ADDIS ABABA UNIVERSITY SCHOOL OF GRADUATE STUDIES

Studies on Salivary Gland Hyperplasia Virus (SGHV) transmission in *Glossina pallidipes* colony using conventional and molecular techniques

By

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LIST OF ABREVATIONS

AAT	African Animal Trypanosomosis
CFU	Colony Forming Units
DFFBB	Difibrinated Fresh Frozen Bovine Blood
DHC	Differential haemocyte counts
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
EDTA	Ethylenediaminetetraacetic acid
EtBr	Ethidium bromide
HAT	Human African Trypanosomosis
HSV	Herpes simplex virus
GC	Guanine -Cytosine
IAEA	International Atomic Energy Agency
IFN	Interferon
Ig	Immunoglobulin
KDa	Kilo Dalton
M	Molar
M cell	Membranous cells
mM	Milli Molar
mRNA	Messenger ribonucleic acid
OD	Optical density
OAU	Organization of African Union
PAATEC	Pan African Tsetse and Trypanosomosis Eradication Campaign
PCR	Polymerase Chain Reaction
PM	Pico molar
rpm	Revolutions Per Minute
QPCR	Quantitative Polymerase Chain Reaction
RBC	Red blood corpuscles

PTA	Phosphotungstic acid
SDS	Sodium dodecyl sulfate
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
SGHV	Salivary Gland Hyperplasia Virus
SIT	Sterile Insect Technique
THC	Total haemocytes
VAP	Virus-associated protein
V/V	Volume by volume
VLP	Virus like particle
WBC	White blood corpuscles
WHO	World Health Organization

Abstract

In Ethiopia five species of tsetse flies were recorded from different parts of the country. *Glossina pallidipes* is one of the five species which cause a fatal disease to human and animal. Sterile Insect Technique (SIT) as a component for tsetse fly control and eradication programme is one of the successful techniques in some insect pest eradication programmes. However, the presence of salivary gland hyperplasia virus affects the application of SIT technique in tsetsecontrol as it limits the mass rearing activity. It is widely accepted that tsetse plays host to salivary gland hyperplasia virus. This study mainly focused on virus free line establishment and the antiviral drug treatment using more than 1800 *G.pallidipes* Tororo and 3072 *G. pallidipes* Arbaminch strains. Prior to the large scale experiments, two preliminary surveys were done on virus free line establishment and antiviral drug treatments. All experiments were analyzed using PCR, dissection and QPCR methods. The preliminary survey on the investigation of the virus free line by dissection showed that 4.45% male flies and 3.09% of female flies were positive for salivary gland hyperplasia, whereas in PCR analysis 95% of the flies were positive for SGHV. In the virus free line establishment a total of 920 female flies were taken by randomly and 13.04% were selected by PCR as positive or negative flies for salivary gland hyperplasia and two out of 120 female flies died. Among 120 flies, 26.7% male and 15% female including the dead flies were negative and 35.8% female and 22.5% male flies were positive by PCR. All screened flies were grouped into four groups as Virus free line I, II, III and IV. In the preliminary survey of the antiviral drug, female flies treated with valacyclovir treated flies produced more pupae than acyclovir treated flies. In the main experiment of the antiviral drugs valacyclovir treated flies showed no significant difference P>.072. But, acyclovir and control showed significant difference P<.000 and P<.005 at P=0.05, respectively.

Key words: *Glossina pallidipes,* SIT, Salivary gland hyperplasia virus, virus free line, Trypanosomosis

DECLARATION

This thesis work is my original work and has not been presented for a degree in any University and all material sources are dully acknowledged.

Name:………………………………………..…………

Signature:.………………………………………..

Date: ……………………………………………….

This thesis has been submitted for examination which my approval as university advisor.

Name…………………………………………

Signature……………………………………

Date……………………………………………..

semi-desert condition along the southern border east of the Rift Valley (Miressa, 2005*).*

G. m. submorsitans, G. pallidipes, G. tachinoides, G. f. fuscipes and G. longipennis have been recorded from Ethiopia, but only four are widespread and of significant economic importance. These are *G. m. submorsitans* and *G. tachinoides*, which have a west to east distribution across Africa south of the Sahara desert, and *G. pallidipes* and *G. f. fuscipes* which often occur together in East Africa. Out of the nine regions of Ethiopia, five (Amhara, Beneshangul-Gumus, Gambella, Oromiya and SNNPR) are infested with more than one species of tsetse fly (Miressa, 2005*).*

Control of tsetse by using SIT would enable livestock intensification to take place and to reduce pressure on ecologically sensitive areas. The main component of SIT package is the mass rearing of tsetse flies which can be affected by pathogens like salivary gland hyperthrophy vurus that is known to occur in tsetse wild flies of *G. pallidipes* and *G. morsitans* (Burtt, 1945; Whitnall, 1934). The presence of virus like particles (VLP) in the epithelial cell of the salivary glands of tsetse, *Glossina pallidipes (*Austen), was reported by Jaenson (1978).

Tsetse are biological vectors of trypanosomes and in the process of feeding, they acquire and transmit small, single-celled protozoa of the genus *Trypanosoma* from infected vertebrate hosts to uninfected animals.

It has been stated by Uilenberg (1998) that the tsetse flies transmit trypanosomes in two ways, *mechanical* and *biological* transmission. Mechanical transmission involves the biting insects which passes the blood forms from an infected animal to another in the course of interrupted feeding. The time between the two feeds is crucial for effective transmission because the trypanosomes die when the blood dries. The term *biological* is used because trypanosomes must reproduce

through several generations inside the tsetse host during the period of incubation, which requires extreme adaptation of the trypanosomes to their tsetse host. Trypanosomes are not transmitted between a pregnant tsetse and her offspring so all new emerged tsetse adults are free of infection. The cycle of biological transmission of trypanosomiasis involves inside the tsetse and inside the vertebrate hosts.

The tsetse vectored trypanosomiases affect various vertebrate species including humans, antelopes, bovine cattle, camels, horses, sheep, goats, and pigs. These diseases are caused by different trypanosome species which may also survive in wild animals such as crocodiles and monitor lizards. The diseases have different distributions across the African continent and are therefore transmitted by different species of tsetse.

The novel idea of controlling insects by sterilisation (Knipling, 1955; Knipling, 1959) suggested that sterility induction, either by gamma irradiation or treatment with chemicals, and subsequent release of sustained numbers of the sterilised insects which lead to the eradication. When irradiated males mate with wild females, embryogenesis is arrested making these females sterile throughout their life. Continuous release of sterile males through many generations progressively reduces the target population that is eventually eliminated. Gamma radiation is currently used for sterility induction and it affects reproduction by affecting sperm chromosomes.

The seriousness of sleeping sickness and trypanosomiasis problem in Africa has brought African Governments together and they have issued a declaration that the year 2001 marked as the start of a Pan-African campaign to eradicate tsetse from the continent. Under the auspices of the OAU the Pan African Tsetse and Trypanosomosis Eradication Campaign (PATTEC) has been initiated.

2.0 Literature review

2.1 Biology and ecology of tsetse fly

Tsetse flies are obligatorily haematophagous insects and become infected with the protozoan when they feed on mammalian blood. They have low reproduction rate in comparison to other insects like mosquito and houseflies, they are well known as k strategist.

