can heat and cool the tubes with the reaction mixture in a very short time.

3.2.7.1.1. Denaturation at 94°C

During the denaturation the double strand melts and opens to single stranded DNA after all enzymatic reaction.

3.2.7.1.2. Annealing

The primers anneal around, annealing being caused by the Brownian motion. Ionic bonds are constantly formed and broken between the single stranded primers and the single stranded template.

The more stable bonds last a little bit longer (primers) that fit exactly on the little piece of double stranded DNA (primers and template.), the polymerase can attach and start copying the template. Once there are a few bases built in, the ionic bond is so strong between the template and the primer that it does not break any more.

3.2.7.1.3. Extension at 72 °C

This is the ideal working temperature for the polymerase. The primers, where there are a few bases built in, already have a stronger ionic attraction to the template than the forces breaking these attractions. Primers that are on positions with on exact match get loose again (because of higher temperature) and don’t give an extension of the fragment. The bases (complementary to

the template) are coupled to the primer on the 3' side (the polymerase add dNTP's from 5' to 3', reading the template from 3' to 5'side, bases are added complementary to the template). Because both of strands are copied during PCR there is exponential increase of the number of copies of the gene.

3.2.7.2. Optimization of PCR Parameters

Various combinations of reaction chemical were tried to optimize the concentration of each component. The PCR parameter, viz. annealing temperature [2(A+T) +4(G+C)] and cycling conditions were optimized to obtain a specific amplified product in sufficient quantity. The reaction volume was kept constant at 50 µl. 4 times volume of reaction mixture was prepared as master mix, and after proper mixing 50 µl reaction mixture was divided in four PCR tubes. The standardized concentrations of components used in the reaction mixture are given in Table 3.2 – 3.4.

Table 3.2: PCR reaction mixture for *RoTat VSG* gene

Components for Master mix	Vol. / reaction	Final conc. / reaction
5X Flexi PCR Buffer	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM of each dNTP
$MgCl_2$ (25 mM)	3 µl	2.5 mM of Mg^{2+}
Primer F	1 µl	10 pM
Primer R	1 µl	10 pM
Template DNA	0.5 µl	100ng
Taq DNA polymerase	0.25 µl	1.5 Units

Distilled Water	33.25 µl	-
Total volume	**50 µl**	-

Table 3.3: PCR reaction mixture for *TevAT1* gene

Components for Master mix	Vol. / reaction	Final conc. / reaction
5X Flexi PCR Buffer	10 µl	1x
dNTP mix (10 mM each)	1 µl	200 µM of each dNTP
$MgCl_2$ (25 mM)	3 µl	2.5 mM of Mg^{2+}
Primer F	1 µl	10 pM
Primer R	1 µl	10 pM
Template DNA/c-DNA	0.5 µl	100ng
Taq DNA polymerase	0.25 µl	1.5 Units
Distilled Water	33.25 µl	-
Total volume	**50 µl**	-

PCR amplification was carried out in thermal cycler using the optimized condition as given in Table 3.2 - 3.4.

3.2.7.3. Protocol for amplification of *RoTat* and *TevAT1* genes of *T. evansi* by PCR

At first, a PCR master mixture, containing all the reaction components except genomic DNA/c-DNA, was prepared in ice under sterile condition. Care was taken to add *Taq* DNA polymerase at the end of preparation. After addition of all the components, the master mix was mixed gently, followed by spinning by table top micro centrifuge. Then, 49.5µl of master mix was added to each pre labeled PCR tubes (eppendorf tube) of 0.2 ml capacity. Thereafter, 0.5µl of good quality genomic DNA/c-DNA was added to each tube. Finally, the PCR tubes were arranged in a preprogrammed thermo cycler. PCR products obtained, after the completion of the programme, were kept at 4^0C in refrigerator for further analysis.

3.2.7.4. PCR programme

Several combinations of PCR programmes were tried before finalizing one programme giving the best amplification of the desired fragments. The standardized programmes for different genes are given below.

Table 3.4: PCR conditions for *RoTat VSG* gene

Step	Temperature	Time	No. of cycle
I. Initial Denaturation	94^{o}C	4 min.	One
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 94^{o}C 51^{o}C 72^{o}C	 30 sec. 45 sec. 1 min. and 30 sec.	 Over all total 36 cycle
III. Final extension	72^{o}C	10 min.	One
IV. Hold	4^{o}C		
Thermal Cycler lid temperature = 105^{o}C			

Table 3.5: PCR conditions for *TevAT1* gene

Step	Temperature	Time	No. of cycle

I. Initial Denaturation	94^{o}C	4 min.	One
II. Cycle (i) Denaturation (ii) Annealing (iii) Synthesis	 94^{o}C 49^{o}C 72^{o}C	 30 sec. 45 sec. 1.30 min.	 Over all total 36 cycle
III. Final extension	72^{o}C	10 min.	One
IV. Hold	4^{o}C		
Thermal Cycler lid temperature = 105^{o}C			

3.2.7.5. Analytical Agarose Gel Electrophoresis

PCR amplified DNA was analyzed by analytical agarose gel electrophoresis as per the procedure described by Sambrook and Russel (2001). The procedure of electrophoresis was same as mentioned in qualitative confirmation of DNA to be analyzed was charged into wells of gel alongside DNA molecular weight marker for the confirmation of molecular size of the DNA bands in relation to molecular weight marker.

3.2.7.6. Elution of DNA from low melting point agarose gel (preparatory)

DNA required for various manipulations was purified after electrophoresing the DNA in preparatory agarose gel. The method of gel preparation and casting were essentially the same as was done with the analytical method except for the usage of 1% Low Melting Point (LMP) Agarose in place of the analytical grade used earlier. The DNA was electrophoresed at a constant low voltage

of 4mV/cm to facilitate clear separation of the DNA bands. The agarose slice containing the DNA band of interest was carefully excised from the gel under UV illumination with the help of a sterile scalpel and used for elution of the DNA within.

3.2.7.7. DNA purification (illustra GFX PCR DNA and Gel Band Purification Kit) method

DNA from LMP agarose slices was purified using illustra GFX PCR DNA and Gel Band Purification Kit involving the following steps

3.2.7.7.1. Sample capture

Capture buffer type 3 was added to the weighed agarose gel slice in accordance to the weight of agarose slice. It was mixed by inversion and incubated at 60^0C for 15-30 minutes until the agarose was completely dissolved. When the agarose was completely dissolved the colour of capture buffer type 3-sample mix was checked and that was yellow. Then it was centrifuged for collecting the liquid at the bottom of the tube.

3.2.7.7.2. Sample binding

One GFX Micro spin column was placed into one collection tube. Then up to 800 µl of capture buffer type 3-sample mix was transferred on to the assembled GFX Micro spin column and collection tube. It was incubated at room temperature for 1 minute and

centrifuged at 16000 x g for 30 seconds. The flow through was discarded by emptying the collection tube and placed the GFX Micro spin column back inside the collection tube. Simple binding steps were repeated until total samples volume was loaded.

3.2.7.7.3. Wash and dry

500 µl Wash buffer type 1 was added to the GFX Microspin column and then the assembled column and collection tube was spined at 16000 x g for 30 seconds. Collection tube was discarded and the GFX Microspin column was transferred to a DNase free 1.5 ml micro centrifuge tube.

3.2.7.7.4. Elution

50 µl elution buffer type 4 was added to the center of the membrane in the assembled GFX Microspin column and simple collection tube and then incubated at room temperature for 1 minute. Thereafter assembled column and sample collection tube was spined at 16000 x g for 1 minute to recover the purified DNA. Purified DNA was stored at -20^{0}C for further use.

3.2.8. Cloning of DNA fragments

Cloning of DNA fragments into pGEM- T Easy vectors involved the following steps, which are described below.

3.2.8.1. Ligation of DNA fragment with pGEM- T Easy vector

The DNA fragment of *RoTat VSG and TevAT1* gene and the pGEM- T Easy vector in which it is to be cloned were digested with appropriate enzyme to generate compatible ends for ligation. The individual DNAs were mixed so as to have a vector and insert DNAs in the ratio of 1:3 in case of sticky ends and 1:5 to 1:10 in case of blunt ends. The ligation was done (as per the

Promega protocol with slight modification) in the reaction volume of 20 µl containing 10µl of 2X Rapid ligation T4 DNA Ligase buffer [400mM Tris-HCl, 100mM $MgCl_2$, 100mM DTT, 5mM ATP (pH 7.8 at 25^0C)], 6 µl PCR product, 2 µl pGEM- T Easy vector and 2 µl of T4 DNA ligase. The contents were vortexed, spun down in a micro centrifuge for 3-5 seconds and incubated for overnight at 4^0C. The ligation mix was used directly for transformation and unused ligation mixture was stored at –20^0C for future use.

3.2.8.2. Transformation

The frozen aliquots of JM109 high efficiency competent cells was removed from -70^oC storage and placed in an ice bath thawing (for 5-10 min.). Competent cells were mixed by gentle flicking the tube. 2 µl ligation mixture and 50 µl JM109 competent cells were mixed in 1.5 ml eppendorf tube and placed the tube in ice for 20 minutes. It was given heat shock at 42^0C for 45 sec before snap cooling on ice for 2 minutes. Immediately thereafter 950 µl of pre warmed SOC (Appendix–III.6) was added to the cells and the suspension was incubated at 37^0C for 1 hr and 30 min. with shaking of 150 rpm in a shaking incubator. 100 µl IPTG and 20 µl X-GAL was spreaded over the surface of LB agar plates with ampicillin (50µg/ml). After incubation of transformation culture 100 µl of each transformation culture was plated onto antibiotic agar plates in duplicate and incubated at 37^0C for overnight (16-20 hr). Colonies that appeared were screened for the presence of plasmids.

3.2.8.3. Screening for recombinants

Both white and blue colonies were grown in the plate. Larger single white colonies were picked up individually and inoculated into LB broth (Appendix–III.3) containing final concentration of ampicilin (50µg/ml) and kept in a water bath cum shaker (at 37^oC and 150 rpm) for 16 hrs. Subsequently, the plasmid DNA was isolated from all the bacterial cultures using the kit.

3.2.8.4. Protocol for plasmid preparation (by using illustra plasmid prep mini spin kit)

3.2.8.4.1. Harvesting of bacterial culture

1.5 ml bacterial culture was taken in eppendorf tube and centrifuged for 30 second at 16000 x g., poured off and supernatant was discarded.

3.2.8.4.2. Lysis

175 µl lysis buffer type 7 was added and pellet was resuspended. Then 175 µl lysis buffer type 8 was added and mixed by gentle inversion till solution became clear and viscous. After 5 min of lysis reaction 350 µl lysis buffer type 9 was added and mixed by gentle inversion till percipitate was evenly dispersed. Then it was centrifuged for 4 min at 16000 x g.

3.2.8.4.3. Plasmid binding

Supernatant was transferred into plasmid mini column inside collection tube. Then it was centrifuged at 30 sec at 16000 x g and flow through was discarded.

3.2.8.4.4. Wash and dry

400 µl Wash buffer type 1 was added to the plasmid mini column, then the assembled column and collection tube were spun at 16000 x g for 30 seconds. Flowthrough and collection tube was discarded and the plasmid mini column was transferred to a new DNase-free 1.5 ml micro centrifuge tube.

3.2.8.4.5. Elution

100 µl elution buffer type 4 was added to the center of the membrane in the assembled plasmid mini column and simple collection tube then incubated at room temperature for 30 sec. Thereafter assembled column and sample collection tube were spun at 16000 x g for 30 sec to recover the purified plasmid DNA. Purified plasmid DNA was kept at -20^{0}C for storage.

3.2.8.5. Confirmation of clones

Confirmation of clones was done by Restriction Enzyme digestion of plasmid DNAs and Colony PCR of plasmid colonies.

3.2.8.5.1. Restriction Enzyme Digestion (to check the insert size)

After checking the quality of the plasmid DNAs in agarose gel electrophoresis, they were subjected to restriction enzyme digestion using *Eco*R1. In 10 µl digestion mixture 5 µl plasmid DNA, 1 µl *Eco*R1 (Promega), 1 µl *Eco*R1 buffer (Promega) and 3 µl ultrapure water was added. After 4 hrs. digestion at 37^{o}C in water bath, 2µl 6X loading dye was added to the mixture and analyzed by running 1.2% agarose gel electrophoresis alongside a DNA molecular weight marker. Release of the expected size fragment confirmed the recombinants.

3.2.8.5.2. Colony PCR

This protocol was designed to quickly screen for plasmid inserts directly from *E. coli* colonies. Colonies were screened for recombinants by colony PCR as per procedure described in Promega protocols. PCR was carried out in the presence of insert

specific primers. The colony PCR reaction mixture was similar to PCR reaction mixture, only template DNA was not added. To each PCR tube containing the PCR reaction, a single colony was added. For each amplification reaction white colonies were added in two PCR tubes and blue colony was added in one tube. A fine yellow pipette tip attached to a pipetter was used to separate a colony from culture and pipetted up and down to mix the colony into PCR reaction mixture (The amount of cells was small, just a touch was done, the small amount required to fill the end of the opening was sufficient). Sufficient mixing of the colony in PCR tube was done for complete cell lysis and high yields. The conditions of amplification applied were similar to those applied for the amplification of the specific genes. The amplified products were analyzed by agarose gel electrophoresis using standard molecular size markers.

3.2.9. Sequencing

Purified plasmids of *RoTat VSG* and *TevAT1* genes were got sequenced from Eurofins Genomics India Pvt Ltd., Whitefield, Bangalore. The sequence obtained was then matched using BLAST (Biological Local Alignment Search Tool) software. After confirmation of the *RoTat and TevAT1* genes nucleotide sequences of *T. evansi* isolated from the host camel (*Camelus dromedarius*), the nucleotide sequences were submitted to GenBank, NCBI database.

3.2.9.1. Sequence analysis

After getting the accession numbers of individual gene sequences Phylogenetic and sequence analysis of the *RoTat VSG* and *TevAT1* genes of *T.evansi* was done. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Phylogenetic tree analysis of *RoTat VSG* and *TevAT1* genes was done by using Neighbor-Joining (NJ) method and maximum parsimony (MP) method and implemented with bootstrap test involving simple stepwise addition.

3.2.9.2. Multiple sequence alignments

The amino acid sequences of the *RoTat VSG* and *TevAT1* genes from *T. evansi* were BLASTed against similar sequences in the public database (http://www.ncbi.nlm.nih.gov/). The sequences of *RoTat VSG* proteins of *T. evansi* (GenBank Accession No. HQ286335, HQ286334, HQ286333 and AF317915), *T. brucei* (GenBank Accession No. L34415, V01387), *T. evansi* (GenBank Accession No. EF067843), *T. brucei* (GenBank Accession No. S62479 and KC613489) were collected and sequence homology between species determined. For sequence homology determination of *TevAT1* protein the sequences of *T. brucei* (GenBank Accession No. AF152369 and AF152370), *T. evansi* (GenBank Accession No. AB124588), *T. equiperdum* (GenBank Accession No. AJ278417 and AJ278418), *T. brucei* (GenBank Accession No. XM838562, XM822640, XM840059 and XM946663). were collected. Multiple sequence alignments of obtained protein sequences of *RoTat VSG* and *TevAT1* genes were performed with Clustal W program version 2.1 at EBI (expasy proteomics tools) with default parameters.

4. RESULTS

Camel trypanosomosis is the most important single cause of economic losses in camel and has been observed in domestic and wild animals and recent reports suggest its ability to infect humans with widest geographical range. Chemotherapeutic, chemoprophylactic and fly control strategies are not quite enough to control this disease and beside the increasing incidence of parasite resistance to available drugs. Recent effort towards the development of a vaccine against *Trypanosoma evansi* has identified several promising candidate vaccine antigens, including non-variant genes of this parasite. Keeping this in view, the present study was undertaken to amplify the *RoTat and TevAT1* genes of *T. evansi* from camels by Polymerase Chain Reaction (PCR), clone the amplicons in a suitable plasmid vector and sequence the genes as these candidate genes could be helpful in future for developing vaccines against the organism. The results obtained are as follows:

4.1. Identification of Camel (*Camelus dromedarius*) infected with 'surra' (*Trypanosoma evansi*) infection

Initially, the camel suffering from 'surra' disease was identified by its gross characteristics and the infected host with *T. evansi* has been shown in Plate 2.

4.2. Stained blood film preparation of *T. evansi*

A Giemsa stained blood smear of *T. evansi* collected from the infected camel was prepared to confirm the infection and has been presented in Plate 3.

4.3. Propagation of *T. evansi* in mice and its purification

The *T. evansi* collected from camel blood were propagated in mice (Giemsa stained blood smear of mice blood shown in Plate 4) and were then purified using DEAE-cellulose chromatography method and were stained with Giemsa stain as shown in Plate 5.

4.4. Isolation and qualitative confirmation of genomic DNA

The total genomic DNA was isolated from the pellets of *T. evansi* using Proteinase K digestion and subsequent phenol-chloroform extraction method as mentioned in materials and methods. The genomic DNA was analyzed in 0.8% analytical agarose gels and was found to be intact without much smearing and has been presented in Plate 6.

