CLASSICO-MOLECULAR STUDIES AND CHARACTERIZATION OF ROTAT 1.2 VSG OF *TRYPANOSOMA EVANSI* IN EQUINES

THESIS

SUBMITTED FOR PARTIAL FULFILLMENT OF THE REQUIREMENTS

FOR THE AWARD OF THE DEGREE

OF

MASTER OF VETERINARY SCIENCE

IN

VETERINARY PARASITOLOGY

By

ANJALI DEVI

Enrollment No-1482/15

COLLEGE OF VETERINARY SCIENCE & ANIMAL HUSBANDRY

U.P. Pandit Deen Dayal Upadhyaya Pashu-Chikitsa Vigyan Vishwavidyalaya

Evam Go Anusandhan Sansthan, Mathura – 281001

(2017)

CERTIFICATE

This is to certify that the thesis entitled **"Classico-molecular studies and characterization of Rotat 1.2 VSG of *Trypanosoma evansi* in equines"** submitted by **Dr. Anjali Devi, Enrollment No. V-1482/15** in partial fulfillment of the requirements for the award of the **Master of Veterinary Science in veterinary parasitology** of the **U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura (U.P.),** India, is a bonafide research work carried out by her under my supervision and guidance and no part of the thesis has been submitted for any other degree or diploma.

13.6.17

Dated: 13.6.17

(Daya Shanker)
Major Advisor and Chairman
Professor and Head
Department of Veterinary Parasitology
DUVASU, Mathura

CERTIFICATE

It is certified that the thesis entitled **"Classico-molecular studies and characterization of Rotat 1.2 VSG of *Trypanosoma evansi* in equines"** submitted by **Dr. Anjali Devi, Enrollment No. V-1482/15** in partial fulfillment of **Master of Veterinary Science** Degree in **Veterinary Parasitology** at College of Veterinary Science and Animal Husbandry, Mathura, and embodies the original work done by the candidate herself. The candidate has carried out her work sincerely and methodically.

We have carefully gone through the contents of the thesis and are fully satisfied with the work carried out by the candidate, which is being presented by her for the award of the Degree of this University.

It is further certified that candidate has completed all the prescribed requirements governing the award of the degree of **Master of Veterinary Science** at U.P. Pt. Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go-Anusandhan Sansthan, Mathura, 281001 (UP), India.

ADVISORY COMMITTEE

S. No.	Name	Status	Signature
1.	Dr. Daya Shanker	**Chairman**	13.6.17
2.	Dr. Amit Kumar Verma	**Member**	
3.	Dr. Rakesh Goel	**Member**	13/6/17
4.	Dr. M.M. Farooqui	**Member/** Dean PG Nominee	

13.6.17

(Signature)
HOD
Date: 13.6.17

13.6.17

(Signature)
Chairman (Advisor)
Date: 13.6.17

U.P. PANDIT DEEN DAYAL UPADHYAYA PASHU CHIKITSA VIGYAN VISHWAVIDYALAYA EVAM GO-ANUSANDHAN SANSTHAN MATHURA-281001 (UP)

VIVA –VOCE REPORT

Name of Student:	**Dr. Anjali Devi**
Enrollment No.:	V-1482/15
Subject:	Veterinary Parasitology
College:	College of Veterinary Science and Animal Husbandry DUVASU, Mathura.
Title of the Thesis:	Classico-molecular studies and characterization of Rotat 1.2 VSG of *Trypanosoma evansi* in equines
Degree:	M .V. Sc

This is to certify that the corrections of the thesis indicated by the external examiner have been incorporated and the viva-voce examination of the student before the advisory committee was found **satisfactory**/ ~~**unsatisfactory**~~. Therefore, the degree of the Master of Veterinary Science **may**/~~**may not**~~ **be** conferred to the candidate.

ADVISORY COMMITTEE

S. No.	Name	Status	Signature
1.	Dr. Daya Shanker	**Chairman**	
2.	Dr. Amit Kumar Verma	**Member**	
3.	Dr. Rakesh Goel	**Member**	
4.	Dr. Mukesh Kumar Srivastava	**Member**/ Dean PG Nominee	

Signature:

Name: Dr. Anish Yadav
Designation: Associate Professor
Address of External Examiner:
Department of Veterinary Parasitology
SKUAST- Jammu, J & K

HOD
Department of Veterinary Parasitology

CERTIFICATE

It is certified that the thesis submitted by **Dr. Anjali Devi** Enrollment No. **V-1482/15**, a Master's student of this Department has been checked and found as per specifications of the format mentioned in the PG Academic Regulation-2009 (revised).

13.6.17

(Daya Shanker)

Head of Department

ACKNOWLEDGEMENTS

This thesis is the result of six months work where I have been accompanied and supported by many people. It is a pleasant aspect that I have now opportunity to express my gratitude to all of them.

Gratitude cannot be seen or expressed, it can only be felt in heart and soul, and is beyond description. Although, thanks are poor expression of the deep debt and gratitude that one feels, yet there is no better way to express it. The publishing of this manuscript is a complete team effort and I would like to express my sincere thanks to all individuals who have played a part in creation of the manuscript. I take this opportunity to express my deep sense of gratitude towards my Guide, **Dr. Daya Shanker,** M.V.Sc., Ph.D., Professor and Head, Department of Parasitology, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalya Evam Go Anusandhan Sansthan, Mathura (U.P.), for his excellent guidance, critical comments, stimulating involvement, constant moral support, constructive counsel and painstaking efforts during the entire period of this study.

Words are not enough to express my gratitude to, **Dr. Vikrant Sudan and Dr. Amit K. Jaiswal** Assistant Professor, Department of Parasitology for constant guidance, leadership, attention to detail, hard work, motivation, precious time, valuable support, encouragement during the entire course of work. This work would not possible without their endless effort to keep me going on. They have stood by me in all the ups and downs of this project. I consider it as a privilege to have worked under his expert guidance and I would remain ever grateful to him for imparting knowledge on all aspects of life.

I express my sincere thanks to **Dr. Amit Singh, Dr. Jitendra Tiwari, Dr. Pradeep Kumar and Dr. Amit Verma,** for their continuous support and strenuous efforts to impart good knowledge in the subject.

I express my sincere thanks to **Dr. K. M. L. Pathak,** Hon'ble Vice Chancellor, **Dr. P.K. Shukla,** Dean PGS and **Dr. S. K. Garg**, Dean of Veterinary faculty, U.P. Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalya Evam Go- Anusandhan Sansthan, Mathura for providing various necessary facilities during entire course of this study.

The assistance provided by the non-teaching staff of the Parasitology, Co.V.Sc and A.H. cannot be forgotten. So I heartily thank to **Mr. Manoj Kumar and Mr. Hemant Kumar** for his technical support**,** while indispensible help by **Mr. Pramod Kumar, Mr. Badan Singh** and **Mr. Heera Singh** for their readily available help, cooperation and for providing conducive environment in the department to carry out my research work.

It’s a great pleasure and pride to remember the valuable time I spent with my seniors and juniors **Dr(s). Ruchi Singh Gaur, Mahendra Kumar Chaudhary, Sanjeev Kumar Verma, Priyanka Dwivedi**. I wish to thank my friends Dr**(s). Takshi Rehalia, Mona Sharma, Ved Prakash Srivastava, Rita Verma, Sanjhi Paliwal,** for their help, care, support and affection in my life.

Words fail to flow when it is time to acknowledge the dedication and sacrifices made by *family members to* bring me to this level in life. The love and affection showered by my **parents** as they are devoted representative of God, father **Shri Vishnu Pal**, and mother **Smt. Sony Devi** and my younger brother **Amit Kumar** for helping me, get through the difficult times, and for all the emotional support, camaraderie, entertainment, and care they provided. This dissertation is simply impossible without their unflagging love and support.

I express my gratitude to **Mr. Ravi Chauhan**, Mathura, for his excellent thesis setting in making the manuscript.

Lastly but above all, I am grateful to Almighty **“GOD”** who has given me courage, patience and motivation to complete the study successfully. A formal statement of acknowledgement will hardly meet the ends of justice in expression of my deeply felt sincere and allegiant gratitude to all who encouraged and helped me during my stay. I feel sorry, if I forgot to mention anyone.

Place: Mathura
Date: 13/6/17

Anjali
(Anjali Devi)

ABBREVIATIONS

°C	:	degree Celsius
%	:	Percent
DNA	:	Deoxyribose nucleic acid
et al.	:	et alii and others
Fig.	:	Figure
gm	:	Grams
Min.	:	Minutes
Ml	:	Millilitre
OIE	:	Office International despizooties
PCR	:	Polymerase chain reaction
Rpm	:	revolution per minute
spp.	:	Species
temp.	:	Temperature
viz.	:	Namely
IFAT	:	Indirect flouroscent antibody test
ELISA	:	Enzyme linked immunosorbent assay
VSG	:	Variant surface glycoprotein
VAT	:	Variant antigen type
CATT	:	Card agglutination test
rRNA	:	Ribosomal ribo nucleic acid
Pg	:	Pico gram
MHCT	:	Micro haematocrit method
P	:	Prevalence
TE-PCR	:	Trypanosome evansi based polymerase chain reaction
WBF	:	Wet blood film
Bp	:	Base pair
EDTA	:	Ethylene diamine tetra acetic acid
LB	:	Luria bertani
TVCC	:	Teaching veterinary clinic complex
µl	:	Microlitre
mM	:	Millimolar

Mg	:	Milligram
SDS	:	Sodium dodecyl sulphate
M	:	Molar
Taq	:	*Thermus aquaticus*
F	:	Forward
R	:	Reverse
Sec.	:	Second
TAE	:	Tris acid Ethylene diamine tetra acetic acid
µg	:	Microgram
V	:	Volt
NFW	:	Nuclease free water
g	:	Gravity
h	:	Hours
Vol	:	Volume
NCBI	:	National Center for Biotechnology Information
CI	:	Confidence interval
i.e	:	That is
&	:	And
ISG-75	:	Invariant surface glycoprotein
ITS-1	:	Internal transcribed spacer

LIST OF TABLES

LIST OF FIGURES

Contents

CHAPTER- 1

INTRODUCTION

Equines hold a special position in livestock sector both for civil and military purpose in view of their multifacet utility. In Northern India, particularly in the states of Jammu and Kashmir, Haryana, Punjab, Uttar Pradesh, Rajasthan and Gujarat, donkeys and horses are extensively used as a mode of transportation and for draught purposes especially in hilly, arid and semi-arid areas where motorable connectivity is inadequate or not feasible. Uttar Pradesh alone contributes 24.31% of India's total equine population of 0.625 million (Livestock Census 2012). Major population of these equids provides livelihood to the landless, small and marginal farmers and other sections of unprivileged rural societies. Approximately, 98% equine in India contribute to the employment and income of the poor farmers and landless laborers mainly through utilization of equines in cart and carriage. The remaining about 2% of the equine population is owned by elite sections of society and is used for sports such as racing, polo and for national security purpose by military and paramilitary forces. Two states *viz*., Uttar Pradesh and Jammu & Kashmir possess 47% of horses/ponies and 40% mules, whereas about 65% donkeys are inhabited in Rajasthan, Uttar Pradesh, Gujarat and Maharashtra of total Indian equine population. Amongst many pathogenic parasitic infections, haemoprotozoan infections inflict enormous losses to equine population in terms of production output. They not only affect health and productivity of equids but also hamper their working efficiency.

Amongst haemoprotozoan infections, trypanosomosis is economically important vector-borne disease of equids (horses, donkeys and mules) in tropical and subtropical parts of the world (Sumbria *et al*., 2015). *T. evansi*, a flagellate kinetoplastid is the causative agent of Surra, (a Hindi word, meaning 'rotten') (Bhatia *et al*., 2016) was discovered by Griffith Evans, in erstwhile Punjab, India (now in Pakistan) in 1880 from the blood of equines and camels (Evans, 1880). In Northern India, *T. evansi* is transmitted mechanically by intermittent biting of insects such as tabanids and stomoxys (Sumbria *et al*., 1998). It is the most prevalent pathogenic *Trypanosome* having the largest geographical distribution in Asia, Africa, South and Central America (Dargantes *et al*., 2009). *T. evansi* infection is widely prevalent throughout India inflicting serious losses in domestic animals (Pathak and Chhabra, 2011). The parasite is having a vast host range undergoing acute phase in dogs and equines while it is seen in chronic form in camels besides affecting cattle and buffalo as reservoir hosts (Pathak and Chhabra, 2011). *T. evansi* is said to have originated from the tsetse transmitted

Trypanosoma brucei brucei, by deletion of kinetoplastic maxicircles (Lun and Desser, 1995; Lai *et al*., 2008).

The Office Internationale des Epizooties (OIE) mentions the disease under list B diseases of significance in horses (OIE, 2012). The main symptoms in equines includes intermittent fever, anaemia, urticarial eruptions (plaques) accompanying febrile paroxysm, oedema of the dependent parts of the body and genitalia (De and Mukherjee, 2006), petechial haemorrhages in visible mucous membranes, tachycardia, staggering gait, jugular pulsation and labored respiration (Bharathi and Padmaja, 2007) besides alterations in antioxidant potential of affected animals (Pandey *et al.,* 2015). Hepatic insult (Varshney and Gupta, 1996) and endocrine dysfunctions (Varshney *et al.,* 1999) are also reported in clinical trypanosomiosis in horses. Transplacental transmission in donkey is also reported (Pathak and Kapoor, 1999). Stress factors such as flooding, intercurrent disease (Gupta *et al*., 2009), vaccination, transport stress (Kalra *et al*., 1994) and malnourishment (Malik *et al*., 2000) often change an inapparent infections into clinical disease.

The disease is usually acute, although some horses may experience chronic manifestations. Mortality rate in horses can be quite high in areas where the disease has been newly introduced (Sellon, 2007). In a field outbreak of surra in equines reported from Mathura (Kumar *et al.,* 1994), 100% morbidity and 66.6% mortality were recorded. Likewise, an acute outbreak in ponies was reported from Jammu (Raina *et al.,* 2000). Surra has also been reported in equines from Maharashtra (Bharkad *et al.,* 2005) and north eastern states of India (Laha and Sasmal, 2008). An overall prevalence rate of 3.7% has been reported from various agroclimatic zones in Punjab (Sumbria *et al*., 2015) and 12.74% from West Bengal (Laha and Sasmal, 2008). The country-wide seroprevalence of *T. evansi* is 4.6% (28/614) in horses from Israel (Berlin *et al*., 2012).

Identifying the carrier status/latent infection is an important criteria in controlling the disease. Detection of clinical disease is generally done by wet film and thin and thick blood smear examination after de-haemoglobinisation in water and later staining, mouse inoculation and various serological methods. The detection of parasites in blood is difficult because parasitaemia is often low and fluctuating, particularly, during the chronic stage of infection and post treatment (Desquesnes *et al*., 2013). The gold standard for diagnosis of trypanosomiosis still remains the microscopical demonstration of parasites in blood smear. However, it suffers from limitations of sensitivity (Singh and Singla, 2012). Nevertheless, detection of the carrier stage is equally important as the carriers play a very critical role as reservoirs of infection and are a constant threat of introduction of a disease in a naïve demographical location. Serological tests like indirect fluorescent antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA) are capable of detecting antibodies in carrier animals and hence are routinely used for

monitoring surveillance and export certification (Sudan *et al*., 2015a). However, they too suffer on the grounds that antibodies can be detected even years after recovery of infections, without any active infection, is prevalent, thereby obscuring the exact picture of prevalence of infection at that particular point (Sudan *et al*., 2015a). Serological methods also suffers from the limitation of cross reactivity and failure to differentiate between current and past infection. For the diagnosis of latent infection, molecular techniques like PCR give a promising result with greater levels of sensitivity and specificity (Bashir *et al*., 2014) than the conventional techniques. PCR is the most accurate tool for the diagnosis of subclinical and latent infections. A number of PCR assays have been standardized *viz*., simples PCR (Parashar *et al*., 2015), duplex PCR (Sudan *et al*., 2015a) and multiplex PCR (Sumbria *et al.*, 2015) in the recent past but still the application of molecular tools in diagnosis of equine surra is limited. Several genes have been investigated and targeted as potential tools for the molecular diagnosis of trypanosomiosis which includes variable surface glycoprotein (VSG) genes (Sengupta *et al*., 2010), repetitive nuclear DNA sequences (Masiga *et al*., 1992), ribosomal DNA (Ijaz *et al*., 1998) and a region from r-RNA internal transcribed spacer 1 (ITS-1) (Taylor *et al*., 2008).

The *Trypanosome* variant surface glycoprotein (VSG) is considered to be an integral part of parasite's surface coat. These VSGs have unique ability to undergo class switching in expression on regular basis which accounts for its immune evasion strategy against the host's immune response (Barry and McCullogh, 2001). Analysis of nuclear DNA, kinetoplast DNA and multilocus isoenzymes (Mathieu-Daude and Tibayrenc, 1994), has suggested that, *T. evansi*, in contrast to the *Trypanosome*s that develop cyclically in the tsetse fly, has a limited variant surface glycoprotein (VSG) antigenic repertoire (Zhang and Baltz, 1994). However, amongst all those VSG repertoires, RoTat 1.2 VSG is a predominant variant antigen type (VAT) expressed in early, middle and late stages of infection (Verloo *et al*., 2001) in many of the *T. evansi* isolates (Verloo *et al*., 2000). Of late, RoTat 1.2 VSG gene was found to be either having some structural variations (Jia *et al*., 2011) or is altogether absent in some *T. evansi Trypanosome* isolates (Ngaira *et al*., 2004; Salim *et al*., 2011). Inspite of being the major determinant of immune evasion process, the host immune system still elicits sufficient level of antibody production against the parasite VSG (Gadelha *et al.*, 2011). The surface epitopes in live *Trypanosome*s are confirmationally labile (Freymann *et al*., 1990) and anti-VSG antibodies are known to recognizes glycosylated and deglycosylated VSGs equally well (Reinwald, 1985). These points make VSG a potent antigen in the diagnosis of *T. evansi* infection.

Consequently, several antibody detection assays have been developed using RoTat1.2 as detecting antigen. These include direct agglutination test (CATT) that uses whole, formaldehyde fixed, Coomassie-stained, freeze-dried RoTat1.2 *Trypanosome*s (Songa and

Hamers, 1988), an indirect agglutination test (LATEX) (Verloo *et al.,* 2000), and an ELISA (Verloo *et al*., 2000) consisting of soluble-form RoTat1.2 VSG purified from the *Trypanosome*s (Payne *et al*., 1991; Monzon *et al*., 1995; Verloo *et al*., 2000; Davison *et al*., 2000).

The recent developments in the molecular techniques have imposed a considerable impact upon the *Trypanosome* identification, characterization, accuracy and reliability at various taxonomic levels (Desquesnes and Davila, 2002). There are ample reports of characterization studies on *Trypanosome*s in general and *T. evansi* in particular. The important molecular studied so far include, oligopeptidase B from *T. b. brucei*, variable surface glycoprotein (VSG) gene of *T. evansi* (Sengupta *et al.,* 2012), ISG-75 gene of *T. b. gambiense* (Tran *et al.,* 2008), hypoxanthine guanine phosphoribosyl transferase gene of *T.b. brucei* (Allen and Ullman, 1993), beta-tubulin gene of *T. evansi* (Li *et al.,* 2007) and actin gene of *T. evansi* (Li *et al.,* 2009). All these molecules are used for phylogenetic analysis between the various isolates.

Keeping above points in mind, the present investigation is designed with the following objectives:

1. Standardization of a PCR based molecular technique employing RoTat 1.2 VSG as molecular target for accurate diagnosis of *T. evansi* infection in equines.
2. Comparison of sensitivity and specificity of RoTat 1.2 VSG PCR vis-à-vis blood smear examination.
3. Molecular characterization and phylogenetic analysis of RoTat 1.2 VSG from *T. evansi* of equine host in suitable prokaryotic cloning system.

CHAPTER-2

REVIEW OF LITERATURE

Trypanosomiosis (surra), caused by *T. evansi* is a major constraint on the health and productivity of domestic animals throughout the tropics and subtropics. The parasite is capable of infecting a wide host range of mammals. *T. evansi* is common in India, owing to favorable environment conditions for the breeding of the fly vectors (Bhatia *et al*., 2016). The incidence of surra usually follows the onset of monsoons, and extends well into the post-monsoon period (Soodan *et al*., 1995). Stress factors such as flooding, intercurrent disease (Gupta *et al*., 2009), vaccination, transport stress (Kalra *et al*., 1994) and malnourishment (Malik *et al*., 2000) usually results in change of apparent infection into clinical disease. Outbreaks of acute disease are recorded in all domestic animals especially those in the high endemicity states like Haryana and Punjab (Gupta *et al*., 2003; Jindal *et al*., 2005).