Tse tse fly belongs to the family Glossinidae. They are crudely similar to other large flies, such as the housefly, *Musca domestica*, but can be distinguished by four characteristics two of which are easy to observe. The four characteristics that definitively separate adult tsetse from other kinds of flies are shown below **(**http://en.wikipedia.org/wiki/tsetse-fly**).**

A. Proboscis: Tsetse has a distinct *proboscis*, a long thin structure attached to the bottom of the head and pointing forward
B. Folded wings: When at rest, tsetse *fold* their wings completely one on top of the other.
C. Hatchet cell: The discal medial ("middle") cell of the wing has characteristic *hatchet* shape resembling a meat cleaver or a hatchet.
D. Branched arista hairs: The antennae have arista with hairs which are themselves *branched.*

The *adenotrophic viviparity* reproduction system of tsetse fly ensures the higher degree of survival of each offspring, but is also the reason why reproductive rates are considerably low in tsetse fly populations.

The first oocyte is ovulated about nine days after emergence of the female (Saunders, 1970) and is fertilized as it enters the uterus. Embryogenesis takes place for three days after which the larva hatches and goes through three larval

instars in the uterus. It is born as a full grown 3rd instar larva and the reproductive tract of adult female includes a uterus which can become large enough to hold the third instars larva at the end of each pregnancy. The first pregnancy cycle takes 18 days under the laboratory conditions, while subsequent cycles take 9 to 10 days (Langley 1977).

The larva pupates in 15 to 30 min and the adult fly emerges after the puparial period which varies according to temperature, but on average is around 30 days at 24°C (Leak, 1999).

Longevity of the adult fly varies greatly according to seasonal factors. Generally for the tsetse population to increase, it is critical that the average female lifespan exceed 36 days. During optimal conditions, female fly can live as long as 3 months, producing as many as 10 offspring during her lifetime (Jordan, 1986).

Under natural conditions tsetse flies inhabit areas where the mean temperatures are around 24 ±1°C and require adequate moisture of 80 ±5 %. They are active for very few hours in a day.

2.2 Tsetse fly anatomy

Tsetse are surrounded by an exoskeleton with internal musculature, a system of trachea which delivers oxygen to the tissues and releases carbon dioxide, an open circulatory system with a simple heart, and a standard digestive system (Leak, 1999).

The adult body is comprised of three, visibly distinct, parts: the head, the thorax, and the abdomen. The head has large eyes, distinctly separated on each side, and a distinct, forward-pointing proboscis attached underneath by a large bulb. The thorax is large, made of three fused segments. Three pairs of legs are attached to

the thorax, as are two wings and two halteres. The abdomen is short but wide and changes dramatically in volume during feeding (Leak, 1999).

The internal anatomy of tsetse is fairly typical of the insects. The crop is large enough to accommodate a huge increase in size during the blood meal feeding since tsetse can take a blood meal weighing as much as themselves. Both sexes of this species are obligatory blood feeders, mainly on vertebrate hosts. Laboratory-reared tsetse flies, which are generally offered blood at frequent intervals, are likely to take smaller blood meal than wild flies, which feeds less frequently.

Female tsetse flies have significant capacity to store nutrients for larval development. The mean wet mass of blood meal taking for a male is 53.9 mg and female 75.3 mg. The feeding interval of tsetse fly varies from 42 hrs to 72 hrs (Leak, 1999).

Tsetse metabolism consists of ingesting vertebrate blood, which is called hematophagy, and digesting this blood to obtain energy and biomass. They have specialized cells that contain bacterial endosymbionts required for survival.

An unusual aspect of tsetse metabolism is the particular pathway which tsetse use for flight which seems to be responsible for the extremely high energy output and elevated flying speeds which tsetse can achieve. Most insects, such as honey bees, consume sugar predominantly for metabolic energy (Leak, 1999). Tsetse, instead, uses a pathway which involves the conversion of the amino acids proline and alanine. The result of this pathway is that tsetse can create large amounts of ATP, but can only sustain this metabolic output for short durations. Tsetse therefore fly at a very high speeds (they are known to be able to follow a car moving at thirty miles per hour), but can only sustain their flight for short durations for about thirty seconds.

2.3 Classification of *Glossina* species and subspecies

Tsetse flies are evidently in the order Diptera, the flies under the genus *Glossina* consists of 23 species and 8 subspecies. It is divided into 3 distinct subgenera or groups, based on the male external genitalia and ecological features (Newstead, 1911).

Currently, the science of systematics is trying to assist the conventional method of biological classification with molecular methods with the understanding of genomic sequencing.

The current classifications place all the tsetse species in a single genus named *Glossina*. Most classifications place this genus as the sole member of the family Glossinidae. The Glossinidae are generally placed within the infraorder Cyclorrhapha which includes the housefly and the blowfly due to the similarity of their developmental biology. This infraorder in turn, is part of the sub-order Brachycera, the stubby flies with reduced antenna.

2.4 Tsetse fly control and eradication

Trypanosomosis can be managed in two ways: The first is to attack the parasite through prophylaxis, by treating the disease or by developing trypanotolerant cattle. The second is to attack the vector (the tsetse fly), and thereby reducing the transmission of the disease.

One of the effective technologies among different methods of non-insecticidal species specific and environmentally friendly technique is the Sterile Insect Technique (SIT). This technique relies on the mating of wild females with sterile male flies.

However, the mating behavior of tsetse flies has a great role in the tsetse control

and eradication. Mating of female tsetse flies is only required once to store sperm in its spermathecae in sufficient quantity such that fertilization can occur over its entire reproductive life. Mating with a sterile male would thus result in no offspring.

Potts (1958) was the first to apply this approach to tsetse and Knipling (1963) proposed that a sterile to wild male ratio of 3:1 would be able to eradicate a stable tsetse population within 1 year. The low reproductive rate of tsetse in the field makes it the ideal candidate for control using this method. For the success of SIT quantity and quality of mass reared flies, good knowledge of tsetse biology and ecology, compatibility and competitive tests of the species, combination of different techniques pre-release suppression, proper application of trypanocidal drugs, Economic feasibility studies are some of the important packages of SIT. Eventhough, SIT has been developed and applied successfully for several insect pests, the above mentioned factors must be considered as the main components.

The eradication of New World screwworm (*Cochliomyia homivorax)* from Libya (Lindquist and Abusowa, 1991) and Central America (Wyss, 1998) Mediterranean fruit fly (*Ceratitis capitata*) and *G. austeni* from Unguja Island, Zanzibar, Tanzania (Vreysen *et al.,* 2000) have more than ever demonstrated the successful application of SIT to eradicate different insects including tsetse flies in an area wide campaign.

2.5 Importance and mode of SGHV transmission.

In addition to several colony growth factors, the tsetse fly survival and fecundity can be also affected the salivary gland hypertrophy viruses. The presence of virus like particles (VLP) in epithelial cells of the salivary glands of tsetse, *G. pallidipes* (Austen), was first reported by Jaensen (1978). Subsequently it has been

partially characterized as a double stranded DNA virus (Odindo *et al.,* 1986).

According to Otieno *et al.* (1980) and Odindo (1982) the disease occurs in most sites where tsetse flies were sampled, although the actual level varied from site to site and from season to season within the same site. Infection occurred in tsetse of various ages, from newly emerged teneral flies to very old tsetse flies.

The tsetse virus causing salivary gland hypertrophy and male reproduction reduction has been shown to infect some tsetse fly species naturally in the wild including *G. pallidipes* and *G. morsitans morsitans* (Whitnall, 1934; Jaenson, 1978; Otieno *et al.,* 1980).

In addition to this, in the ovarioles of virus infected *G. pallidipes,* it has been observed that, the majority of the germaria were affected by degeneration and severe necrosis and virus particles were seen within the germarial cells suggesting that this virus may be transmitted transovarially to the progeny by an infected female. The degenerative changes may affect the development of the larva resulting from such an egg. Sang *et al. (*1997) demonstrated that flies infected by this virus had difficulty in feeding and this affected their survival significantly.