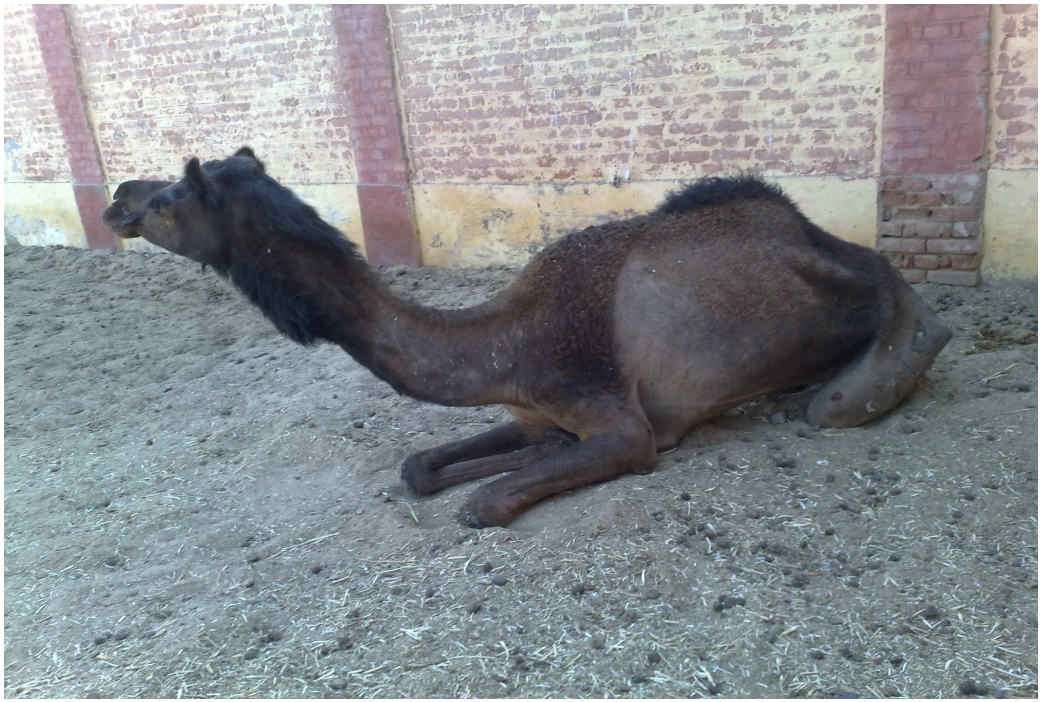


Plate 2. Camel infected with ‘surra’ disease

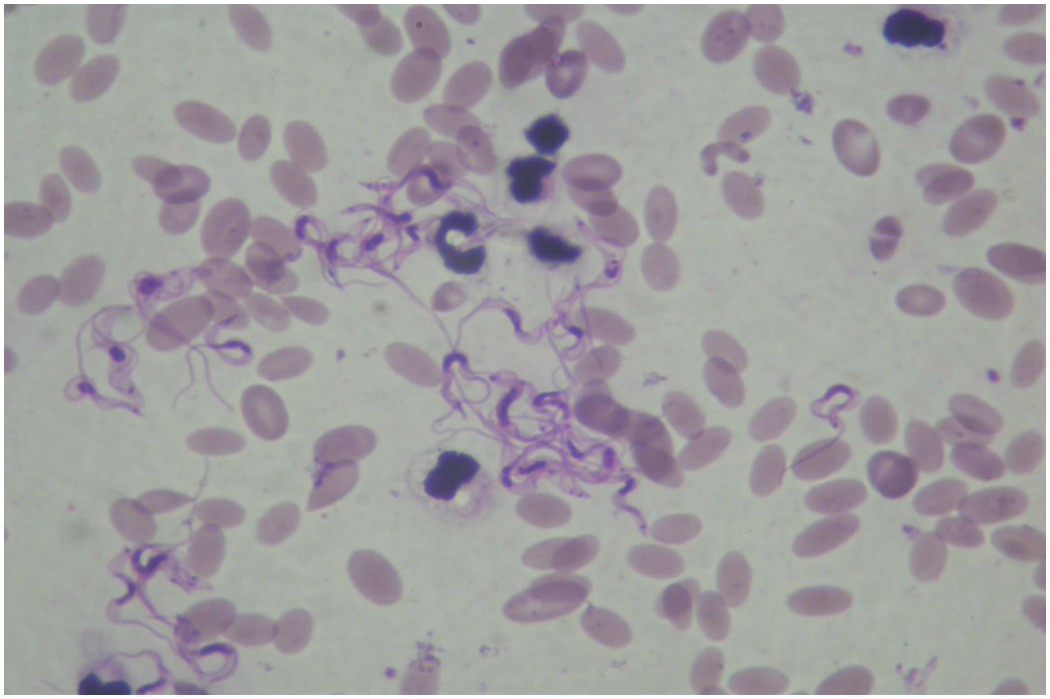


Plate 3. Giemsa stained blood smear of *T. evansi* from camel

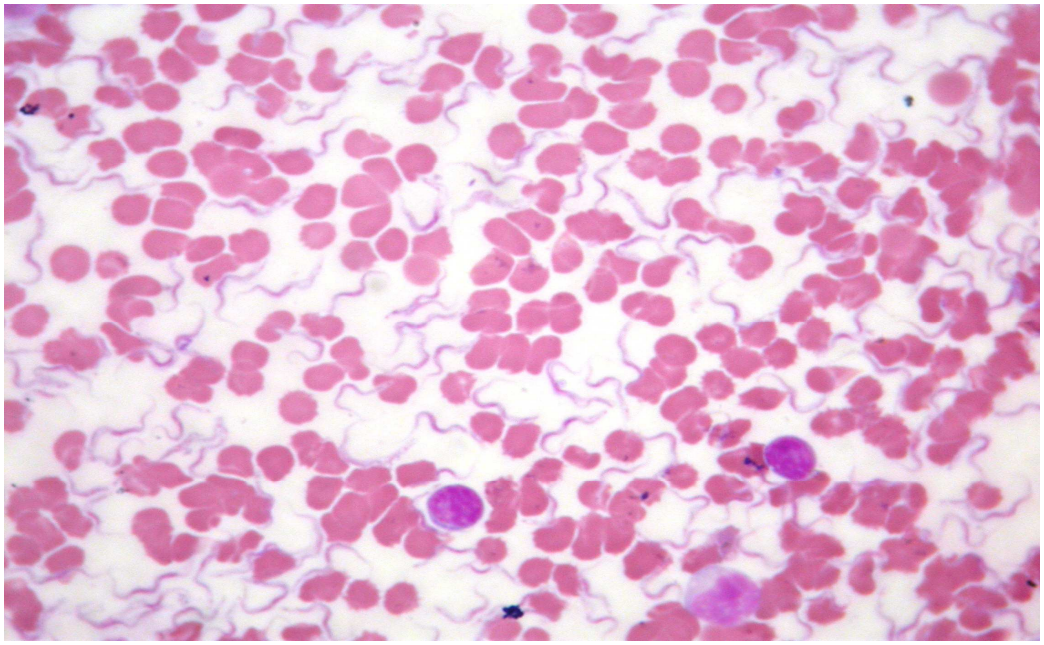


Plate 4.Giemsa stained blood smear of *T. evansi* from mice

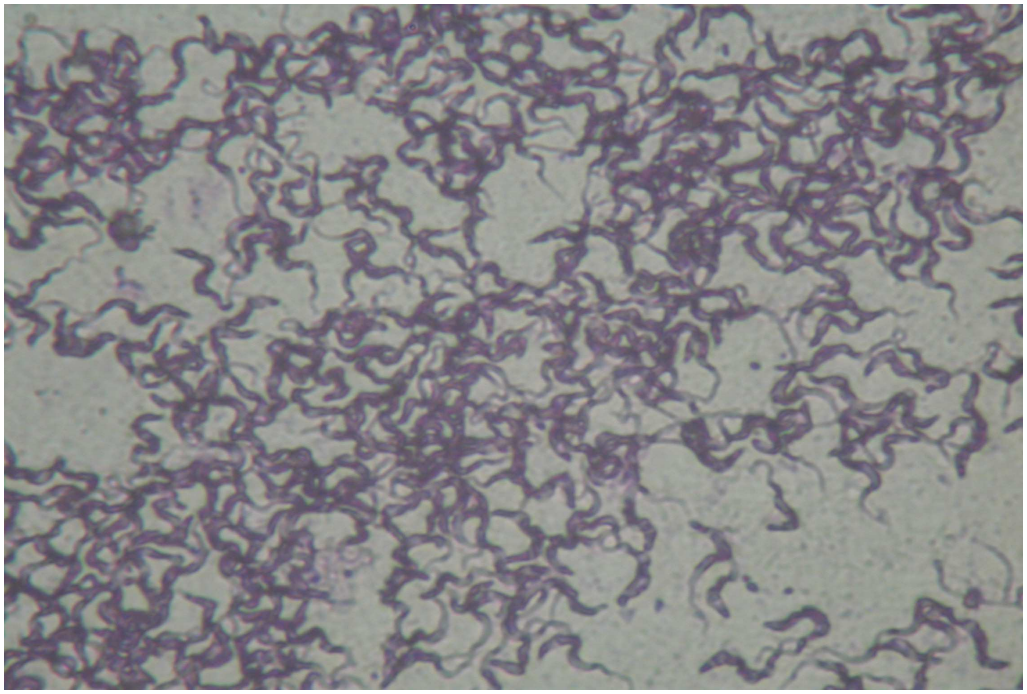


Plate 5. Purified and Giemsa stained *T. evansi*

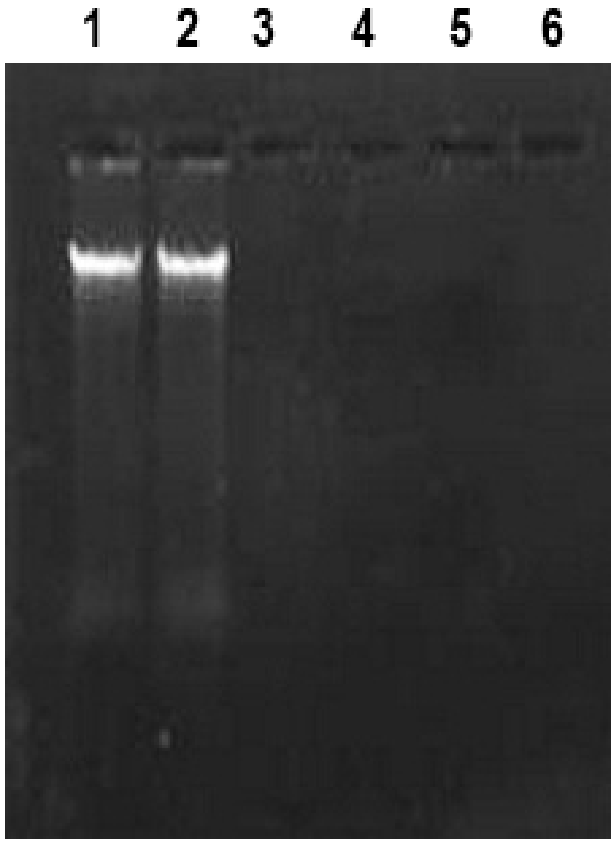


Plate 6. **Ethidium bromide stained 0.8% agarose gel showing genomic DNA of *Trypanosoma evansi* in lane 1 and 2**

4.5. Amplification of *RoTat* and *TevAT1* genes of *T. evansi* by PCR

Total genomic DNA was isolated from the pellets of *T. evansi* and used as template for amplification by PCR. Gene specific forward and reverse primers were used for amplification and the amplicons analyzed by agarose gel electrophoresis as per protocol mentioned in the materials and methods. To confirm the size of amplicons, the sample (amplicon) was run on gel electrophoresis using molecular weight marker (10 kbs plus DNA ladder). The amplification band of *RoTat* gene was obtained in between 1000 bp and 3000 bp (1450 bp) which has been presented in Plate 7. The amplification band of *TevAT1* gene was obtained in between 500 bp and 1500 bp (1413 bp) which has been presented in Plate 8.

4.6. Cloning of RoTat *VSG* and *TevAT1* genes of *T. evansi* into pGEM-T Easy vector

The amplicons (obtained from genomic DNA amplification) were purified from the LMP agarose gel and ligated with pGEM-T Easy vector (Promega). 100 µl of transformation culture was plated onto X-gal-IPTG-Ampicillin agar plate. There were several white colonies along with a few blue colonies (plate 9). The blue colonies represent the presence of vector alone but few blue

colonies may contain vector with insert. The white colonies represented recombinant clones of carrying insert in the plasmid. The white colonies were screened for the presence of vector with insert.

4.6.1. Confirmation of clones by restriction digestion

Plasmid DNAs were extracted from positive colonies grown in LB medium containing ampicillin, digested with *Eco*RI and analyzed by 1.2% analytical agarose gel electrophoresis using 10 kb plus molecular weight marker. Two well separated DNA bands were seen in case of plasmids isolated from positive colonies upon digestion with *Eco*RI (lanes 2 & 3), the less intense lower band may correspond to the insert. Release of DNA fragments of around 1450 bp for *RoTat VSG gene and* 1413 bp for *TevAT1* gene was found after restriction enzyme digestion, the results of which have been presented in Plate 10 and 11.

4.6.2. Confirmation of clones by Colony PCR

Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies and results were analyzed by agarose gel electrophoresis using 10 kb plus molecular weight marker. For *RoTat VSG* gene amplifications was found in wells of white colonies but for *TevAT1* gene amplification was also found in blue colony (Plate 12 and 13).

4.7. Sequencing

After confirmation of clones of *RoTat* and *TevAT1* genes the plasmid DNAs along with their respective forward and reverse primers were sent to Eurofins Genomics India Pvt Ltd., Whitefield, Bangalore, for getting the sequences. The coding sequences of *RoTat* and *TevAT1* genes according to the results obtained were of 1450 bp and 1413 bp, respectively. These sequences were then matched using BLAST software. After confirmation of the *RoTat* and *TevAT1* genes nucleotide sequences of *T. evansi* isolated from the host camel (*Camelus dromedarius*), the sequences were submitted to GenBank, national center for biotechnology informations (NCBI) database to which the assigned accession numbers are KF313141for *RoTat VSG gene* (Plate 14) and KF280206 for *TevAT1* gene (Plate 17).

4.7.1. Sequence analysis

Sequence analysis revealed that the length of the coding sequences of *RoTat VSG* and *TevAT1* genes of *T. evansi* from Bikaner, India were 1450 bp and 1413 bp, respectively. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Tree topology is based on the Neighbor-Joining (NJ) method with100% bootstrap values. The NJ, bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic tree analysis of *RoTat VSG* and *TevAT1* gene using maximum parsimony (MP) also showed same topology as NJ method. The results, along with the assigned accession numbers and the names of accession, countries and workers who submitted them are presented in Plate 15a, 15b, 18a, 18b, and Tables 4.1 to 4.4.

4.7.2. Multiple sequence alignment

Multiple sequence alignment of obtained protein sequences of *RoTat VSG* and *TevAT1* genes was performed with Clustal W at EBI (expasy proteomics tools). The results of amino acid alignments (Clustal 2.1 multiple sequence alignment) are presented in Plate 16(page 82-84) and 19(page 90-92). The asterisks below the alignments indicate the positions of highly conserved amino acid residues.

The obtained *RoTat VSG* protein sequence of *T. evansi* was of 483 amino acids (GenBank Accession No. KF313141) which showed 100% amino acid sequence identity of *T. evansi*, GenBank Accession No. HQ286335, GenBank Accession No HQ286334 and GenBank Accession No HQ286333, 99% of T. evansi GenBank Accession No. AF317915. and *T. brucei*, GenBank Accession No. L34415, 65% of *T. brucei* (GenBank Accession No. V01387, 58% of *T. brucei* (GenBank Accession No. EF067843 , 56% of *T.brucei* (GenBank Accession No.KC613489) and 55% *T. evansi* (GenBank Accession No. S62479).

Obtained *TevAT1* protein sequence of *T. evansi* was of 463 amino acids (GenBank Accession No. KF280206) which showed 99% amino acid sequence identity of *T. brucei*, GenBank Accession No. AF152369, *T. evansi* GenBank Accession No. AB124588 and *T. equiperdum* GenBank Accession No.AJ278417, 98% of *T. brucei* (GenBank Accession No. AF152370), *T. equiperdum* (GenBank Accession No. AJ278418, *T. brucei* (GenBank Accession No. XM838562) and *T.brucei* (GenBank Accession No. XM840059) , 66% of *T. brucei* (GenBank Accession No. XM946663) and *30% of T. brucei* (GenBank Accession No. XM822640).

Obtained *RoTat VSG* gene sequence of *T. evansi* was of 1450 bp (GenBank Accession No. KF313141) which showed99% homology towards *T. evansi*, GenBank Accession No. HQ286335, HQ286334, HQ286333 and AF317915, 98.6% similarities was found with *T brucei.* GenBank Accession No. L34415 and V01387. 69.5% homology was found between obtained *RoTat VSG* sequence and *T. brucei*, GenBank Accession No. KC613489 and 68.5 homology with *T. brucei* GenBank Accession No.S62479. Lower homology was found between the obtained *RoTat VSG* gene sequence and *T. evansi*, GenBank Accession No. EF067843.

Obtained *TevAT1* gene sequence of *T. evansi* was of 1413 bp (GenBank Accession No. KF280206) which showed 99.9% homology with *T. brucei*, GenBank Accession No. AF152369, 99.8% with *T. equiperdum* GenBank Accession No.AJ278417, 99.7 with *T. evansi* GenBank Accession No. AB124588, 99.3% with *T. equiperdum* (GenBank Accession No. AJ278418, 99.2% with *T. brucei* (GenBank Accession No. AF152370, 73.8% with *T. brucei* (GenBank Accession No. XM838562), 71% with *T. brucei* (GenBank Accession No. XM822640), 68.5% with *T. brucei* (GenBank Accession No. XM946663) and 65.8% with *T.brucei* (GenBank Accession No. XM840059).