The gold standard for diagnosis of trypanosomiosis still remains the microscopical demonstration of parasites in blood smear. However, this method suffers from limitations of sensitivity (Singh and Singla, 2012). It also fails to detect infection in cases where parasitaemia is often low and fluctuating, particularly, during the chronic stage of infection and post treatment cases (Desquesnes *et al*., 2013).Serological techniques enzyme-linked immunosorbent assay (ELISA) are no doubt capable of detecting antibodies in carrier animals and hence are routinely used for monitoring surveillance and export certification (Singh *et al*., 2014). But they too suffers on the grounds that antibodies can be detected even years after recovery from infection (though no active infection is prevalent) thereby obscuring the exact picture of prevalence of infection at that particular point (Sudan *et al*., 2015). Nevertheless, detection of this carrier stage is equally important as the carriers play a very critical role as reservoirs of infection and are a constant threat of introduction of a disease in a naïve demographical location. This stresses the relevance of molecular PCR-based techniques. PCR is the most accurate tool for the diagnosis of subclinical and latent infections of trypanosomiosis. A number of PCR techniques have been standardized *viz*., simple PCR (Parashar *et al*., 2015), duplex PCR (Sudan *et al*., 2015) and multiplex PCR in the recent past but still, the application of molecular tools in diagnosis of equine surra is very limited especially in Indian scenario (Sumbria *et al*., 2015).

Equines too suffer from trypanosomiosis. The disease usually runs in acute phase in horses.Diagnosis of *T. evansi* infection in a horse stable of Eastern Region of India on the basis of examination of Giemsa stained blood smears revealed a high percentage (12.74%) of

infection (Laha and Sasmal, 2008). An outbreak reported from Mathura (Kumar *et al*., 1994) resulted in 100% morbidity with 66.6% mortality. Similar fatal outbreak was reported in ponies from Jammu (Raina *et al*., 2000). Likewise, reports of surra in equine are frequent from Maharashtra (Bharkad *et al*., 2005) and eastern parts of India (Laha and Sasmal, 2008).

PCR for diagnosis of trypanosomiosis

PCR based detection of *T.evansi* has undergone a great expansion during the last 20 years. Though PCR has been reported to be more sensitive than conventional parasitological techniques (Desquesnes, 1997; Masake *et al*., 1997), however, most of PCR assays have not been validated under field conditions for the diagnosis of natural infection (Donelson *et al*., 1998).Most of the PCR based studies are limited to experimental infections a number of important molecules have been targeted like kinetoplast DNA (Donelson *et al.,* 1998), repetitive sequence DNA (Artama *et al*., 1992; Wuyts *et al*., 1995), ribosomal DNA (Ijaz *et al*., 1998) and internal transcribed spacer 1 (ITS-1) region of r-RNA (Taylor *et al*., 2008). Besides these molecular targets, there are a few commonly used sets of primers like TBR (Masiga *et al*., 1992), TEPAN (Panyim *et al*., 1993), pMUTEC (Wuyts *et al*., 1994), ESAG (Holland *et al*., 2001a),TRYP4 and TRYP1 (Desquesnes *et al*., 2001). PCR based on variable surface glycoprotein (VSG) have been shown to be more sensitive and specific than other targets reported from different parts of the world (Verloo *et al*., 2001). Omanwar (1998) optimized a PCR with threshold sensitivity level of 0.5 pg of *T. evansi* DNA. Pruvot *et al*. (2010) compared the performance of six primer pairs-TBR1/2 (Masiga *et al*., 1992), ESAG 6/7 (Holland *et al*., 2001a,b), TEPAN1/2 (Panyim *et al*., 1993), pMUTEC F/R (Wuyts *et al*., 1994), TRYP1R/S (Desquesnes *et al*., 2001) and TRYP4R/S using serial dilution of purified *T.evansi* DNA, from rats and Thai dairy cattle. Results revealed TBR1/2 primer set was able to detect 0.01 pg of purified DNA and a parasitaemia below one parasite per mL in rat blood.

Ravindran *et al*. (2008) compare two methods *viz.*, PCR and blood smear examination for sensitive and specific detection of *T. evansi* in camels, donkeys and dogs. Out of 131 blood samples (61 camels, 44 donkeys and 26 dogs) examined, 26 samples (21 camels, 3 donkeys and 2 dogs) were detected positive by PCR. Blood smear examination revealed *T. evansi* organisms in only two camels while rest all others were found negative by blood smear examination.

Muieed *et al*. (2010) tested PCR in comparison with microscopic examination for the diagnosis of *T. evansi* infection (surra) in horses in Lahore, Pakistan, and found 16% infection by PCR. However, microscopic examination revealed only 5% infection.

Aslam *et al*. (2010) compared parasitological (micro-haematocrit method, MHCT), serological (ELISA) and molecular (PCR) methods for diagnosis of trypanosomal infection in

equines. Results showed that higher number of positive samples ($p < 0.05$) were detected with PCR (30.8%) compared with either ELISA (21.6%) or MHCT (17.5%). The sensitivity and specificity of PCR which was found to be 100% and 58.97%, respectively in comparison to ELISA which gave 85.7% sensitivity and 79.5% specificity.

Rudramurthy *et al*. (2013) sequenced ISG-75 gene using PCR for the diagnosis of trypanosomiosis in carrier animals.

Shyam *et al*. (2013) detected *T. evansi* in cattle, buffaloes and equines in the state of Haryana by blood smear examination, and DNA-detecting (TE-PCR) tests. Out of 205 field blood samples tested, only 2% were positive for *T. evansi* by blood smear while PCR detected 60.49% samples positive**.**

Nadeem *et al*. (2010) studied the prevalence of trypanosomiosis in equine in District Gujranwala by using indirect fluorescent antibody technique and thin smear method. Blood samples were collected from a total of 200 horses and donkeys of different ages and either sex. Giemsa stained smears revealed infection in 2% population and IFAT revealed 6 % population.

Sumbria *et al*. (2015) studied multiplex PCR for detection of *T. evansi* and *Theileria equi* in 108 equids from Punjab by targeting variable surface glycoproteins of *T. evansi* and found 3.7 % prevalence. Sharma *et al*. (2013) employed specific duplex PCR on 411 blood samples of dairy animals from 9 districts of Punjab, India. The overall prevalence by duplex PCR was found to be 36.49 % & by Giemsa stained thin blood smear it was found to be 0.73 % .

Sudan *et al*. (2015a) developed a duplex PCR for simultaneous detection of *T.evansi* and *T.annulata* in buffaloes from Mathura and found prevalence of 53.73% and 26.25 % respectively. The sensitivity and specificity of duplex PCR was found to be at par with simple PCR.

Taylor *et al*. (2008) developed TaqMan real time PCR assay for the detection of *T. evansi* targeting the internal transcribed spacer 1 (ITS-1) region of rRNA using blood from infected rats. It was found to be highly sensitive, and being able to detect less than one genomic equivalent of *T. evansi*.

Kumar *et al*. (2013) developed, an antibody-ELISA using whole cell lysate antigen prepared from purified *Trypanosome*s and used it for seroprevalence study of *T. evansi* in equids. The maximum seroprevalence of 19.69% for *T. evansi* infection was observed in equids of Uttar Pradesh state with an overall seroprevalence of 11.36% in North and North-

western regions of India. Sudan *et al*. (2015b) found the seroprevalence of 19.38% by using native whole cell ELISA on cattle from Uttar Pradesh.

Work done on VSG

The variant surface glycoprotein (VSG) of *Trypanosome* plays an important part of its body surface coat, and are expressed in early, middle and late stages of infection there by, contributing a major diagnostic value. Analysis of nuclear DNA, kinetoplast DNA and multilocus isoenzymes (Mathieu-Daude and Tibayrenc, 1994) have suggested that *T. evansi*, in contrast to the cyclically developing *Trypanosome*s in the tsetse fly, has a limited variant surface glycoprotein (VSG) antigenic repertoire. RoTat 1.2 VSG is a predominant variant antigen type (VAT) expressed in all *T. evansi* stocks examined so far (Verloo *et al*., 2000). Alongside, RoTat 1.2 VSG is again thought to be expressed during early, middle and late stages of infection (Robinson *et al*., 1999) thereby conferring it to be a potent molecule for diagnosis of surra (OIE, 2012).

Rogé *et al*. (2013) cloned RoTat 1.2 VSG in *Pichia (P.) pastoris*. The M5 strain of this yeast has an engineered N-glycosylation pathway resulting in homogenous $Man_5GlcNAc_2$N-glycosylation which resembles the pre-dominant Man9-5GlcNAc2 oligomannose structures in *T. brucei*.

Lejon *et al*. (2005) cloned and expressed *T. evansi* variable surface glycoprotein RoTat 1.2 into *Spodoptera frugiperda* and *Trichoplusia ni* (insect) cells and used it in ELISA, the reactivity of the recombinant RoTat 1.2 VSG was found similar to that of native RoTat 1.2 VSG. An indirect agglutination reagent was also 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, were 89.3 % and 99.1% respectively using a 1:4 serum dilution.

Ngaira *et al*. (2005) detected *T. evansi* by using diagnostic tests based on the variant surface glycoprotein (VSG) 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 for the detection of non-RoTat 1.2 *T. evansi*. Genomic DNA samples from different *Trypanosome*s were tested including 32 *T. evansi*, 10 *T. brucei*, 3 *T. congolense* and 1 *T. vivax*. 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.

Sengupta *et al*. (2012) amplified, cloned and expressed N-terminal protein of RoTat 1.2 VSG in prokaryotic system. Rudramurty *et al*. (2015) developed ELISA employing recombinant invariant surface glycoprotein (rISG) 75 for serodiagnosis of bovine trypanosomiosis. The sensitivity and specifivity of this ELISA was found to be 98.47% and 99.10% respectively. Sengupta *et al*. (2010) designed EXP3F/4R primer and amplified the 1.4 kb of VSG gene of *T. evansi* and studied the phylogenetic relationship by *in silico* analysis. Sengupta *et al*. (2014) expressed VSG of *T. evansi* in prokaryotic system (*E. coli*) and used it in immuno blot and ELISA. The expressed protein showed 95.6% sensitivity, 98.0% specificity and 0.93 Cohen's kappa value, when compared with standard antigens.

Molecular characterization of *Trypanosoma evansi*

The recent developments in the molecular techniques have imposed a considerable impact upon the *Trypanosome* identification, characterization, accuracy and reliability at various taxonomic levels (Desquesnes and Davila, 2002). There are ample reports of characterization studies on *Trypanosome*s in general and *T. evansi* in particular. The important molecules that are studied include oligopeptidase B from *T. b. brucei*, variable surface glycoprotein (VSG) gene of *T. evansi* (Sengupta *et al*., 2012), ISG-75 gene of *T. b. gambiense* (Tran *et al*., 2008), hypoxanthine guanine phosphoribosyl transferase gene of *T.b. brucei* (Allen and Ullman, 1993), beta-tubulin gene of *T. evansi* (Li *et al*., 2007) and actin gene of *T. evansi* (Li *et al*., 2009). In addition, analyses of the internal transcribed spacer (ITS) region (Areekit *et al*., 2008; De Oliveira *et al*., 2008; Khuchareontaworn *et al*., 2007) and diversity of the transferrin receptor encoding gene located at expression-site-associated (ESAGs6 and 7) genes (Mekata *et al*., 2009; Witola *et al*., 2005; Isobe *et al*., 2003) are utilized in molecular classification of *Trypanosome*s. All these molecules are used for phylogenetic analysis between the various isolates. It has been demonstrated that the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of the rDNA are useful targets for species delineation and for inferring phylogenetic relationships of *Trypanosoma* spp. (Amer *et al*., 2011; Khuchareontaworn *et al*., 2007). They are particularly useful when the availability of morphological characters is limited. Reportedly, microsatellites markers (Biteau *et al*., 2000), amplified restriction fragment length polymorphism analyses (Masiga *et al*., 2006) and intersequence simple repeat PCR (Njiru *et al*., 2007) are also utilized in molecular identification of *Trypanosoma*.

The conserved nature of RoTat 1.2 makes it an important molecule to study phylogeny. Though such studies have been done in buffaloes so far (Sengupta *et al*., 2010), none of such study has been attempted on equine population from India. With all this information in the background, the present study was conducted to evaluate the PCR technique to detect the trypanosomal antigens in the naturally infected equines and to compare the PCR with the

blood smear examination. Alongside, the characterization and phylogenetic analysis of RoTat 1.2 VSG was planned to be studied by its cloning in prokaryotic vector host and subsequent sequencing.

CHAPTER-3

MATERIALS AND METHODS

Glasswares and plastic wares

All glassware used for the present study were purchased from Borosil, Schott Duran, Axygen, and Eppendorff. Microscopic glass slides and coverslips were purchased from Himedia. All the tips were procured from Tarsons. PCR tubes and micro centrifuge tubes were procured from Himedia.

Chemicals

Phenol (Banglore Gene i); Phenol: chloroform : isoamyl alcohol (25:24:1) (SRL Laboratories); chloroform : isoamyl alcohol (24:1), Tris EDTA, Giemsa stain, Glycerol, EDTA, Sodium acetate, Nuclease free water, Proteinase K, Sodium dodecyl sulphate, Ampicillin, Calcium chloride (All from Himedia); Agarose (Affumetrix, USA); Ethidium bromide (Amresco); Methanol (Qualigens), absolute ethanol (Changshu Yanguan, China); 100bp DNA molecular weight marker (New England Biolabs); DH5α, LB agar, LB broth (Puregene).

Kits

Clonejet PCR cloning kit; Gene Jet plasmid miniprep kit; Gene Jet Gel Extraction and DNA clean up micro kit (All from Fermentas)

Instruments

Autoclave (Scientech); Vortexer, Deep freezer (-20˚C), pH meter, Incubator (All from Remi); Microcentrifuge (Tarsons); Micropippetes (Axiva); Thermal cycler (BioRad); Weighing balance (SDFCL); Horizontal agarose gel electrophoresis apparatus with power supply, U.V. translluminator (Banglore Gene i); Microscopes (Labomed).

Sampling procedure and data collection

A cross-sectional design in which data was collected at a single point and time (Thrusfield, 2005) was used in the instant study. The population of study constituted the horses that were brought for routine clinical camps organized by Brooke Hospital for Animal in Mathura, Unnao and Raebareli alongside those animals which are brought to Teaching Veterinary Clinical Complex (TVCC), DUVASU, Mathura.

Sampling and parasitological techniques

Blood samples from a total of 86 susceptible horses randomly from different localities of district Mathura, Unnao and Raebareli and screened for the presence of *T. evansi* through PCR and conventional thin smear method. On spot thin blood smears were made, fixed and thereafter brought to the laboratory of Department of Parasitology, DUVASU, Mathura, for routine blood examination upon Giemsa staining. The various haemoprotozoa were identified using the standard keys provided by Soulsby (1982). Data was collected on the horse's demographic characteristics (age, gender, etc.). The age of the presented horses was determined based on body condition scores and by asking the owners. Horses were subjectively evaluated on two scale level as 'unhealthy' or 'healthy'. Horses were grouped into two age categories. Horses under 5 year of age were classed as young and those beyond 5 year were classed as adult.

Statistical analysis

For the epidemiological studies, the prevalence (p) of horses harbouring each parasite was calculated as p = d/n, where d is the number of horses diagnosed as having a given parasite at that point in time and n is the number of horses at risk (examined) at that point in time (Thrusfield, 2005). Alongside, single and multiple infections were also calculated.

DNA isolation

DNA was isolated from blood using standard phenol chloroform method with minor modifications. Briefly, 200 µL of blood was added into equal volume in TE lysis solution consisting of 10 mM of Tris acid and 1 mM of EDTA. Then 5 µL of 25 mg/mL of Proteinase K and 50 µL of 10% SDS were added to it and the sample was kept at 65 ^{o}C for 4 hours. Thereafter, the DNA was extracted as per standard phenol chloroform protocol (Sambrook and Russel, 2001). Briefly, 500 µL phenol was added to it and was mixed gently and incubated at room temperature for 15 minutes. The micro centrifuge tubes were then centrifuged at 3500 rpm for 15 minutes, and the supernatant was collected. Equal amount of phenol: chloroform: isoamyl alcohol (25:24:1) was added to the supernatant and centrifuged at 10,000 rpm for 5 minutes. Thereafter supernatant was again collected and equal amount of chloroform: isoamyl alcohol (24:1) was added and again centrifuged at 10,000 rpm for 5 minutes. Then, the supernatant was collected and 3 M sodium acetate (10µL/100mL) and chilled ethanol was added to the supernatant twice the volume and the sample was left at -20^{o}C for overnight for precipitation of DNA. Next day it was centrifuged at 10,000 rpm for 2 minutes. The pellet was washed with 75% alcohol and again centrifuged at 10,000 rpm for 2 minutes. The pellet was finally air dried before re-suspension into 100 µL of Nuclease Free Water (NFW).

Polymerase chain reaction (PCR) based amplification of RoTat1.2 VSG 1 gene

Primers for RoTat 1.2 VSG 1 were custom synthesized using nucleotide pattern from a highly conserved region of the published sequence of VSG (Gaur *et al*., 2016). This pair of primers was used for the PCR amplification product of 681-bp. Details for primers design including position of nucleotides, nucleotide sequences, and expected PCR products are shown in Table 1.The PCR reaction was set up into 25 µL volume containing 12.5 µL PCR Master Mix (0.05/ µL Taq DNA polymerase in reaction buffer, 4 mM $MgCl_2$, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 1.5 µL of each primer (RoTat 1.2 VSG F/ R), 5 µL of the DNA template and total volume was made up to 25 µL using Nuclease free water. The thermocycling conditions used for amplification of the RoTat 1.2 VSG 1 gene were as follows:

Step 1: Initial denaturation	:	95ºC for 2 minutes.	
Step 2: Denaturation	:	94ºC for 45 sec.	40 cycle
Step 3: Annealing	:	48ºC for 60 sec.	
Step 4: Extension	:	72ºC for 5 minutes.	
Step 5: Final extension	:	72ºC for 10 minutes.	

The amplified amplicons were analyzed by agarose gel electrophoresis in 1.5 % agarose gel.

Agarose gel electrophoresis

Agarose gel (1.5%) was prepared by boiling the agarose (Affumetrix, USA) in an appropriate volume of 1X TAE buffer. It was then allowed to cool to about 50^{o}C and ethidium bromide (10 µg/mL) was added to a final concentration of 0.5 µg/mL. About 50 mL of agarose gel was poured into the casting tray and was allowed to solidify at room temperature for 20 min following insertion of the comb. 10 µL of the PCR samples were loaded in each well of the gel along with a 100 bp DNA molecular weight marker (New England biolab) in a designated well. Electrophoresis was performed at 85 V for 45 min. After sufficient migration, the gel was examined in a gel documentation system and images were stored.

Sensitivity and specificity assay

In order to check of the specificity of these primers, PCR was performed on DNA of the confirmed *T. evansi* infected blood (microscopic observation of blood smears and confirmed by PCR) for *Babesia* sp. and *Theileria* sp. using standard primers (d'Oliveira *et al.*, 1995; Singh *et al.*, 2007).

Statistical analysis

The results of the PCR were compared with that of blood smear examination. The sensitivity and specificity of PCR was calculated and compared with that of blood smear examination, using online softwares (http://graphpad.com/quickcalcs /kappa1.cfm). The kappa value, hence calculated, was compared and results were formulated.

Molecular characterization of RoTat 1.2 VSG 1 and VSG 2 gene

Two portions of the RoTat 1.2 VSG gene *viz.*, VSG 1 and VSG 2 were PCR amplified using DNA of the confirmed *T. evansi* infected horse (microscopic observation of blood smears and confirmed by PCR). Oligonucleotide primers targeting the RoTat 1.2 VSG of *T. evansi* (VSG 1 F/R and VSG 2 F/R) were custom synthesized (Gaur *et al*., 2016; Claes *et al*., 2004). The PCR reactions were set up into 25 µL volume containing 12.5 µL of Green PCR Master Mix (0.05/µL Taq DNA polymerase in reaction buffer, 4 mM MgCl2, 0.4 mM dATP, 0.4 mM dCTP, 0.4 mM dGTP, and 0.4 mM dTTP), 1.5 µL of each primer (VSG 1 F/R; VSG 2 F/R; 20 pmol of each primer) and 2 µL of the extracted DNA template. The total volume of the PCR mix was made up to 25 µL using nuclease-free water. The PCR conditions for the RoTat VSG 1 gene were:

Step 1: Initial denaturation	:	95ºC for 2 minutes.	
Step 2: Denaturation	:	94ºC for 45 sec.	40 cycle
Step 3: Annealing	:	48ºC for 60 sec.	
Step 4: Extension	:	72ºC for 5 minutes.	
Step 5: Final extension	:	72ºC for 10 minutes.	

Likewise, the PCR conditions for the RoTat VSG 2 gene were

Step 1: Initial denaturation	:	94ºC for 4 minutes.	
Step 2: Denaturation	:	94ºC for 60 sec.	35 cycle
Step 3: Annealing	:	59ºC for 60 sec.	
Step 4: Extension	:	72ºC for 60 sec.	
Step 5: Final extension	:	72ºC for 5 minutes.	