The mode of horizontal transmission in the field is not well known, but in the laboratory the most likely mode of transmission is vertical, either trans-ovarial or through the milk gland, and horizontal through the membrane feeding, venereal or from environmental contamination. Further more, when normal female are mated to either normal male or those with SGH, the resultant progeny are normal (Jaenson, 1986).

Jaenson (1978) and Odindo (1982) demonstrated the possibility of the vertical transmission from parent to offspring of the tsetse virus in the field. Infection of wild males of *G. pallidipes* by the DNA virus of tsetse leads to total arrest of spermatogenesis and degeneration, resulting in spermless testicular follicules

(Jura *et.al.*, 1988), hence complete sterility.

The paired salivary glands of *G. pallidipes* are long, cylindrical organs each divided in to three morphologically and functionally distinct parts, distal muscle bounded secretory region, Proximal non-muscle bounded absorptive portion and non-muscular duct (Kokwaro *et al*.,1991).

The salivary glands of *G. pallidipes* consist of a single- layered, flattened epithelium, out side which is a layer of muscle. The lumen is filled with a darkly stained homogeneous secretion (Kokwaro *et al*., 1991). Testicular degeneration, and ovarian abnormalities was first detected in the tsetse fly *G. pallidipes* (Jaenson, 1978).

The enlargement of the salivary glands was first reported for a tsetse species in *Glossina pallidipes* Austien in Zululand, South Africa (Withnall, 1934). The increase in size was due to a cellular proliferation of the glandular epithelial cells and hypertrophy of their nuclei and cytoplasm. Nuclear and cytoplasmic inclusions were present in the enlarged cells, but were not found in cells of normal size-glands (Thomas and Jaenson, 1978).

Odindo (1988) reported that the major symptom of infection is enlargement of the salivary glands, which results from hyperthplasia and hyperthrophy of the epithelial cells which can affect both male and female tsetse flies.

The enveloped viruses associated with the salivary gland hyperplastic symptom observed in various dipterans are included within this group. These viruses, referred to as salivary gland hyperplasia viruses (SGHV), have been detected in the tsetse *Glossina spp.* and house fly *M. domestica*. These viruses infect the salivary gland cells, inducing cell proliferation and hypertrophy. The resulting enlarged (hyperplastic) salivary glands can be readily discerned by dissection. SGHV infection doesn’t impair host feeding or vector and flight behaviors, but it doesn’t impact the reproductive potential of infected flies. The SGHV infect adult

tsetse flies had an increased incidence of either abnormal ovarian development or abnormally small, aspermic testes. Similarly, 95% of field collected female house flies that harbored SGHV infections showed no signs of normal ovarian development Boucias and Pendland (1998**)**.

In the same publication it has been demonstrated that, virus particles have been isolated successfully from hyperplastic glands and subjected to biochemical analysis. These viruses are composed of a complex of structural peptides and contain a linear dsDNA genome. Restriction endonuclease profiles have demonstrated that the housefly SGHV possesses a 137 kbp genome. Laboratory bioassays have shown that the SGHV can be transmitted *per os* to healthy adult flies. Surveys of tsetse and house fly populations demonstrated that this virus is wide spread and undergoes significant seasonal fluctuations in host populations.

The virus has only been partially characterized and shown to be rod shaped with a double–stranded DNA which appeared to be linear, 57 nm wide by 700 to 1300 nm long (Odindo *et al.*, 1986). However, the virus could not be placed in any of the exiting taxonomic grouping of DNA viruses (Sang *et al*,. 1997). These VLP in hypertrophied glands described as baculovirus-like, although their average length in thin section (597 to 708 nm) was much in excess of that normally associated with members of the baculovirus group.

These viruses, like the baculoviruses, replicate in the nuclei of the host cells, producing progeny rod shaped nucleocapsids containing a relatively large ds DNA genome. However, unlike the members within Baculoviridae, these viruses appear to posses a linear ds DNA genome. In the majority of cases, infection by these viruses results in chronic debilitative disease.

Odindo (1988) reported that the tsetse virus compares closely to the baculovirus-like particles of the rhino beetle, *Oryctes rhinoceros* which causes a lethal infection

in larvae. It also infects adults, with the gut cells being the center of the viral replication.

The length of the DNA was estimated to be 185-220 Kbp (IAEA, 2005) and Odindo *et al*. (1986) also demonstrated that the genome size is ranging from 20.4 and 34.0 Kbp.

2.6 Insect virus classification

It has been stated in Boucias and Pendland (1998**)** that **i**nsect viruses are heterogeneous and are placed in most of the major viral taxa. Unlike other biological entities, virus classification is non-Linnaean and lacks the subphyla, class, order, and suborder hierarchical divisions. The International Committee on Taxonomy of Viruses (ICTV) has currently placed most known viruses into a hierarchical level of family (suffix-*viridae*) and in certain cases subfamily (*-virinae*) and genus (*-virus*). According to the classification, viruses can be placed into one of seven groups depending on a combination of their nucleic acid (DNA or RNA), strandedness (single-stranded or double-stranded), and method of replication. Classifying viruses according to their genome indicates that those in a given category will all behave in a similar fashion, offering some indication of how to proceed with further research. Viruses can be placed in one of the seven following groups,

Group I: double-stranded DNA viruses

Viruses possess double-stranded DNA and include such virus families as *Ascoviridae, Baculoviridae (*Occluded Baculoviruses, Nonoccluded Baculoviruses, Unclassified Enveloped, Rod-Shaped dsDNA viruses, *Nudivirius, Iridoviridae, Polydnaviridae, and Poxiviridae* (chickenpox and smallpox).

Group II: single-stranded DNA viruses

Group III: double-stranded RNA viruses

Group IV: positive-sense single-stranded RNA viruses

Group V: negative-sense single-stranded RNA viruses

Group VI: reverse transcribing RNA viruses

Group VII: reverse transcribing DNA viruses

2.7 Insects vs. vertebrate immunity

Insects do not possess the ability to produce antibodies (immunoglobulins) and do not use immunoglobulin as recognition molecules in the classical sense, against foreign antigen and hence antigenic memory appears to be lacking i.e. (non-memory type). Further, they do not produce alpha/beta interferons (IFN - α / β) in response to viral infections. Nevertheless, they are capable of "immune" reactions, which appears to be predominantly cellular in nature, several haemolymph induced antibacterial proteins have been reported to be broad spectrum in their action, which are produced in insects in response to bacterial challenger and of shorter duration in nature. This would suggest that analogy to the phenomenon of immunity in vertebrate may be inappropriate, and hence immunity in insects is different from immunity in vertebrates (http://www.fbae.org/channels/Biotech_in_plant_Disease_Control/insect _resistance.html).

2.8 Specific defense mechanisms/immunity/ Cellular immunity

In a cellular defense mechanism, unlike vertebrate which has red blood corpuscles (RBC) and white blood corpuscles (WBC) in a closed circulatory system, insects with open body cavity lack lymphocytes, the major source of vertebrate immunity to virus infection. But, they have only free blood cells called haemocytes. Different types of blood cells have important roles in the protection

of insects against invading microorganisms. Hence, identification and classification of various types of insect blood cells based on the structure and function is important (Gotz & Boman, 1985).

Among the six major group of insect haemocytes in recognizing the "self" (Isografts) and non-self (Allografts), plasmocytes and granulocytes, are the major effector cells and they react to foreign invaders either by phagocytosing like microorganisms or nodulating and encapsulating the objects too large to be individually engulfed,viz. metazoan parasite by way of haemocytes attaching and forming many layers which often become melanotic, thereby causing the death of the parasitoid through starvation and or anoxia mechanism, (Gotz and Boman, 1985).