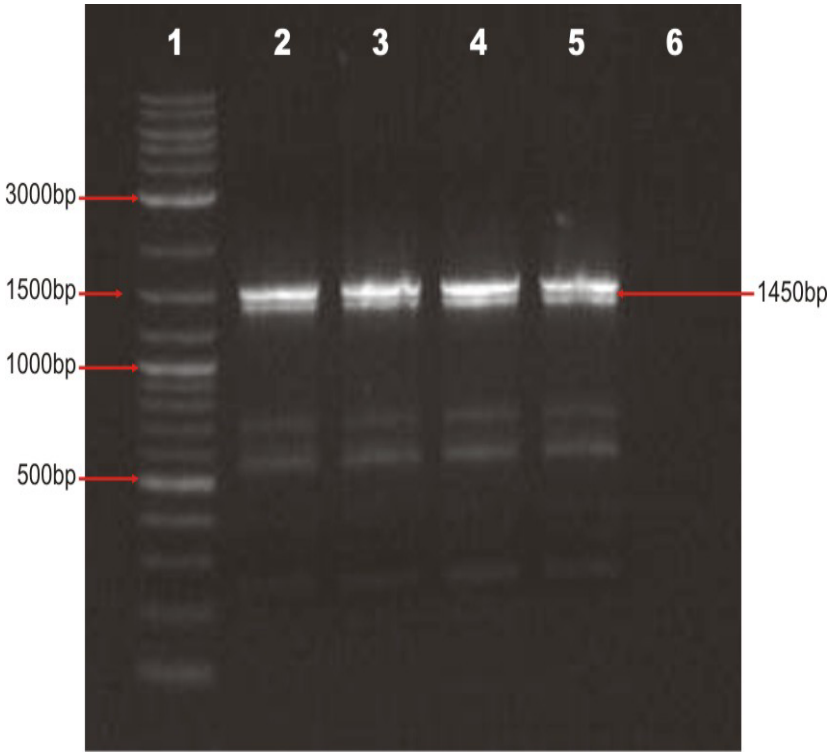


Plate .7. Amplification of *RoTat* VSG gene of *T. evansi* by PCR

Lane1. 10 Kb plus DNA Ladder **Lane2 – 5. Amplicons**

Lane 6. ***control***

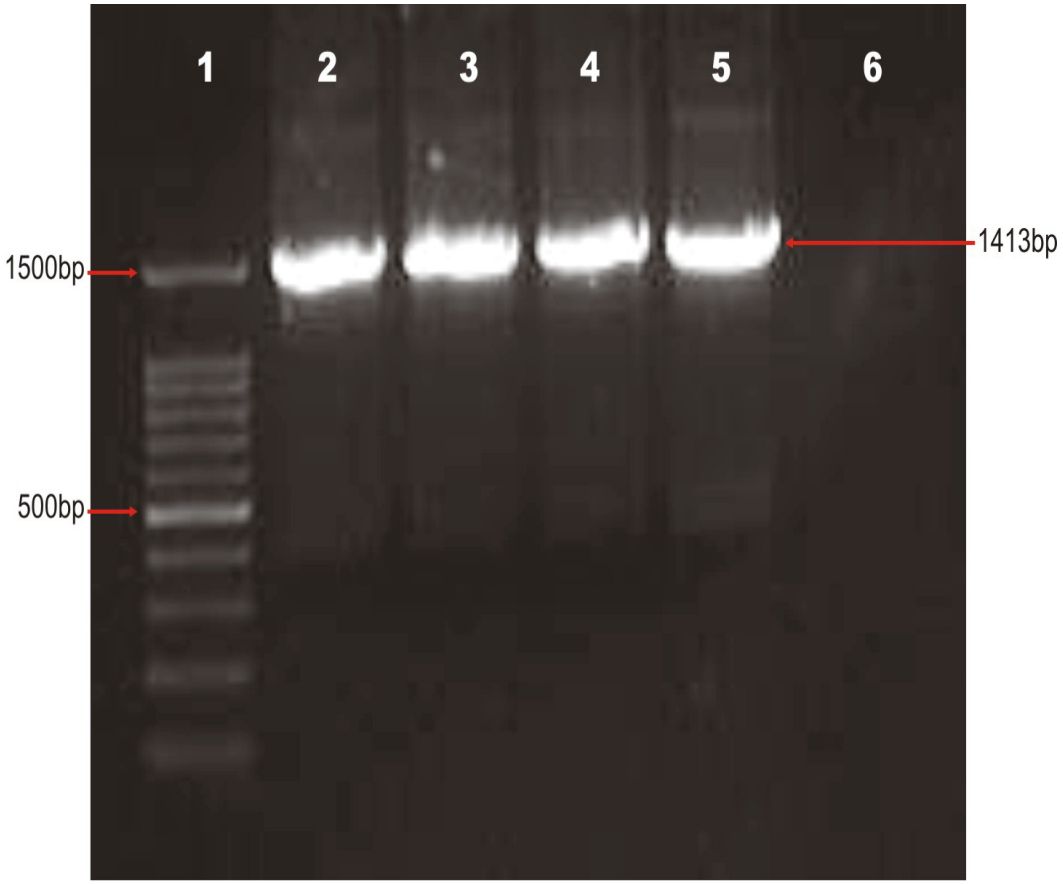


Plate 8. Amplification of *TevAT1* gene of *T. evansi* by PCR
Lane 1. 10 Kb plus DNA Ladder
Lane 2 – 5. Amplicons

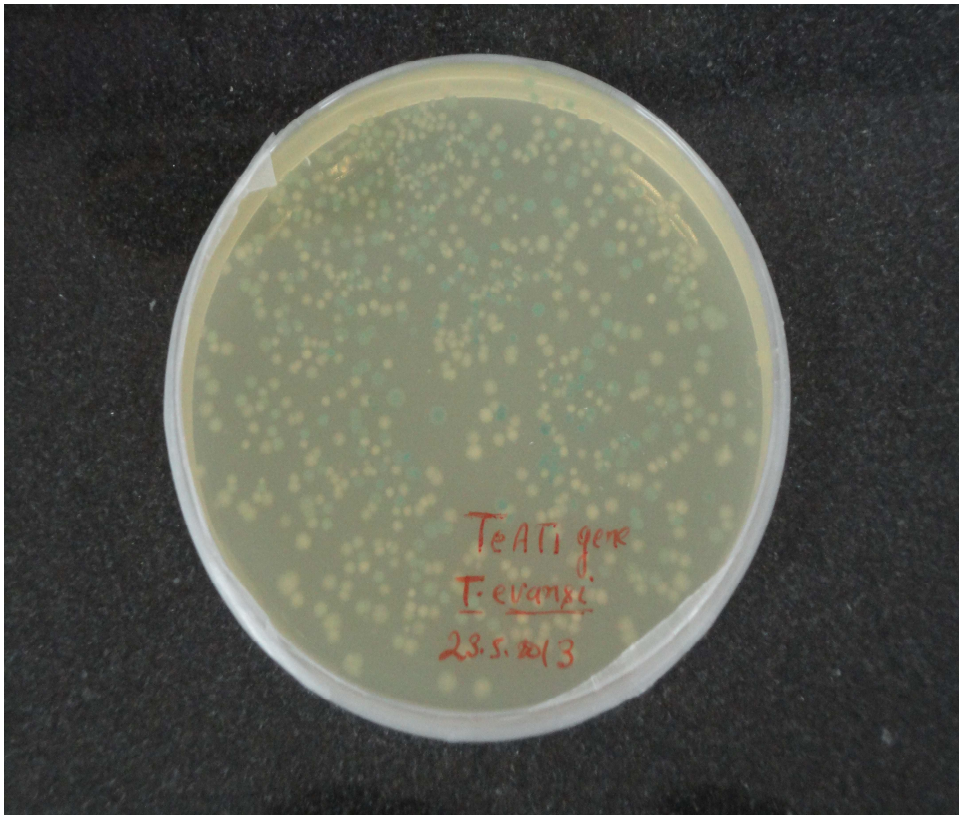


Plate 9. Appearance of Blue and white colonies in LB agar plate showing culture of *Trypanosoma evansi adenosine transporter 1* gene of *T. evansi*

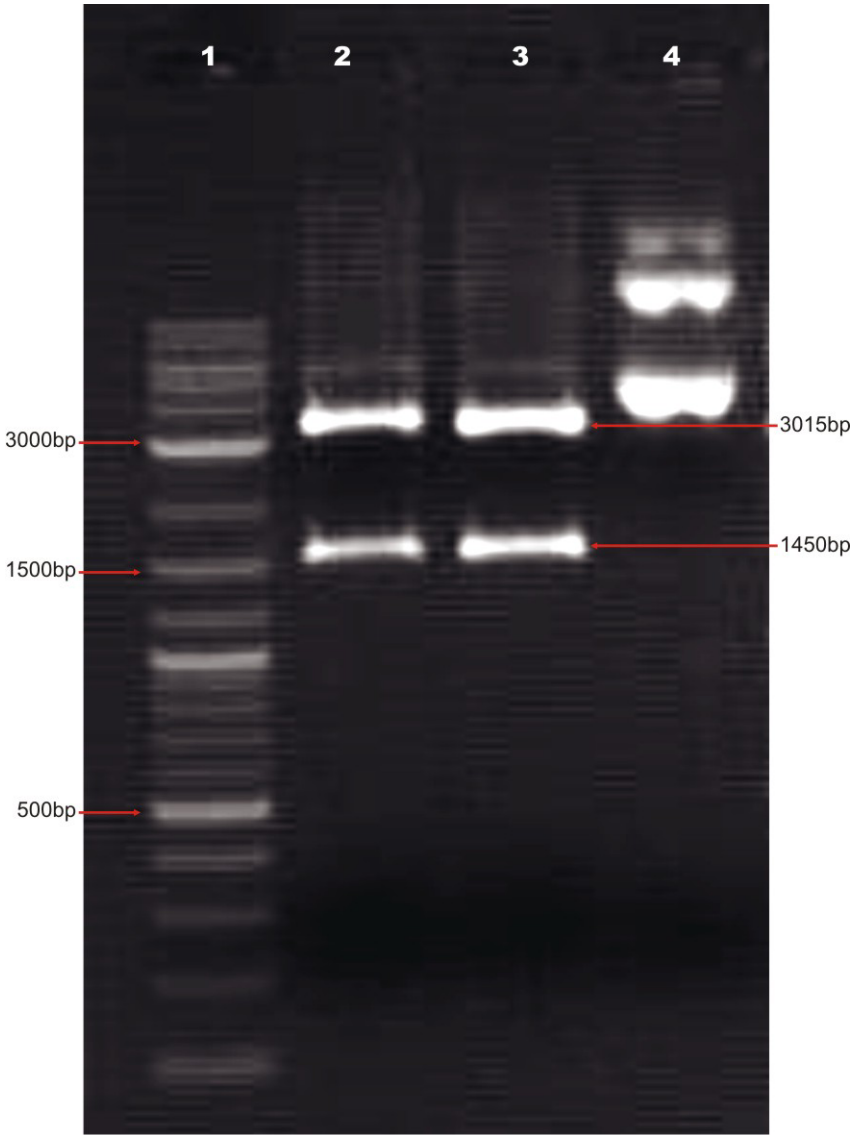


Plate 10. *RoTat VSG* gene fragments of *T. evansi* after restriction digestion of *RoTat VSG* gene plasmid

Legends

Lane 1. 10 Kb plus DNA Ladder **Lane 2 – 3. *RoTat* VSG gene clone**

Lane 4. Uncut plasmid

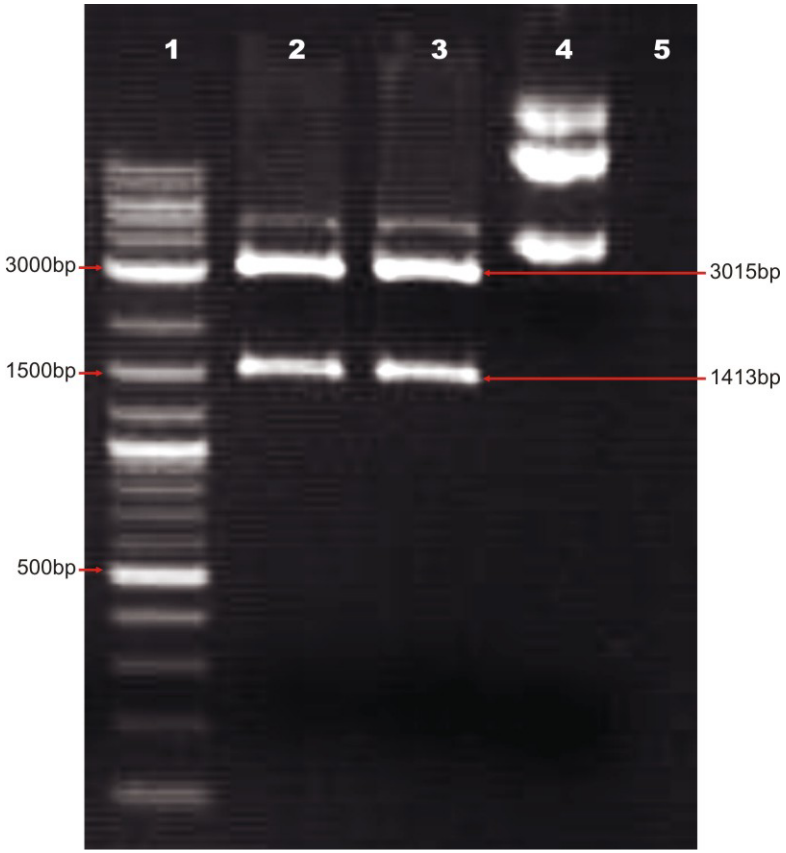


Plate 11. *TevAT1* gene fragments of *T. evansi* after restriction digestion of *TevAT1* gene plasmid

Legends

Lane 1. 10Kb plus DNA Ladder **Lane 2 – 3. *TevAT1* gene clone**

Lane 4. Uncut plasmid

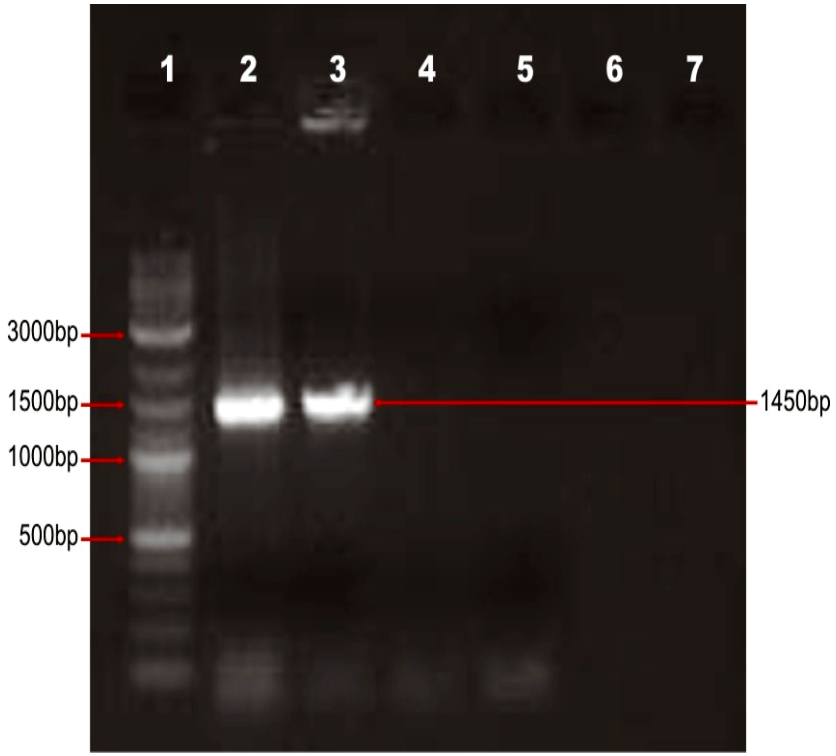


Plate 12. Amplification of *RoTat* VSG gene of *T. evansi* by Colony-PCR

Lane 1. 10 Kb plus DNA Ladder Lane 2 – 3. PCR reaction with white colony shows Amplification
Lane 4. PCR reaction with blue colony shows absence of Amplification
Lane 5. Control

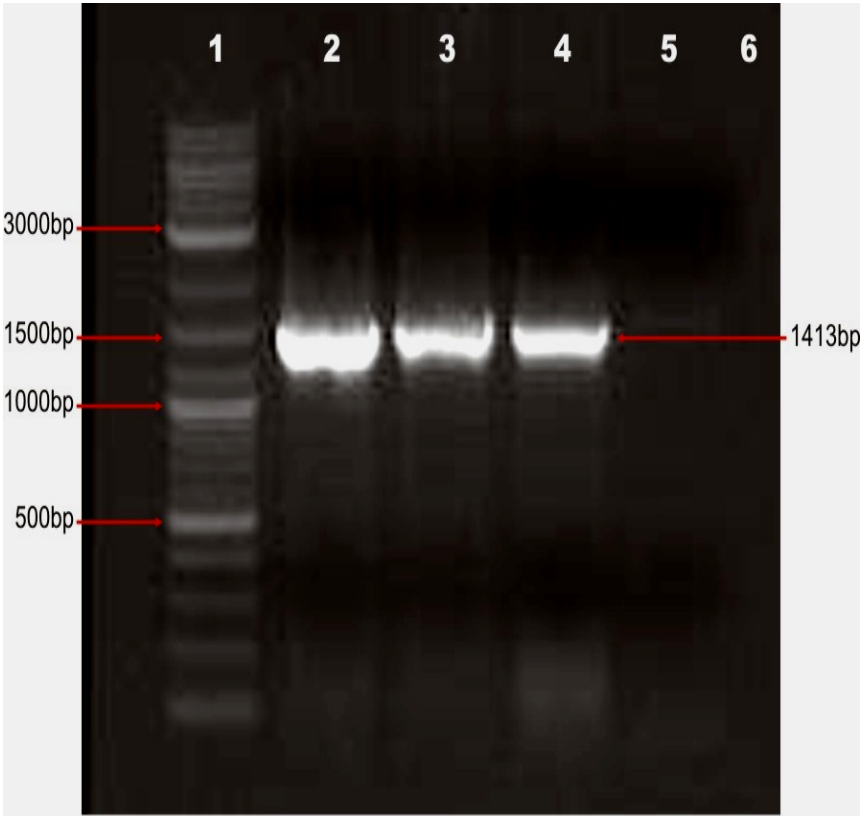


Plate 13. Amplification *TevAT1* gene of *T. evansi* by Colony-PCR

Lane 1. 10 Kb plus DNA Ladder Lane 2 – 3. PCR reaction with white colony shows Amplification
Lane 4. PCR reaction with blue colony shows Amplification
Lane 5. Control

```
ORIGIN
       1 atggactgcc atacaaagga gacactaggg gtcacacaat ggaggcgatc aacgatgcta
      61 acactatcac tgctttacgc catcactcca gcggacggcg ccaaagaagc ccttgaatac
     121 aaaacttgga caaaccactg cggactggcg gccacactga gaaaggttgc cggtggagta
     181 ttaacgaaac tgaaaagcca cattaactac cggcaaaaac tggaagaaat ggaaacgaag
     241 ctacgaatct acgcactaaa aggagacgga gtgggagagc aaaaatcagc ggagatacta
     301 gctacgacgg ccgccttaaa gcgacaaaaa gcactcacag cagaagaagc aaatttgaaa
     361 acagcggtga aggcggcagg attcgcaggc gaaggagcgg cagccgtcag cagctacctg
     421 atgacactcg ggacactgac aacaagcgga tctgcgcact gcctaagcaa cgaaggcggc
     481 aacggtgacg gaaaagacca acttgcgccg aaaggctgcc ggcacggcac agaagcagac
     541 ttcgacgcag gagccggccc ggcggaatct gaagtagccg acagcggctt cgcgcaagta
     601 ccaggcaaac aggacggagc aaacgcaggc caagcaaaca tgtgcgcatt gttcacacac
     661 caagcaacgc cgcacagctc acagggcata tacataaccg gggcgaaccc aaaaccttca
     721 ttcgggtacg gcatgctgac aatcggcacg acggaccaga ccatcggctt gaaactttcg
     781 gacataaagg gcaaacaagc agacagcgcg cagaaattct ggagcagctg ccacgcagca
     841 gtcaaagccg cccaggatat gaaggcagac ccagccctaa aggtcgacca gacgctccta
     901 gctgttcttg tggcttctcc ggagatggcg gaaatactga aactagaagc ggcagcatca
     961 cagcaaaaag gaccagagga agtgacgatc gacctagcca ccgagaaaaa caattatttc
    1021 ggaaccaaca acaacaaact agagccgctc tggactaaaa tcaaaggaca gaatatagtt
    1081 gacctggcgg cgaccaaagg cagcacgaaa gagttaggaa cagtcacaga cacggccgag
    1141 ctacaaaaac ttttaagtta ttattacacg gtcaacaaag aagaacagaa aaaaacagcg
    1201 gagaaaataa ctaaactcga aaccgaacta gcagatcaaa aaggcaaatc ccctgaaagc
    1261 gagtgcaata aaatatctga ggaacccaaa tgcaacgagg acaagatatg cagttggcat
    1321 aaggaggtta aagcgggaga aaagcactgc aaatttaact caacaaaagc aaaagaaaag
    1381 ggggtctctg taacacgaac tcaaactgca ggaggaaccg aagcgacaac agataaatgc
    1441 aaagggaaat
```