The details of primers alongside expected PCR products are shown in Table 1 & 2. The amplified amplicon was analyzed by agarose gel electrophoresis in 1.5 % agarose gel (using the method described above).

Purification of the PCR product

The PCR products of the respective genes were purified using GeneJet PCR purification kit (Fermentas) following manufacturer's protocol. The reaction mixture volume was adjusted to 200 µL with nuclease-free Water. 100 µL of Binding Buffer was added to it,

Table 1: Primer sequence for VSG 1 and VSG 2 gene along with expected amplicon size

Primer	**Primer sequence**	**Amplicon size**
VSG 1 F	5'GGGAATTCATGCAAACCAAGGCGCTCGTTGGCGT3'	681bp (Gaur *et al.* (2016)
VSG 1 R	5'CGGGAATTCCTTGATGTTGCTGGTCGCGATTTTGATC3'	
VSG2 F	5'GCG GGG TGT TTA AAG CAA TA 3'	204 bp (Claes *et al*. (2004)
VSG 2 R	5'ATT AGT GCT GCG TGT GTT CG 3'	

Table 2: Thermal cycling conditions for VSG 1 and VSG 2 gene

Thermal cycling conditions					
	Initial denaturation	**Denaturation**	**Hybridization**	**Extension**	**Termination**
PCR with VSG 1 gene	95°C, 120 sec	94°C, 45 sec	48°C, 60 sec	72°C, 300 sec	72°C, 600 sec
		× 40 cycles			
PCR with VSG 2 gene	94°C, 240 sec	94°C, 60 sec	59°C, 60 sec	72°C, 60 sec	72°C, 300 sec
		× 35 cycles			

and was mixed thoroughly by pipetting. 300 µL of ethanol was then added and mixed by pipetting. The mixture was then transferred to the DNA Purification Micro Column preassembled with a collection tube. The column was centrifuged for 60 seconds at 14,000 × g. Flow-through was discarded. The DNA Purification Micro Column was again placed back into the collection tube and 700 µL of Wash Buffer was added to the DNA Purification Micro Column and centrifuged at 60 seconds at 14,000 × g. Flow-through was again discarded and the purification column was put back into the collection tube. Centrifuge the an additional 1 minute at 14,000 × g to completely remove residual Wash Buffer. The DNA Purification Micro Column was transferred into a clean 1.5 mL microcentrifuge tube. 30 µL of Elution Buffer was added to the center of the DNA Purification Micro Column membrane. and it was centrifuged for 1 minute at 14,000 × g . The purified DNA was stored at -20°C.

Preparation of *Escherichia coli* DH5-α competent cells

Fresh competent cells were prepared following the protocol of Sambrook and Russel (2001). *E. coli* DH5- α (glycerol stock, Puregene) cells were streaked on a LB agar plate and incubated overnight at 37^{o}C. A single colony was picked up from the plate and inoculated into 10 mL LB broth and incubated at 37^{o}C with constant shaking for overnight. 100 µL of the overnight culture was inoculated to a fresh 100 mL LB broth and was grown until mid log phase. The culture was kept on ice for 1 h and the cells were pelleted by centrifugation at 6,000 rpm for 10 min. The cell pellet was resuspended in 1/3 vol of 100 mM calcium chloride and was incubated on ice for 30 min. The cells were harvested by centrifugation at 6,000 rpm and again resuspended in 2 mL of 100 mM calcium chloride. 15% glycerol was added to the cell suspension before aliquoting in a sterile 1.5 mL microfuge tube. The competent cells thus prepared were stored at – 80^{o}C until use.

Ligation

Ligation reaction for cloning of RoTat 1.2 VSG 1 and VSG 2 genes into InsTAclone CloneJet (Fermentas) cloning vector was carried following manufacturer's protocol. Firstly, the blunting reaction was done on ice

Component	:	Volume
2x reaction buffer	:	10 µL
PCR product (respective product)	:	2 µL
Nuclease free water	:	5 µL
DNA blunting enzyme	:	1 µL
Total volume	:	18 µL

Incubate the mixture at 70ºC for 5 min. then chill on ice. Ligation reaction was seted on ice after adding the following to the blunting reaction mixture

Component	:	Volume
pJET1.2/blunt cloning vector	:	1 µL
T4 DNA ligase	:	1 µL
Total volume	:	20µL

Incubate the ligation mixture on room temperature for 5 minutes. Use the ligation mixture directly for transformation by adding ligation mixture on DH5α.

Transformation of DH5-α competent cells

An aliquot (250 µL) of the frozen competent cells was thawed on ice for 30 min. To this 20 µL of the ligation mixture was added and mixed gently and then further incubated on ice for 15-20 min. The cells were then subjected to heat shock at 42^{o}C for 90 sec, and immediately chilled on ice for 10 min. The cells were supplemented with 800 mL of fresh autoclaved LB broth and incubated for 1 h at 37^{o}C. 200 µL of the transformed cells were then plated on LB agar plates supplemented with ampicillin (100 µg/mL). The plates were then allowed to dry under laminar flow and subsequently incubated at 37^{o}C for 12 -14 h for the development of colonies.

Confirmation by colony PCR

Half of a single colony (in triplicate for each gene) was picked up with a sterile loop and transferred to a microfuge tube containing 30 µL of nuclease free water. It was boiled for 10 min and immediately placed on ice for 5 min. After spinning at 13,000 rpm for 1 min to pellet the cell debris, 5 µL of the clear supernatant was used as template for the PCR reaction using gene specific primers. The PCR amplification was carried out following standardized protocol and the amplicons were visualized by ethidium bromide stained agarose gel electrophoresis as described earlier.

Plasmid isolation from confirmed colonies

Single colony of each gene that was confirmed by colony PCR was picked up from the plate and inoculated into 10 mL LB broth supplemented with ampicillin (100 µg/mL) and incubated at 37^{o}C with constant shaking for overnight. Thereafter, the plasmid for each gene was isolated using plasmid isolation kit Fermentas following manufacturer's protocol. The overnight grown cells were pelleted by centrifuging at 6000 rpm for 10 min and were resuspended in resuspension solution. The bacteria were resuspended completely by vortexing until no cell clumps remain. The cell suspension was transferred to a microcentrifuge tube. 250 µL of the lysis solution was added and mixed thoroughly by inverting the tube 4-6 times

until the solution becomes viscous and slightly clear. 350 µL of the Neutralization Solution was added and mixed immediately and thoroughly by inverting the tube 4-6 times. Centrifugation was done for 5 min to pellet cell debris. The supernatant was transferred to the supplied GeneJET spin column by decanting. It was centrifuged for 1 min at 10000 rpm. Flow-through was discarded and the column back was placed into the same collection tube. 500 µL of the Wash Solution was added to the GeneJET spin column and centrifuged for 60 seconds at 10000 rpm and the flow-through was discarded. Place the column back into the same collection tube. The wash procedure was repeated using 500 µL of the Wash Solution. Flow-through was again discarded and was centrifuged for an additional 1 min to remove residual Wash Solution. The GeneJET spin column was transferred into a fresh 1.5 mL microcentrifuge tube. 100 µL of the Elution Buffer was added to the center of GeneJET spin column membrane to elute the plasmid DNA and was incubated for 2 min at room temperature and centrifuged for 2 min. The purified plasmid DNA was stored at -20°C. Thereafter the plasmids were outsourced for custom sequencing from the using M13 universal primers.

Phylogenetic analysis

The sequence information for each gene was received and the sequences were submitted in NCBI to receive the respective accession numbers. Thereafter, the respective sequences were compared with known sequences in the pubmed using online available softwares (Gene Tool, Mega 5.0 and DNA Star). The sequences were analyzed and nucleotide homology as well as divergence for each gene was calculated using above said softwares.

CHAPTER-4

RESULTS

Among the haemoprotozoan diseases, trypanosomiosis is one of the major constraints affecting the production performance of domestic animals including equines throughout the world as well as India. The prevalence of trypanosomiosis varies from region to region due to diversity in agro-climatic conditions and environmental factors like temperature, rainfall and humidity, which plays an important role in the development and survival of parasitic stages. The present study was undertaken to examine the prevalence pattern of trypanosomiosis in equines in and around Mathura region by blood smear examination and PCR. Cloning and characterization of RoTat 1.2 VSG gene was also taken alongside.

Blood smear examination

Eighty six equine samples were screened by peripheral blood smear examination as per the standard procedure using giemsa staining technique (Jain, 1993). Out of 86 equines under study, 3 were found to positive for trypanosomiosis with prevalence rate of 3.48%. Alongside, anaplasmosis, babesiosis and microfilariosis were also detected in 2.32%, 6.97% and 4.65% animals (Table 3). The area wise prevalence rate pattern of these haemoprotozoan infections is given in the Table 4.

Amongst the different age groups, maximum prevalence rate for trypanosomiosis were found in animals with over 5 years of age (4.16 %) than in animals under 5 years of age (2.70 %). Anaplasmosis and babesiosis followed the reverse trend with prevalence rates of 2.7% and 10.81 % in groups of under 5 years, respectively whereas, the prevalence rates of these two infections in animals over 5 years were recorded 2.08% and 4.16%, respectively, while microfilariosis was found only in adult animals with over 5 year age (Table 5).

So far as sex wise distribution of prevalence rates is concerned, males were found to be more infected (5.55%) than females (2.00%). Similar trend was found in babesiosis with prevalence rates of 8.69% and 4.00% in male and female counterparts, respectively. Only females and no males were found to be affected by anaplasmosis and microfilariosis (Table 6).

Single infection was found in 13.95% animals while mixed infections were recorded in 1.16% animals (Table 7).

PCR based detection

The PCR-based assay afforded sensitive and specific detection of *T. evansi* in all naturally infected equines. The PCR produced a 681-bp PCR product (Fig. 2b). The DNA

from other blood parasites failed to produce the primary amplification products accounting for its high specificity.

Comparison of PCR and blood smear examination

When compared with blood smear examination, PCR detected 8 cases positive in comparison to blood smear examination, which detected only 3 positive cases (Table 8). Keeping blood smear examination as a gold standard for detecting actual number of confirmed positive cases, PCR was found to be 100 % sensitive and 93.98% specific based on their kappa values estimation (Table 9).

PCR amplification, molecular cloning and molecular characterization of the RoTat 1.2 VSG 1 gene of horse isolates of *T. evansi*

RoTat 1.2 VSG 1 gene was amplified from the genomic DNA of horse isolates of *T. evansi* using the specific forward and reverse primers. The amplicons were resolved as a single band of 862 bp (Fig. 3a). It was further purified for ligation into cloning vector (Fig. 4a). The selection of positive colonies was performed by colony PCR using the specific primers.

Data analysis

The sequence hence generated was submitted to NCBI and an accession number KY457408 was obtained. The nucleotide sequence revealed 100.0% (Fig. 9a) sequence homologies with that of isolates from Egypt (Accession numbers JX888091 and KF726106). However, with the nucleotide similarity with camel and buffalo sequences from India it revealed 99.8% and 99.6% homologies, (Accession numbers JX134605 and EF495337), respectively. Alongside, it also showed 99.4% homology with Kenyan isolate (Accession number AF317914). Likewise, it also showed 99.3%, 100 % and 98.7 % homologies in protein patterns with various isolates from India, Egypt and Kenya, respectively (Fig : 9b). A phylogenetic association, for analyzing the identity between strains and testing the robustness of the association, was done using the online bootstrap method (http://blast.ncbi.nlm.nih.gov/) to delineate its relationship with other referral stains (Fig 10 a & b).

PCR amplification, molecular cloning and molecular characterization of the RoTat 1.2 VSG 2 gene of horse isolates of *T. evansi*

VSG 2 gene was amplified from the genomic DNA of horse isolates of *T. evansi* using the specific forward and reverse primers. The amplicons were resolved as a single band of 204 bp product (Fig. 3b). It was further purified for ligation in CloneJet cloning vector (Fig.4b). The selection of positive colonies was performed by colony PCR using the specific primers. The result of colony PCR was again checked by agarose gel electrophoresis.

Data analysis

The sequence, hence generated, was submitted to NCBI and an accession number KY457409 was obtained. A phylogenetic association, for analyzing the identity between strains and testing the robustness of the association, was done using the online bootstrap method (http://blast.ncbi.nlm.nih.gov/) to delineate its relationship with other referral stains (Fig. 11). The nucleotide sequence revealed 100.0% sequence homologies with all the other known *T. evansi* sequences *viz.*, buffalo, camel and horse isolates from Karnataka and Bikaner, India (EF495337, JX134605, AB259839) ; Kenyan isolate (AF317914); camel and cattle isolates from Egypt (JX888091, KF726106), respectively.

Table 3: Percent prevalence of various haemoprotozoan infections

Total no. of sample	*Trypanosoma*	*Trypanosome* %	*Anaplasma*	*Anaplasma* %	*Babesia*	*Babesia* %	*Microfilariae*	*Microfilairae* %	Total
86	3	3.48	2	2.32	6	6.97	4	4.65	15

Table 4: Area wise distribution of various haemoprotozoan infections

Area	Total no. of sample	*Trypanosoma*	*Trypanosome* %	*Anaplasma*	*Anaplasma* %	*Babesia*	*Babesia* %	*Microfilariae*	*Microfilariae* %	Total
Unnao	6	0	0	0	0	0	0	0	0	0
Raebareli	14	0	0	2	14.28	6	42.85	0	0	8
Baldev	29	1	3.44	0	0	0	0	1	3.44	2
Raya	15	0	0	0	0	0	0	3	20.00	3
Nagla	13	1	7.69	0	0	0	0	0	0	1
DUVASU	9	1	11.11	0	0	0	0	0	0	1

Table 5: Age wise distribution of various haemoprotozoan infections

Age	Total no. of sample	*Trypanosoma*	*Trypanosome* %	*Anaplasma*	*Anaplasma* %	*Babesia*	*Babesia* %	*Microfilariae*	*Microfilariae* %	Total
0-5 yr	38	1	2.70	1	2.70	4	10.81	0	0	6
>5yr	48	2	4.16	1	2.08	2	4.16	4	8.33	9

Table 6: Sex wise distribution of various haemoprotozoan infections

Sex	Total no. of sample	*Trypanosoma*	*Trypanosome* %	*Anaplasma*	*Anaplasma* %	*Babesia*	*Babesia* %	*Microfilarae*	*Microfilarae* %	Total
Male	36	2	5.55	0	0	4	8.69	0	0	6
Female	50	1	2.00	2	4.00	2	4.00	4	8.00	9

Table 7: Nature of haemoprotozoan infections

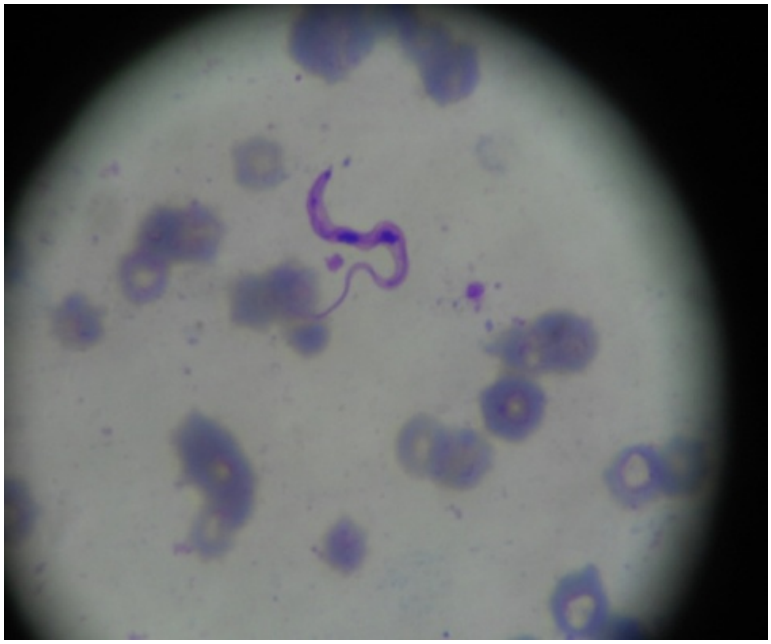
Total no. of sample	Single infection	Single infection %	Mixed infection	Mixed infection %	Total
86	12	13.95	1	1.16	13

Table 8: Comparative evaluation of blood smear examination and PCR

Total samples tested	**86**
Number of samples positive by blood smear examination	**3**
Number of samples positive by PCR	**8**
Number of samples positive by both blood smear examination and PCR	**3**
Number of samples positive by blood smear examination but negative by PCR	**NONE**
Number of samples positive by PCR but negative by blood smear examination	**5**
Number of samples negative by both blood smear examination and PCR	**78**

Table 9: Kappa value prediction of PCR with blood smear examination.

TEST	**BLOOD SMEAR**					
PCR	**POSITIVE**	**NEGATIVE**	**TOTAL**	**SENSITIVITY (95%CI)**	**SPECIFICITY (95%CI)**	**KAPPA VALUE**
POSITIVE	**3**	**5**	**8**	**100 % (29.24% to 100.00%**	**93.98 % (86.50% to 98.02%**	**Kappa=0.521** **SE of kappa= 0.182** **95% confidence interval from 0.164 to 0.879**
NEGATIVE	**0**	**78**	**78**			
TOTAL	**3**	**83**	**86**			



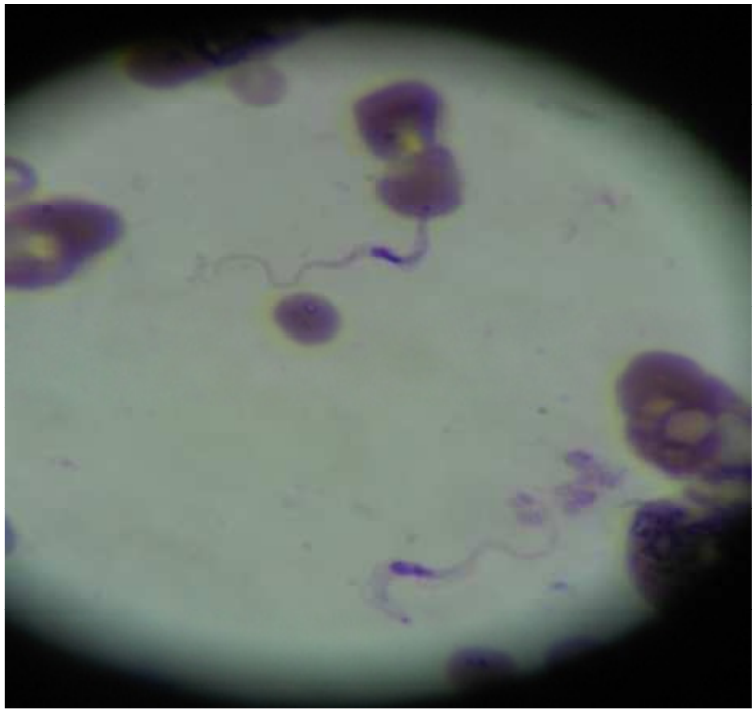


Fig. 1: Microscopic view of *Trypanosoma evansi* in horse blood (1000 magnification)

Fig 2a: RoTat 1.2 VSG based PCR on tested equine samples

M: Marker lane, 1-25: Different samples

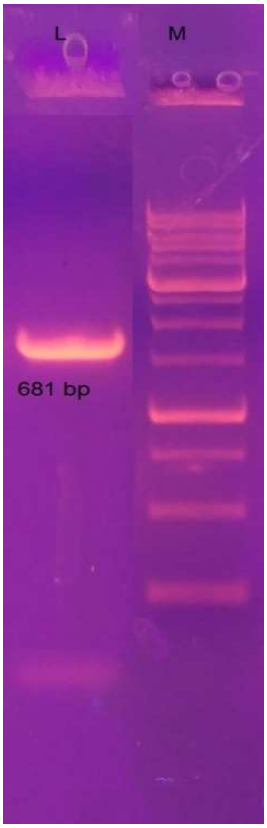
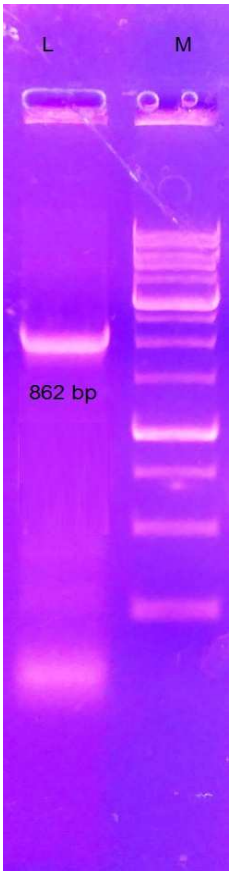


Fig 2b: standardization of RoTat 1.2 VSG based PCR amplification of confirmed positive *T. evansi* DNA