Changes in total haemocytes (THC) during growth and development of healthy insects have been reported by a number of workers (Narayanan, 1976) Drastic reduction in number of haemocytes during various microbial infections has also been reported by several workers. Infection by *B. bassiana* results in a gradual suppression of the phagocytic competence of circulating haemocytes and alteration in both total and differential haemocyte counts (DHC) has been reported in the case of virus (Narayanan, 1979).

2.9 Antiviral drugs and virus replication

The discovery of antiviral drugs has been largely fortuitous. Spurred on by success with antibiotics, drug companies launched huge blind-screening programmes with relatively little success. Lead compounds were modified by chemists in an attempt to improve bioactivity. Solubility, stability, availability and activity are all important (http://www.amazon.co.uk). Antiviral drugs are designed to attack viruses at every stage of their life cycles. Viral life cycles vary in their precise details depending on the species of virus, but they all share a

general pattern and it has been stated in Davidson (1972) that the replication of the virus can be considered in the following stages,

1. adsorption of the virus into the host cell.
2. penetration of the viral nucleic acid into the cell,
3. development of virus specific functions, alteration of cell functions, replication of the nucleic acid and synthesis of other virus constituents,
4. assembly of the progeny virus particles,
5. release of viral particles from the cell.

The best time to attack a virus is as early as possible in its life cycle. One approach is to interfere with the ability of a virus to get into a target cell. The virus has to take a sequence of actions to do this, beginning with binding to a specific "receptor" molecule on the surface of the host cell and ending with the virus "uncoating" inside the cell and releasing its payload. Viruses that have a lipid envelope must also fuse their envelope with the target cell, or with a vesicle that transports them into the cell, before they can uncoat.

The stage of viral replication can be inhibited in two ways:

1. Using agents which mimic the virus-associated protein (VAP) and bind to the cellular receptors.

2. Using agents which mimic the receptor and bind to the VAP. This strategy of designing drug can be very expensive. The process of generating anti-idiotypic antibodies is not fully understood. And it has very poor pharmacokinetics.

The key to success in drug development is specificity, understanding Molecular biology including viral replication and producing specific targets for inhibition and deliberate design of drugs.

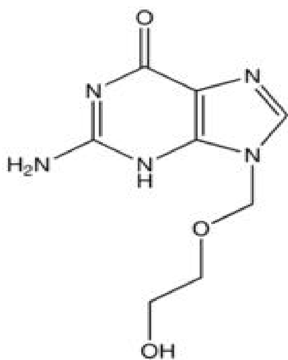
Any stage of virus replication can be a target for a drug. But, drug must be more toxic to virus than to the host. The smaller the value of the chemotherapeutic index number the better result can be obtained.

$$CHEMOTHRRAPUTIC\ INDEX = \frac{Dose\ of\ drug\ which\ inhibits\ virus\ replication}{Dose\ of\ drug\ which\ is\ toxic\ to\ host}$$

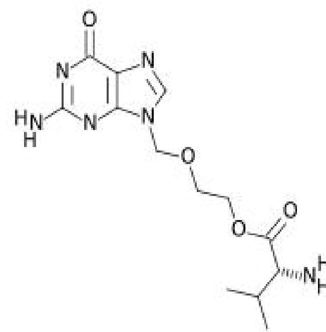
2.10 Acyclovir and valacyclovir

Acyclovir is a nucleoside analogue, and is effective against herpesvirus infections. The first antiviral drug to be approved for treating HIV is also a nucleoside analogue. These nucleoside analogues are in fact pro-drugs, since they need to be phosphorylated before becoming effective. The cell DNA polymerase is less sensitive to it than the viral DNA polymerase.

Valacyclovir is a prodrug of acyclovir with more favorable bioavailability and it has been used in human medicine as antiviral drug for the management of herpes simplex and herpes zoster (shingles).



Acyclovir



Valacyclovir

2.10.1 Antiviral drugs mode of action

Acyclovir is selectively converted into a monophosphate form by viral thymidine kinase, which is far more effective in phosphorylation than cellular thymidine kinase. Subsequently, the monophosphate form is further phosphorylated into the active triphosphate form, aciclo-GTP, by cellular kinases. Aciclo-GTP is a very potent inhibitor of viral DNA polymerase; it has approximately 100 times higher affinity to viral than cellular polymerase. Its monophosphate form also incorporates into the viral DNA, resulting in chain termination. It has also been shown that the viral enzymes cannot remove aciclo-GMP from the chain, which results in inhibition of further activity of DNA polymerase. Aciclo-GTP is fairly rapidly metabolised within the cell, possibly by cellular phosphatases (www.wikipedia, the free encyclopedia).

3.0 Hypothesis and Objectives

3.1 Hypothesis

- The present study has been anticipated with the hypothesis that there is a virus in *G. pallidipes*. This virus hinders the tsetse colony size through damaging the male and female reproductive organs.

3.2 General objective

- To establish virus free line from *G. pallidipes* virus infected colony and to develop protection of virus development in *G. pallidipes*.

3.3 Specific objective

- To investigate the presence and the impact of the SGHV in the colony.
- To study the preventing ways of the SGHV transmission by testing antiviral drugs against SGHV.
- To establish virus free line.

4.0 Material and Methods

4.1 Tsetse flies samples

The tsetse flies were obtained from two insectaries, Kaliti–Ethiopia and Seibersdorf-Vienna. *G. pallidipes* Arbaminch strain is originated from the southern part of Ethiopia Nechsar National park and the *G. pallidipes* Tororo strain is originated from pupae collected in Tororo, Uganda in 1975 and initially colonized in the Netherlands. It was transferred to the Seibersdorf laboratory in 1982 and has been maintained on an *in vitro* rearing system.

Tsetse flies were kept at 24±1 ^{O}C temperature, 80±5% relative humidity and 12:12 light/dark cycle. All flies were fed on Difibrinated Fresh Frozen Bovine Blood (DFFBB), three days in a week and 10 min/day. They fed through silicon membrane under aluminum tray. The animal body temperature was animated using the black plastic mat with the temperature of 38±1^{O}C.

4.1.1 Salivary gland dissection

The flies were immobilised by chilling at 4°C and dissections were carried out with out saline solution by cutting off the wings and placing the fly on the ventral surface on a microscope slide. While holding the thorax under the dissecting microscope, the first abdominal tergite was removed to reveal the salivary glands. The salivary gland contents were observed for the presence of abnormal (enlarged and milky stages) of the gland. Female flies were dissected at the end of fourth reproductive cycles.

4.1.2 Viral purification and DNA extraction

Enlarged salivary glands were collected from *G.pallidipes* Arbaminch strain and

Tororo strain flies, homogenised in Tris buffer (50 Mm, pH 7.8), and clarified twice by centrifugation for 10 min at 3000g. The supernatant was layered on 20-60% liniear sucrose gradiant and centrifuged for 1h at 27,000 g. The viral band was taken and washed in Tris buffer by centrifugation for 1 h at 150,000 rpm and the viral pellet was resuspended in Tris buffer.

Viral DNA was extracted from purified virus by incubating the viral suspension at 55°C for three hours with proteinase K (0.2 mg/ml final concentration) and 1 % sarkosyl, followed by two phenol/chloroform extractions. The DNA was precipitated from the aqueous phase by adding 0.7 volume isopropanol, mixing and holding at-20°C overnight and centrifuging for 10 min. at 16,000 g. The pellet DNA was washed with 70% cold ethanol, briefly air dried and resuspended in Tris-EDTA buffer (10 mM Tris, 1mM EDTA, pH 7.8).