Plate 14. The coding sequence of *RoTat VSG* gene of *T. evansi* isolated from *Camelus dromedarius*

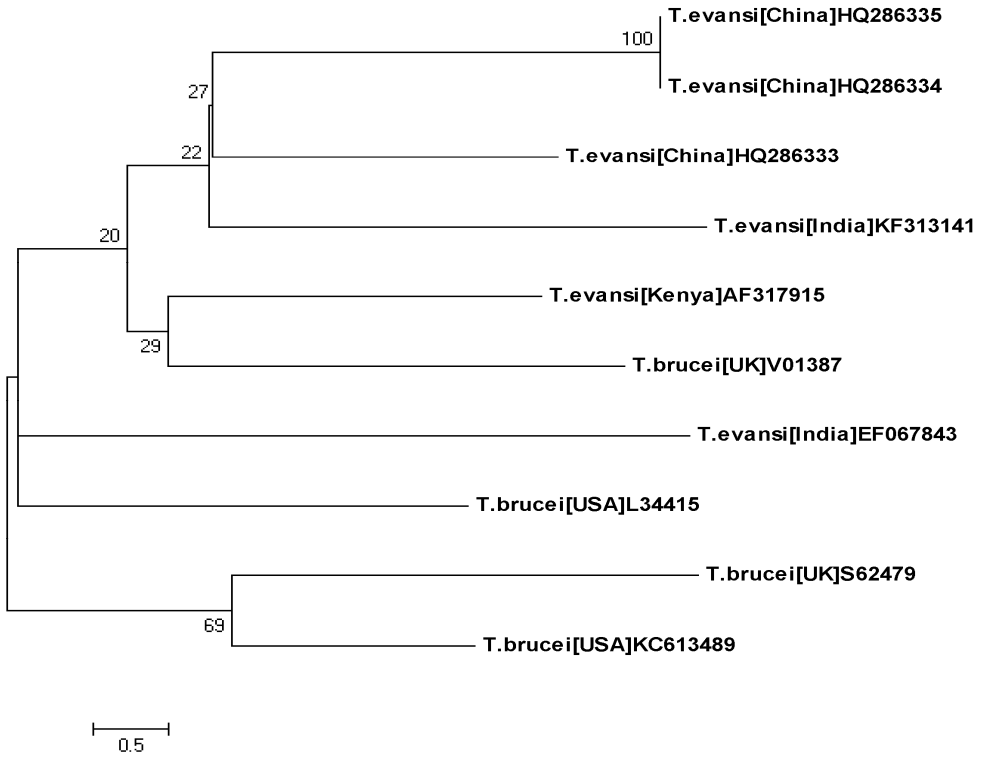


Plate 15a. Phylogenetic tree analysis of *RoTat VSG* gene using the Neighbor-Joining method

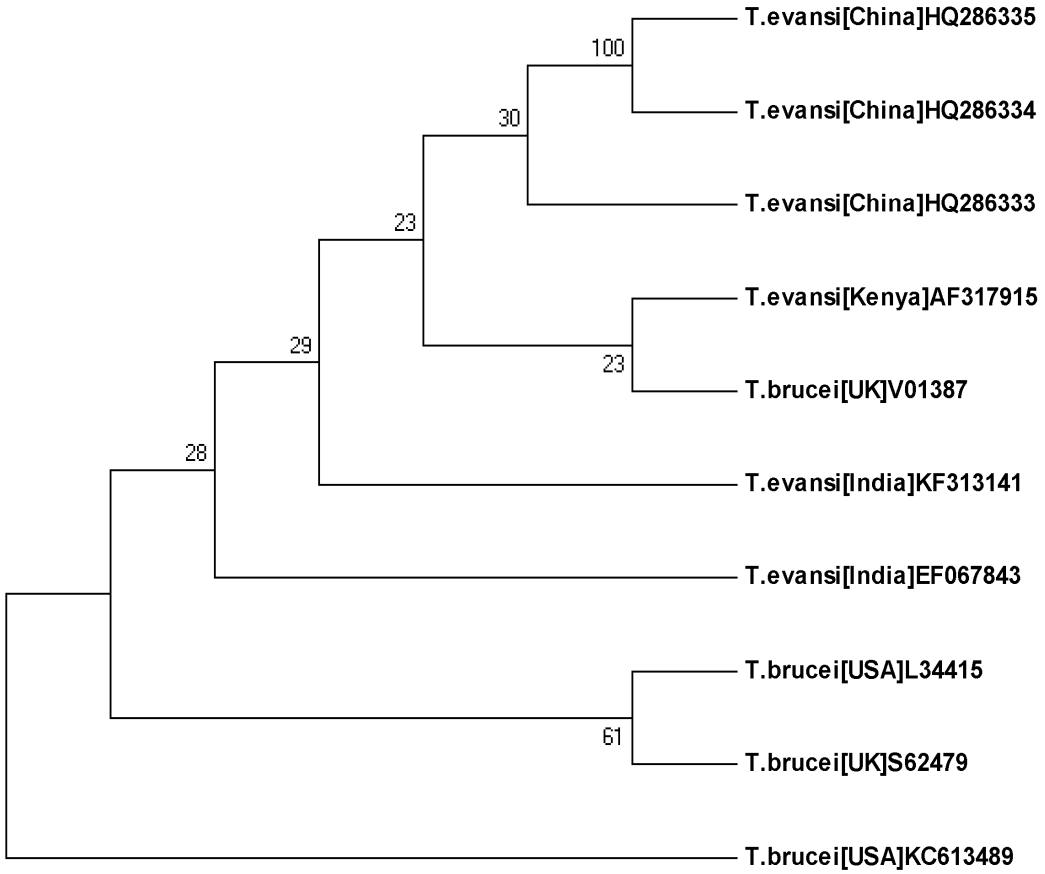


Plate 15b. Phylogenetic tree analysis of *RoTat VSG* gene using maximum parsimony method

Table 4.1: Sequence identity of RoTat VSG gene of *T. evansi* with other allied species

Identity of *RoTat VSG* gene for *Trypanosoma evansi*	*T.evansi* [India] KF313141	*T.evansi* [China] HQ286335	*T.evansi* [China] HQ286334	*T.evansi* [China] HQ28333	*T.* evansi[Kenya] EF317915	*T.brucei* [UK] L34415	*T.brucei* [UK] V01387	*T.evansi* [India] EF06743	T.brucei [UK] S62479	*T.brucei* [USA] KC613489
T.evansi [India] KF313141	**	99	99	99	99	98.6	98.6	39.2	68.5	69.5
T.evansi [China] HQ286335	99	**	96.2	97.5	95.6	95.9	96	41.6	75.8	65.9
T.evansi [China] HQ286334	99	96.2	**	95.6	96.7	95.1	96.9	39.7	75.8	66.4
T.evansi [China] HQ286333	99	97.5	95.6	**	97.2	98.7	95.5	40.5	75.9	66
T.evansi [Kenya]	99	95.6	96.7	97.2	**	95.4	95.6	40	75.4	66.3

AF317915										
T.brucei [UK] L34415	98.6	95.9	95.1	98.7	95.4	**	96.3	40.3	96.2	63
T. brucei [UK] V01387	98.6	96	96.9	95.5	95.6	96.3	**	41.1	74.5	66.3
T. evansi [India] EF06743	39.2	41.6	39.7	40.5	40	40.3	41.1	**	24.6	37.5
T.brucei [UK] S62479	68.5	75.8	75.8	75.9	75.4	96.2	74.5	24.6	**	60.7
T. brucei [USA] KC613489	69.5	65.9	66.4	66	66.3	63	66.3	37.5	60.7	**

Table 4.2: RoTat VSG gene for different species as submitted by various workers

Sl. No.	Identity of *RoTat VSG* gene for *Trypanosoma evansi*	Accession No.	Collection Country	Reference
1.	*T. evansi*	KF313141	India	Yagi *et al.* (2013)

2.	*T. evansi*	HQ286335	China	Jia *et al.* (2012)
3.	*T. evansi*	HQ286334	China	Jia *et al.* (2012)
4.	*T. evansi*	HQ286333	China	Jia *et al.* (2012)
5.	*T. evansi*	AF317915	Kenya	Urakawa *et al.* (2001)
6.	*T.brucei*	L34415	UK	Field and Boothroyd .(1996)
7.	*T.brucei*	V01387	UKl	Boothroyd *et al.*(1993)
8.	*T.evansi*	EF067843	India	Baidya *et al.* (2006)
9.	*T.brucei*	S62479	UK	Bromidge *et al.* (1993)
10.	T. brucei	KC613489	USA	Cross *et al.* (2013)

```
                                10         20         30         40         50
                                 |         |          |          |          |
T.brucei_USA_L34415              MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.brucei_UK_V01387               MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.evansi_China_HQ286335          MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.brucei_UK_S62479               MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.evansi_China_HQ286333          MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.evansi_China_HQ286334          MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.evansi_India_KF313141          MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.evansi_Kenya_AF317915          MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA
T.brucei_USA_KC613489            MDCQNRAAIALVKWKAATAAAVALLYVAVTASGSYEALEYTTWSTHCGLA
T.evansi_India_EF067843          ----------MVYRNLLQLTVLKVLLIVLIVEATHFGVKYELWQPECELT
                                       :.   .     .: :*     ...:   .::*  *   .* *:
Prim. cons.                      MDCHTKETLGVTQWRRSTMLTLSLLYAITPADGAKEALEYKTWTNHCGLA

                                60         70         80         90        100
                                 |         |          |          |          |
T.brucei_USA_L34415              ATLRKVAGGVLTKLKSHISYRKKLEEMETKLRIYALKGDGVGEQKSAEIL
T.brucei_UK_V01387               ATLRKVAGGVLTKLKSHISYRKKLEEMETKLRIYALKGDGVGEQKSAEIL
T.evansi_China_HQ286335          ATLRKVAGGVLTKLKSHINYRQKLEEMETKLRIYALKGDGVGEQKSAEIL
T.brucei_UK_S62479               ATLRKVAGGVLTKLKSHINYRQKLEEMETKLRIYALKGDGVGEQKSAEIL
T.evansi_China_HQ286333          ATLRKVAGGVLTKLKSHINYRQKLEEMETKLRIYALKGDGVGEQKSAEIL
T.evansi_China_HQ286334          ATLRKVAGGVLTKLKSHINYRQKLEEMETKLRIYALKGDGVGEQKSAEIL
T.evansi_India_KF313141          ATLRKVAGGVLTKLKSHINYRQKLEEMETKLRIYALKGDGVGEQKSAEIL
T.evansi_Kenya_AF317915          ATLRKVAGGVLTKLKSHINYRQKLEEMETKLRIYALKGDGGGEQKSAEIL
T.brucei_USA_KC613489            ATPRKVHGGILAKLESQISYRNKLEEMEAKLRIYGLKRAGGDEQTTVDML
T.evansi_India_EF067843          AELRKTAGVAKMKVNSDLNSFKTLKLTKMKLLTFAAKFPESKEALTLRAL
                                 *  **. *    *::*.:.  :.*:  : **  :. *     *  :   *
Prim. cons.                      ATLRKVAGGVLTKLKSHINYRQKLEEMETKLRIYALKGDGVGEQKSAEIL

                               110        120        130        140        150
                                 |         |          |          |          |
T.brucei_USA_L34415              ATTAALMRQKALTPEEANLKTALKAAGFAGEGAAAVSSYLMTLGTLTTSG
T.brucei_UK_V01387               ATTAALMRQKALTPEEANLKTALKAAGFAGEGAAAVSSYLMTLGTLTTSG
T.evansi_China_HQ286335          ATTAALMRQKALTPEEANLKTALKAAGFAGEGAAAVSSYLMTLGTLTTSG
T.brucei_UK_S62479               ATTAALMRQKALTPEEANLKTALKAAGFAGEGAAAVSSYLMTLGTLTTSG
T.evansi_China_HQ286333          ATTAALMRQKALTPEEANLKTALKAAGFAGEGAAAVSSYLMTLGTLTTSG
T.evansi_China_HQ286334          ATTAALMRQKALTPEEANLKTALKAAGFAGEGAAAVSSYLMTLGTLTTSG
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T.evansi_India_KF313141   ATTAALKRQKALTAEEANLKTAVKAAGFAGEGAAAVSSYLMTLGTLTTSG
T.evansi_Kenya_AF317915   ATTAALMRQKALTAEEANLKTAVKAAGFAGEGAAAVSSYLMTLGTLTTSG
T.brucei_USA_KC613489     ASTAALMRMASLKEEKTNMQTALIAVVFASEGAAAVSSYLMTIGSLTHNT
T.evansi_India_EF067843   EAAVNTDLRALRDNIANGIDRAVRATAYASEAAGALFSGIQTLHDAN-DG
                           ::.             .:. *: *. :*.*.*.*: * : *:   . .
Prim. cons.               ATTAALMRQKALTPEEANLKTALKAAGFAGEGAAAVSSYLMTLGTLTTSG

                                160       170       180       190       200
                                  |         |         |         |         |
T.brucei_USA_L34415        SAHCLSNEGGDGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.brucei_UK_V01387         SAHCLSNEGGDGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.evansi_China_HQ286335    SAHCLSNEGGNGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.brucei_UK_S62479         SAHCLSNEGGDGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.evansi_China_HQ286333    SAHCLSNEGGNGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.evansi_China_HQ286334    SAHCLSNEGGNGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.evansi_India_KF313141    SAHCLSNEGGNGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.evansi_Kenya_AF317915    SAHCPSNEGGNGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV
T.brucei_USA_KC613489      QTYCLSNTAKSGNGKAELSTAGCRHGKTSDYKAGIGPDEQEVDASGFTKI
T.evansi_India_EF067843    ATYCLSASGQGSNGNAAMASQGCKPLALPELLTEDSYNTDVISDKGFPKI
                            ::* *  . ..:*:  ::. **:    .:  :  .   . :  .**.::
Prim. cons.                SAHCLSNEGGNGDGKDQLAPKGCRHGTEADFDAGAGPAESEVADSGFAQV

                                210       220       230       240       250
                                  |         |         |         |         |
T.brucei_USA_L34415        PGKQDGANAGQANMCALFTHQATPHSSQGIYITGAQTKPSFGYGMLTIGT
T.brucei_UK_V01387         PGKQDGANAGQANMCALFTHQATPHSSQGIYITGAQTKPSFGYGMLTIGT
T.evansi_China_HQ286335    PGKQDGANAGQANMCALFTHQATPHSSQGIYITGAQTKPSFGYGMLTIGT
T.brucei_UK_S62479         PGKQDGANAGQANMCALFTHQATPHSSQGIYITGAQTKPSFGYGMLTIGT
T.evansi_China_HQ286333    PGKQDGANAGQANMCALFTHQATPHSSQGIYITGAQTKPSFGYGMLTIGT
T.evansi_China_HQ286334    PGKQDGANAGQANMCALFTHQATPHSSQGIYITGAQTKPSFGYGMLTIGT
T.evansi_India_KF313141    PGKQDGANAGQANMCALFTHQATPHSSQGIYITGANPKPSFGYGMLTIGT
T.evansi_Kenya_AF317915    PGKQDGANAGQANMCALFTHQATPHSSQGIYITGANPKPSFGYGMLTIGT
T.brucei_USA_KC613489      TGKTGAANTGETSKCGLFTHQGNPESAAGIFITAASSKPSFGYGMLKISA
T.evansi_India_EF067843    SALTTTQGQGKRSECGLFQAAGGAQASNTGVQFSGGSKINLGLGAIVATA
                           ..     . *: . *.**   . ..::      .. .* .:* * :   :
Prim. cons.                PGKQDGANAGQANMCALFTHQATPHSSQGIYITGAQTKPSFGYGMLTIGT

                                260       270       280       290       300
                                  |         |         |         |         |
```

```
T.brucei_USA_L34415       TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADP-ALKVDQTL
T.brucei_UK_V01387        TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADP-ALKVDQTL
T.evansi_China_HQ286335   TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADP-ALKVDQTL
T.brucei_UK_S62479        TDQTIGLKLSDIKGKQA--------------------------------
T.evansi_China_HQ286333   TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADP-ALKVDQTL
T.evansi_China_HQ286334   TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADP-ALKVDQTL
T.evansi_India_KF313141   TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADP-ALKVDQTL
T.evansi_Kenya_AF317915   TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADP-ALKVDQTL
T.brucei_USA_KC613489     QDQTAAQKLSDIKGKTEDDDQTFWSSCHAAVKAGKDMQAEP-PLKVDQTL
T.evansi_India_EF067843   AQQPTRPDLSDFSGPARAQADTLYGKAHAAINALRQLAQGPRPGQTEVET
                           :*.   .***:.*
Prim. cons.               TDQTIGLKLSDIKGKQADSAQKFWSSCHAAVKAAQDMKADPRALKVDQTL

                                310       320       330       340       350
                                  |         |         |         |         |
T.brucei_USA_L34415        LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKNNYFGTNNNKLEP
T.brucei_UK_V01387         LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKNNYFGTNNNKLEP
T.evansi_China_HQ286335    LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKNNYFGTNNNKLEP
T.brucei_UK_S62479         --------------------------------------------------
T.evansi_China_HQ286333    LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKNNYFGTNNNKLEP
T.evansi_China_HQ286334    LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKNNYFGTNNNKLEP
T.evansi_India_KF313141    LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKNNYFGTNNNKLEP
T.evansi_Kenya_AF317915    LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKTNYFGPNNNKLEP
T.brucei_USA_KC613489      LTVLVASTEMQYILKLEAAASEQKGPEEVTIDLASAKKTYFGSDNNKLEP
T.evansi_India_EF067843    MKLLAQKTAALESIKFQLAARTGKKPSDYKEDEN-LKTEYFGKTESNIEP

Prim. cons.                LAVLVASPEMAEILKLEAAASQQKGPEEVTIDLATEKNNYFGTNNNKLEP

                                360       370       380       390       400
                                  |         |         |         |         |
T.brucei_USA_L34415         LWTKIKGQNIVDLAATKGS-TKELGTVTDTAELQKLLSYYYTVNKEEQKK
T.brucei_UK_V01387          LWTKIKGQNIVDLAATKGS-TKELGTVTDTAELQKLLSYYYTVNKEEQKK
T.evansi_China_HQ286335     LWTKIKGQNIVDLAATKGS-TKELGTVTDTAELQKLLSYYYTVNKEEQKK
T.brucei_UK_S62479          --------------------------------------------------
T.evansi_China_HQ286333     LWTKIKGQNIVDLAATKGS-TKELGTVTDTAELQKLLSYYYTVNKEEQKK
T.evansi_China_HQ286334     LWTKIKGQNIVDLAATKGS-TKELGTVTDTAELQKLLSYYYTVNKEEQKK
T.evansi_India_KF313141     LWTKIKGQNIVDLAATKGS-TKELGTVTDTAELQKLLSYYYTVNKEEQKK
T.evansi_Kenya_AF317915     LWTKIKGQHIVDLAATKGS-TKELGTVTGTAELQKLLSYYYTVNKEEQKK
T.brucei_USA_KC613489       LWTKIKGENVVDLTKAKGS-TKELGTVTDTTELHKLLSYYYTVRKEKQKK
```

```
T.evansi_India_EF067843   LWNKVKEEKVKGADPEDPSKESKISDLNTEEQLQRVLDYYAVATMLKLAK

Prim. cons.               LWTKIKGQNIVDLAATKGSKTKELGTVTDTAELQKLLSYYYTVNKEEQKK


                                410       420       430       440       450
                                  |         |         |         |         |
T.brucei_USA_L34415        TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK
T.brucei_UK_V01387         TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK
T.evansi_China_HQ286335    TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK
T.brucei_UK_S62479         --------------------------------------------------
T.evansi_China_HQ286333    TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK
T.evansi_China_HQ286334    TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK
T.evansi_India_KF313141    TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK
T.evansi_Kenya_AF317915    TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK
T.brucei_USA_KC613489      TAEQVEKLETELAAQKGKSPEAECNKIFEEPKCNEDKICSWHKDVKAGEE
T.evansi_India_EF067843    QTEDIAKLETELADQRGKSPETECNKISEEPKCNDENICSWHKDVKNGEN

Prim. cons.               TAEKITKLETELADQKGKSPESECNKISEEPKCNEDKICSWHKEVKAGEK


                                460       470       480       490       500
                                  |         |         |         |         |
T.brucei_USA_L34415        HCKFNSTKAKEKGVSVTQTQTAGGTEATTDKCKGKLEDTCKKESNCKWEN
T.brucei_UK_V01387         HCKFNSTKAKEKGVSVTQTQTAGGTEATTDKCKGKLEDTCKKESNCKWEN
T.evansi_China_HQ286335    HCKFNSTKAKEKGVSVTQTQTAGGTEATTDKCKGKLEDTCKKESNCKWDG
T.brucei_UK_S62479         --------------------------------------------------
T.evansi_China_HQ286333    HCKFNSTKAKEKGVSVTQTQTAGGTEATTDKCKGKLEDTCKKESNCKWEN
T.evansi_China_HQ286334    HCKFNSTKAKEKGVSVTQTQTAGGTEATTDKCKGKLEDTCKKESNCKWEN
T.evansi_India_KF313141    HCKFNSTKAKEKGVSVTRTQTAGGTEATTDKCKGK--------------
T.evansi_Kenya_AF317915    HCKFNSTKAKEKGVSVTQTQTAGGTEATTDKCKGKLEDTCKKESNCKWEN
T.brucei_USA_KC613489      NCQFNSTKAKEKGVSVTQTQTVGGTETTTDKCKGKGEKDCK-SPDWKWEG
T.evansi_India_EF067843    NCKFNSTKAKEKGVSVTQTQTTGGTETTTEKCKDKKKDDCK-SPDCNWDG

Prim. cons.               HCKFNSTKAKEKGVSVTQTQTAGGTEATTDKCKGKLEDTCKKESNCKWEN


                                510       520       530
                                  |         |         |
T.brucei_USA_L34415         NACKDSSILVTKKFALS---AAAFAALLF--
T.brucei_UK_V01387          NACKDSSILVTKKFALTVV-SAAFVALLF--
```

```
T.evansi_China_HQ286335   KECKDSSILVNKQFALS---AAAFAALLF--
T.brucei_UK_S62479        ------------------------------
T.evansi_China_HQ286333   NACKDSSILVTKKLALS---AAAFAALLF--
T.evansi_China_HQ286334   NACKDSSILATKKFALSVV-SSAFVALLF--
T.evansi_India_KF313141   ------------------------------
T.evansi_Kenya_AF317915   NACKDSSILVTKKLALS---AAAFAALLF--
T.brucei_USA_KC613489     ETCKGSSFLVTKVLAVIFCCFYGFDSILNFF
T.evansi_India_EF067843   KQCKDSSILVNKHFALS-VVSAAFVALLF--

Prim. cons.               NACKDSSILVTKKFALSVV2AAAFAALLFFF
```