M: Marker lane, L: Lane with 681bp specific amplicon



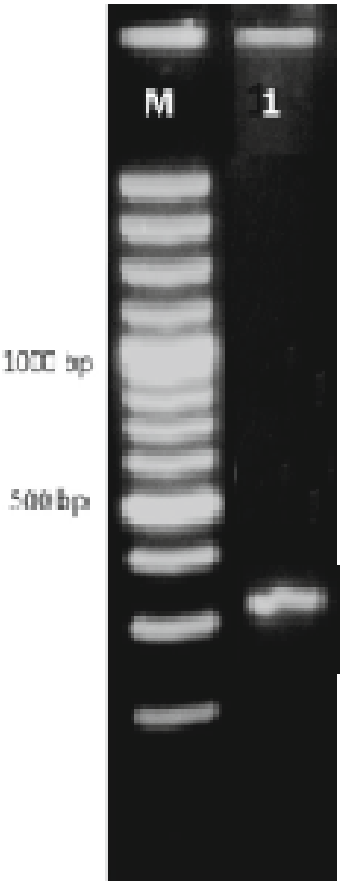


Fig 3a: RoTat 1.2 VSG 1 based PCR amplification of *T. evansi* DNA
M: Marker lane
L: Lane with 862bp specific amplicon

Fig 3b: RoTat 1.2 VSG 2 based PCR amplification of *T. evansi* DNA
M: Marker lane
1: Lane with 204bp specific amplicon

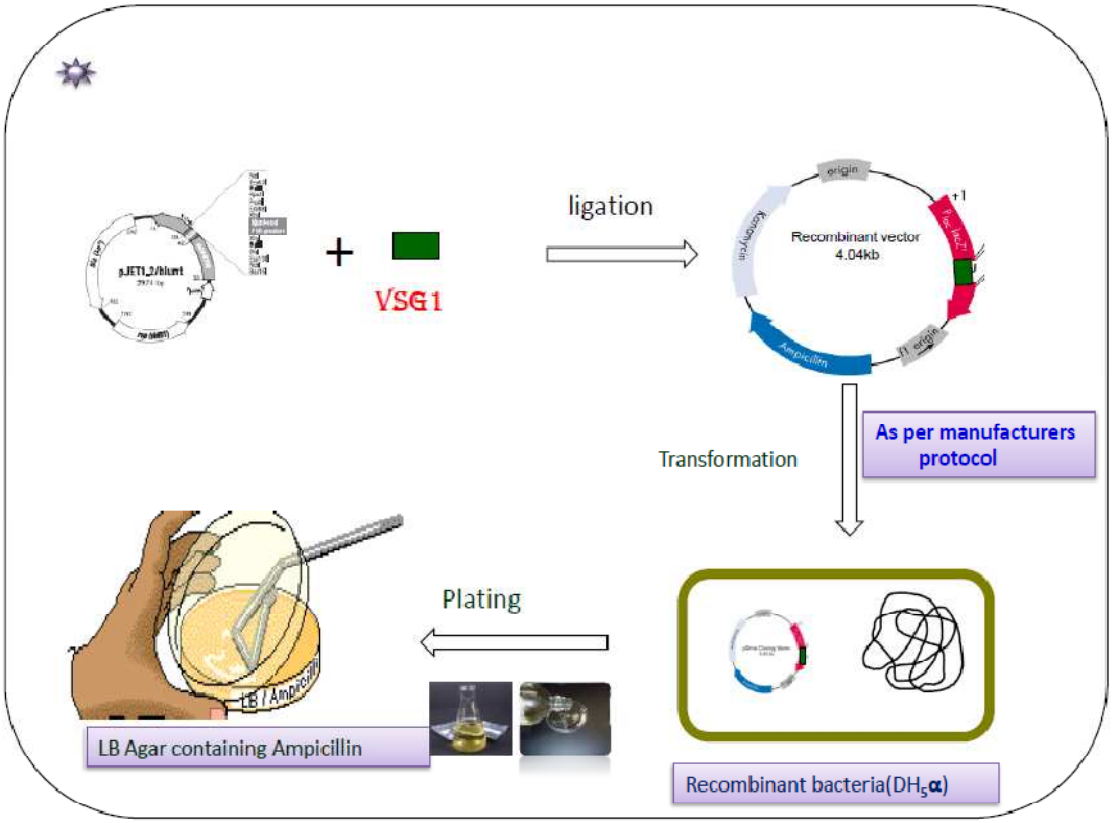


Fig 4a: Schematic diagram of cloning of RoTat 1.2 VSG 1 gene

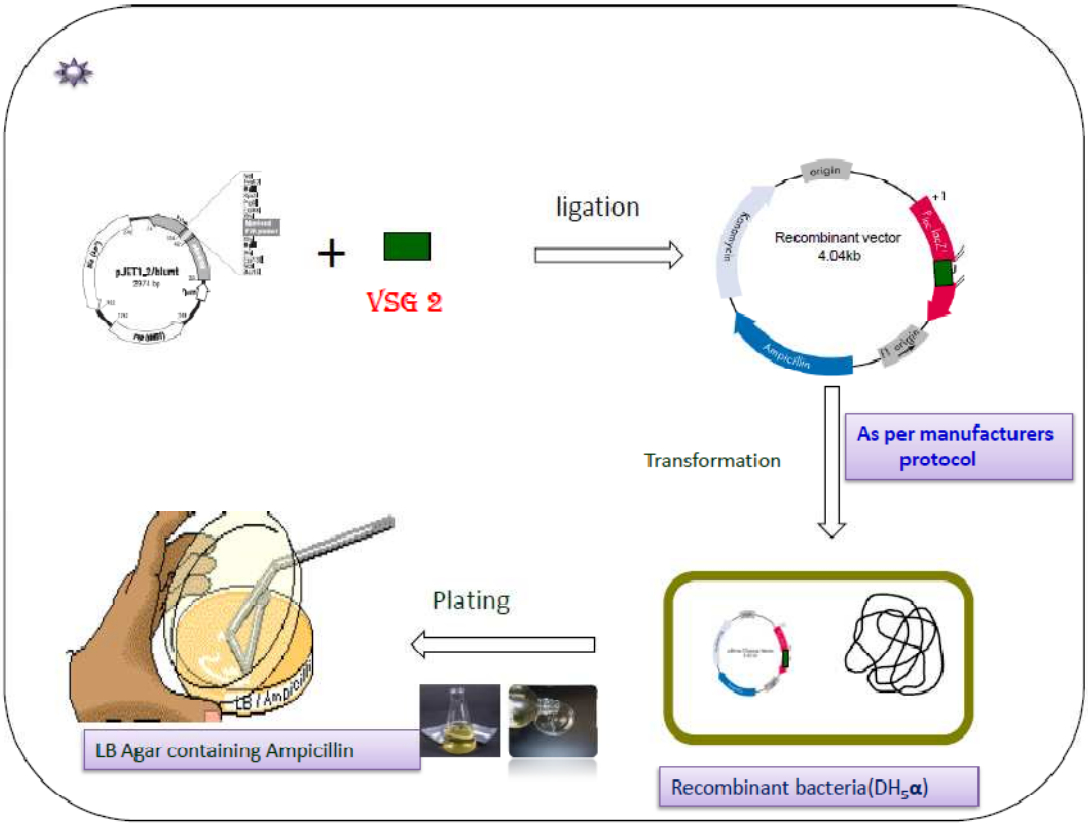


Fig 4b: Schematic diagram of cloning of RoTat 1.2 VSG 2 gene

Fig 5: competent cells DH5α

Fig 6: LB broth

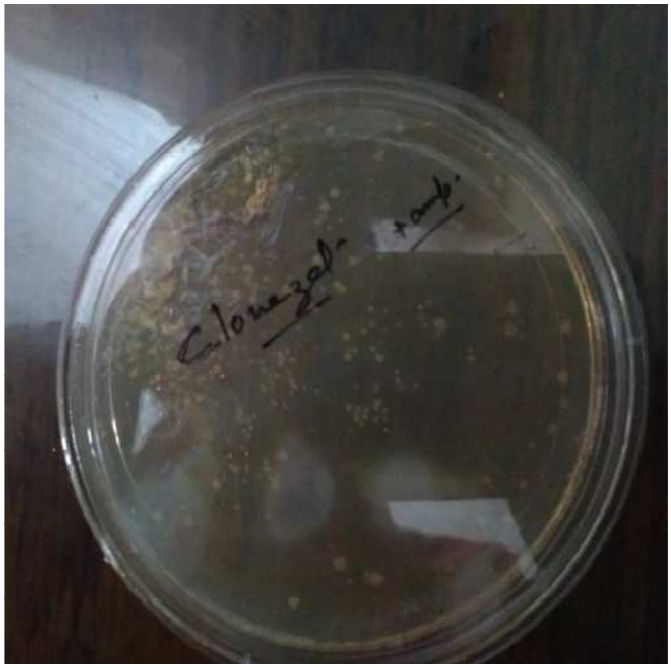


Fig 7: Positive colonies of RoTat1.2 VSG 1 gene on CloneJet vector

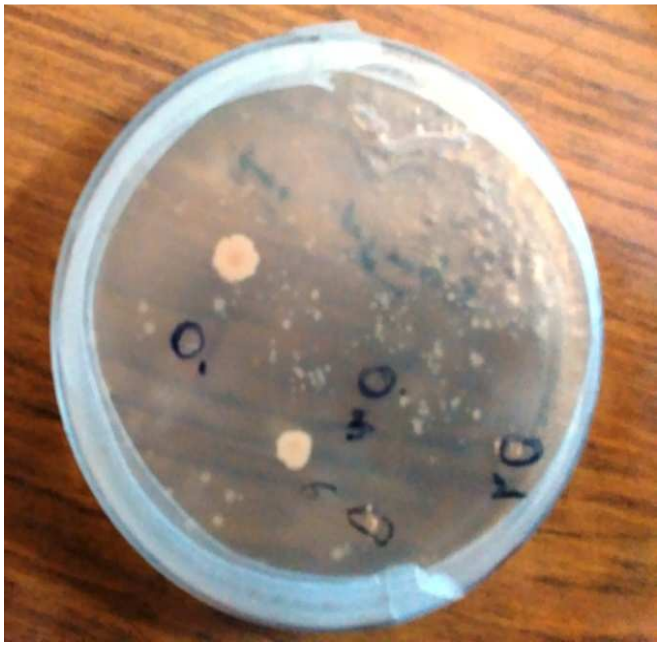


Fig 8: Positive colonies of RoTat1.2 VSG 2 gene on CloneJet vector

	1	2	3	4	5	6		
1		99.8	99.4	100.0	100.0	99.6	1	KY457408.1 Mathura
2	0.2		99.8	96.4	99.8	95.9	2	JX134605.1 Bikaner
3	0.4	0.2		100.0	100.0	99.6	3	AF317914.1 Kenya
4	0.0	0.2	0.0		100.0	99.6	4	Jx888091.1 Egypt
5	0.0	0.2	0.0	0.0		99.6	5	KF726106.1 Egypt
6	0.4	0.7	0.4	0.4	0.4		6	EF495337.1 Karnataka
	1	2	3	4	5	6		

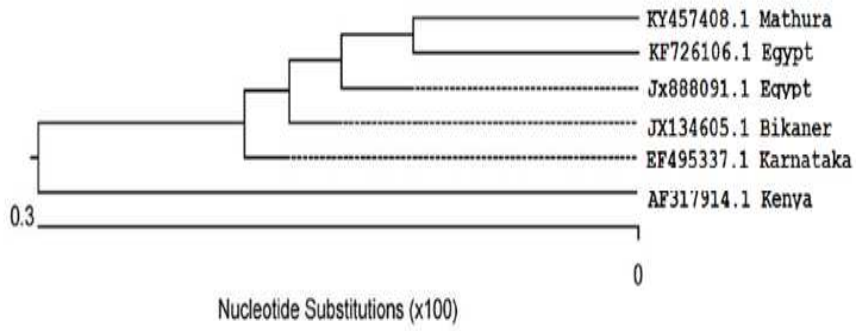
(a)

Percent Identity

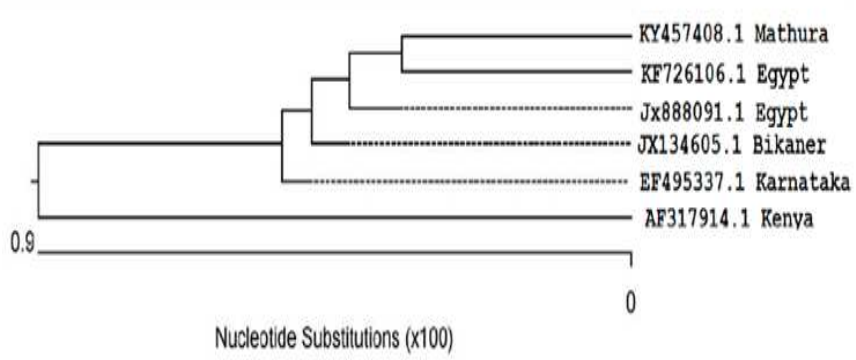
	1	2	3	4	5	6		
1		99.3	99.3	100.0	98.7	100.0	1	KY457408.1 Mathura
2	0.7		99.3	95.9	94.6	99.3	2	JX134605.1 Bikaner
3	0.7	0.7		100.0	98.7	100.0	3	EF495337.1 Karnataka
4	0.0	0.7	0.0		98.7	100.0	4	Jx888091.1 Egypt
5	1.3	2.1	1.3	1.3		98.7	5	AF317914.1 Kenya
6	0.0	0.7	0.0	0.0	1.3		6	KF726106.1 Egypt
	1	2	3	4	5	6		

(b)

Fig. 9: Divergence table of RoTat 1.2 VSG 1Mathura equine isolate with other known sequences; 9(a): Nucleotide homologies; and 9(b): Protein homologies.



(a)



(b)

Fig. 10: Phylogenetic tree of RoTat 1.2 VSG 1 Mathura equine isolate with other known sequences; 10(a): Nucleotide substitution; and 10(b): Protein substitution

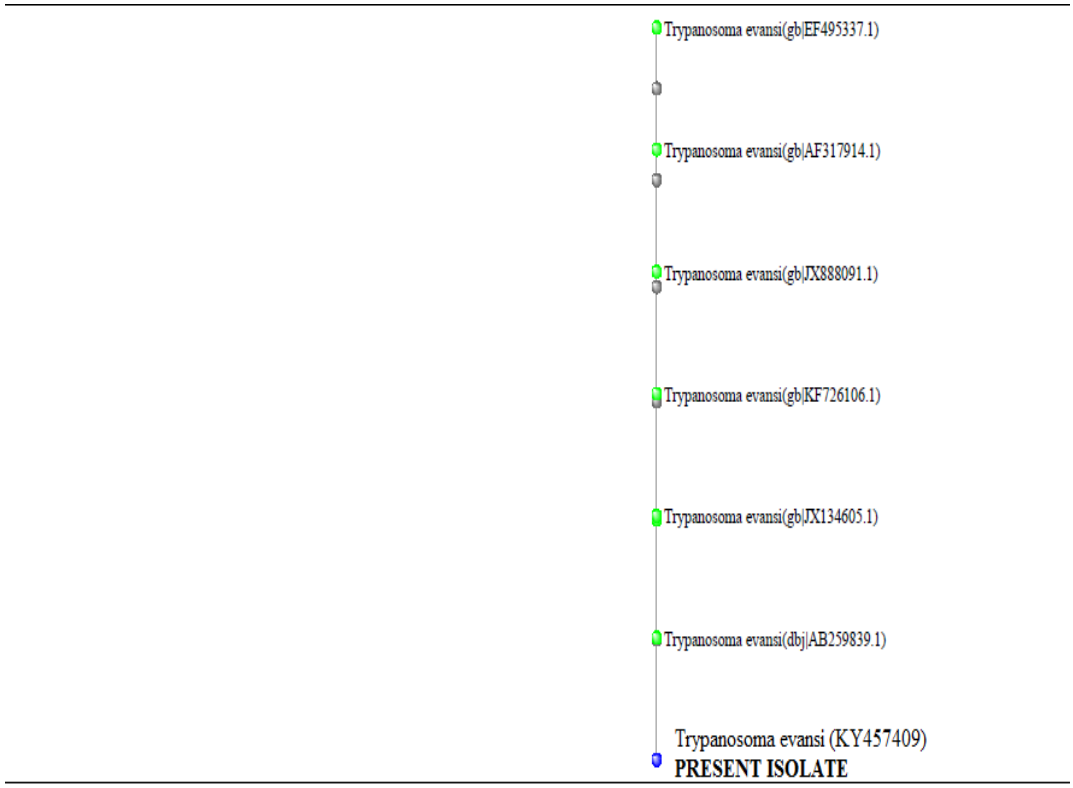
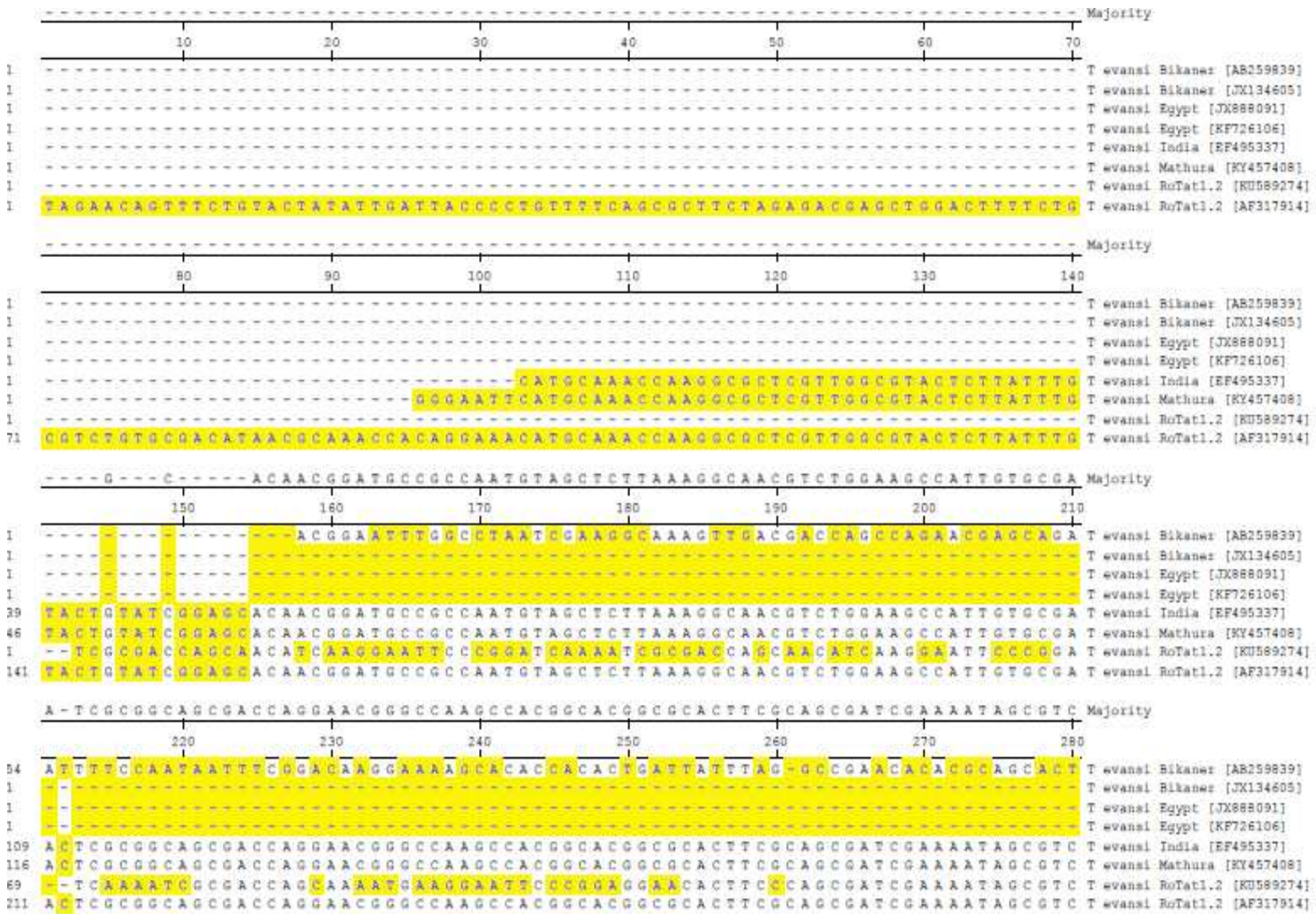
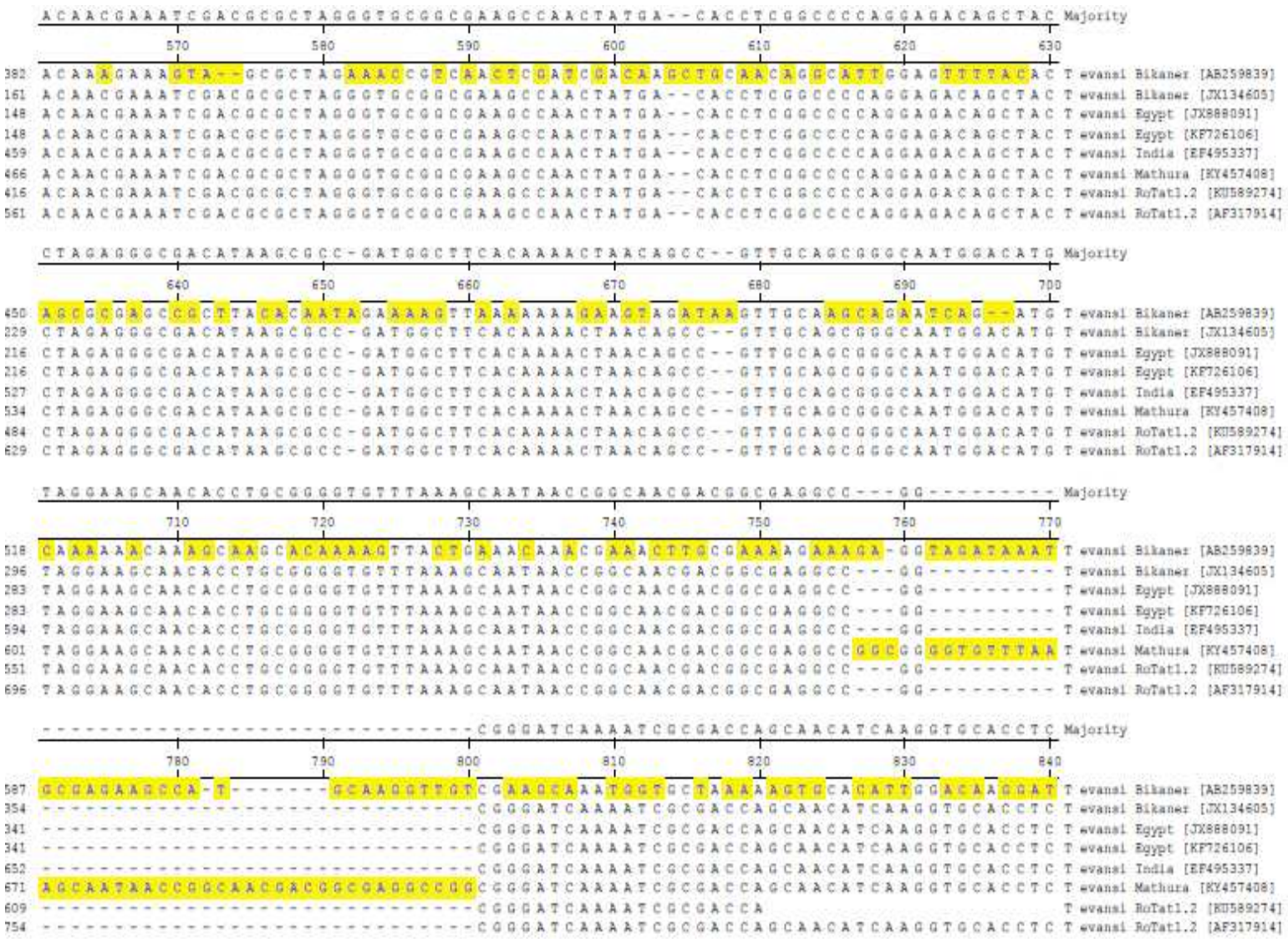
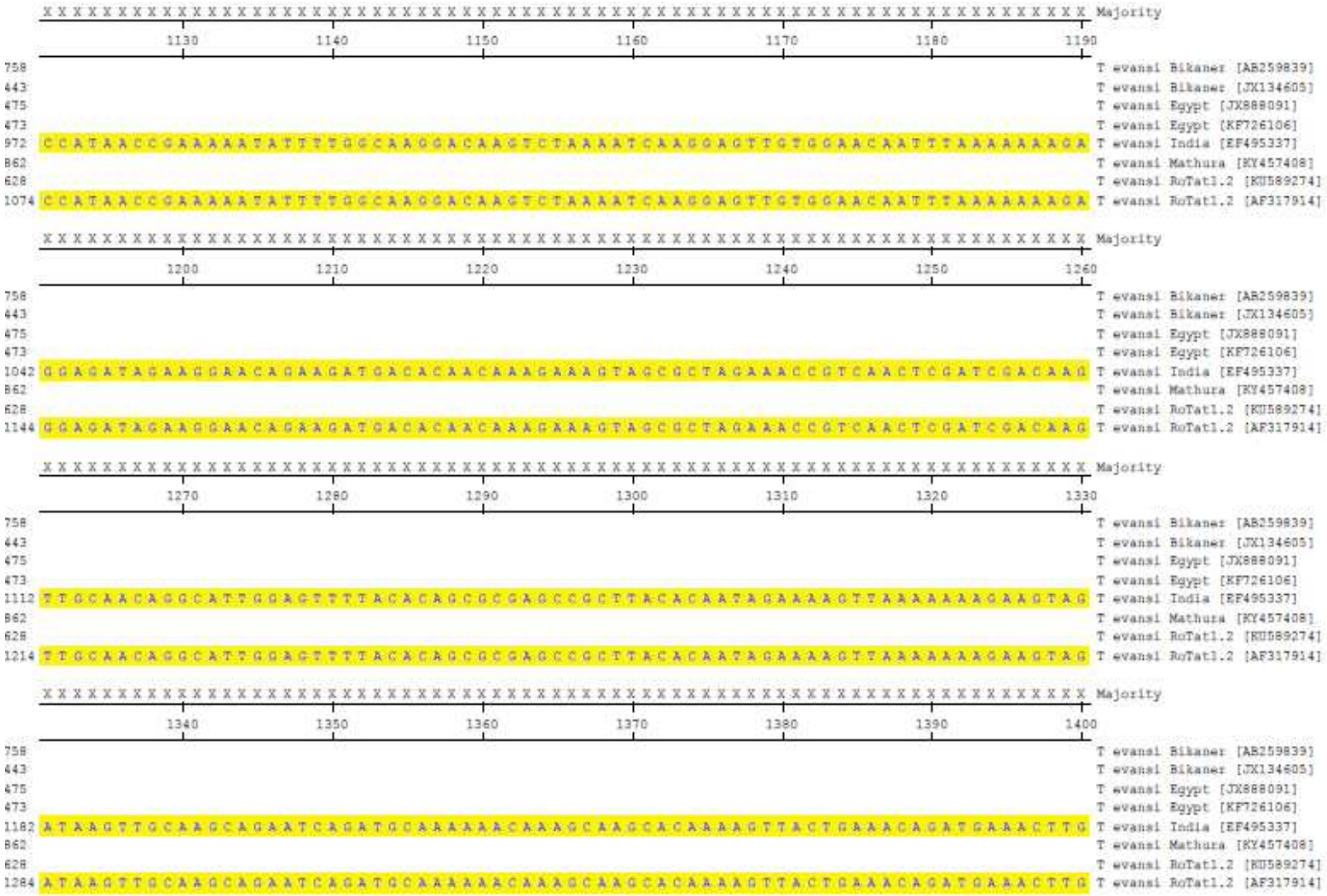