4.1.3 Morphological study of the virus

PTA stained salivary gland hypertrophy virus of *G. pallidipes* ArbaMinch strain was observed under electron microscopy.

4.2 Samples from leg

In order to keep the fly with its maximum reproduction rate, the sampling method was based on middle parts of legs /left and right/. At a time one part of legs were cut from coxae using fine scissor and preserved in 1.5ml of Eppendorf tube. Each sample was labeled on the individual tube according to the number of the individual tsetse, date of collection and sex.

4.2.1 DNA extraction, purification and amplification

The total DNA from a leg was taken and extracted using a modified method of Bender *et al, (*1983). Leg of the individuals was placed in 1.5 ml Eppendorf tubes, dipped in liquid nitrogen and then homogenised in 250 µl of grinding buffer containing 0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris, 0.05 M EDTA and 0.5% SDS. The Eppendorf tubes were incubated in a water bath for 30 minutes at 65 ^{O}C, 70 µl of warm 8 M potassium acetate was then added, well mixed and the tubes were then incubated on ice for 30 minutes followed by centrifugation at maximum speed for 15 min. The supernatant was removed to a new tube. 1ml of 100% ethanol was then added and the tube left at room temperature for 30 min followed by centrifugation at 15000 rpm for 15 min. The DNA pellet was washed in 70% ethanol, dried and re-suspended in 10 µl of TE buffer and incubated for 30 minutes at 55°C.

4.2.2 ZR-96 Genomic DNA kit ™

Based on the solid tissue DNA extraction protocol, each leg was homogenized using 250 µl of genomic lysis buffer and centrifuged at 5000-10000 rpm for 5 minutes. Then 250 µl of gDNA wash buffer was added in to each well of the silicon-A plate ™ and centrifuged at 2500-5000 rpm for 5 minutes. The flow from the collection plate was discarded and this procedure was repeated once.

Finally it was mounted in the silicon-A plate ™ onto the elution plate, after all these procedures it was added 30 µl of DNA elution buffer and centrifuged at 2500-5000 rpm for 5 minutes to elute the DNA and stored at 20°C.

4.2.3 Purity and yield of DNA (NanoDrop®)

The purity of the extracted DNA was assessed spectrophotometrically by calculating A_{260} / A_{280} ratios in a NanoDrop® ND-1000, full-spectrum UV/Vis spectrophotometer.

4.2.4 DNA amplification

For SGHV PCR templet amplification 1.0 µl of reverse and forward primers and 1.5 µl target templet DNA was added in to the 22.5 µl master mix. The amplifications were performed by Hot Start Taq DNA polymerase PCR kit (ABgene, UK) according to the manufacturer specifications.

Each PCR reaction was prepared in 1.1 X PRE-Aliquated PCR Master Mix, 25 µl Reaction of 0.2 ml low profile Thermo-Fast 96 plates, which includes: 0.625 units Thermoprime plus DNA polymerase, 75 mM Tris-HCl (pH 8.8 at 25°C), 20 mM$(NH_4)_2SO_4$, 2.0 Mm$MgCl_2$, 0.01%(v/v) Tween 20, 0.2 mM of each dNTP.

4.2.4.1 Primers

The sets of primers with following nucleotide sequences were used the forward and reverse amplification of the target region (Annex 7). The amplification of the virus by PCR, followed the application of specific primer (Bossin, 2004), with a final concentration of 0.2 mM which amplifies a region about 400 base pairs long, the probability of generating a break in this specific region in all viruses present in the sample, and thereby preventing amplification by PCR, is very low.

GpSGHV 1F 5’GCTTCAGCATATTATTCCGAACATAC 3’
GpSGHV 1R 5’GATCCTGTTCGCGTAAACCA 3’
GpSGHV 2F 5’ CTTGTCAGCGCCACGTACAT 3’
GpSGHV 2R 5’GCATTCACAGCATCCCAATTTT 3’

The PCR program which was optimized for tsetse GpSGHV 1F and tsetse GpSGHV 1R with seven steps is as follow:

Step 1, Denaturation 94 ^{O}C for 2 minutes

Step 2, Denaturation 94 ^{O}C 45 seconds

Step 3, Annealing 55 ^{O}C 45 seconds,

Step 4, elongation 72 ^{O}C for 1 minute.

Step 2-4, repeating /cycling/for 35 times.

Step 5, elongation 72 ^{O}C 5 minutes,

Step 6, storage 4 ^{O}C infinite times

Step 7, end of the program

4.2.5 Agarose gel preparation and visualization

2% agarose gel with 5 µl of ethidium bromide was placed in a plastic tray, edges taped and Allowed to solidify. The solidified gel was placed in electrophoresis apparatus and filled with **1X** TAE buffer. 1% DNA loading dye was added to the DNA sample and loaded in the gel wells. 5-10 µl of 1 kb ladder marker (500 ng/µl) was loaded and run the gel at 100 volts constant at room temperature. Each of the PCR products was visualized under ultraviolet light and pictures were taken from all gel samples.

4.3 QPCR (Real time PCR)

Real-time PCR had been used in this experiment to quantify the load of viral DNA

per leg and SYBR Green® was used to detect the accumulation of the double-stranded viral DNA product.

The SYBR Green with this instrument performed a melting curve analysis and determined the melting temperature, T_m, which permitted the detection of different amplification products based upon the %G+C content and length of the amplification product. The amplified product detection was completed in two hours.

In the real time PCR 1.25 ml of SYBR green, Standard DNA templates, serial dilution in 1: 100, 1000 from each target DNA sample, Elution buffer, filtered ddH_2O, QPCR thermal circler were used for amplification reaction.

The main steps for real time PCR were as follows,
Step 1, 90 µl of elution buffer was added in to each PCR wall.
Step 2, 10 µl of target DNA was added into the next 90 µl to get 10^{-2}.
Step 3, 10 µl target DNA was added into the next 90 µl to get 10^{-3}.
Step 4, Samples from 10^{-2} were taken into three wall of the QPCR plate
Step 5, 1.25 ml of SYBR green distributed into 96 walls of the QPCR plate.
Step 6, Standards located horizontally from 1-3 and vertically A-G, of the plate.
Step 7, Negative controls located from 1-3 and vertically H8 of the plate.
Step 8, DNA templet was added in plates vertically from 4-12 and horizontally from A-H
Step 9, Positive controls located vertically from 10-12 and horizontally on H of the plate
Step 10, QPCR plate was sealed and inserted into *icycler* thermocycler machine.
Step 11, Final result of the real time PCR obtained in two hours time.

4.4 Isolation of virus free line

118 mated male and female flies were held separately in 59 individual tubes and fed on clean DFFBB. After the fourth reproduction cycle of a female, the flies were checked for virus infection by salivary gland dissection. Pupae from virus negative and positive female flies were kept separately and the progeny from virus negative females were used for further study on transmission.

4.4.1 Screening of SGHV positive or negative flies

The diagnostic criterion used to screen virus free *G. pallidipes* was the detection of hypertrophied salivary glands virus by PCR using the specific primers which can produce specific bands for positive samples. 920 male and female flies were taken and examined by PCR.

4.4.2 Mating of the flies

The selected 59 female and 59 male flies were not sexually mature at the time of screening. Seven days old female was mated with 10 days old male fly. These days were the time at which a maximum insemination rates can be achieved. Male and female flies were released in to the transparent mating box. After copulation flies were placed in individual tubes.

4.5 Antiviral drugs

Acyclovir and valacyclovir were prepared with good quality of DFFBB at the dose of 0.3 mg in 1 ml of DFFBB. These antiviral drugs were tested in preliminary survey in 72 female and 18 male *G. pallidipes* Tororo strain flies.