Plate 16. Multiple sequence alignment of *RoTat VSG* Amino acid sequences with Clustal W.

```
ORIGIN
        1 atgctcgggt ttgactcagc caatgaattc atcgtctacg tcaccttcct cttcttcgga
       61 atgtcggtgg tggtggtgac aaactccatc ttttcgatgc cattcttctt catcgagtac
      121 tacaagtatg cgcaggggaa acctgatgca aagccagagg acccgaagtt ctggaaacat
      181 atgtttacct actacagtat tgcagcgttc ctcgtagagt tggttttggc gtcgctcatg
      241 cttacgccaa tcggacggcg gatctctgta accgttcgcc tcggtgtagg tcttgtcatt
      301 ccaattgtgt tggtattctc cgtgatgatg gttactatcg ttacgacaac agaaaccggt
      361 gccaaggtga ccatcatgct cattgctatc gcaaatggcg tagcgatgac gctttgcgat
      421 gctggaaacg ccgcactcat cgccccgttt ccaacgaaat tttatagctc cgtcgtgtgg
      481 ggtatcgctg tgtgcggcgt cgtcacatct ttcttctcga tcgtcataaa agcatccatg
      541 ggaggcggtt atcacaacat gctcatacag tcgcgcatat actttggatt ggtcatgttt
      601 atgcaggtga tatcttgcgc ccttttagtg ttgctaagga agaaccctta cgcccaaaag
      661 tacgcggcag agttccgata tgcagcgagg aaagggattg atgataaggg cgcagatggt
      721 gacgaaggaa acggcgcagc aaaagggccg gccgatcagg atgatgaccc ccacggaggc
      781 gatgatactg acaaaggaaa tgtaatgacc gccactgtag atcctgacac aatgaaggac
      841 atggaccagg tggaaaacat cacgacttcg cagcagatgt taatggcaag ggtatggaat
      901 gtgttctggc gcgtttggcc catgctgttc gcatgcttca tggttttctt caccacattt
      961 ctcgtctacc ctgccgtgta cttcgccatc aaggcagata cgggtgacgg ctggtacttg
     1021 acgatcgctg ccgcattgtt caatttgggt gatttcttgt cgcgtctttg ccttcagttc
     1081 aaagccttac acgtctcacc gcggtgggtt ctgattggga catttgcgcg tatgctgctc
     1141 attatcccac ttgtgctttg cgtgcgaagc atcatcaccg gcccttggct cccttacatt
     1201 cttgtccacg cttggggctt cacgtacggt tattatggtg gaatatcaca aatctacgcg
     1261 ccgcgcaccg gctcactgac aacagctggc gagcggtctc ttgccgcaaa ttggactatc
     1321 atttcgctcc tgggtggcat cttcgttggc gccatgttcg ccctggctgt caatgagggg
     1381 ctttccaagt agcactaatc gtcatacgaa tac
```

Plate 17. The coding sequence of *TevAT1* gene of *T. evansi* isolated from *Camelus dromedarius*

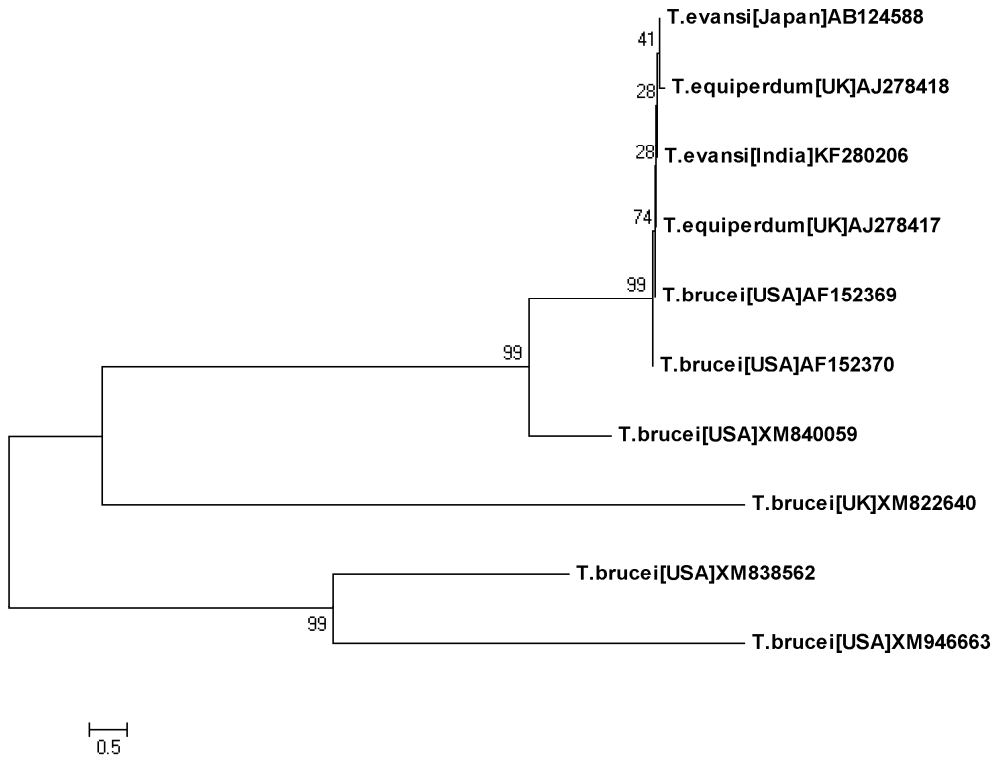

Plate 18a. Phylogenetic tree analysis of *TevAT1* gene using the Neighbor-Joining method

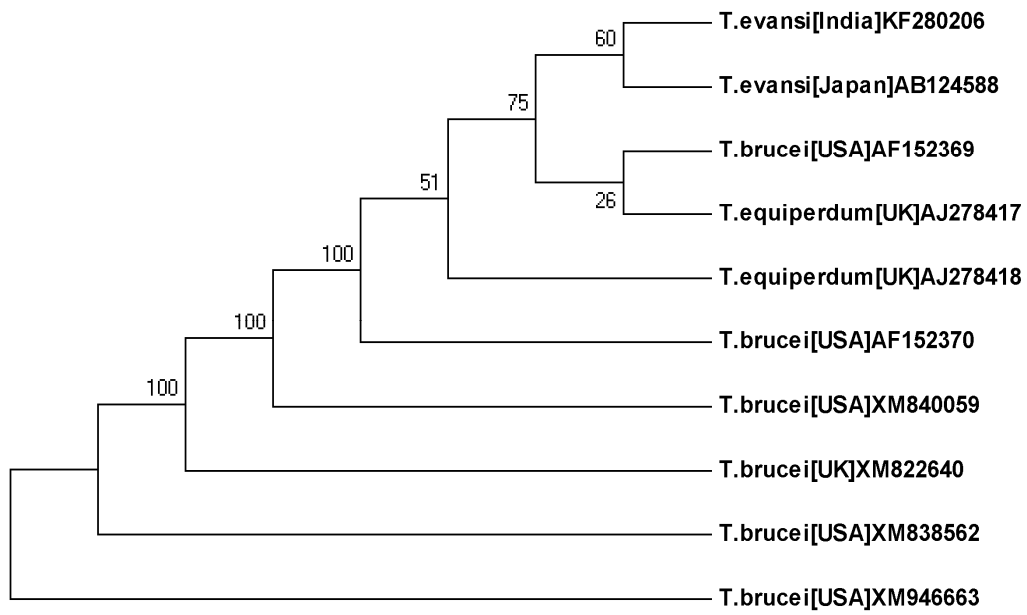

Plate 18b. Phylogenetic tree analysis of *TevAT1* gene using maximum parsimony method

Table 4.3: Sequence identity of *TevAT1* gene of *T. evansi* with other allied species

Identity of TevAT1 gene for *Trypanosoma evansi*	*T.evansi* [India] KF280206	*T.brucei* [USA] AF152369	*T.evansi* [Japan] AB124588	*T.equiperdum* [UK] AJ278417	*T.brucei* [USA] AF152370	*Tequiperdum* [UK] AJ278418	*T.brucei* [USA] XM838562	*T.brucei* [UK] XM822640	*T.brucei* [USA] XM840059	*T.brucei* [USA] XM946663
T.evansi [India] KF280206	**	99.9	99.7	99.8	99.2	99.3	73.8	71	66.5	65.8
T.brucei [USA] AF152369	99.9	**	99.7	99.9	99.3	99.4	73.9	69.7	68.5	65.9
T.evansi [Japan] AB124588	99.7	99.7	**	99.6	99	99.1	73.7	69.8	68.4	66
T.equiperdum [UK] AJ278417	99.8	99.9	99.6	**	99.2	99.3	73.8	71.6	68.1	65.9
T.brucei [USA] AF152370	99.2	99.3	99	99.2	**	98.2	73.3	69.4	68.4	66.1
Tequiperdum [UK] AJ278418	99.3	99.4	99.1	99.3	98.2	**	73.4	71.6	68.2	65.8

T.brucei [USA] XM838562	73.8	73.9	73.7	73.8	73.3	73.4	**	68.6	66.4	64.1
T.brucei [UK] XM822640	71	69.7	69.8	71.6	69.4	71.6	68.6	**	65.7	65.1
T.brucei [USA] XM840059	68.5	68.5	68.4	68.1	68.4	68.2	66.4	65.7	**	73.1
T.brucei [USA] XM946663	65.8	65.9	66	65.9	66.1	65.8	64.1	65.1	73.1	**

Table 4.4: *TevAT1* gene for different species as submitted by various workers

Sl. No.	Identity of *TevAT1* gene for *Trypanosoma evansi*	Accession No.	Collection Country	Reference
1.	*T.evansi*	KF280206	India	Yagi *et al.* (2013)
2.	*T.brucei*	AF152369	USA	Maser *et al.* (1999a)