Fig. 11: Phylogenetic tree of RoTat 1.2 VSG 2 with other known sequences


Majority
10 20 30 40 50 60 70
T evansi Bikaner [AB259839]
T evansi Bikaner [JX134605]
T evansi Egypt [JX888091]
T evansi Egypt [KF726106]
T evansi India [EF495337]
T evansi Mathura [KY457408]
T evansi RoTat1.2 [KU589274]
T evansi RoTat1.2 [AF317914]
Majority
80 90 100 110 120 130 140
T evansi Bikaner [AB259839]
T evansi Bikaner [JX134605]
T evansi Egypt [JX888091]
T evansi Egypt [KF726106]
T evansi India [EF495337]
T evansi Mathura [KY457408]
T evansi RoTat1.2 [KU589274]
T evansi RoTat1.2 [AF317914]
Majority
150 160 170 180 190 200 210
T evansi Bikaner [AB259839]
T evansi Bikaner [JX134605]
T evansi Egypt [JX888091]
T evansi Egypt [KF726106]
T evansi India [EF495337]
T evansi Mathura [KY457408]
T evansi RoTat1.2 [KU589274]
T evansi RoTat1.2 [AF317914]
Majority
220 230 240 250 260 270 280
T evansi Bikaner [AB259839]
T evansi Bikaner [JX134605]
T evansi Egypt [JX888091]
T evansi Egypt [KF726106]
T evansi India [EF495337]
T evansi Mathura [KY457408]
T evansi RoTat1.2 [KU589274]
T evansi RoTat1.2 [AF317914]


ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC Majority
570 580 590 600 610 620 630
382 ACAAAGAAAGTA--GCGCTAGAAACCGTCAACTCGATCGACAAGCTGCAACAGGCATTGGAGTTTTACAC T evansi Bikaner [AB259839]
161 ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC T evansi Bikaner [JX134605]
148 ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC T evansi Egypt [JX888091]
148 ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC T evansi Egypt [KF726106]
459 ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC T evansi India [EF495337]
466 ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC T evansi Mathura [KY457408]
416 ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC T evansi RoTat1.2 [KU589274]
561 ACAACGAAATCGACGCGCTAGGGTGCGGCGAAGCCAACTATGA--CACCTCGGCCCCAGGAGACAGCTAC T evansi RoTat1.2 [AF317914]
CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG Majority
640 650 660 670 680 690 700
450 AGCGCGAGCCGCTTACACAATAGAAAAGTTAAAAAAAAGAAGTAGATAAGTTGCAAGCAGAATCAG--ATG T evansi Bikaner [AB259839]
229 CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG T evansi Bikaner [JX134605]
216 CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG T evansi Egypt [JX888091]
216 CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG T evansi Egypt [KF726106]
527 CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG T evansi India [EF495337]
534 CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG T evansi Mathura [KY457408]
484 CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG T evansi RoTat1.2 [KU589274]
629 CTAGAGGGCGACATAAGCGCC-GATGGCTTCACAAAACTAACAGCC--GTTGCAGCGGGCAATGGACATG T evansi RoTat1.2 [AF317914]
TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCC---GG---------- Majority
710 720 730 740 750 760 770
518 CAAAAAACAAAGCAAGCACAAAAGTTACTGAAACAAACGAAACTTGCGAAAAGAAAGA-GGTAGATAAAT T evansi Bikaner [AB259839]
296 TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCC---GG---------- T evansi Bikaner [JX134605]
283 TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCC---GG---------- T evansi Egypt [JX888091]
283 TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCC---GG---------- T evansi Egypt [KF726106]
594 TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCC---GG---------- T evansi India [EF495337]
601 TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCCGGCGGGGTGTTTAA T evansi Mathura [KY457408]
551 TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCC---GG---------- T evansi RoTat1.2 [KU589274]
696 TAGGAAGCAACACCTGCGGGGTGTTTAAAGCAATAACCGGCAACGACGGCGAGGCC---GG---------- T evansi RoTat1.2 [AF317914]
----------CGGGATCAAAATCGCGACCAGCAACATCAAGGTGCACCTC Majority
780 790 800 810 820 830 840
587 GCGAGAAGCCA-T-------GCAAGGTTGTCGAAGCAAATGGTGCTAAAAAGTGCACATTGGACAAGGAT T evansi Bikaner [AB259839]
354 ----------CGGGATCAAAATCGCGACCAGCAACATCAAGGTGCACCTC T evansi Bikaner [JX134605]
341 ----------CGGGATCAAAATCGCGACCAGCAACATCAAGGTGCACCTC T evansi Egypt [JX888091]
341 ----------CGGGATCAAAATCGCGACCAGCAACATCAAGGTGCACCTC T evansi Egypt [KF726106]
652 ----------CGGGATCAAAATCGCGACCAGCAACATCAAGGTGCACCTC T evansi India [EF495337]
671 AGCAATAACCGGCAACGACGGCGAGGCCGGCGGGATCAAAATCGCGACCAGCAACATCAAGGTGCACCTC T evansi Mathura [KY457408]
609 ----------CGGGATCAAAATCGCGACCA T evansi RoTat1.2 [KU589274]
754 ----------CGGGATCAAAATCGCGACCAGCAACATCAAGGTGCACCTC T evansi RoTat1.2 [AF317914]


GCACACGGCCTAATCGAAGGCAAAGTTGACGACCAGCCAGAACGAGCAGAATTTTCCAATAATTTCGGAC Majority
850 860 870 880 890 900 910
649 GAGGCCAAAAAATTAGAAGAAAAGACAGATGGTAAAACAAACACCACAGGAAGCAATTCTCTTCTCATCA T evansi Bikaner [AB259839]
394 GCACACGGCCTAATCGAAGGCAAAGTTGACGACCAGCCAGAACGAGCAGA T evansi Bikaner [JX134605]
381 GCACACGGCCTAATCGAAGGCAAAGTTGACGACCAGCCAGAACGAGCAGAATTTTCCAATAATTTCGGAC T evansi Egypt [JX888091]
381 GCACACGGCCTAATCGAAGGCAAAGTTGACGACCAGCCAGAACGAGCAGAATTTTCCAATAATTTCGGAC T evansi Egypt [KF726106]
692 GCACACGGCCTAATCGAAGGCAAAGTTGACGACCAGCCAGAACGAGCAGAATTTTCCAATAATTTCGGAC T evansi India [EF495337]
741 GCACACGGCCTAATCGAAGGCAAAGTTGACGACCAGCCAGAACGAGCAGAATTTTCCAATAATTTCGGAC T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
794 GCACACGGCCTAATCGAAGGCAAAGTTGACGACCAGCCAGAACGAGCAGAATTTTCCAATAATTTCGGAC T evansi RoTat1.2 [AF317914]
AAGGAAAAGCACACCACACTGATTAXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX Majority
920 930 940 950 960 970 980
719 AAGCTTCCCCTCTTTTTCTTGCGGTTTTGCTGAATTCGTA T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
451 AAGGAAAAGCACACCACACTGATTA T evansi Egypt [JX888091]
451 AAGGAAAAGCACACCACACTGAT T evansi Egypt [KF726106]
762 AAGGAAAAGCACACCACACTGATTATTTAGGCCGAACACACGCAGCACTAATCAATCTGAAGAGGTTGGA T evansi India [EF495337]
811 AAGGAAAAGCACACCACACTGATTATTTAGGCCGAACACACGCAGCACTAAT T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
864 AAGGAAAAGCACACCACACTGATTATTTAGGCCGAACACACGCAGCACTAATCAATCTGAAGAGGTTGGA T evansi RoTat1.2 [AF317914]
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX Majority
990 1000 1010 1020 1030 1040 1050
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
832 AATGGAGAAGGTACCGGAACTCACAGAAGAAACCCTTAAGACTTTAGCAGACGACCCGGCCGCAACGGCA T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
934 AATGGAGAAGGTACCGGAACTCACAGAAGAAACCCTTAAGACTTTAGCAGACGACCCGGCCGCAACGGCA T evansi RoTat1.2 [AF317914]
XXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXXX Majority
1060 1070 1080 1090 1100 1110 1120
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
902 ACCCTAAACGTTGAGGAATGCGCACGAACAAGCAACAAGAAGATAACAACAACAGAACCACCGAAACCGC T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1004 ACCCTAAACGTTGAGGAATGCGCACGAACAAGCAACAAGAAGATAACAACAACAGAACCACCGAAACCGC T evansi RoTat1.2 [AF317914]


Majority
1130 1140 1150 1160 1170 1180 1190
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
972 CCATAACCGAAAAATATTTTGGCAAGGACAAGTCTAAAATCAAGGAGTTGTGGAACAATTTAAAAAAAGA T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1074 CCATAACCGAAAAATATTTTGGCAAGGACAAGTCTAAAATCAAGGAGTTGTGGAACAATTTAAAAAAAGA T evansi RoTat1.2 [AF317914]
Majority
1200 1210 1220 1230 1240 1250 1260
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
1042 GGAGATAGAAGGAACAGAAGATGACACAACAAAGAAAGTAGCGCTAGAAACCGTCAACTCGATCGACAAG T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1144 GGAGATAGAAGGAACAGAAGATGACACAACAAAGAAAGTAGCGCTAGAAACCGTCAACTCGATCGACAAG T evansi RoTat1.2 [AF317914]
Majority
1270 1280 1290 1300 1310 1320 1330
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
1112 TTGCAACAGGCATTGGAGTTTTACACAGCGCGAGCCGCTTACACAATAGAAAAGTTAAAAAAAAGAAGTAG T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1214 TTGCAACAGGCATTGGAGTTTTACACAGCGCGAGCCGCTTACACAATAGAAAAGTTAAAAAAAAGAAGTAG T evansi RoTat1.2 [AF317914]
Majority
1340 1350 1360 1370 1380 1390 1400
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
1182 ATAAGTTGCAAGCAGAATCAGATGCAAAAAACAAAGCAAGCACAAAAGTTACTGAAACAGATGAAACTTG T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1284 ATAAGTTGCAAGCAGAATCAGATGCAAAAAACAAAGCAAGCACAAAAGTTACTGAAACAGATGAAACTTG T evansi RoTat1.2 [AF317914]

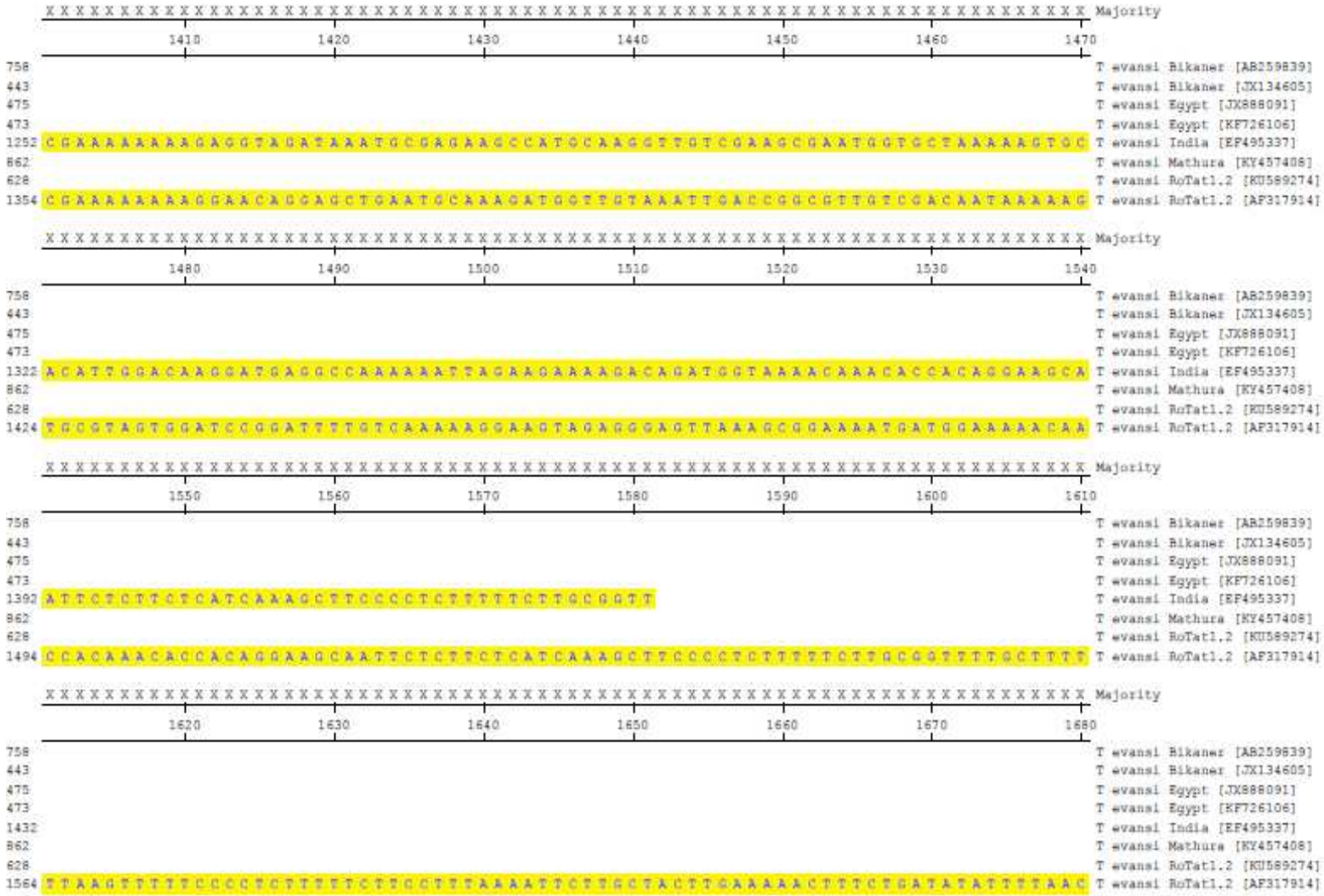

Majority
1410 1420 1430 1440 1450 1460 1470
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
1252 CGAAAAAAAAGAGGTAGATAAATGCGAGAAGCCATGCAAGGTTGTCGAAGCGAATGGTGCTAAAAAGTGC T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1354 CGAAAAAAAAGGAACAGGAGCTGAATGCAAAGATGGTTGTAAATTGACCGGCGTTGTCGACAATAAAAAG T evansi RoTat1.2 [AF317914]
Majority
1480 1490 1500 1510 1520 1530 1540
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
1322 ACATTGGACAAGGATGAGGCCAAAAAATTAGAAGAAAAGACAGATGGTAAAACAAACACCACAGGAAGCA T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1424 TGCGTAGTGGATCCGGATTTTGTCAAAAAGGAAGTAGAGGGAGTTAAAGCGGAAAATGATGGAAAAACAA T evansi RoTat1.2 [AF317914]
Majority
1550 1560 1570 1580 1590 1600 1610
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
1392 ATTCTCTTCTCATCAAAGCTTCCCCTCTTTTTCTTGCGGTT T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1494 CCACAAACACCACAGGAAGCAATTCTCTTCTCATCAAAGCTTCCCCTCTTTTTCTTGCGGTTTTGCTTTT T evansi RoTat1.2 [AF317914]
Majority
1620 1630 1640 1650 1660 1670 1680
758 T evansi Bikaner [AB259839]
443 T evansi Bikaner [JX134605]
475 T evansi Egypt [JX888091]
473 T evansi Egypt [KF726106]
1432 T evansi India [EF495337]
862 T evansi Mathura [KY457408]
628 T evansi RoTat1.2 [KU589274]
1564 TTAAGTTTTTCCCCTCTTTTTCTTGCTTTAAAATTCTTGCTACTTGAAAAACTTTCTGATATATTTTAAC T evansi RoTat1.2 [AF317914]

Fig 12: Nucleotide substitution in RoTat VSG 1 gene Mathura isolate with other known sequences across the globe

CHAPTER-5

DISCUSSION

Trypanosomiosis in equines is a serious cause of economic losses in livestock industry. The occurrence of trypanosomiosis is mainly related to vector population and climatic condition prevailing in that particular area. The present study was done with objective of finding out the prevalence of trypanosomiosis in equines in areas and around Mathura, RoTat 1.2 VSG based molecular detection in equines and molecular characterization along with phylogenetic analysis of RoTat 1.2 VSG equine isolates.