4.5.1 Preparation of antiviral drug solution

Acyclovir and Valacyclovir were selected for this experiment and both drugs were prepared in clean hood by solving the in double distilled and filtered water with 100% DFFBB.

Blood with the QFC 1.0 was taken from the laboratory blood processing unit, this blood was mixed with the antiviral solution and stored in -20°C. Three times in a week blood was taken out from -20°C and before feeding of the experimental flies the blood was thawed in risen water.

4.6 Preliminary survey on antiviral drugs

This preliminary study carried out with no replication and antiviral drugs were prepared with DFFBB of 0.3 mg/ml. A total of 72 female and 18 male flies were taken from the main rearing facility. The first 24 female and 6 male flies were treated with Acyclovir, the second 24 treated with Valacyclovir and the third treatment was treated with DFFBB mixed with 2 µL of double distilled water.

All female flies were mated in the ration of 1:4 male to female at the age of 7 days of female and 10 days of male from the day of their emergence. They were assessed for 35 days.

4.7 Antiviral drug in population study

This pre-post randomized design of experiment, carried out with the total of 360 (1:4) male to female flies ratio were taken and for the initial virus study 72 female and 18 male flies' and whole genomic DNA was extracted from left middle leg and the initial viral DNA status of the fly was examined by QPCR. 216 female and 54 male were handled for further study on population and they were handled in middle and small size tsetse cages. Flies were inseminated on their 7:10 female to male maturation time. The initial 90 flies of viral DNA load was compared with 30, 60 and 80 days of female flies.

4.8 Antiviral drug in individual fly study

This pre-post randomized stratified design of experiment, carried out on the day of emergence. A total of 432 male and female flies middle left leg were taken as an initial viral DNA load study. All fly legs were stored in -20°C and the whole genomic DNA was extracted using the ZR genomic kit. Each fly remained with five legs were kept in the individual cage for mating and productivity.

Flies were mated by using mating tube on the 7th and 10th day age of the female and male, respectively. After 11±2 days pupae were collected and sorted by classes and recorded in the daily sheet. On days 30, 60 and 80 right middle leg was taken from replication1, 2 and 3, respectively and DNA was extracted to compare with initial viral DNA load.

4.9 Transmission study in F1 generation

The rate of vertical transmission determined by isolating newly emerged F1 flies in individual tube. 24 flies were taken from each treatment with the sample size of 96 Acyclovir, Valacyclovir, Control and mixed treated flies (Acyclovir 1-8, Valacyclovir 9-16 and Control 17-24). The flies were dissected on the day of there emergence to determine the presence of salivary gland hypertrophy. Legs were taken and analyzed by QPCR.

4.10 Parameters measured and statistical analysis

4.10.1 Parameters measured (Feldmann, 1994)

Survival

$$\%\ daily\ mortality = \frac{No\ of\ dead\ females\ X\ 100}{No\ of\ females\ survived\ last\ mortality\ X\ No\ of\ days\ elapsed\ last\ check}$$

Fecundity

$$P/F/10d = \frac{No\ of\ pupae\ produced\ during\ previous\ x\ 10}{No\ females\ recorded\ surviving\ previous\ week\ X\ 7}$$

Weight –frequency distribution for *G.pallidipes* of pupae

A	B	C	D	E
>23 mg	23-29mg	29-33 mg	33-37mg	37mgand above

Emergence rate

$$\%\,Emergence = \frac{No\ of\ emergence}{No\ of\ pupae} X100$$

Feeding response

$$\%\ Feeding\ response = \frac{Full\ X\ 2 + Half\ X\ 1 + Not\ fed\ X\ 0}{number\ of\ flies\ X\ 2}\quad X\ 100$$

Quality factor calculations

For the calculation of the blood quality (QF)
First reproduction cycle
FS 18 Number of females surviving on day 18
FS 25 Number of females surviving on day 25
PT Total number of produced pupae
PA Number of A class pupae
PB Number of B class pupae
PC Number of C Class pupae
PD Number of D Class pupae
PE Number of E class pupae

Second reproductive cycle

E + I Number of inseminated females on day 25 with early pregnancy stages in utero.

II + III Number of inseminated females on day 25 with late pregnancy stages in utero

BL Number of inseminated females on day 25 with oviduct blockage

AB Number of inseminated females on day 25 that aborted, empty uterus, follicle next in sequence to ovulate is not mature

Calculation of the blood quality factor

QF= Positive parameters from first reproductive cycle + Positive parameters from second reproductive cycle –negative parameters from first reproductive cycle- Parameters from second cycle / numbers of females.

$$QF = \frac{[FS25 + PT + (PB\ X0.3) + (PD\ X0.5) + (PE\ X0.6) + (E + 1\ X0.3) + (II + III\ X\ 0.6) - (PA\ X0.2) - (AB\ X0.5) - (BL\ X1.0)]}{[FS18 + FS25]}$$

4.10.2 Statistical analysis

Analyses were performed by one-way analysis of variance (Anova) SPSS Version 13.0, Bio-Rad *icycler* version 3.1 and survival and life table analysis.

These software analyzed the viral DNA load in the leg of individual and population samples (triplicate measurements) in each group and expressed as mean ± SE. This the SE indicates biological variation in addition to the parametric measurement variation.

5.0 Results

5.1 Enlarged salivary gland and Salivary gland hyperplasia virus

A total of 3072 *G. pallidipes* Arbaminch strain were dissected and 3.55% flies were positive for salivary gland hyperplasia by dissection (Fig.1).

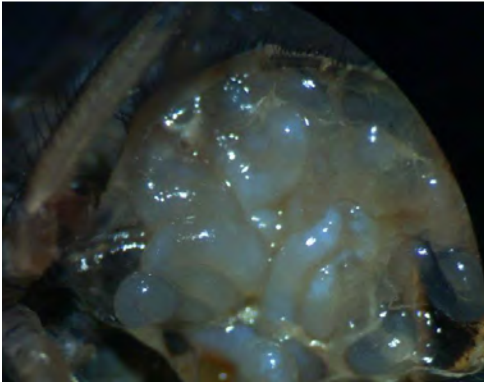


Fig. 1. Enlarged salivary gland(x25)

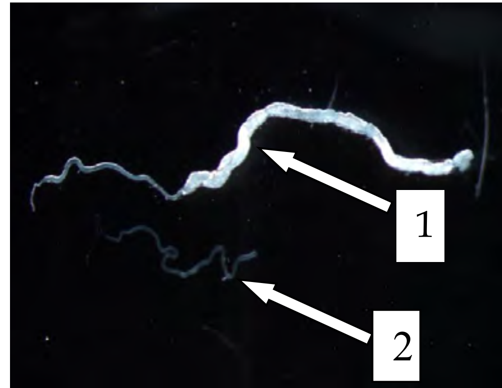


Fig. 2. Enlarged (1) normal (2) glands(x12.5)

The enlarged salivary glands (Fig.2) were used for virus purification. Under electron microscopy the purified virus (Fig. 3 and 4) preparation showed to be rod-like particles 700-1000 nm in length and 50 nm in diameter.