3.	*T.evansi*	AB124588	Japan	Witola *et al.* (2004)
4.	*T.equiperdum*	AJ278417	UK	Leadsham *et al.* (2001)
5.	*T.brucei*	AF152370	USA	Maser *et al.* (1999b)
6.	*T.equiperdum*	AJ278418	UK	Leadsham *et al.* (2001)
7.	*T.brucei*	XM838562	USA	Berriman *et al.* (2012)
8.	*T.brucei*	XM822640	UK	Berriman *et al.* (2012)
9.	*T.brucei*	XM840059	USA	Berriman *et al.* (2012)
10.	*T.brucei*	XM946663	USA	Berriman *et al.* (2012)

```
                                10         20         30         40         50
                                 |          |          |          |          |
T.evansi_India_KF280206     --MLGFDSANEFIVYVTFLFFGMSVVVVTNSIFSMPFFFIEYYKYAQGKP
T.evansi_Japan_AB124588     --MLGFDSANEFIVYVTFLFFGMSVVVVTNSIFSMPFFFIEYYKYAQGKP
T.brucei_USA_AF152369       --MLGFDSANEFIVYVTFLFFGMSVVVVTNSIFSMPFFFIEYYKYAQGKP
T.equiperdum_UK_AJ278417    --MLGFDSANEFIVYVTFLFFGMSVVVVTNSIFSMPFFFIEYYKYAQGKP
T.brucei_USA_AF152370       --MLGFDSANEFIVYVTFLFFGMSVVVVTNSIFSMPFFFIEYYKYAQGKP
T.equiperdum_UK_AJ278418    --MLGFESANEFIDYVTFLFFGMSVVVVTNSIFSMPFFFIEYYKYAQGKP
T.brucei_USA_XM838562       -MMLGFESVSEFTVYITFIFFGMSAVVVTTSIFSIPFFFIEYYKYAQGDP
T.brucei_UK_XM822640        --MLGFGSVHELLAYITFMCFGMSVMVVSNTVLSFLEFFLQFYLFAAKDG
T.brucei_USA_XM840059       --MLGFYSVPEFVVYVTFIFFGMSVMNVTNAIYSIPFFFKEYYKFAQGDA
T.brucei_USA_XM946663       MAMLGFESTAEFFVYLTFIFFGMSVMNVTNAIYSNYNFFSEYYKFVKKQE
                              **** *. *:  *:**: ****.: *:.:: *   ** ::* :.  .
Prim. cons.                 M2MLGFDSANEFIVYVTFLFFGMSVVVVTNSIFSMPFFFIEYYKYAQGKP

                                60         70         80         90        100
                                 |          |          |          |          |
T.evansi_India_KF280206     D---AKPEDPK-FWKHMFTYYSIAAFLVELVLASLMLTPIGRRISVTVRL
T.evansi_Japan_AB124588     D---AKPEDPK-FWKHMFTYYSIAAFLVELVLASLMLTPIGRRISVTVRL
T.brucei_USA_AF152369       D---AKPEDPK-FWKHMFTYYSIAAFLVELVLASLMLTPIGRRISVTVRL
T.equiperdum_UK_AJ278417    D---AKPEDPK-FWKHMFTYYSIAAFLVELVLASLMLTPIGRRISVTVRL
T.brucei_USA_AF152370       D---AKPEDPK-FWKHMFTYYSIAAFVVELVLASLMLTPIGRRISVTVRL
T.equiperdum_UK_AJ278418    D---AKPEDPK-FWKHMFTYYSIAAFLVELVLASLMLTPIGRRISVTVRL
T.brucei_USA_XM838562       N---AEAEDQR-FWNNVFTYYNATTFLVEFLLTLFMLTNLGRRIPLAVRL
T.brucei_UK_XM822640        ENIKLETEEQKFFWKNVFTYYLATTFIVECLVVSLMLTNFGKRIPITLRL
T.brucei_USA_XM840059       D---VQPKDEG-FWKHMFTYYNVVVYTMQVLLEAFMLTPLGRRFPIRWRL
T.brucei_USA_XM946663       K---AEPENES-FWKHMFTYYNVVVFTMQVVLEAFMLTPLGRRIPISWRL
                            .    :.::   **:::****  ..: :: ::  :*** :*:*:.:  **
Prim. cons.                 DNIKAKPEDPKFFWKHMFTYYSIAAFLVELVLASLMLTPIGRRISVTVRL

                              110        120        130        140        150
                                 |          |          |          |          |
T.evansi_India_KF280206     GVGLVIPIVLVFSVMMVTIVTTTETGAKVTIMLIAIANGVAMTLCDAGNA
T.evansi_Japan_AB124588     GVGLVIPIVLVFSVMMVTIVTTTETGAKVTIMLIAIANGVPMTLCDAGNA
T.brucei_USA_AF152369       GVGLVIPIVLVFSVMMVTIVTTTETGAKVTIMLIAIANGVAMTLCDAGNA
T.equiperdum_UK_AJ278417    GVGLVIPIVLVFSVMMVTIVTTTETGAKVTIMLIAIANGVAMTLCDAGNA
T.brucei_USA_AF152370       GVGLVIPIVLVFSVMMVTIVTTTETGAKVTIMLIAIANGVAMTLCDAGNA
T.equiperdum_UK_AJ278418    GVGLVIPIVLVFSVMMVTIVTTTETGAKVTIMLIAIANGVRMTLCDAGNA
```

```
T.brucei_USA_XM838562      GAGLILSILAVFVVIMVTIIKTTETGAKVTIMLVGVINGVAATLCDTGNG
T.brucei_UK_XM822640       YIGLVFPIILVFSVMMVTIGKTTETGARVTIILIGLINGASTALCSSGAV
T.brucei_USA_XM840059      TFGLAVPIVEIIVILVIPVVRTSEDAAKAAMMMIAFIGGVSKTLCDSGNA
T.brucei_USA_XM946663      IFGLTIPMVEIIVILVIPAVGGSENGAIATMMMVAFVGGISKTLCDSSNA
                             ** ..:: :: ::::.    :* .* .::::.. .*   :**.:.
Prim. cons.                GVGLVIPIVLVFSVMMVTIVTTTETGAKVTIMLIAIANGVAMTLCDAGNA

                                 160       170       180       190       200
                                   |         |         |         |         |
T.evansi_India_KF280206    ALIAPFPTKFYSSVVWGIAVCGVVTSFFSIVIKASMGGGYH-------NM
T.evansi_Japan_AB124588    ALIAPFPTKFYSSVVWGIAVCGVVTSFFSIVIKASMGGGYH-------NM
T.brucei_USA_AF152369      ALIAPFPTKFYSSVVWGIAVCGVVTSFFSIVIKASMGGGYH-------NM
T.equiperdum_UK_AJ278417   ALIAPFPTKFYSSVVWGIAVCGVVTSFFSIVIKASMGGGYH-------NM
T.brucei_USA_AF152370      ALIAPFPTKFYSSVVWGIAVCGVVTSFFSIVIKTSMEGGYH-------NM
T.equiperdum_UK_AJ278418   ALIAPFPTKFYSSVVWGIAVCGVVTSFFSIVIKASMEGGYH-------NM
T.brucei_USA_XM838562      ALISPFPTKFFSAVVWGVAVCGIITSFFSIVIKASMESNYE-------SM
T.brucei_UK_XM822640       ALAGPFPTKFLSAYVWGVSVCGVITSTFAIVIKASTESNFKRTEDRVASR
T.brucei_USA_XM840059      ALVGPFPTKFYGAVVWGLGISGLLTSFMSIIIKVSMDDSFS-------SL
T.brucei_USA_XM946663      ALAGPFPTKFYGAIVWGLAISGLMTSFLAIVIQASMDSSFT-------SK
                           ** .****** .: ***:.:.*::** ::*:*:.*  ..:         .
Prim. cons.                ALIAPFPTKFYSSVVWGIAVCGVVTSFFSIVIKASM2GGYHRTEDRVANM

                                 210       220       230       240       250
                                   |         |         |         |         |
T.evansi_India_KF280206    LIQSRIYFGLVMFMQVISCALLVLLRKNPYAQKYAAEFRYAARKGIDDKG
T.evansi_Japan_AB124588    LIQSRIYFGLVMFMQVISCALLVLLRKNPYAQKYAAEFRYAARKGIDDKG
T.brucei_USA_AF152369      LIQSRIYFGLVMFMQVISCALLVLLRKNPYAQKYAAEFRYAARKGIDDKG
T.equiperdum_UK_AJ278417   LIQSRIYFGLVMFMQVISCALLVLLRKNPYAQKYAAEFRYAARKGIDDKG
T.brucei_USA_AF152370      LIQSRIYFGLVMFMQVISCALLVLLRKNPYAQKYAAEFRYAARKGIDDKG
T.equiperdum_UK_AJ278418   LIQSRIYFGLVMFMQVISCALLVLLRKNPYAQKYAAEFRYAARKGIDDKG
T.brucei_USA_XM838562      LTQSRIFFGLVVLLEVVSCILLVLLRKNPYAMKYAAEFRYAARERTN---
T.brucei_UK_XM822640       LTQSRIYFGLVMIMQSISCGLLLLLRKNPYAMKYTADFRYAARKGNAVEG
T.brucei_USA_XM840059      LTQSRIYFGLIMLLQVIACILLVLLRKNPYAMRYAAELRFDAKKSGTKDS
T.brucei_USA_XM946663      NTQSQIYFGLVMLLQVVACVLLVLLRKNPYAIKYAAEFRYAARKDGVTG-
                             **:*:***:::::: ::* **:******** :*:*::*: *::
Prim. cons.                LIQSRIYFGLVMFMQVISCALLVLLRKNPYAQKYAAEFRYAARKGIDDKG

                                 260       270       280       290       300
                                   |         |         |         |         |
```

```
T.evansi_India_KF280206     ---ADGDEGNGAAKGPADQDDDPHGGDD-TDKGNVMTATVDPDTMKDMDQ
T.evansi_Japan_AB124588     ---ADGDEGNGAARGPADQDDDPHGGDD-TDKGNVMTATVDPDTMKDMDQ
T.brucei_USA_AF152369       ---ADGDEGNGAAKGPADQDDDPHGGDD-TDKGNVMTATVDPDTMKDMDQ
T.equiperdum_UK_AJ278417    ---ADGDEGNGAAKGPADQDDDPHGGDD-TDKGNVMTATVDPDTMKDMDQ
T.brucei_USA_AF152370       ---AGGDEGNGAAKGPADQDDDPHGGDD-TDKGNVMTATVDPDTMKDMDQ
T.equiperdum_UK_AJ278418    ---AGGDEGNGAAKGPADQDDDPHGGDD-TDKGNVMTATVDPDTMKDMDQ
T.brucei_USA_XM838562       ---ACENKESGASNGPAEQDEDSVAIDNNTTKGNVMTVTVDPDTMKDTDQ
T.brucei_UK_XM822640        DDAGDDNEPSSLGKGPADQDDDLKADCN-AGKSNVMTSTVDPDTMRDTDQ
T.brucei_USA_XM840059       --NGLVDVADARGTGPADEECEREADER--SDINVMNATTDPDTMRDTDQ
T.brucei_USA_XM946663       --DRADGEFDAKGTGPADEN--RYPDEK--ENKNVLNADIDPDDMRDTDQ
                                  .  .. . ***:::           . **:.   *** *:* **
Prim. cons.                 DD3ADGDEGNGAAKGPADQDDDPHGGDDNTDKGNVMTATVDPDTMKDMDQ

                                  310        320        330        340        350
                                    |          |          |          |          |
T.evansi_India_KF280206     VENITTSQQMLMARVWNVFWRVWPMLFACFMVFFTTFLVYPAVYFAIKAD
T.evansi_Japan_AB124588     VENITTSQQMLMARVWNVFWRVWPMLFACFMVFFTTFLVYPAVYFAIKAD
T.brucei_USA_AF152369       VENITTSQQMLMARVWNVFWRVWPMLFACFMVFFTTFLVYPAVYFAIKAD
T.equiperdum_UK_AJ278417    VENITTSQQMLMARVWNVFWRVWPMLFACFMVFFTTFLVYPAVYFATKAD
T.brucei_USA_AF152370       VESITTSQQMLMARVWNVFWRVWPMLFACFMVFFTTFLVYPAVYFAIKAD
T.equiperdum_UK_AJ278418    VENITTSQQMLMARVWNVFWRVWPMLFACFMVFFTTFLVYPAVYFAIKAD
T.brucei_USA_XM838562       VEDITNSQQMLKAKVSVVLKRVWPMLAAGFLAFSTTFLVYPGVFFAVKTD
T.brucei_UK_XM822640        VENITNSQQMLKASALSVFRRVWPMLAVCFIAFFTAFLIYPGVFFAVKLG
T.brucei_USA_XM840059       LENMTNAKQMLDASVMVVAKRIWPMLVSCFFVFFATLLVFPGVFFAVKTD
T.brucei_USA_XM946663       VEGTTNAQQMLDASVMVVVKRIWPMLVACFFVFFATLLVFPGVFIAAKTG
                            :*. *.::*** * .  *  *:****   *:.* :::*::*.*::* * .
Prim. cons.                 VENITTSQQMLMARVWNVFWRVWPMLFACFMVFFTTFLVYPAVYFAIKAD

                                  360        370        380        390        400
                                    |          |          |          |          |
T.evansi_India_KF280206     TG-DGWYLTIAAALFNLGDFLSRLCLQFKALHVSPRWVLIGTFARMLLII
T.evansi_Japan_AB124588     TG-DGWYLTIAAALFNLGDFLSRLCLQFKALHVSPRWVLIGTFARMLLII
T.brucei_USA_AF152369       TG-DGWYLTIAAALFNLGDFLSRLCLQFKALHVSPRWVLIGTFARMLLII
T.equiperdum_UK_AJ278417    TG-DGWYLTIAAALFNLGDFLSRLCLQFKALHVSPRWVLIGTFARMLLII
T.brucei_USA_AF152370       TG-DGWYLTIAAALFNLGDFLSRLCLQFKALHVSPRWVLIGTFARMLPII
T.equiperdum_UK_AJ278418    TG-DGWYLTIAAALFNLGDFLSRLCLQFKALHVSPRWVLIGTFARMLLII
T.brucei_USA_XM838562       VP-NGWYMTITAAMFHFGDFLSRLLLQFKRLQPSPRYVVVGTFARVFLII
T.brucei_UK_XM822640        PDDNGWYMVIIPMMFNLGDFVARLFVQFKTLHASPLFVVIGTFARLLLVI
T.brucei_USA_XM840059       VP-SGWYFTIVAAMYNLGDFLSRLVLQFKRLHPSPRGVVIGTFSRLLVIP
```

```
T.brucei_USA_XM946663      DT-SGWYFTVVVAMFNLGDFLSRLVLQFKQLHVSPRMVMIGSFARALLII
                              .***:.:   ::::***::** :*** *: **  *::*:*:* : :
Prim. cons.                TGDDGWYLTIAAALFNLGDFLSRLCLQFKALHVSPRWVLIGTFARMLLII


                                 410       420       430       440       450
                                   |         |         |         |         |
T.evansi_India_KF280206    PLVLCVRSIITGPWLPYILVHAWGFTYGYYGGISQIYAPRTGSLTTAGER
T.evansi_Japan_AB124588    PLVLCVRSIITGPWLPYILVHAWGFTYGYYGGISQIYAPRTGSLTTAGER
T.brucei_USA_AF152369      PLVLCVRSIITGPWLPYILVHAWGFTYGYYGGISQIYAPRTGSLTTAGER
T.equiperdum_UK_AJ278417   PLVLCVRSIITGPWLPYILVHAWGFTYGYYGGISQIYAPRTGSLTTAGER
T.brucei_USA_AF152370      PLVLCVRSIITGPWLPYILVHAWGFTYGYYGGISQIYAPRTGSLTTAGER
T.equiperdum_UK_AJ278418   PLVLCVRSIITGPWLPYILVHAWGFTYGYYGGISQIYAPRTGSLTTAGER
T.brucei_USA_XM838562      PLVFCVRGIIGGTLLPYILSFLWGLTYGYFGGMALIHTPRTGSLTAAGER
T.brucei_UK_XM822640       PIVLCAYSVIKGTTFPYILCFLWSLTYGYVGGLAGVYAPRTGSLTTAGER
T.brucei_USA_XM840059      LLALCVYDVISGPWVPYVLCLIWGLTNGYFGGMSMIYGPRTGSLTTAGQR
T.brucei_USA_XM946663      PLSLCAAGTVTGVWLPYIVSLLWGLTNGYFGGLSMIYGPRTGSLTTAGQR
                            : :*. . : *  .**::   *.:* ** **:: :: *******:**:*
Prim. cons.                PLVLCVRSIITGPWLPYILVHAWGFTYGYYGGISQIYAPRTGSLTTAGER

                                 460       470       480
                                   |         |         |
T.evansi_India_KF280206    SLAANWTIISLLGGIFVGAMFALAVNEGLSK----
T.evansi_Japan_AB124588    SLAANWTIISLLGGIFVGAMFALAVNEGLSK----
T.brucei_USA_AF152369      SLAANWTIISLLGGIFVG----------------
T.equiperdum_UK_AJ278417   SLAANWTIISLLGGIFVGAMFALAVNEGLPK----
T.brucei_USA_AF152370      SLAANWTIISLLGGIFVGAMFALAVNEGLPK----
T.equiperdum_UK_AJ278418   SLAANWTIISLLGGIFVGAMFALAVNEGLPK----
T.brucei_USA_XM838562      SLAANCAVIAILCGLFSGSMLALAVKEGLPQ----
T.brucei_UK_XM822640       SLAANWAVSSLLFGIFAGCMCALGVNSALPKDESQ
T.brucei_USA_XM840059      SLAAICINLALLLGLFGGAMSAMAVIKALPH----
T.brucei_USA_XM946663      SLAAICINVALLMGLFVGAMFALAVKEGLPK----
                           ****     ::* *:* *
Prim. cons.                SLAANWTIISLLGGIFVGAMFALAVNEGLPKDESQ
```

Plate 19. Multiple sequence alignment of *TevAT1* Amino acid sequences with Clustal W.

5. DISCUSSION

Camel trypanosomosis caused by *Trypanosoma evansi* is the most widely distributed pathogenic mechanically transmitted vector borne haemoprotozoan disease of domestic livestock and wild animals in India. The Office of International Epizootics (OIE) mentioned this disease under list B diseases of significance in horses. The geographical area affected by trypanosomosis caused by *T. evansi* is greater than area affected by tsetse borne trypanosomosis in Africa. Surra is responsible for causing severe

anaemia, oedema, immunosuppression and various neurological disorders resulting into death of the affected animals. It is a potential killer of livestock and causes economic losses to the farmers in terms of morbidity, mortality, abortion, infertility and reduced milk yield. The disease is seasonal and the incidence is higher during rainy and post rainy seasons due to preponderance of *Tabanus* flies.

Despite the impact of trypanosome species on human and livestock health, the full diversity of this organism is poorly understood and therefore therapeutic strategies have their limitations. Trypanosomes have the capacity for antigenic variation which is the basis of their ability to escape the host immune response and because of this prospects for the development of a vaccine against trypanosomiasis have been considered poor. Control of the disease remains a challenge as chemotherapy has only modest success in disease treatment. The resistance of parasite to current drugs is increasingly becoming a major problem while there are no prospects for the development of new anti-trypanosome drugs. Drug tolerance develops in the field is derived basically from under-dosing due to incorrect estimation of body weight. A high incidence of trypanosomosis in conjunction with the irregular use of prophylactic and therapeutic drugs also favours the emergence of drug resistant trypanosome. Thus drug resistant parasites may emerge in any situation where prophylaxis and therapy are inadequate for the degree of tsetse fly challenge. The misuse of drugs leads consequently first to individual resistance and then to area resistance. Besides, the lack of vaccine against this organism due to its antigenic variations. the present study was undertaken in purpose that it could be usefull in improvement of diagnostic tools and helpful for understanding the problem of drug resistance, development of more effective drugs and evolution of vaccine.