Prevalence of trypanosomiosis in equines from areas in and around Mathura

Blood samples from 86 horses that were brought for routine clinical camps organized by Brooke Hospital for Animal in Mathura, Unnao and Raebareli alongside those animals which are brought to Teaching Veterinary Clinical Complex (TVCC), DUVASU, Mathura were screened for peripheral blood smear examination. Out of 86 samples examined, 3 (3.48%) samples were found positive for trypanosomiosis based on blood smear examination.

There are few reports of trypanosomiosis in equines on basis of blood smear examination alone though few more studies are there based on serology and molecular PCR based diagnosis. Diagnosis of *T. evansi* infection in a horse stable of Eastern Region of India on the basis of examination of Giemsa stained blood smears revealed a high percentage (12.74%) of infection (Laha and Sasmal, 2008). An outbreak reported from Mathura (Kumar *et al*., 1994) resulted in 100% morbidity and 66.6% mortality. Similar fatal outbreak was reported in ponies from Jammu (Raina *et al*., 2000). Likewise reports of surra in equine are frequent in Maharashtra (Bharkad *et al*., 2005) and eastern parts of India (Laha and Sasmal, 2008).

Trypanosomiosis was found to be more likely affecting the animals which were of adult group as maximum prevalence rate for trypanosomiosis were found in animals with over 5 years of age (4.16 %) than in animals under 5 years of age (2.70%). Although such literature is not present in the Indian context pertaining to equines yet a similar pattern was seen by Sinha *et al*. (2006) in cattle and buffaloes affected with trypanosomiosis. The possible reason for this can be attributed to the fact that young ones are more susceptible to infection than the older animals (Bhatia *et al.*, 2016).

So far as sex wise distribution of prevalence rates is concerned, males were found to be more infected (5.55%) than females (2.00%). This can be very much explained by the fact that male horses are more commonly used for riding and draught purposes making them more prone to stress and subsequent flaring of the infection.

Comparative study between blood smear and PCR

Although different techniques have been developed, demonstration of organisms by light microscopy is routinely used for specific detection of trypanosomiosis in infected animals. According to Herbert and Lumsden (1976), when the parasite concentration is less than 2,500,000 parasites per mL are present in blood samples, microscopic detection is not feasible. Hence, blood smear examination proved to be of limited value in diagnosis of subacute or chronic cases. In the present study only 3 animals were identified positive by microscopy out of 86 samples tested.

Molecular diagnostic techniques particularly PCR is a promising technique for the diagnosis of *Trypanosome* infection which is based on the detection of specific DNA sequences of *Trypanosome*s. PCR technique uses thermostable DNA polymerase and specific oligonucleotide primers to conduct repeated cycles of DNA *in vitro* on a small amount of template DNA. Using this technique, a specific segment of the *Trypanosome* DNA can be amplified over a million times which makes the subsequent detection of that specific segment much easier. Although the detection of a single DNA molecule is possible, detection levels to a minimum of 5 *Trypanosome*s by PCR assay are well documented (Diall *et al*., 1992; Artama *et al*., 1992). Amplification of repetitive *T. evansi* specific DNA sequence was possible even with DNA of a single *Trypanosome* (Panyim *et al*., 1993; Viseshakul and Panyim, 1990). Omanwar *et al.*(1999) had reported that PCR amplification of DNA using parasite specific primers represented a potentially powerful tool for epidemiological studies in animal trypanosomiosis. Generally the disease surra is chronic in nature in most of the animals but follows an acute phase in equines (Bhatia *et al.*, 2016), still it is matter of common fact that very often the recovered animals exhibit low levels of fluctuating parasitaemia for years and serve as carriers for the disease. Several PCR methods have been developed for diagnosis of trypanosomiosis, targeting different genes likes kinetoplast DNA, repetitive sequence DNA (Artama *et al*., 1992; Wuyts *et al*., 1995), ribosomal DNA (Ijaz *et al*., 1998). The variant surface glycoprotein (VSG) of *Trypanosome* plays an important part of its body surface coat and are expressed in early, middle and late stages of infection there by, contributing a major diagnostic value.

The total number of blood sample tested was 86, out of which 3 samples were found positive by blood smear and 5 by PCR. 3 samples were found to be positive by both these tests and 78 were found negative by both of them. None of the samples that was found positive by blood smear and negative by PCR. The sensitivity (95%CI) and specificity (95%CI) of PCR method was 100% and 93.98% in comparison to giemsa staining method with kappa value of 0.521. Similar study was also done in horses in Pakistan by Muieed *et al.* (2010), wherein 100 horses with suspected *T. evansi* infection (Surra) were examined by

microscopic examination, *i.e.* Giemsa stained smear method, and PCR. With the stained blood smear method, 5 out of the 100 horses were found positive for *T. evansi* infection. With PCR using TBR1 and TBR2 primers, 16 positive cases were found positive. These results indicate clearly that the PCR assay is a much more sensitive detector of *T.evansi* infection than microscopic examination of Giemsa stained smears. A similar pattern study was carried out in India by Ravindran *et al*. (2008), where in Giemsa stained blood smears upon microscopical examination detected only 2 camels as positive for the presence of *T. evansi*. Smears from donkeys and dogs did not reveal any protozoan organisms. Out of 131 blood samples from different animals tested, 21 camels (including 2 blood smear positive camels), 3 donkeys and 2 dogs showed specific PCR signals as a distinct 227 bp band on agarose gel. Also Parashar *et al*. (2015) reported PCR to be much efficient that blood microscopy in detection of trypanosomiosis in buffaloes from Mathura. In their study, PCR detected 49 cases positive in comparison to blood smear examination, which detected 38 positive cases.

In addition, molecular evidence of *T. evansi* DNA can be tested using PCR with a number of primers specific for the subgenus *Trypanozoon* or to species levels (Desquesnes and D´avila, 2002). Despite being relatively expensive and technical, PCR is generally used to improve the sensitivity of the detection. Comparative studies have led to the recommendation of the Phenol-Chloroform method (Sambrook and Russell, 2001) as the most sensitive DNA preparation method (Pruvot *et al*., 2010). If a right diagnostic molecule is chosen the combination of this method provide a sensitivity of around 5–10 trypanosomes/mL of blood (or other fluid).

No doubt, the conventional parasitological techniques will always remain important for understanding the biology, ecology and molecular epidemiology of different strains of the parasites yet there is need of a highly sensitive test that can detect the lowest levels of parasitaemia (Singh *et al*., 2004) . Blood smear examination proved to be of limited value in diagnosis of subacute or chronic cases. Herbert and Lumsden (1976) reported non feasibility of microscopic detection when less than 2,500,000 parasites per mL are present in blood samples. Again, antibodies to *T. evansi* infection persist after drug treatment, complicating the differentiation of patent infection from non-patent infections using serological methods. Hence, polymerase chain reaction, free of these hurdles, specifically amplifies genetically defined regions of the genome of the infectious agent. Although the detection of a single DNA molecule is possible, detection levels to a minimum of 5 trypanosomes by PCR assay are well documented in literal use (Diall *et al*., 1992; Artama *et al*., 1992). Amplification of repetitive *T. evansi* specific DNA sequence was possible even with DNA of a single *Trypanosome* (Panyim *et al*., 1993; Viseshakul and Panyim, 1990). *T. evansi* genome size is considered to be between 0.05 and 0.1 pg of DNA (Panyim *et al*., 1993). Using PCR it is

possible to detect 0.01 pg of purified DNA which is approximately equivalent to about 0.1 parasite (Pruvot *et al*., 2010).

The described PCR assay provides simple, rapid, sensitive and specific method for detection of *T. evansi* in naturally infected horses as well as in other species also and can be recommended for inclusion in survey and control programmes. Selection of the universal primers in the instant study was based on the observation that the RoTat 1.2 VSG is the most conserved VSG of *T. evansi* known so far.

Molecular characterization of RoTat 1.2 VSG

With the advent of latest molecular techniques, a sea change has been observed in characterization, accurate identification and reliability at various taxonomic levels in the field of *Trypanosome*s study (Desquesnes and Davila, 2002). There are ample reports of characterization studies on *Trypanosome*s in general and *T. evansi* in particular. Amongst these studies, the molecular targets that are being employed are oligopeptidase B from *T. b. brucei* (Rae *et al*., 2006), variable surface glycoprotein (VSG) gene of *T. evansi* (Sengupta *et al*., 2012), ISG-75 gene of *T. b. gambiense* (Tran *et al*., 2008), hypoxanthine guanine phosphoribosyl transferase gene of *T.b. brucei* (Allen and Ullman, 1993), beta-tubulin gene of *T. evansi* (Li *et al*., 2007) and actin gene of *T. evansi* (Li *et al*., 2009). All these molecules are used for phylogenetic analysis between the various isolates. It has been demonstrated that the first and second internal transcribed spacers (ITS-1 and ITS-2, respectively) of the rDNA are useful targets for species delineation and for inferring phylogenetic relationships of *Trypanosoma* spp. (Amer *et al*., 2011; Khuchareontaworn *et al*., 2007), particularly when the availability of morphological characters is limited. Reportedly, microsatellites markers (Biteau *et al*., 2000), amplified restriction fragment length polymorphism analyses (Masiga *et al*., 2006), and intersequence simple repeat PCR (Njiru *et al*., 2007) are utilized in molecular identification of *Trypanosoma*.

T. evansi possess marked importance in tropical and subtropical areas of the world owing to its economic impact. The parasite surface is covered by a thick uniform coat of VSG. These VSGs shields the invariant surface proteins that are present beneath them from the host's immune system effectors by preventing complement activation (Turner *et al*., 1985). These VSGs are known to be expressed during early, middle and late stages of infection (Verloo *et al*., 2001). Even with being regarded as the potent weapon to evade host's immune response, yet the host elicits sufficient levels of antibody production against these VSGs (Gadelha *et al*., 2011). These points make VSG a potent antigen in the diagnosis of *T.evansi* infection. Amongst all the studied VSGs, RoTat 1.2 VSG is the predominant variant antigen type and is known to be expressed in many *T. evansi* stocks (Verloo *et al*., 2000) like cattle (Sengupta *et al*., 2012), buffalo (Sengupta *et al*., 2010) and camel (Roge *et al*., 2013)

across many parts of the globe. Of late, RoTat 1.2 VSG gene was found to be absent in some *T. evansi* isolates (Ngaira *et al*., 2004; Salim *et al*., 2011).

The present study not only gives the first report of presence of RoTat 1.2 VSG from *T. evansi* isolates of equine origin but also gives a fair idea about s phylogenetic relationship of the equine isolates from Mathura with others RoTat 1.2 VSG isolates available throughout the globe. In our study, we characterized two RoTat 1.2 VSG repetoires, *viz*., VSG 1 and VSG 2. While VSG 1 was found to be conserved in *T. evansi*, VSG 2 is specific for T*rypanozoan* genus. The RoTat1.2 VSG 1 *T. evansi* horse isolate used in the present study showed 100% homology with that from camel isolate from Egypt and gave comparatively lesser homologies with that of Trypanosome isolates of camel and buffalo origin from India. The sequence hence generated, was submitted to NCBI and an accession number KY457408 was obtained. The nucleotide sequence revealed 100.0% sequence homologies with that of isolates from Egypt (Accession numbers JX888091 and KF726106). However, the nucleotide similarity with camel and buffalo sequences from India revealed 99.8% and 99.6% homologies, (Accession numbers JX134605 and EF495337), respectively. Alongside, it also showed 99.4% homology with Kenyan isolate (Accession number AF317914). Likewise, it also showed 99.3%, 100% and 98.7% homologies in protein patterns with various Trypanosome isolates from India, Egypt and Kenya, respectively. Hence, it can be very much concluded that the present *T. evansi* isolates was more phylogenetically closer to Egyptian isolates than the Indian and the Kenyan couterparts. The finding is significant from molecular evolutionary point of view.

Of late, RoTat 1.2 VSG gene was found to be absent in some *T. evansi* isolates (Ngaira *et al*., 2004; Salim *et al*., 2011) and it also showed marked variations in fewer isolates (Jia *et al*., 2011). Hence, there is need for identification of such a molecule which is conserved in all the isolates. The present truncated fragment of RoTat 1.2 VSG 2 is a good alternative in this regard. The sequence hence generated, was submitted to NCBI and an accession number KY457409 was obtained. The nucleotide sequence revealed 100.0% sequence homologies with all the other known *T. evansi* sequences *viz.,* buffalo, camel and horse isolates from Karnataka and Bikaner, India (EF495337, JX134605, AB259839) ; Kenyan isolate (AF317914); camel and cattle isolates from Egypt (JX888091, KF726106), respectively. Since the present truncated portion was showing 100% homology with other isolates, thereby suggesting, that it could be used as a good alternative both in molecular detection using PCR as well as in serology using recombinant proteins. However, it will give an idea about the general prevalence of trypanosomiosis and not the species as the used primers are *Trypanozoan* genus specific. Further research in this regard is thereby, warranted before using this molecule for molecular and serological studies.

CHAPTER-6

SUMMARY AND CONCLUSIONS

Trypanosomiosis, caused by the haemo-flagellate protozoan *Trypanosoma evansi* is a major constraint on the health and productivity of domestic animals throughout the tropics and subtropics. *T. evansi* is the common and the most prevalent *Trypanosome* of livestock in India. The parasite is capable of infecting a wide host range including almost all land mammals. It is transmitted mechanically through the bite of blood sucking flies. It is endemic in most parts of the Indian sub-continent, and epizootics have occurred particularly in bovines with mortality rate ranging from 20% to 90%. Widespread morbidity in the form of progressive emaciation, anaemia, oedema, pyrexia, lowered weight gains, lowered milk yield, lowered work capacity and abortions, results in heavy economic losses to the livestock owners. The Office Internationale des Epizooties listed it as the list B disease among notifiable diseases of significance.

In present study, prevalence of trypanosomiosis was studied in equines from clinical camps organized by Brooke Hospital for Animal in Mathura, Unnao and Raebareli alongside those animals which are brought to Teaching Veterinary Clinical Complex (TVCC), DUVASU, Mathura. A total of 86 animals were screened for trypanosomiosis using giemsa stained thin blood smears out of which 3 were found positive for trypanosomiosis.

Trypanosomiosis was found to be more likely affecting the animals which were of adult group as maximum prevalence rate for trypanosomiosis were found in animals with over 5 years of age (4.16 %) than in animals under 5 years of age (2.70%). So far as sex wise distribution of prevalence pattern is concerned, males were found to be more infected (5.55%) than female couterparts (2.00%).

No doubt, the conventional parasitological techniques will always remain important for understanding the biology, ecology and molecular epidemiology of different strains of the parasites yet there is need of a highly sensitive test that can detect the lowest levels of parasitaemia. Although different techniques have been developed, demonstration of parasite by light microscopy is routinely used for specific detection of infected animals. Blood smear examination proved to be of limited value in diagnosis of subacute or chronic cases. Molecular diagnostic techniques particularly PCR is a promising technique for the diagnosis of Trypanosome infection which is based on the detection of specific DNA sequences of Trypanosomes. The Phenol-Chloroform method is the most sensitive DNA preparation

method (or other fluid). The primer used in the study was RoTat 1.2 VSG F/R. These primers target the most commonly conserved VSG repetoire in *T. evansi*.

The total blood samples screened in this study were 86 out of which 3 were positive in blood smear and 5 in PCR and 3 samples were found positive by both test. There was no such sample which was positive by blood smear and negative by PCR. The sensitivity (95%CI) and specificity (95%CI) of PCR method was 100% and 93.98% in comparison to giemsa staining method with kappa value of 0.521. The described PCR assay provides simple, rapid, sensitive and specific method for detection of *T. evansi* in naturally infected horses as well as in other species also and can be recommended for inclusion in survey and control programmes. Selection of this primer set in the instant study was based on the observation that the RoTat 1.2 VSG is the most conserved VSG of *T. evansi* known so far.

The parasite surface is covered by a thick uniform coat of VSG which shields the invariant surface proteins that are present beneath them from the host's immune system effectors by preventing complement activation. These VSGs are known to be expressed during early, middle and later stages of infection. These points make VSG a potent antigen in the diagnosis of *T. evansi* infection. Amongst all the studied VSGs, RoTat 1.2 VSG is the predominant variant antigen type and is known to be expressed in many *T. evansi* stocks like cattle, buffalo and camel across many parts of the globe. However, there are no reports of its presence in equines.

The present study not only gives the first report of presence of RoTat 1.2 VSG from *T. evansi* isolate of equine origin but also suggests phylogenetic relationship of the equine isolate from Mathura with others RoTat 1.2 VSG isolates available throughout the globe.The study characterized two RoTat 1.2 VSG repetoires, *viz*., VSG 1 and VSG 2. The RoTat1.2 VSG 1 *T. evansi* horse isolates used in the present study showed 100 % homology with that from camel isolates from Egypt and yielded comparatively lesser homologies with that of isolates of camel and buffalo origin from India. The sequence was submitted to NCBI and an accession number KY457408 was obtained. Hence, it can be very much concluded that the present stock was more phylogenetically closer to Egyptian isolates than the Indian and the Kenyan counterparts. This finding is significant from molecular evolutionary point of view.

Of late, RoTat 1.2 VSG gene was found to be absent in some *T. evansi* isolates and it also showed marked variation in fewer isolates. Hence, there is need for identification of a molecule which is conserved in all the isolates. The present truncated fragment of RoTat 1.2 VSG 2 is a good alternative in this regard. The sequence was submitted to NCBI and an accession number KY457409 was obtained. The nucleotide sequence revealed 100.0% sequence homologies with all the other known *T. evansi* sequences *viz.,* buffalo, camel and

horse isolates from Karnataka and Bikaner, India; Kenyan isolate; camel and cattle isolates from Egypt, respectively. Since it showed full homologies with other sequences, it can be used as a good alternative both in molecular detection using PCR as well as in serology using recombinant proteins.

ABSTRACT

Trypanosomiosis (Surra), caused by the *Trypanosoma evansi* is a major constraint in the health and productivity of domestic animals throughout the tropics and subtropics. The disease is mainly transmitted mechanically through the bite of blood sucking flies. Widespread morbidity in the form of progressive emaciation, anaemia, oedema, pyrexia, lowered weight gains, lowered milk yield, lowered work capacity and abortions, results in heavy economic losses to the livestock owners. The Office Internationale des Epizooties listed it as the list B disease among notifiable diseases of significance. In present study, prevalence of trypanosomiosis was screened in equines from clinical camps organized by Brooke Hospital for Animal in Mathura, Unnao and Raebareli alongside those animals which are brought to Teaching Veterinary Clinical Complex (TVCC), DUVASU, Mathura. A total of 86 animals were screened for trypanosomiosis using Giemsa stained thin blood smears out of which 3 were found positive for trypanosomiosis. Trypanosomiosis was found to be more likely affecting the animals which were of adult age group So far as sex wise distribution of prevalence rates is concerned, males were found to be more infected (5.55%) than females (2.00%). DNA was isolated from these samples by phenol chloroform method. The primer used in the study was RoTat 1.2 VSG F/R. These primers target the most commonly conserved VSG repetoire in *T. evansi*. Out of 86 samples tested, 3 were found to be positive by blood microscopy and 5 by PCR. Again 3 samples were found positive both with microscopy as well as PCR. There was no such sample which was positive by blood smear and negative by PCR. The sensitivity (95%CI) and specificity (95%CI) of PCR method was 100% and 93.98% in comparison to giemsa staining method with kappa value of 0.521.