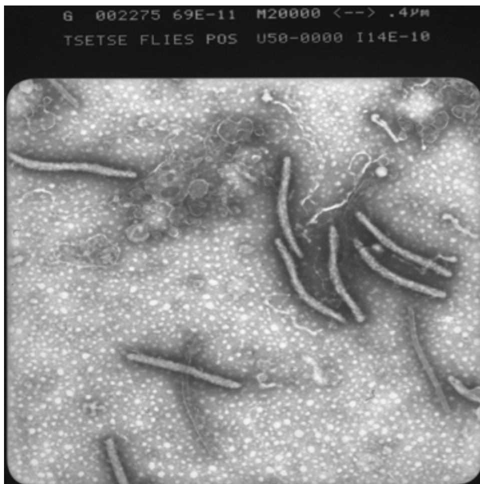


Fig. 3. *G.pallidipes* Tororo SGHV (4µm)

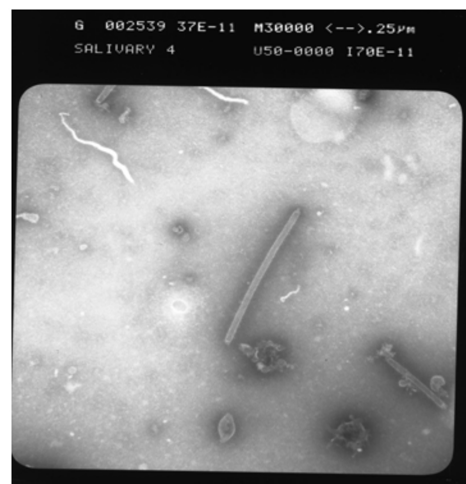


Fig. 4. *G.pallidipes* Arbaminch SGHV (25µm)

5.2 Virus free line establishment

Out of 920 flies, 120 were screened by PCR as positive or negative for salivary gland hyperplasia virus (Fig.5). From PCR result, 32 males and 18 females including two dead flies were screened as negative for SGHV. 27 male and 43 female flies screened as positive for SGHV. Excluding two dead flies, a total of 118 male and female flies were involved in the experiment (Table 1).

Table 1. Virus free line establishment (VFL)

Date of emergence	30/05/2006	VFL	Male	Female	Pupae	Female survived
Date of mating	9/06/2006	VFL I	16	16	22	13
Date of separation	14/06/2006	VFL II	16	16	19	14
Date of male dissection	15/06/2006	VFL III	16	16	24	13
Date of female dissection	7/8/2006	VFL IV	11	11	14	9
Last pupae collection	4/8/2006	Total	59	59	79	49

As shown in Table 1, all screened flies (Annex 8) were grouped based on PCR sample of Figure 5 selection criterion into four groups as virus free line one, two, three and four (VFL I, VFL II, VFL III, VFL IV).

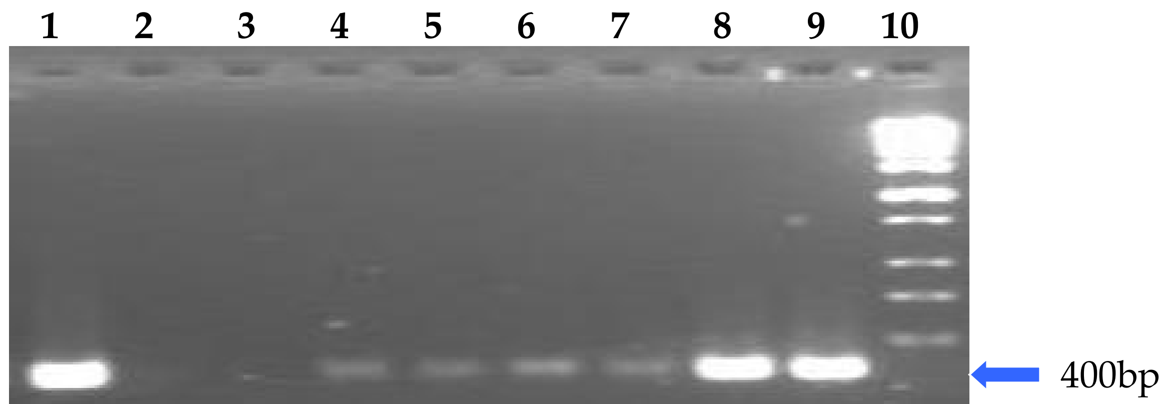


Fig. 5. PCR products from *G. pallidipes* Tororo strain leg samples

1: Positive control, 2: Negative control, 3:Negative, 4:5:6:7:8:9: Positive, 10: 1Kb ladder

VFLI comprised 32 negative flies with equal number of female and male. VFL II, had 16 negative male and 16 positive female flies. VFL III, 32 positive flies and VFLIV had 22 highly positive male and female flies. Seven days old male was mated with 10 days old female fly. Male flies were removed from the cage after 72 hours of mating.

As shown in Table 1, 79 pupae were collected from 59 female flies. In 64 days of the experiment 16.95& female flies died. There was significant difference in survival between groups, P<.023 (Annex 5)

5.2.1. Virus free line one

The virus free line one that consists 32 flies (16 male and 16 female) showed negative for the presence of salivary gland hyperplasia virus by PCR and 12.5 % of the VFL were positive by dissection. The result showed that 18.75 % of male flies positive for salivary gland hyperplasia by dissection.

37.5 % of the female flies did not produced pupae in four cycles. Female fly with cage number 1 aborted on the first reproduction cycle. Fly cage number 4 male mated with female cage number 4 produced two high (E class) pupae and fly cage number 6 male mated with female cage number 6 did not produce pupa in four cycles (Table 2).

Three positive male flies for salivary gland hyperplasia were taken for further examinations on QPCR and the result showed that 2.25E + 03, 9.31 E+05 and 8.90 E+05 viral DNA load was recorded from flies 1, 4 and 6 respectively. All mated female flies with the positive male showed to be negative (Table 2).

18.75 % of the female flies died after a total of four pupae production. All of them produced three high (E class) pupae during the first reproduction cycle and female from cage number 7 produced one average (C class) pupae on the second reproduction cycle.(Table 2).

On the first cycle nine out of sixteen female flies produced nine pupae. Eight of the females produced the highest quality (E class) of pupae, one fly produced one average (C class) pupae. On the second cycle four pupae were produced and two of them high class (E class) pupae, one good (D class) class and one average (C class) pupae. On the third reproduction cycle three flies produced three high quality (E class) pupae. On the fourth cycle 6 pupae were produced and five pupae were with the highest quality (E class) and one average (C class) pupa. Pupa from cage number two was collected one average pupa (C class).

The highest quality and quantity of pupae production were collected from fly cage number 11 which showed four high (E class) pupae. Both male and female flies were negative for PCR and dissection. Female flies from cage number 1, 5, 8 and 10 didn't produce pupa in 64 days of the experiment. But, they were negative by dissection and on PCR. Male fly cage number 1 mated with female fly cage number 1 showed negative on PCR, but positive by dissection.

Table 2. Virus free line one (VFL I)

							Reproduction Cycle			
Cage No	SG Male dissection	PCR Male	Pupae	QPCR	SG Female dissection	PCR female	1	2	3	4
1	Positive	Negative	Abortion	2.25E +03	Negative	Negative				
2	Negative	Negative	2		Negative	Negative	C			C
3	Negative	Negative	0		Negative	Negative				
4	Positive	Negative	2	9.31 E+05	Negative	Negative	E			E
5	Negative	Negative	0		Negative	Negative				
6	Positive	Negative	0	8.90 E+05	Negative	Negative				
7	Negative	Negative	2		Died	Negative	E	C		
8	Negative	Negative	0		Negative	Negative				
9	Negative	Negative	1		Died	Negative	E			
10	Negative	Negative	0		Negative	Negative				
11	Negative	Negative	4		Negative	Negative	E	E	E	E
12	Negative	Negative	3		Negative	Negative	E	E		E
13	Negative	Negative	3		Negative	Negative		D	E	E
14	Negative	Negative	1		Died	Negative	E			
15	Negative	Negative	1		Positive	Negative	E			
16	Negative	Negative	3		Negative	Negative	E		E	E
			22							

Male fly from cage number 4 mated with female fly cage number 4 showed negative on PCR, but positive by dissection and the female fly showed negative on PCR and dissection. The female produced two high quality (E class) pupae.