Trypanosomes undergo antigenic variation that enables them to evade the host‘s immune system (Barry and Turner, 1991; Baron, 1996; Donelson *et al.*, 1998). VSG covers the entire parasite including the flagellum presumably as a protective shield against host antibodies (Englund *et al.*, 1982; Pays *et al.*, 2001). This protective coat which determines the antigenic features of the parasite, is easily recognized by the host and is highly immunogenic.

RoTat 1.2 VSG is a predominant variant antigen type thought to be expressed in all *T. evansi* stocks examined so far (Verloo *et al.*, 2001). It is the diagnostic antigen in CATT/*T. evansi*, a direct agglutination test produced by the Laboratory of Serology, Institute of Tropical Medicine, Antwerp, Belgium. *RoTat 1.2* gene based PCR test (Urakawa *et al*., 2001) has been considered as reliable for it detects both dyskinetoplastic and kinetoplastic isolates (Claes *et al.,* 2004). However, recent reports of the occurrence of *RoTat 1.2* negative isolates in Kenya (Ngaira *et al.,* 2004) may deny this marker the expected universality.

During the present study initially, *T.evansi* were isolated from the naturally infected camel at NRCC, Bikaner. The parasites propagated in laboratory animals (mice/rats) after injecting intra-peritoneally. When parasite counts reached 10^8/ml, the mice were sacrificed and the infected blood was collected from the heart using anticoagulant heparin. Subsequently the trypanosomes were purified from the infected blood by diethylaminoethyl cellulose (DEAE) chromatography. Genomic DNA extraction of *T. evansi* was carried out through the phenol-chloroform extraction followed by ethanol precipitation (Sambrook and Russel, 2001). Following this, the desired genes, *RoTat* VSG and *TevAT1* were amplified employing PCR. The PCR product (amplicons) thus obtained were cloned into suitable vector (pGEM-T Easy vector). The genes were sequenced from purified plasmid of the obtained clones.

The *RoTat* VSG gene of *T. evansi* was amplified from DNA, using primers designed from published sequences (Accession No. HQ286335 using the primer designing tool at NCBI, (Forward primer 5' CACTGCTTTACGCCATCACTC 3'and Reverse primer 5' GCATTCTTTTCCATCCCATTTGC 3'. Polymerase chain reaction of 50µl was used which contained 10 µl of 10x buffer, 1 µl of 10 mM of dNTPs, 25mM MgCl2 (3 µl), 1 µl of each nucleotide primer, 0.5µl of DNA sample and 0.25µl of Taq DNA polymerase. Amplified PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide and visualized under UV illuminator. The specificity of the amplicon was confirmed by running it along with standard DNA molecular weight marker (10 kb DNA ladder). In the agrose gel DNA profile, the expected size of the amplicon (1450 bp) was obtained. Absence of primer-dimer in the DNA profile was proving as the primer concentration of 10 pmole used in the PCR reaction was highly optimal.

In the present study, newly designed primers for amplification of *T evansi RoTat1.2* VSG gene was confirmed as specific amplicon. The obtained products was 1450bp and it appeared as a partial sequence. Gene encoding the members of this protein family have already been found in several species of *Trypanosoma*. Jia *et al.* (2012) reported *T. evansi VSG R3D15,VSG R2D15, VSG R1D12 genes size were* 1745 bp, 1715bp and 1727bp. Similarly, For *RoTat VSG* gene Urakawa *et al.* (2001a) reported *T. evansi* AnTat3.3 VSG gene size 1732bp and Baidya *et al.* (2006) reported *T. evansi* VSG gene size 1689bp.There were variable sizes of the VSG gene from Trypanosoma group of protozoa as evidenced from NCBI database. Field and Boothroyd (1996) reported *T. b .brucei* VSG gene size 2233bp and Cross *et al.* (2013) reported it as 2187bp. Boothroyd *et al.*(1993) reported *T. brucei* VSG gene size 1704bp in contrast to Bromidge *et al.* (1993) who reported *T. brucei* VSG gene size 1147bp in the form of partial sequence.

The developments in the molecular techniques have had considerable input into trypanosome identification, characterisation and diagnosis, accuracy and reliability at various taxonomic levels (Desquesnes and Davila, 2002). PCR-based methods reported by various workers are most reliable and used for sensitive detection of the parasite directly from the blood samples. The use of specific DNA probes to detect *T. evansi* in blood from the carrier animal was found to have several advantages over conventional microscopical, serological and animal inoculation tests (Omanwar *et al*., 1999).

The present study revealed that *RoTat* VSG gene accession No (KF 313141) was appropriated marker for detection of *T.evansi* strains in camels except the Kenyan *T. evansi* type B. This finding agrees with Claes *et al.* (2004) in their study in Variable Surface Glycoprotein (VSG) *RoTat* 1.2 PCR as diagnostic tool for the detection of *T. evansi* infection. A total of 39 different trypanosome stocks were tested using the *RoTat* 1.2 based Polymerase Chain Reaction (PCR). The expression of VSG in the early, middle and late stages of infection favours its use in early diagnosis (in prepatency) as well as in carrier status where the

conventional microscopy fails to diagnose (Robinson *et al*., 1999). This would make the use of VSG-based diagnosis more feasible in *T. evansi* infection than in cyclically transmitted trypanosome species.

Nijiru *et al*. (2006) designed PCR primers to the type B minicircle sequence amplifying a 436 bp fragment. The sensitivity of the *T. evansi* type B primers, as assessed through DNA titration, was 1 trypanosome (0.1 pg). Out of the 185 field blood samples analysed with the type B minicircle primers, two more *T. evansi* type B were detected from Isiolo (SA17) and Samburu (SA201) regions of Kenya.

A remarkable degree of sequence similarity was found among VSGs in gene Data Bank and NCBI databases. In some cases the homology was greater between VSGs from different species than between VSGs of the same species. In the present study obtained *RoTat VSG* gene sequence showed 99% homology towards *T. evansi*, GenBank Accession No. HQ286335, HQ286334, HQ286333 and AF317915. 98.6% similarities was found with *T brucei.* GenBank Accession No. L34415 and V01387. However, 69.5% homology was found between obtained *RoTat VSG* sequence and *T. brucei*, GenBank Accession No. KC613489 and 68.5% homology with *T. brucei* GenBank Accession No.S62479. Lower homology was found between the obtained *RoTat VSG* gene sequence and *T. evansi*, GenBank Accession No. EF067843. Urakawa *et al*. (2001b) observed that DNA sequences homologous to the diagnostic RoTat 1.2 VSG gene were present in the genomes of other trypanosomes as also found in the present sequence analysis of the same gene.

Phylogenetic tree analysis *of RoTat VSG gene* by using the neighbor- joining method revealed that *Trypanosoma evansi,* GenBank Accession No. HQ286335, HQ286334 and HQ286333 were more closely related to *T. evansi* GenBank Accession No. KF313141 than other documented species in this study. *T. evansi* GenBank Accession No. AF317915 and *T. brucei* GenBank Accession No. V01387 were placed as two sub cluster of one mega cluster. *T. brucei* GenBank Accession No. S62479 and *T. brucei* GenBank Accession No. KC613489 as two sub cluster of one mega cluster. The earlier study in *T. evansi* by Sengupta *et*

al. (2012) showed the nucleotide sequence of 681 bp gene encodes *RoTat 1.2 VSG* and was highly conserved and homologous to the VSG gene sequence of published isolates GenBank Accession No. AF317914, AB259839 and EF495337. It had an open reading frame for a protein of 227 amino acids. The previous report by Sengupta *et al*. (2010) of .a partial sequence of *T.evansi* of VSG gene 1432 bp, accession No. AF317914 was amplified by RT-PCR of mRNA. Sequencing of the amplified fragment showed that the amplicon is specific and the nucleotide and amino acid sequences had a high percentage of similarity with the published sequences of *T. evansi* isolates. Similarity search showed that the sequence had 99% homology with *T. evansi* isolate reported from India accession No. AB259839 and 95% with *T. evansi* isolate from Kenya accession No AF317914. Sequencing and phylogenetic analysis showed the close genetic relationship with the *T. evansi* isolates and not with other trypanosomes.

Amer *et al*. (2011) reported alignment of nucleotide sequence of VSG gene variants cloned from Egyptian *T. evansi* isolates and the reference sequence GenBank Accession No. AF317914 clone *RoTat 1.2 VSG.* phylogenetic tree analysis of *T. evansi* from Egypt were done based on partial sequence of ribosomal RNA and VSG RoTat 1.2 gene markers. Phylogenetic relationships of Egyptian isolates of *Trypanosoma* parasites with other salivarian trypanosomes based on ITS2 rRNA.

Cellular uptake of the major trypanocidal drugs is thought to occur through an adenosine transporter. The adenosine transporter-1 gene, *TbAT1*, encoding a P2-like nucleoside transporter has previously been cloned from *Trypanosoma brucei brucei*, and when expressed in yeast, it showed very similar substrate specificity to the P2-nucleoside transporter, but could not transport diamidines (pentamidine and diminazene). They have cloned and sequenced a similar gene (*TevAT1)* from *Trypanosoma evansi* and found it to have 99.7% identity to the *TbAT1* gene.

The number of anti-trypanosomal preparations available is limited and their value in disease control and eradication is reduced by the development of drug resistance in trypanosome populations (Leach and Roberts, 1981).

In the present study the *TevAT1* gene of *T. evansi* was amplified from DNA, using primers newly designed, (Forward primer 5' CGGGTTTGACTCAGCCAATGA 3' and Reverse primer 5' CGTTTTACGTTTATGTCGTGACC 3'). The amplicon obtained in the PCR reaction would be the specific target region as there was no amplification in the negative control included in the reaction (negative control included all the components of the PCR mix without template). The specificity of the amplicon was confirmed by running it along with standard DNA molecular weight marker (10kb DNA ladder). In the agrose gel DNA profile, the expected size of the amplicon (1413 bp) was obtained. Absence of primer-dimer in the DNA profile was proving as the primer concentration of 10 pmole used in the PCR reaction was highly optimal. The amplicon was purified by running LMP agarose gel electrophoresis and subsequent DNA purification by using illustra GFX PCR DNA and Gel Band Purification Kit. The pGEM-T Easy vector (Promega) used for the cloning was highly efficient vector as there was clear cut blue-white selection and maximum number of white colonies in comparison to blue colonies in the LB agar plate. The plasmid DNA isolated from the white colonies released a DNA fragment of 1413 bp. The DNA fragment released upon EcoRI digestion of the plasmid DNA extracted from white colonies was confirmed by running it along with standard DNA molecular weight marker (10kb ladder). It was proving that the DNA fragment amplified in the PCR reaction was of expected size (1413bp).

For the screening of bacterial colonies harbouring recombinant plasmids, colony PCR was employed. In the colony PCR, the amplicon of expected size (1413 bp) was obtained from both the white colonies and from the blue colonies.

Similar work was conducted by Witola *et al*., (2004) in Japan, who had cloned and sequenced *TevAT1* gene from *Trypanosoma evansi* and found it to have 99.7% identity to the *TbAT1* gene. Liao and Shen, (2010) studied the antrycide-resistance of *Trypanosoma brucei evansi* in China in some aspects in the last recent several years, the analysis of quinapyramine-sensitive situation of *T. b. evansi* in China, biological characteristics of *T. b. evansi* population in quinapyramine-resistance and biological materials of quinapyramine-resistance in *T. b. evansi* population. They collected 12 *T.b*. *evansi* isolates from buffaloes,

horses, mules and camels across nine provinces of China. At genetic level, the gene, *TbAT1*, was amplified from the *T. b. evansi* isolates sensitive to quinapyramine-sensitivity but the *T. b. evansi* isolates with quinapyramine-resistance using not only the RT-PCR technique, but also PCR technique.

For *TevAT1* gene the documented other related species gene sequences were reported in various sizes. Maser *et al.* (1999) repoted *T.brucei* strain STIB427 and strain STIB777 adenosine transporter 1(AT1) gene size 1493bp. Witola *et al.* (2004) reported *TevAT1* gene size 1493bp. Leadsham *et al.* (2001) reported *T. equiperdum* AT1 strain BoTat 1.1 and *T. equiperdum* AT1 of strain BoTat 1.1 drug resistant genes size were 1392 for both genes. Berriman *et al.* (2012) reported *T.brucei* AT (Tb927. 3.590), *T.brucei. brucei* strain 927/4 AT1, *T.brucei. brucei* strain 927/4 GUTat10. 1 AT1and *T.brucei. brucei* strain 927/4 GUTat10. 1 AT2 genes size were 1389bp, 1449bp, 1392bp and1389bp.

During the present study, the obtained *TevAT1* gene sequence showed 99.3% homology with *T. equiperdum*, GenBank Accession No. AJ278418, 99.2% with *T. brucei*, GenBank Accession No. AF152370. Higher homology was found between the obtained *TevAT1* gene sequence and *T. brucei*, GenBank Accession No. AF152369 and AJ278417. 73.8% sequence similarity was found between obtained *TevAT1* gene sequence and *T. brucei*, GenBank Accession No. XM838562. Slightly lower homology was documented between the obtained *TevAT1* gene sequence and *T. brucei*, GenBank Accession No. XM822640. lower homology was showed between the obtained *TevAT1* gene sequence and *T. brucei*, GenBank Accession No. XM840059 and *T.brucei*, GenBank Accession No. XM946663.

Based on the above homology and according to the neighbor- joining phylogenetic tree analysis of *TevAT1* gene*, T. brucei,* GenBank Accession No. XM838562 and *T. brucei,* GenBank Accession No. XM946663 were placed as two sub cluster of one mega cluster. *T. evansi,* GenBank Accession No. AB124588, KF280206, *T. equiperdum,* GenBank Accession No. AJ278418, AJ278417 and *T. brucei,* GenBank Accession No. AF152369 and AF152370 as six sub cluster of one mega cluster. *T. brucei,*

GenBank Accession No. XM822640 as sub cluster of one mega cluster. The earlier report by Anne *et al*. (2008) in cloning and sequencing of *TbAT1* gene of *T*. *brucei* in Uganda revealed that the patient isolate *TbAT1* sequences were 99% identical to the *TbAT1* gene sequence of the wild type *T. brucei* reference strain STIB 427, Genbank Accession No. AF152369. The *TbAT1* sequence of the positive control STIB 950 was 99% identical to the *TbAT1* gene of the melarsen oxide cysteamine resistant *T. brucei* reference strain STIB 777R, Genbank Accession No. AF152370.

In the present study the results indicated that the sequence identity of obtained *RoTat VSG* and *TevAT1* nucleotide sequences of *T. evansi* with other trypanosomes species was highly conserved in the most of trypanosomes species. The results of amino acid sequences of obtained *RoTat VSG* and *TevAT1* proteins of *T. evansi* observed high level of homology with amino acid sequence of respective proteins of other trypanosomes species.

Sequence analysis of the gene is the most appropriate method for the confirmation of specificity of the target region of any gene. Therefore aforementioned experiments are attempted to characterize these genes of *T. evansi* from Indian dromedaries. With the sequencing of these genes and expression of protein it can make a great impact on the discovery of new protective antigen, and moreover may be a guide line for developing vaccine to control of trypanosomosis in India.

Though the results from the present studies are encouraging, further work has to be carried out for more investigation and characterization of *TevAT1* gene. These are :

1) Analysis of *TevAT1* gene of *T. evansi* isolates by a PCR /RFLP based method using restriction endonuclease enzyme sfaNI and detection of mutations to differentiate the drug sensitive and resistance *Trypanosoma evansi* isolate of camels.

2) A fluorescence based method for detection of P2 transporter activity appears to be promising option assisting in the determination of the specific drug treatment.

6. SUMMARY AND CONCLUSION

Trypanosoma evansi, a kinetoplastid haemoprotozoan with considerable economic importance, affects a wide range of hosts. The cameline surra occurs in an acute or chronic debilitating form causing high morbidity and mortality. Control of trypanosomosis may be aimed against either the fly or the trypanosome. Trypanocides have been widely used to control the disease. The number of drugs is limited and most of these drugs developed resistance that lead to the failure of treatment. Both the prophylactic as well as the therapeutic aspects of trypanosomosis in animals need alternative approaches. Currently there is no vaccine against the trypanosomes due to antigenic variation that causes by the parasites. Some alternative approaches have been

initiated but still more detailed and comprehensive strategies should be investigated. Recent effort towards the development of a vaccine against *Trypanosoma evansi* has identified several promising recombinant protein based vaccine for *Trypanosoma evansi* .

The present study entitled “Identification and molecular characterization of Variable Surface Glycoprotein and Adenosine transporter genes of *T. evansi* of camel origin” was undertaken to characterize the above mentioned genes of *Trypanosoma evansi* of camel from Indian sub-continent at molecular level. This study could be helpful for understanding the pathogenesis of trypanosomosis and drug resistant concept for control camel trypanosomosis.

The present study was carried out at the National Research Centre on Camel,(NRCC) Bikaner, Rajasthan, India. Initially, the camel suffering from ‘surra’ disease at the NRCC was identified by its clinical signs. Then confirmed of *T. evansi* infection in camel blood by Giemsa stained blood smear examination. These parasites were then propagated in albino mice. Thereafter massive parasitimic mice were dissected to collect *T. evansi*. DEAE cellulose chromatography was used to gain the pellets of trypanosomes from blood of mice. The pellets were then subjected for whole genomic DNA isolation using proteinase K digestion coupled with Phenol: Chloroform: Isoamyl alcohol extraction and subsequent ethanol precipitation. The quality of the DNA was resolved by running agarose gel electrophoresis and genomic DNA was found to be intact without much smearing.

RoTat VSG and *TevAT1* genes of *T. evansi* were amplified from genomic DNA isolated from the pellets of trypanosomes using gene specific primer sequences designed from published sequences (Accession No. HQ286335 for *RoTat VSG* and Accession No. AB124588 for *TevAT1* gene). For *RoTat VSG* gene (1450 bp) amplification, forward 5’ CACTGCTTTACGCCATCACTC 3’and reverse 5’ GCATTCTTTTCCATCCCATTTGC 3’primer sequences were used and for *TevAT1* gene (1413 bp) amplification, forward 5’ CGGGTTTGACTCAGCCAATGA 3’and reverse 5’ CGTTTTACGTTTATGTCGTGACC 3’ primer sequences were used.