The study characterized two RoTat 1.2 VSG repetoires, *viz.*, VSG 1 and VSG 2. The RoTat 1.2 VSG 1 *T. evansi* horse isolates (KY457408) used in the present study showed cent percent homology with that from camel isolates from Egypt and yielded comparatively lesser homologies with that of isolates of camel and buffalo origin from India. RoTat 1.2 VSG 2 (KY457409) revealed 100.0% homology with other isolates of buffalo, camel and horse isolates from Karnataka and Bikaner, India; Kenyan isolate; camel and cattle isolates from Egypt.

BIBLIOGRAPHY

Allen, T.E. and Ullman, B. (1993). Cloning and expression of the hypoxanthine-guaninephosphoribosyl transferase gene from *Trypanosoma brucei*. *Nucleic Acids Res.* **21**: 5431–38.

Amer, S.; Ryu, O.; Tada, C.; Fukuda, Y.; Inoue, N. and Nakai, Y. (2011). Molecular identification and phylogenetic analysis of *Trypanosoma evansi* from dromedary camels (*Camelus dromedarius*) in Egypt, a pilot study. *Acta Trop.* **117**:39–46.

Areekit ,S.; Singhaphan, P.; Kanjanavas, P.; Khuchareontaworn, S.; Sriyapai, T.; Pakpitcharoen, A. and Chansiri, K. (2008). Genetic diversity of *Trypanosoma evansi* in beef cattle based on internal transcribed spacer region. *Infect. Genet. Evol.* **8**:484–488.

Artama, W.T.; Agay, M.W. and Donelson, J.E. (1992). DNA comparisons of *Trypanosoma evansi* (Indonesia) and *Trypanosoma brucei* spp. *Parasitol.* **104**:67-74.

Aslam, A.; Chaudhary, Z.I.; Habib ur, R.; Ashraf, K.; Ahmad, N.; Yaqub, T.; Maqbool, A. and Shakoori, A.R. (2010). Comparative Evaluation of Parasitological, Serological and DNA Amplification Methods for Diagnosis of Natural Trypanosomal Infection in Equines. *Pakistan J. Zool*. **42(4)**:371-376.

Barry, J.D. and McCullogh, R. (2001). Antigenic variation in trypanosomes: enhanced phenotypic variation in a eukaryotic parasite. *Adv. Parasitol.* **4**:1–70.

Bashir, S.; Bakheit, M.A. and Sugimoto, E. (2014). Molecular detection of equine trypanosomosis in the Sudan. *Vet. Parasitol*. **200**: 246–250.

Berlin, D.; Nasereddin, A.; Azmi, K.; Ereqat, S.; Abdeen, Z.; Eyal, O. and Baneth, G. (2012). Prevalence of *Trypanosoma evansi* in horses in Israel evaluated by serology and reverse dot blot. *Res. Vet. Sci.* **93(3)**: 1225–1230.

Bharathi, S. and Padmaja, K. (2007). Trypanosomiasis in equine – A case report. *Indian J. Vet. Med*. 27:72.

Bharkad, G. P.; Bhikane, A.U.; Raote, Y.V.; Markendeya, N.M. and Khan, M.A. (2005). Surra in a Kathiawari mare -A case report. *Intas Polivet.* **6**: 205–06.

Bhatia, B.B.; Pathak, K.M.L. and Juyal P.D. (2016). Textbook of Veterinary Parasitology. Kalyani Publishers, New Delhi. Pp 376.

Biteau, N.; Bringaud, F.; Gibson, W.; Truc, P. and Baltz, T. (2000). Characterization of *Trypanozoon* isolates using a repeated coding sequence and microsatellite markers. *Mol. Biochem. Parasit*. **105**:185–201.

Claes, F.; Radwanska, M.; Urakawa, T.; Majiwa, P.A.O.; Goddeeris, B. and Büscher, P. (2004). Variable Surface Glycoprotein RoTat 1.2 PCR as a specific diagnostic tool for the detection of *Trypanosoma evansi* infections. *Kinetoplastid Biol. Dis.* doi:10.1186/1475-9292-3-3.

d'Oliveira, C.; Van-derWeide, M.; Habela, A.; Jacquiet, P. and Jongejan, F. (1995). Detection of *Theileria annulata* in blood samples of carrier cattle by PCR. *J. Clin. Microbiol.* **33(10)**: 2665–2669.

Dargantes, A.P.; Mercado, R.T.; Dobson, R.J. and Reid, S.A. (2009). Estimating the impact of *Trypanosoma evansi* infection (surra) on buffalo population dynamics in southern Philippines using data from cross-sectional surveys. *Int. J. Parasitol.* **39**: 1109–1114.

Davison , H.C.; Thrusfield, M.V.; Husein, A.; Muharsini, S.; Partou- tomo, S.; Rae, P. and Luckins , A.G. (2000). The occurrence of *Trypanosoma evansi* in buffaloes in Indonesia, estimated using various diagnostic tests. *Epidem. Inf.* **124**:163–172.

De Oliveira, L.A.; Da Silva, S.S.; Herrera, H.; Gama, C.; Cupolillo, E.; Jansen, A. and Fernandes, O. (2008). *Trypanosoma evansi*: molecular homogeneity as inferred by phenetical analysis of ribosomal internal transcribed spacers DNA of an eclectic parasite. *Exp. Parasitol*. **118**:402–407.

De, U.K. and Mukherjee, R. (2006). Trypanosomiasis in equine and its management – A case report. *Indian Vet. J*. **83**:72.

Desquesnes, M. 1997. Evaluation of a simple PCR technique for the diagnosis of *Trypanosoma vivax* infection in the serum of cattle in comparison to parasitological techniques and antigen–enzyme-linked immunosorbent assay. *Acta Trop*. **65**: 139-148.

Desquesnes, M. and Davila, A.M.R. (2002). Application of PCR-based tools for detection and identification of animal trypanosomes: a review and perspectives. *Vet. Parasitol.* **109:** 213–31.

Desquesnes, M.; Hilzmuller, P.; Lai, De.; Dargantes, A.; Lun, Z. and Jittaplapong, S. (2013). *Trypanosoma evansi* and Surra: A Review and Perspectives on Origin, History, Distribution, Taxonomy, Morphology, Hosts, and Pathogenic Effects. *Hindawi Publishing Corporation BioMed. Res. Int.* ,Volume 2013, Article ID 194176, 22.

Desquesnes, M.; McLaughlin, G.; Zoungran, A. and Davila, A.M.R. (2001).Detection and identification of *Trypanosoma* of African livestock through a single PCR based on internal transcribed spacer 1 of rDNA. *Int. J. Parasitol*. **31**:610-614.

Diall, O.; Banjyana-Songa,E.; DE VOS-Dbenahman, N.; Muyldermans, S.; Meirvenne-N VAN, Hammers,R.; DE-Vos, D.;Van-Meirvenne and Uilenberg, G. (1992). Detection and strain identification of *Trypanosoma evansi* by PCR amplification of a kinetoplast minicircle DNA sequence for use in diagnosis and epidemiology of camel trypanosomiasis. In: Resistance or Tolerance of Animals to Disease and Veterinary Epidemiology and Diagnostic Methods. Proceedings of EEC Contractants Workshop 2-6 November 1992, Rethymor, Crete, Greece.

Donelson, J.E.; Artama, W.T.; Mikami, T. and Hirumi, H. (1998). Diagnosis of *Trypanosoma evansi* by the polymerase chain reaction (PCR). J. *Protozool. Res.* **8**: 204-213.

Evans, G. 1880. Report on Surra disease in Dera Ismail Khan. Punjab Government Military Department No. 493, p 446.

Freymann, D.; Down, J.; Carrington, M.; Roditi, I.; Turner, M. and Wiley, D**. (**1990). A resolution structure of the N-terminal domain of a variant surface glycoprotein from *Trypanosoma brucei*. *J. Mol. Biol*. **216(1)**:141-60.

Gadelha, C.; Holden, J.M.; Allison, H.C.; Field, M.C. and Jennifer, M. (2011). Spe-cializations in a successful parasite: what makes the blood streamform African trypanosomes so deadly? *Mol. Biochem. Parasitol.* **179**:51–58.

Gaur, R.S.; Sudan, V.; Jaiswal, A.K.; Singh, A. and Shanker, D. (2016). Classico-molecular targeting of oligopeptidase B, cysteine protease and variable surface glycoprotein (VSG) genes of *Trypanosoma evansi* . *J. Parasit. Dis*. **41(1)**:51–54 .DOI 10.1007/s12639-016-0748-7.

Gupta, M.P.; Kumar, H. and Singla, L.D. (2009). Trypanosomosis concurrent to tuberculosis in black bucks. *Indian Vet. J*. **86**: 727–28.

Gupta, M.P.; Singla, L.D.; Singh, K.B.; Mohan, R. and Bal, M.S. (2003). Recrudescence of trypanosomosis following administration of dexamthasone in bovines. *Indian Vet. J.* **80**: 360–61.

Herbert, W.J. and Lumsden, W.H. (1976). *Trypanosoma brucei*: a rapid "matching" method for estimating the host's parasitemia. *Exp. Parasitol*. **40 (3)**: 427–431.

Holland, W.G.; Claes, F.; My, L.N.; Thanh, N.G.; Tam, P.T.; Verloo, D.; Buscher, P.; Goddeeris, B. and Vercruysse, J. (2001a). A comparative evaluation of parasitological tests and a PCR for *Trypanosoma evansi* diagnosis in experimentally infected water buffaloes. *Vet. Parasitol*. **97(1)**: 23–33.

Holland,W.G.; My L.N.; Dung, T.V.; Thanh, N.G.; Tam, P.T.; Vercruysse, J. and Goddeeris, B.M. (2001b). The influence of *T.evansi* infection on the immuno-responsiveness of experimentally infected water buffaloes. *Vet. Parasitol*. **102**:225-234.

Ijaz, M.; Nur, E.; Kamal, M.; Mohamed, A. and Dar, F. (1998). Comparative studies on the sensitivity of polymerase chain reaction and microscopic examination for the detection of *Trypanosoma evansi* experimentally infected mice. *Comp. Immun. Microbiol. Infect. Dis*. **21**: 215–223.

Isobe, T.; Holmes, E.C. and Rudenko, G. (2003). The transferrin receptor genes of *Trypanosoma equiperdum* are less diverse in their transferrin binding site than those of the broad-host range *Trypanosoma brucei*. *J. Mol. Evol*. **56**:377–386.

Jain, N.C. (1993). Essentials of veterinary hematology. Lea and Febiger, Philadelphia, pp 66–67.

Jia, Y.; Zhao, X.; Zou, J. and Suo, X. (2011). *Trypanosoma evansi*: Identification and characterization of a variant surface glycoprotein lacking cysteine residues in its C-terminal domain. *Exp. Parasitol.* **127**: 100-106.

Jindal, N.; Gupta, S.L.; Batra, M. and Singh, R. (2005). A note on the prevalence of surra in bovines in Haryana. *Indian Vet. J.* **82:**1114–15.

Kalra, S.; Dhaliwal, P.S. and Juyal, P.D. (1994). Trypanosomiasis in a 23 - day old calf (Holstein-Friesian). *Indian Vet. J.* **71**: 191–92.

Khuchareontaworn, S.; Singhaphan, P.; Viseshakul, N. and Chansiri, K. (2007). Genetic diversity of *Trypanosoma evansi* in buffalo based on internal transcribed spacer regions. *J. Vet. Med. Sci*. **69**:487–493.

Kumar, A.; Saxena, S.C.; Sharma, S.D. and Joshi, B.P. (1994). Epidemiology and therapeutic studies on a field outbreak of equine trypanosomiasis. *Indian Vet. J.* **1**: 74–76.

Kumar, R. ; Kumar, S.; Khurana, S.K. and Yadav, S.C. (2013). Development of an antibody-ELISA for seroprevalence of *Trypanosoma evansi* in equids of North and North-western regions of India. *Vet. Parasitol.* **196(3–4)**:251–257.

Laha, R. and Sasmal, N.K. (2008). Endemic status of *Trypanosoma evansi* infection in a horse stable of eastern region of India – a field investigation. *Trop. Anim. Health Prod.* **40**: 357–61.

Lai, D.H.; Hashimi, H.; Lun, Z.R.; Ayala, F.J. and Lukes, J. (2008). Adaptations of *Trypanosoma brucei* to gradual loss of kinetoplast DNA: *Trypanosoma equiperdum* and *Trypanosoma evansi* are petite mutants of *T. brucei*. *Proc. Nat. Acad. Sci.* U.S.A. **105 (6)**:1999–2004.

Lejon, V.; Claes, F.; Verloo, D.; Maina, M.; Urakawa, T.; Majiwa, P.A.O. and Bu¨scher, P. (2005). Recombinant RoTat 1.2 variable surface glycoprotein as antigen for diagnosis of *Trypanosoma evansi* in dromedary camels. *Int. J. Parasitol.* **35** :455–460.

Li, S.Q.; Fung, M.C.; Reid, S.A.; Inoue, N. and Lun, Z.R. (2007). Immunizationwith recombinant beta-tubulin from *Trypanosoma evansi* induced protein *T. evansi*, *T. equiperdum* and *T.b. brucei* infection in mice. *Parasit. Immunol.* **29:** 191–99.

Li, S.Q.; Yang, W.B.; Lun, Z.R.; Ma, L.J.; Xi, S.M.; Chen, Q.L.; Song, X.W.; Kang, J. and Yang, L.Z. (2009). Immunization with recombinant actinfrom *Trypanosoma evansi* induces protective

immunity against *T.evansi, T. equiperdum* and *T.b. brucei* infection. *Parasitol. Res.* **104**:429–35.

Livestock Census (2012). 19th all india livestock census, department of animal husbandry,dairying and fisheries, ministry of agriculture, GOI.

Lun, Z.R. and Desser, S.S. (1995). Is the broad range of hosts and geographical distribution of *Trypanosoma evansi* attributable to the loss of maxicircle kinetoplast DNA? *Parasitol. Today*, **11 (4)**: 131–133.

Malik, B.S.; Chaudhri, S.S. and Gupta, R.P. (2000).Effect of different levels of nutrition on experimental bubaline trypanosomosis. *Indian J. Anim. Sci.* **70**: 559–62.

Masake, R.A.; Majiwa, P.A.O.; Moloo, S.K.; Makau, J.M.; Njuguna, J.T.; Maina, M.; Kabata, J.; Moiyoi, O.K. and Nantulya, V.M. (1997). Sensitive and specific detection of *Trypanosoma vivax* using the polymerase chain reaction. *Exp. Parasitol.* **85**: 193-205.

Masiga, D.K.; Ndungu, K.; Tweedie, A.; Tait, A. and Turner, C.M. (2006). *Trypanosoma evansi*: genetic variability detected using amplified restriction fragment length polymorphism (AFLP) and random amplified polymorphic DNA (RAPD) analysis of Kenyan isolates. *Exp. Parasitol.* **114**:147–153.

Masiga, D.K.; Smyth, A.J.; Hayes, P.; Bromidge, T.J. and Gibson, W.C. (1992). Sensitive detection of trypanosomes in tsetse flies by DNA amplification. I*nt. J. Parasitol.* **22**: 909-918.

Mathieu-Daude, F. and Tibayrenc, M. (1994). Isoenzyme variability of *Trypanosoma brucei* sl.: genetic, taxonomic and epidemiological significance. *Exp. Parasitol*. **78**: 1–19.

Mekata, H.; Konnai, S.; Witola, W.H.; Inoue, N.; Onuma, M. and Ohashi, K. (2009). Molecular detection of *Trypanosomes* in cattle in South America and genetic diversity of *Trypanosoma evansi* based on expression-site-associated gene 6. *Infect. Genet. Evol.* **9**:1301–1305.

Monzon, C.M.; Jara, A. and Nantulya, V.M. (1995). Sensitivity of antigen ELISA test for detecting *Trypanosoma evansi* antigen in horses in the subtropical area of Argentina. *J. Parasitol.* **81**:806–808.

Muieed, M.A.; Chaudhary, Z.I. and Shakoori, A.R. (2010). Comparative studies on the sensitivity of polymerase chain reaction (PCR) and microscopic examination for the detection of *Trypanosoma evansi* in horses. *Turk. J. Vet. Anim. Sci*. **34(6)**: 507-512.

Nadeem, A.; Aslam, A.; Chaudhary, Z.I.; Ashraf, K.; Saeed, K.; Ahmad, N.; Ahmed, I. and Rehman, H. ur. (2010). Indirect Fluorescent Antibody Technique based Prevalence of Surra in Equines ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE JOURNAL).

Ngaira, J.M.; Njagi, E.N.; Ngeranwa, J.J. and Olembo, N.K. (2004). PCR amplification of RoTat 1.2 VSG gene in *Trypanosoma evansi* isolates in Kenya. *Vet. Parasitol.* **120**: 23–33.

Ngaira, J.M.; Olembo, N.K.; Njagi, E.N.M. and Ngeranwa, J.J.N. (2005). The detection of non-RoTat 1.2 *Trypanosoma evansi. Exp. Parasitol*. 110*:* 30–38.

Njiru, Z.K.; Constantine, C.C.; Gitonga, P.K.; Thompson, R.C. and Reid, S.A. (2007). Genetic variability of *Trypanosoma evansi* isolates detected by inter-simple sequence repeat anchored-PCR and microsatellite. *Vet. Parasitol*. **147**:51–60.

Office Internationale Des Epizooties (OIE) (2012). *Trypanosoma evansi* infection (surra). OIE Terrestrial Manual Chapter 2.1.17.

Omanwar S. (1998). Amplification of kinetoplast and nuclear DNA for the detection of *Trypanosoma evansi*. M.V.Sc. Thesis submitted to Deemed University, IVRI, Izatnagar, India.

Omanwar, S.; Rao, J.R.; Basagoudanavar, S.H.; Singh, R.K. and Butchaiah, G. (1999). A simple and highly sensitive detection of *Trypanosoma evansi* by DNA amplification from crude blood samples collected on filter papers. *J. Vet. Parasitol.* **13**: 27-29.

Pandey, V.; Nigam, R.; Jaiswal, A.K.; Sudan, V.; Singh, R.K. and Yadav, P.K. (2015). Haemato-biochemical and oxidative status of buffaloes naturallyinfected with *Trypanosoma evansi*. *Vet. Parasitol*. **212(3-4)**:118-22. doi: 10.1016.

Panyim, S.; Viseshakul, N.; Luxananil, P.; wuyts, N. and Chokesajjawatee, N. (1993). A PCR method for highly sensitive detection of *Trypanosoma evansi* in blood sample. In: Proceeding of EEC contractants workshops, "Resistance or Tolerance of animal to disease and veterinary epidemiology and diagnostic method,Rethymno, Greece. 138-143.

Parashar, R.; Shanker, D.; Sudan, V. and Jaiswal, A.K. (2015). PCR-based diagnosis of surra-targeting mini-chromosomal satellite DNA for unraveling the cryptic epizootiology of bubaline trypanosomosis. *Indian J. Anim. Sci*. **85 (4)**: 43–00.

Pathak, K.M.L. and Chhabra, M.B.(2011). Trypanosomosis of livestock in India: A review of two decades. *Indian J. Anim. Sci*. **81 (7)**: 653–660.

Pathak, K.M.L. and Kapoor, M. (1999). Transplacental transmission of *Trypanosoma evansi* in a donkey. *Indian Vet. J.* **76**: 179.

Payne, R.C.; Sukanto, I.P.; Djauhari, D.; Partoutomo, S.; Wilson, A.J.; Jones, T.W.; Boid, R. and Luckins, A.G. (1991). *Trypanosoma evansi* infection in cattle, buffaloes and horses in Indonesia. *Vet. Parasitol.* **38**: 109–119.

Pruvot, M.; Kamyingkird, K.; Desquesnes, M.; Sarathapan, N. and Jittapalapong, S.(2010). A comparison of six primer sets of detection of *Trypanosoma evansi* by polymerase chain reaction in rodents and Thai livestock. *Vet. Parasitol*. **171**: 185-193.

Rae, D.; Hazell, C.; Andrew, N.W.; Morty, R.E. and Fulop, V. (2006). Expression,purification and preliminary crystallographic analysis of oligopepti-dase B from *Trypanosoma brucei*. Acta Crystallogr. *F. Struct. Biol. Cryst.Commun.* **62**: 808–810.

Raina, R.; Raina, A.K. and Bhadwal, M.S.(2000). Outbreak of surra in buffaloes and ponies. *Indian J. Vet. Med*. **20**: 32.