Polymerase chain reaction was optimized using $MgCl_2$ concentration (2.5mM) and primer annealing temperature which was 51^0C and 49^0C for *RoTat VSG* and *TevAT1* genes, respectively. Amplified PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide (0.5µg/ml) and visualized under UV light. Subsequently, the amplicons of expected size were purified from the 1% low melting agarose gel using illustra GFX PCR DNA and Gel Band Purification Kit. The DNA fragment of interest was then ligated to the pGEM- T Easy vector as per Promega protocol with slight modification using cloning kit. Following overnight incubation at 4^0C, the ligated mixture was transformed into JM109 cells by heat shock method and was plated onto LB agar containing the final concentration of ampicillin 50 µg/ml, IPTG 10 mM and X gal 20 mg/ml. The plate was incubated for overnight (16-20 hrs) at 37^0C. Both white and blue colonies were grown in the plate. Larger single white colonies were picked up individually and inoculated into LB broth containing final concentration of Ampicilin 50 µg/ml and kept in a water bath cum shaker (at 37^oC and 150 rpm) for 16 hrs. Subsequently, the plasmid DNA was isolated from all the bacterial cultures by using illustra plasmid prep mini spin kit.

After checking the quality of the plasmid DNAs in agarose gel electrophoresis, they were subjected to restriction enzyme digestion using *Eco*RI. After 4 hrs. digestion at 37^oC in water bath, the mixture was analyzed by running agarose gel electrophoresis alongside a DNA molecular weight marker. Release of the expected size fragment confirmed the recombinants. It was found that the release of DNA fragments around 1450 bp for RoTat VSG, gene, and 1413 bp for *TevAT1* gene in the 1.2% agarose gel incorporated with ethidium bromide stain.

Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies in the presence of insert specific primers and results were analyzed by agarose gel electrophoresis using 10 kb plus molecular weight marker. For *RoTat VSG* gene amplifications were found in wells of white colonies but for *TevAT1,* gene amplification was found in wells of white and blue colonies.

The plasmid DNAs along with their respective forward and reverse primers were sent to Eurofins Genomics India Pvt Ltd., Whitefield, Bangalore, for getting the sequences. The coding sequences of *RoTat VSG* and *TevAT1* genes according to the results obtained were of 1450 bp and 1413 bp, respectively. These sequences were then matched using BLAST software. After confirmation of the *RoTat VSG* and *TevAT1* genes nucleotide sequences of *T. evansi* isolated from the host camel, the sequences were submitted to GenBank, NCBI database to which the assigned accession numbers are KF313141 for *RoTat VSG gene* and KF280206 for *TevAT1* gene. Sequence analysis revealed that the length of the coding sequences of *RoTat VSG* and *TevAT1* genes of *T. evansi* from Bikaner, India were 1450 bp and 1413 bp, respectively. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Tree topology of *RoTat VSG* and *TevAT1* gene is based on the Neighbor-Joining method and maximum parsimony with100% bootstrap values. The NJ, bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. Phylogenetic tree analysis of *RoTat VSG* and *TevAT1* gene using maximum parsimony (MP) also showed same topology as NJ method.

Multiple sequence alignment of obtained protein sequences of *RoTat VSG* and *TevAT1* genes was performed with Clustal W (Clustal 2.1) at EBI.

Obtained *RoTat VSG* protein sequence of *T. evansi* was of 483 amino acids (GenBank Accession No. KF313141) which showed 100% amino acid sequence identity with *T. evansi* GenBank Accession No. HQ286335, GenBank Accession No HQ286334 and GenBank Accession No HQ286333, 99% of *T. evansi* GenBank Accession No. AF317915 and *T. brucei* GenBank Accession No. L34415, 65% of *T. brucei* (GenBank Accession No. V01387, 58% of *T. brucei* (GenBank Accession No. EF067843 , 56% of *T.brucei* (GenBank Accession No.KC613489) and 55% *T. evansi* (GenBank Accession No. S62479).

Obtained *TevAT1* protein sequence of *T. evansi* was of 463 amino acids (GenBank Accession No. KF280206) which showed 99% amino acid sequence identity of *T. brucei* GenBank Accession No. AF152369 *T. evansi* GenBank Accession No. AB124588 and *T. equiperdum* GenBank Accession No.AJ278417, 98% of *T. brucei* (GenBank Accession No. AF152370), *T. equiperdum* (GenBank Accession No. AJ278418, *T. brucei* (GenBank Accession No. XM838562) and *T.brucei* (GenBank Accession No. XM840059) , 66% of *T. brucei* (GenBank Accession No. XM946663) and *30% of T. brucei* (GenBank Accession No. XM822640).

Sequence analysis of the gene is the most appropriate method for the confirmation of specificity of the target region of any gene. Therefore aforementioned experiments were attempted to characterize these genes of *T. evansi* from Indian dromedaries. With the sequencing of these genes and expression of protein it can make a great impact on the discovery of new protective antigen, and moreover may be a guideline for developing vaccine to control of trypanosomosis in India.

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Identification and Molecular Characterization of Variable Surface Glycoprotein and Adenosine Transporter 1 genes of *Trypanosoma evansi* of Camel Origin

Ph.D. Thesis

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Major Advisor : **Dr. G. S. Manohar**

ABSTRACT

The present study was carried out to isolate the Rode Trypanozoon Antigen Type VSG and Trypanosoma evansi adenosine transporter 1 genes of *Trypanosoma evansi* using PCR, then clone the amplicons in a suitable plasmid vector and characterize the above genes through sequencing. For this study, suspected *Trypanosoma evansi* infected camel was confirmed by examination of Giemsa stained camel blood smear . After confirming infection, the *T. evansi* were propagated in Swiss albino mice and at peak parasitaemia the mice were dissected for collecting blood. Purification of trypanosomes from blood of mice was done by using DEAE cellulose chromatography. DNA extraction was done from collected pellets of *Trypanosoma evansi* using the phenol-chloroform extraction followed by ethanol precipitation.

The desired amplicons of *RoTat VSG* and *TevAT1* genes were successfully amplified by PCR using gene specific primers. Amplified PCR products were analyzed on 1.2% agarose gel stained with ethidium bromide and identified on the basis of size of the *RoTat VSG* and *TevAT1* genes i.e (1450 and 1413, respectively). The amplicons obtained were purified employing illustra GFX PCR DNA and Gel Band Purification Kit. The purified DNA fragment was ligated to the pGEM- T Easy vector and ligated mixture was transformed into *Escherichia coli* JM109 strains. The cells containing recombinant plasmid could be identified on the basis of white/blue colony selection on LB agar containing X-Gal, IPTG and ampicillin. Screening of recombinants was done by Restriction Enzyme digestion of plasmid DNAs using *Eco*RI and confirmed on the basis of genes size, i. e 1450 bp for *RoTat VSG* and 1413 bp for *TevAT1* gene. Colony PCR was done for quick screening of plasmid inserts directly from *E. coli* colonies in the presence of insert specific primers. Then the plasmid DNAs were sequenced and coding sequences of *RoTat VSG* and *TevAT1* genes according to the results obtained were of 1450 bp, and 1413 bp, respectively. The phylogenetic and sequence analysis was done by use of Clustal X and MEGA5 softwares. Tree topology of *RoTat VSG* and *TevAT1* gene was based on the Neighbor-Joining method and maximum parsimony with 100% bootstrap values. Multiple sequence alignment of obtained protein sequences of *RoTat VSG* and *TevAT1* genes was performed with Clustal W (Clustal 2.1) at EBI. Identified *RoTat VSG* and *TevAT1* gene sequences showed a close homology with most of the other *Trypanosoma spp*. gene sequences in the public database at National Center for Biotechnology Informations (NCBI).

ऊँट से पृथक ट्रीपैनोसोमा इवान्सी के वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर 1 जीन की पहचान और आन्विक अभिलक्षण

पीएच.डी. शोध ग्रंथ,

i'kq ijthoh foKku foHkkx]

i'kq fpfdRlk ,oa i'kq foKku egkfo|ky;]

jktLFkku i'kq fpfdRlk ,oa i'kq foKku fo'ofo|ky;]

chdkusj&334 001

शोधकर्ता- **रेहाब अली इब्राहीम यागी**

मुख्य उपादेष्टा- **डॉ. जी. एस. मनोहर**

अनुक्षेपण

वर्तमान अध्ययन में *ट्रीपैनोसोमा इवान्सी* के वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन को पी.सी.आर. का उपयोग कर अलग किया गया। उसके बाद एक उपयुक्त प्लाज्मिड वेक्टर में एमप्लीकोंस को क्लोन किया गया और अनुक्रमण के माध्यम से उपरोक्त जीनों की विशेषता का अध्ययन किया गया। ऊंट खून के स्टेन्ड स्मीयर की परीक्षा के द्वारा आकृति के आधार पर संदिग्ध *ट्रीपैनोसोमा इवान्सी* संक्रमित ऊंट की पुष्टि की गई। संक्रमण की पुष्टि करने के बाद ऊंट रक्त से एकत्र *ट्रीपैनोसोमा इवान्सी* को स्विस अल्बिनो चूहों में विस्तारित किया गया और जब चूहों में भारी संक्रमण हो गया तब चूहों को विच्छेदित कर दिल क्षेत्र से रक्त एकत्र की गई। चूहों के रक्त से ट्रीपैनोसोमा की शुद्धि के लिए डी.इ.ए.इ. सेल्यूलोज़ क्रोमैटोग्राफी किया गया।

फिनॉल क्लोरोफॉर्म निस्सारण और उसके बाद इथेनॉल अपघतन के द्वारा संग्रहित *ट्रीपैनोसोमा इवान्सी* की पेल्लेट्स (गोलियों) से डी.एन.ए. निष्कर्षण किया गया। वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन को जीन विशिष्ट प्राइमरों का उपयोग करते हुए पी.सी.आर. द्वारा प्रवर्धित किया गया। प्रवर्धित पी.सी.आर. उत्पादों को इथीडीएम ब्रोमाइड से चिह्नित 1.2% अगारोस जेल पर विश्लेषण किया गया और वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन के आकार के आधार पर पहचान की गई। इलुस्टरा जी.एफ.एक्स. पी.सी.आर. डी.एन.ए. और जेल बैंड

शोधन किट द्वारा 1% कम पिघलने वाले एगारोज जेल से अपेक्षित आकार के एमप्लीकोंस को शोधित किया गया। दिलचस्पी के डीएनए टुकड़े को पी.जी.इ.एम.टी. ईजी वेक्टर में क्लोन करने के लिए लायगेट किया गया और लायगेटेड मिश्रण को *ई. कोलाई* जे.एम.109 उपभेदों में तब्दील किया गया। रीकॉम्बीनैंट प्लाज्मिड युक्त कोशिकाओं को लुरीया बर्तानी अगार जिनमें एक्सगाल, आई.पी.टी.जी. और एम्पीसिलीन होता ह नीली/सफेद कॉलोनी के आधार पर पहचाना गया। *इको* आर1 का उपयोग कर प्लाज्मिड डी.एन.ए. के प्रतिबंध एंजाइम पाचन द्वारा रिकोम्बिनेंट्स की जाँच की गयी और पाया गया कि वेरीयेबल सरफेस ग्लाइकोप्रोटीन जीन के लिए 1450 बेस जोड़ी और एडीनोसिन ट्रांसपोर्टर जीन 1413 बेस जोड़ी के आसपास डी.एन.ए. के टुकड़े रिलीज हुए हैं। जीन विशिष्ट प्राइमरों की उपस्थिति में सीधे *ई. कोलाई* से प्लाज्मिड आवेषण के त्वरित जांच के लिए कॉलोनी पी.सी.आर. किया गया। वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन की क्लोन के पुष्टि के बाद प्लाज्मिड डी.एन.ए. का अनुक्रम किया गया और प्राप्त परिणामों के अनुसार वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन के कोडिंग अनुक्रम क्रमशः 1450 बेस जोड़ी और 1413 बेस जोड़ी के पाये गये। वंशावली और अनुक्रम विश्लेषण क्लसटल एक्स और मेगा 5 सॉफ्टवेयर के उपयोग के द्वारा किया गया। वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन की ट्री टोपोलॉजी (सांस्थिति) 100% बूटस्ट्रप मूल्यों के साथ पड़ोसी शामिल होने और अधिकतम कृपणता की विधि पर आधारित ह वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन के प्राप्त प्रोटीन अनुक्रमों का एकाधिक अनुक्रम संरेखण इ.बी.याई. में क्लसटल डब्ल्यू (क्लसटल 2.1) के साथ किया गया। पहचान किये गये वेरीयेबल सरफेस ग्लाइकोप्रोटीन और एडीनोसिन ट्रांसपोर्टर जीन अनुक्रमों ने अन्य प्रकार के *ट्रायपनोसोमा* जीन अनुक्रमों के साथ एक करीबी अनुरूपता दिखाई।

9. APPENDICES

APPENDIX – I

1. Agarose Gel Electrophoresis buffer

1.1. TAE buffer (50x)

Stock solution:

Tris base	121 gm
Glacial acetic acid	28.55 gm
0.5 M EDTA acid (pH 8.0)	50 ml
Water upto	500 ml

Working concentration of TAE buffer (1x)

TAE buffer	10 ml
Water	490 ml

1.2. TE (Tris/EDTA) Buffer

Tris-HCl (pH 7.5) 10 mM

EDTA 1 mM

Make from 1M stock of Tris-HCl (pH 7.5) and 500 mM stock of EDTA (pH 8.0).

Working solution:

1M Tris-HCl (pH 7.5) 1.0 ml

500mM EDTA (pH 8.0) 0.2 ml

Water to 100 ml

2. Phosphate Buffered Saline buffer (1X), pH 7.4

NaCl 8.0 gm

$Na_2HPO_4.2H_2O$ 1.44 gm

KH_2PO_4 0.24 gm

KCl 0.20 gm

Distilled water to make 1000 ml

3. Trypanosome separatic fer (PSG buffer, pH 8.0)

३०१०ैवसनजपवद ।

Na_2HPO_4 (anhydrous) 8.000 gm

$NaH_2PO_4.2H_2O$ 0.780 gm

NaCl 4.250 gm

Distilled water to make 1000 ml

३०२०ैवसनजपवद ठ

Glucose solution dextrose 10 gm

Distilled water to make 400 ml

Just before use 6 parts of solution A was mixed with 4 part of solution B

4. Proteinase K buffer

Tris base (ph 8)	100mM
EDTA	10mM
NaCl	50mM
SDS	2%
β Mercapto ethanol	20mM

APPENDIX – II

1. Giemsa stain

T.evansi pellet fixed with methnol for 5 minutes and air-dried for 5 min

Giemsa stain	1 ml
Distilled water	9 ml

Slides were stained for 45 min.

2. 6X loading dye

Bromophenol blue	0.25%
Xylene cyanol	0.25%
Glycerol	50%
EDTA	2 mM

3. Ethidium bromide solution (10 mg/ ml)

Ethidium bromide	0.2gm
Sterile Water	20 ml

4. Agarose (0.8%)

Agarose	800 mg
TAE buffer	100 ml

5. Agarose (1.2%)

Agarose	1.2 gm
TAE buffer	100 ml

APPENDIX – III

1. Phenol: Chloroform: Iso amyl alcohol; 25:24:1

Phenol	250 ml
Chloroform	240 ml
Iso amyl alcohol	100 ml

2. Alcohol (70%)

Alcohol	70 ml
Water	30 ml

3. Luria Bertani (LB) Medium (500 ml)

Trypton	1.0% (5 gm)
Yeast extract	0.5% (2.5 gm)
NaCl	0.5% (2.5 gm)

Adjust the pH to 7.0 with NaOH

For LB plates, add 1.5% (7.5 gm) agar to the LB broth and autoclave.

4. Luria Bertani (LB) Agar (500 ml)

Trypton	1.0% (5 gm)
Agar	1.5% (7.5 gm)
Yeast extract	0.5% (2.5 gm)
NaCl	0.5% (2.5 gm)

Adjust the pH to 7.0 with NaOH

5. LB plates with ampicillin/ IPTG/ X- Gal

LB plates with ampicillin were made by adding ampicillin to a final concentration of 50µg/ml after cooling of LB agar to 50^0C. Then 100 µl of 100mM IPTG and 20 µl of 20mg/ml X- Gal was spreaded over the surface of the LB- ampicillin plate and allowed to absorb for 30 minutes at 37ºC prior to use.

6. SOC medium (100ml)

Trypton	2 gm

Yeast extract	0.5 gm
NaCl (1M)	1 ml
KCl (1M)	0.25 ml
Mg+2 stock (2M)	1ml
Glucose (2M)	1ml

2M Mg^{+2} stock (100ml)

$MgCl_2.6H_2O$	20.33 gm
$MgSO_4.7H_2O$	24.65 gm

7. X-GAL solution

X-GAL	20mg/ml

Dissolved in 100% N,N dimethyl formamide.

20 µl X-GAL was used for 25-30ml LB agar medium in LB plate.

8. IPTG solution

IPTG 100mM

Dissolved in distilled water.

100µl IPTG was used for 25-30ml LB agar medium in LB plate.

9. Ampicillin solution

Ampicillin 50µg/ml

Dissolved in distilled water.

To make 25ml of LB broth medium containing 50µg/ml Ampicillin, 125 µl of 10mg/ml ampicillin stock was used.

For 500 ml LB agar 2.5 ml of 10 mg/ml ampicillin stock was added, ensure that LB agar cooled to 50^{o}C before adding ampicillin.

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Place: Bikaner

Date: *Rehab Ali Ibrahim Yagi*

Introduction

Review of Literature

MATERIALS AND METHODS

RESULTS

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LITERATURE CITED

ABSTRACT

(ENGLISH AND HINDI)

APPENDIX

This thesis is dedicated to my late father whom I lost while pursuing my Ph.D. programme at Rajasthan University of Veterinary and Animal Sciences, Bikaner, India. “May almighty God rest his soul in eternal peace”.