Ravindran, R.; Rao, J.R.; Mishra, A.K.; Pathak, K.M.L.; Babu, N.; Satheesh, C.C. and Rahul, S. (2008). *Trypanosoma evansi* in camels, donkeys and dogs in India: comparison of PCR and light microscopy for detection – short communication *Veterinarski Arhiv*. **78 (1)**: 89-94.

Reinwald, E. (1985). Role of carbohydrates within variant surface glycoprotein of *Trypanosoma congolense*: protection against proteolytic attack. E*ur. J. Biochem.* **151**: 385–391.

Robinson, N.P.; Burman, N.; Melville, S.E. and Barry, J.D. (1999). Predominance of duplicative VSG gene conversion in antigenic variation in African trypanosomoses. *Mol. Cell. Biol*. **19**: 5839–5846.

Roge, S.; Van Reeta, N.; Odiwuora Tran, T.; Schilderman, K.; Vandamme, S.; Vandenberghe, I.; Verveckene, W.; Gillingwater, K.; Claes. F.; Devreese, B.; Guisez, Y. and Buscher, P. (2013). Recombinant expression of trypanosome surface glycoproteins in *Pichia pastoris* forthe diagnosis of *Trypanosoma evansi* infection. *Vet. Parasitol.* **197**:571–579.

Rudramurthy, G.R.; Sengupta, P.P.; Balamurugan, V.; Prabhudas, K. and Rahman, H. (2013). PCR based diagnosis of Trypanosomiasis exploring invariant surface glycoprotein (ISG) 75 gene. *Vet. Parasitol.* **193** :47– 58.

Rudramurthy, G.R.; Sengupta, P.P.; Metilda, B.; Balamurugan, V.; Prabhudas, K. and Rahman, H. (2015). Development of an Enzyme immunoassay using recombinant invariant surface glycoprotein (rISG) 75 for serodiagnosis of bovine trypanosomosis. *Indian J. Exp. Biol.* **53**:7-15.

Salim, B.; Bakheit, M.A.; Kamau, J.; Nakamura, I. and Sugimoto, C. (2011). Molecular epidemiology of camel trypanosomiasis based on ITS1rDNA and RoTat 1.2 VSG gene in the Sudan. *Parasit. Vect.* **4**: 31.

Sambrook ,J. and Russell, D.W. (2001). Molecular cloning: A laboratory Manual. Cold spring Harbor Laboratory Adviser. 33-37.

Sellon, D.C. (2007). Surra-miscellaneous parasitic infections. In: Sellon, D.C., Long, M.T. (Eds.), Equine Infectious Diseases. Saunders Elsevier, St. Louis, MO, pp. 475–476.

Sengupta, P.P.; Balumahendiran, M.; Balamurugan, V.; Rudramurthy, G.R. and Prabhudas, K. (2012). Expressed truncated N-terminal variable surfaceglycoprotein (VSG) of *Trypanosoma evansi* in *E. coli* exhibits immuno-reactivity. *Vet. Parasitol.* **187**: 1–8.

Sengupta, P.P.; Balumahendiran, M.; Suryanaryana, V.V.S.; Raghavendra, A.G.; Shome, B.R.; Gajendragad, M.R. and Prabhudas, K.(2010). PCR-based diagnosis of surra-targeting VSG gene: Experimental studies in small laboratory rodents and buffalo. *Vet. Parasitol*. **171**: 22–31.

Sengupta, P.P.; Rudramurthy, G.R.; Ligi, M.; Roy, M.; Balamurugan, V.; Krishnamoorthy, P.; Nagalingam, M.; Singh, L. and Rahman, H. (2014). Sero-diagnosis of surra exploiting recombinant VSG antigenbased ELISA for surveillance. *Vet. Parasitol.* **205**: 490–498.

Sharma, A.; Singla, L.D.; Tuli, A.; Kaur, P.; Batth, B.K.; Javed, M. and Juyal, P.D. (2013). Molecular Prevalence of *Babesia bigemina* and *Trypanosoma evansi* in Dairy Animals from Punjab, India, by Duplex PCR: A Step Forward to the Detection and Management of Concurrent Latent Infections. *BioMed Res. Int.* Volume 2013 Article ID 893862, 8 pages.

Shyam, K.P.; Gupta, S.K.; Singh, A.; Chaudhary, S.S. and Gupta, J.P. (2013). detection of *Trypanosoma evansi* in whole blood of domestic animals by DNA amplification method. *Indian J. Anim. Res.* **47 (5)**: 456-459.

Singh, H.; Mishra, A.K.; Rao, J.R. and Tewari, A.K. (2007). A PCR assay for detection of *Babesia bigemina* infection using clotted blood in bovines. *J. App. Anim. Res.* **32(2)**: 201–02.

Singh, H.; Tewari, A.K.; Mishra, A.K.; Maharana, B.R.; Sudan, V.; Raina, O.K. and Rao, J.R.(2014). Detection of antibodies to *Toxoplasma gondii* in domesticated ruminants by recombinant truncated SAG2 enzyme-linked immunosorbent assay. *Trop. Anim. Health Prod*. doi: 10.1007/s11250-014-0703-5.

Singh, N.; Pathak, K.M. and Kumar, R.(2004). A comparative evaluation of parasitological, serological and DNA amplification methods for diagnosis of natural *Trypanosoma evansi* infection in camels. *Vet. Parasitol*. **126**: 365-73.

Singh, V. and Singla, L.D.(2012). Trypanosomosis in cattle and buffaloes from latent carrier status to clinical form of disease: Indian scenario. *Integrated Research Approaches in Vet. Parasitol.* (Eds) Shankar, D.; Tiwari, J.; Jaiswal, A.K. and Sudan, V. DUVASU, Mathura: 10–18.

Sinha, B.S.; Verma, S.P.; Mallick, K.P.; Samantaray, S.; Kumar, B. and Kumar, R.P. (2006). Study on epidemiological aspects of bovine trypanosomosis in some districts of Bihar. *J. Vet. Parasitol.* **20(1)**: 69-71.

Songa, E.B. and Hamers, R. (1988). A card agglutination test (CATT) for veterinary use based on an early VAT RoTat1.2 of *Trypanosoma evansi. Ann. Soc. Belge. Med. Trop.* **68:** 233–240.

Soodan, J.S.; Singh, K.B.; Juyal, P.D. and Khahra, S.S. (1995). Incidence of *Trypanosome evansi* infection in equines in Punjab State. *J. Vet. Parasitol*. **9**: 133–34.

Soulsby, E.T.L. 1982. Helminthes, Arthropods, And Protozoa Of Domesticated Animals, 7th Ed. London, Bailliere and Tindal.

Sudan, V.; Jaiswal, A.K.; Parashar, R. and Shanker, D. (2015a). A duplex PCR-based assay for simultaneous detection of *Trypanosoma evansi* and *Theileria annulata* infections in water buffaloes. *Trop. Anim. Health Prod*. **47**: 915–919.

Sudan, V.; Tewari, A.K. and Singh, H. (2015b). A native whole cell lysate antigen (WCLA) based ELISA for the sero-detection of surra in Indian cattle. *Indian J. Anim. Sci*. **85 (6)**: 601–603.

Sumbria, A.L.; Mihok, S. and Oyieke, F.A. (1998). Mechanical transmission of *Trypanosoma evansi* and *T. congolense* by *Stomoxy sniger* and *S. taeniatus* in a laboratory mouse model. *Med. Vet. Entomol*. **12**: 417–422.

Sumbria, D.; Singla, L.D.; Sharma, A.; Bal, M.S. and Kumar, S. (2015). Multiplex PCR for detection of *Trypanosoma evansi* and *Theileria equi* in equids of Punjab. *India. Vet. Parasitol*. **211**: 293–299.

Taylor, T.K.; Boyle, D.B. and Bingham, J. (2008). Development of a TaqMan PCR assay for the detection of *Trypanosoma evansi* the agent of surra. *Vet. Parasitol*. **153**: 255–264.

Thrusfield, M.V. (2005). Veterinary epidemiology. 3. Oxford: Blackwell Science; pp. 234–238.

Tran, T.; Buscher, P.; Vandenbussche, G.; Wyns, L.; Messens, J. and Greve, H.D. (2008). Heterologous expression, purification and characterization of the extra cellular domain of trypanosome invariant surface glycopro-tein ISG75. *J. Biotech*. **135**: 247–54.

Turner, M.J.; Cardoso de Almeida, M.L.; Gurnett, A.M.; Raper, J. and Ward, J. (1985). Biosynthesis, attachment and release of variant surface glycoproteins of the African trypanosome. *Current Topics Microbiol. Immunol.* **117**: 23–55.

Varshney, J.P. and Gupta, A.K. (1996). Haematobiochemical changes in clinical trypanosomiasis with reference to liver function indices. Centaur **13**: 13–16.

Varshney, J.P.; Varshney, V.P. and Dwivedi, S.K. (1999). Endocrine dysfunctions in clinical trypanosomosis in horses. *J. Vet.Parasitol.* **13**: 33–35.

Verloo, D.; Holland, W.; My L.N.; Thanh, N.G.; Tam, P.T.; Goddeeris, B.; Vercruysse, J. and Buscher, P. (2000). Comparison of serological tests for *Trypanosoma evansi* natural infections in water buffaloes from North Vietnam. *Vet. Parasitol*. **29**: 87–96.

Verloo, D.; Magnus, E. and Buscher, P. (2001). General expression of RoTat 1.2 variable antigen type in *Trypanosoma evansi* isolates from different origin. *Vet. Parasitol.* **97**: 183–189.

Viseshakul, N. and Panyim, S. (1990). Specific DNA probe for the sensitive detection of *Trypanosoma evansi*. *Southeast Asian J. Trop. Med. Pub. Hlth*. **21**: 21-27.

Witola, W.H.; Sarataphan, N.; Inoue, N.; Ohashi, K. and Onuma, M. (2005). Genetic variability in ESAG6 genes among *Trypanosoma evansi* isolates and in comparison to other Trypanozoon members. *Acta Trop*. **93**:63–73.

Wuyts, N.; Chokesajawatee, N. and Panyim, S. (1994). A simplified and highly sensitive detection of *Trypanosoma evansi* by DNA amplification. *Southeast Asian J. Med. Publ. Hlth.* **25**: 266-271.

Wuyts, N.; Chokesajjawatee, N.; Sarataphan, N. and Panyim, S. (1995). PCR amplifcation of crude blood on microscope slides in the diagnosis of *Trypanosoma evansi* infection in dairy cattle. *Annls. Soc. Belg. Med. Trop*. **75**: 229-237.

Zhang, Z.Q. and Baltz, T. (1994). Identification of *Trypanosoma evansi*,*Trypanosoma equiperdum* and *Trypanosoma brucei brucei* using repetitive DNA probes. *Vet. Parasitol.* **53**: 197–208.

STAINING CHEMICALS

Giemsa stain

Giemsa powder 3.8 g

Methyl alcohol (Acetone free) 250.0 ml

Warm glycerine 250.0 ml

The stain was diluted 1:20 in PBS (pH 7.2).

DNA ISOLATION CHEMICALS

T E Lysis solution

10mM of Tris acid

1mM of EDTA

Tris-HCl (10mM pH 8.0)

Tris base 12.11 g

Dissolve in 800 ml distilled water. Adjust the pH to 8.0 by conc. HCl. Make the olume to 1 l. Autoclave and store at 4°C

Ethylene diamine tetra acetate (EDTA) (1mM, pH 8.0)

EDTA 292.24 g

Distilled water 800 ml

Stir vigorously on a magnetic stirrer to mix. Adjust the pH to 8.0 and make the volume to 1000 ml. Autoclave and store at 4°C. This is 1 M solution. Add 1ml of this solution to 99 ml distilled water to get 1mM EDTA.

Proteinase K (25mg/ml)

Proteinase K	25 mg
Nuclease free water	1 ml

SDS (10%)

SDS	1g
Distilled water	10 ml

3 M Sodium Acetate

Sodium acetate trihydrate 4.08g dissolved in 6 ml of distilled water. Adjust pH to 5.2 using glacial acetic acid thereafter make final volume upto 10 ml using distil water.

AGAROSE ELECTROPHORESIS CHEMICALS

Ethylene diamine tetra acetate (EDTA) (0.5M, pH 8.0)

EDTA 18.61 g

Distilled water 80 ml

Stir vigorously on a magnetic stirrer to mix. Adjust the pH to 8.0 and make the volume to 100 ml. Autoclave and store at 4°C

Ethidium bromide (10 mg/ml)

Dissolve 0.2 g ethidium bromide in 20 ml water. Mix well and store at 4°C in dark

Tris-acetate EDTA (TAE) buffer (50X) for 1 litre

Tris 242 g

EDTA (0.5 M) 100 ml

Acetic acid 57.1 ml

Store at 4°C.

Working solution was made to 1X for gel electrophoresis.

CLONING CHEMICALS

Luria Bertani (LB) Broth

Bacto Tryptone 1 g

NaCl 1 g

Yeast Extract 0.5 g

Add distilled water to make up volume to 100 ml. Adjust pH to 7.5 by NaOH. Sterilize by autoclaving.

Luria Bertani (LB) Agar

LB Agar was prepared by adding 2% agar to LB medium.

Ampicillin

Ampicillin powder 100 mg

Sterile distilled water 1 ml

Filter sterilize and store at 4°C.

100mM Calcium Chloride Solution

$CaCl_2.6H_2O$ 54 g

Distilled Water 200 ml

Sterilize by passing through a 0.22 μm filter. This is 1 M solution. dilute it 10 times to get 100mM solution.

Trypanosoma evansi isolate CJ-1 variable surface glycoprotein (VSG) gene, partial cds

GenBank: KY457408.1

```
LOCUS       KY457408                862 bp    DNA     linear   INV 02-APR-2017
DEFINITION  Trypanosoma evansi isolate CJ-1 variable surface glycoprotein (VSG)
            gene, partial cds.
ACCESSION   KY457408
VERSION     KY457408.1
KEYWORDS    .
SOURCE      Trypanosoma evansi
  ORGANISM  Trypanosoma evansi
            Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae;
            Trypanosoma.
REFERENCE   1  (bases 1 to 862)
  AUTHORS   Devi,A., Shanker,D., Sudan,V., Jaiswal,A. and Singh,A.
  TITLE     Direct Submission
  JOURNAL   Submitted (10-JAN-2017) Department of Parasitology, U P Pandit Deen
            Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anus,
            College of Vety. Sciences & Animal Husbandry, Mathura, Uttar
            Pradesh 281001, India
COMMENT     ##Assembly-Data-START##
            Sequencing Technology :: Sanger dideoxy sequencing
            ##Assembly-Data-END##
FEATURES             Location/Qualifiers
     source          1..862
                     /organism="Trypanosoma evansi"
                     /mol_type="genomic DNA"
                     /isolate="CJ-1"
                     /db_xref="taxon:5697"
     gene            <1..>862
                     /gene="VSG"
     mRNA            <1..>862
                     /gene="VSG"
                     /product="variable surface glycoprotein"
     CDS             <1..>862
                     /gene="VSG"
                     /codon_start=3
                     /product="variable surface glycoprotein"
                     /protein_id="ARB51110.1"
                     /translation="EFMQTKALVGVLLFVLYRSTTDAANVALKGNVWKPLCELAAATR
                     NGPSHGTAHFAAIENSVETYTKLKLKLLIYAAAKGSTTEASAARGLAAAADRHIRAAA
                     TTAKDKSRVILPAVAYGGEVAGAISSALKFLKHAVGNSKFCVGKADGTNADGNNEIDA
                     LGCGEANYDTSAPGDSYLEGDISADGFTKLTAVAAGNGHVGSNTCGVFKAITGNDGEA
                     GGVFKAITGNDGEAGGIKIATSNIKVHLAHGLIEGKVDDQPERAEFSNNFGQGKAHHT
                     DYLGRTHAAL"
ORIGIN
        1 gggaattcat gcaaaccaag gcgctcgttg gcgtactctt atttgtactg tatcggagca
       61 caacggatgc cgccaatgta gctcttaaag gcaacgtctg gaagccattg tgcgaactcg
      121 cggcagcgac caggaacggg ccaagccacg gcacggcgca cttcgcagcg atcgaaaata
      181 gcgtcgaaac gtacactaag ttaaaactaa agctcttgat ttacgcggcg gccaaaggca
      241 gcaccaccga agcaagcgca gcaagagggt tagcagcggc cgcagataga cacatacgag
      301 cagcggccac cacggcgaaa gacaaaagca gggtaattct gcccgcagtt gcctatggcg
      361 gcgaagtcgc aggggcgatt tcatcggcgc taaaatttct aaagcacgcg gttggcaaca
      421 gcaagttttg tgtgggcaaa gccgacggca caaatgccga cggtaacaac gaaatcgacg
      481 cgctagggtg cggcgaagcc aactatgaca cctcggcccc aggagacagc tacctagagg
      541 gcgacataag cgccgatggc ttcacaaaac taacagccgt tgcagcgggc aatggacatg
      601 taggaagcaa cacctgcggg gtgtttaaag caataaccgg caacgacggc gaggccggcg
      661 gggtgtttaa agcaataacc ggcaacgacg gcgaggccgg cgggatcaaa atcgcgacca
      721 gcaacatcaa ggtgcacctc gcacacggcc taatcgaagg caaagttgac gaccagccag
      781 aacgagcaga attttccaat aatttcggac aaggaaaagc acaccacact gattatttag
      841 gccgaacaca cgcagcacta at
//
```

Trypanosoma evansi isolate CJ-2 variable surface glycoprotein (VSG) gene, partial cds

GenBank: KY457409.1

LOCUS KY457409 205 bp DNA linear INV 02-APR-2017
DEFINITION Trypanosoma evansi isolate CJ-2 variable surface glycoprotein (VSG) gene, partial cds.
ACCESSION KY457409
VERSION KY457409.1
KEYWORDS .
SOURCE Trypanosoma evansi
ORGANISM Trypanosoma evansi
Eukaryota; Euglenozoa; Kinetoplastida; Trypanosomatidae; Trypanosoma.
REFERENCE 1 (bases 1 to 205)
AUTHORS Devi,A., Shanker,D., Sudan,V., Jaiswal,A. and Singh,A.
TITLE Direct Submission
JOURNAL Submitted (10-JAN-2017) Department of Parasitology, U P Pandit Deen Dayal Upadhyaya Pashu Chikitsa Vigyan Vishwavidyalaya Evam Go Anus, College of Vety. Sciences & Animal Husbandry, Mathura, Uttar Pradesh 281001, India
COMMENT ##Assembly-Data-START##
Sequencing Technology :: Sanger dideoxy sequencing
##Assembly-Data-END##
FEATURES Location/Qualifiers

```
   source        1..205
                 /organism="Trypanosoma evansi"
                 /mol_type="genomic DNA"
                 /isolate="CJ-2"
                 /db_xref="taxon:5697"
   gene          <1..>205
                 /gene="VSG"
   mRNA          <1..>205
                 /gene="VSG"
                 /product="variable surface glycoprotein"
   CDS           <1..>205
                 /gene="VSG"
                 /codon_start=3
                 /product="variable surface glycoprotein"
                 /protein_id="ARB51111.1"
                 /translation="GVFKAITGNDGEAGGIKIATSNIKVHLAHGLIEGKVDDQPERAE
                 FSNNFGQGKAHHTDYLGRTHAAL"

ORIGIN
      1 gcggggtgtt taaagcaata accggcaacg acggcgaggc cggcgggatc aaaatcgcga
     61 ccagcaacat caaggtgcac ctcgcacacg gcctaatcga aggcaaagtt gacgaccagc
    121 cagaacgagc agaattttcc aataatttcg gacaaggaaa agcacaccac actgattatt
    181 taggccgaac acacgcagca ctaat
//
```

CV OF STUDENT

NAME	:	ANJALI DEVI
DATE OF BIRTH	:	15th APRIL 1990
PLACE OF BIRTH	:	FATEHPUR (U.P)
FATHER'S NAME	:	Shri. VISHNU PAL
MOTHER'S NAME	:	Smt. SONY DEVI
PERMANENT ADDRESS	:	VILL-PARADAN, POST- SELAWAN, DIST- FATEHPUR U.P.
MOBILE NO.	:	8005088611, 8542819866
E-MAIL	:	vety.anjali@gmail.com

ACADEMIC QUALIFICATION:

Degree	University/Board	Year Passing	Percentage of Marks	Subject
B.V.Sc. & A.H.	College of Veterinary Science and Animal Husbandry, DUVASU (MATHURA)	2015	76	All Veterinary & Animal Science Sub.

UNDERTAKING OF COPY RIGHT

I **Dr. Anjali Devi** Enrolment No V-1482/15 undertake that I give copy right to the DUVASU, Mathura of my thesis entitled **"Classico-molecular studies and characterization Rotat 1.2 VSG of *Trypanosoma evansi* in equines".**

I also undertake that patent, if any, arising out of research work conducted during the programme shall be filed by me only with due permission of the competent authority of DUVASU, Mathura (UP).

Anjali

Signature of student