Male fly cage number 6 mated with female fly cage number 6 showed negative on PCR, but positive by dissection, the female fly showed negative on PCR and by dissection produced no pupa.

Male fly cage number 15 mated with female fly cage number 15 showed negative both by PCR and dissection and the female fly showed negative on PCR, but positive by dissection. The female produced one high (E class) quality pupae.

5.2.2. Virus free line two

The virus free line two had a total of 32 flies. 16 negative male and 16 positive female for salivary gland hyperplasia virus on PCR. 25% of the dissected male flies were positive for salivary glands hyperplasia by dissection.

Based on the dissection results male fly cage number 3, 9, 11, 12 were negative by PCR, but positive by dissection for salivary gland hyperplasia. 50% of the female did not produce pupae. Female flies mated with fly cage number 3, 9 and 12 did not produce pupae. Female fly cage number 11 mated with female cage number 11 produced one good (D class) pupae on the second reproductive cycle.

Four male flies which showed positive for salivary gland hyperplasia on dissection were taken for further examinations on QPCR and 2.41 E+05, 4.60 E+05, 2.28 E+02, 5.09 E+05 viral DNA load was recorded from flies 3, 9, 11, and 12, respectively.

Two female flies from cage number 7 and 13 died during the experiment. Female fly with the cage number 7 produced one good (D class) and one high quality (E class) pupae in the first and second reproduction cycle.

On the first reproduction cycle 5 pupae were good (D class) and two high (E class) qualities. On the second reproductive cycle two good (D class), two high (E class) pupae were collected. On the third cycle there were two average (C class) pupae, one good (D class) and two high (E class) quality pupae. On the fourth cycle one average (C class), one good (D class) and one high (E class) quality pupae were collected.

The highest quality and quantity of pupae production was recorded from cage number 5, three out of four pupae were in high (E class) and one in good (D class).

There was no pupa from a total of 7 female flies (cage number, 1, 3, 4, 9, 12, 15 and 16) in 64 days. Fly with cage number 1, 15 and 16 were negative both by dissection and on PCR. But, flies number 3, 9, 11, 12 were negative on PCR, but positive for salivary gland hyperplasia.

Table 3. Virus free line two (VFL II)

							Reproduction cycle			
Cage No	SG Male dissection	PCR male	pupae	QPCR	SG Female dissection	PCR female	1	2	3	4
1	Negative	Negative	0		Negative	Positive				
2	Negative	Negative	1		Negative	Positive	D			
3	Positive	Negative	0	2.41 E+05	Negative	Positive				
4	Negative	Negative	0		Negative	Positive				
5	Negative	Negative	4		Negative	Positive	D	E	E	E
6	Negative	Negative	2		Negative	Positive	D		C	
7	Negative	Negative	2		Died	Positive	E	D		
8	Negative	Negative	4		Negative	Positive	D	E	D	C
9	Positive	Negative	0	4.60 E+05	Negative	Positive				
10	Negative	Negative	3		Negative	Positive	D		E	D
11	Positive	Negative	1	2.28 E+02	Negative	Positive		D		
12	Positive	Negative	0	5.09 E+05	Negative	Positive				
13	Negative	Negative	0		Died	Positive				
14	Negative	Negative	2		Negative	Positive	E		C	
15	Negative	Negative	0		Negative	Positive				
16	Negative	Negative	0		Negative	Positive				
			19							

Male fly cage number 3 mated with female fly cage number 3 showed negative by PCR, but positive by dissection. The female fly showed negative on PCR and dissection. There was no pupa production, but survived for 64 days.

Male fly cage number 9 mated with female fly cage number 9 showed negative by PCR but positive by dissection. The female fly showed negative on PCR and dissection. There was no pupa production but they survived for 64 days.

Male fly cage number 11 mated with female fly cage number 11 showed negative by PCR but positive by dissection. The female fly showed negative on PCR and dissection and produced 1 good quality (D class) pupa.

Male fly cage number 12 mated with female fly cage number 12 showed negative by PCR but positive by dissection. The female fly showed negative on PCR and dissection, but there was no pupa production and 12.5% mortality of female flies was recorded during the experimental in 64 days.

5.2.3. Virus free line three

Virus free line three consisted of 32 flies (16 positive males and 16 positive females) showed positive for the presence of salivary gland hyperplasia virus by PCR. 6.25% of female populations were positive for salivary gland hyperplasia by dissection.

Female fly from cage number 9 mated with cage number 9 male showed positive on dissection, but the male was negative on PCR and dissection. 25% of the female population did not produced pupae and 18.75% mortality was recorded.

In the first and second reproduction cycles three female flies died. These are cage numbers 1, 10 and 15. Female fly mated with male cage number cage 1 produced one average (C class) on first cycle. Male cage number 10 mated with female cage number 10 produced one good (D class) pupa on the first cycle. Female cage

number 15 mated with male cage number 15 produced high (E class) quality pupae on the second reproduction cycle.

Out of 16 female flies 11 different pupae were collected on the first reproductive cycle. These pupae ranged from average (C class) to high (E class) quality pupae. On the second cycle only three pupae were collected and two in good (D class) and one high (E class). On the third cycle there were 5 high (E class) quality pupae.

On the fourth cycle one good (D class) and four high (E class) pupae were collected.

Male fly cage number 9 mated with female fly cage number 9 showed negative by PCR but positive by dissection. Female cage number 9 showed negative on PCR and dissection and they survived for 64 days, but there was no pupa production.

Table 4. Virus free line three (VFL III)

							Reproduction Cycles			
Cage No	SG male dissection	PCR male	pupae	QPCR	SG Female dissection	PCR female	1	2	3	4
1	Negative	Positive	1		Died	Positive	C			
2	Negative	Positive	2		Negative	Positive	E		E	
3	Negative	Positive	4		Negative	Positive	E	D	E	C
4	Negative	Positive	0		Negative	Positive				
5	Negative	Positive	2		Negative	Positive	D			E
6	Negative	Positive	2		Negative	Positive	C			E
7	Negative	Positive	2		Negative	Positive	D		E	
8	Negative	Positive	0		Negative	Positive				
9	Negative	Positive	0	Not taken	Positive	Positive				
10	Negative	Positive	1		Died	Positive	D			
11	Negative	Positive	2		Negative	Positive	D		E	
12	Negative	Positive	0		Negative	Positive				
13	Negative	Positive	1		Negative	Positive	D			
14	Negative	Positive	4		Negative	Positive	E	D	E	E
15	Negative	Positive	1		Died	Positive		E		
16	Negative	Positive	2		Negative	Positive	E			E
			24							

The best quality and quantity of pupae production was recorded from fly cage number 3 and 14, each of them produced four pupae. Fly cage number 3 produced one average (C class), one good (D class) and two high (E class). Fly cage number 14 produced one good (D class) and three high (E class) quality pupae. Three was no pupae production from female fly cage number 4, 8, 9 and 12 (Table 4).

5.2.4. Virus free line four

As shown in Table 5, a total of 22 flies (11 male and 11 female) were grouped as VFL III. All flies were highly positive by PCR for the Salivary gland hyperplasia virus and 18.18%, 9.09% of male and female flies, respectively were positive by dissection for salivary gland hyperplasia.

Male flies from cage number 1, 4 and 5 were positive by dissection for salivary gland hyperplasia. Male fly positive by PCR and dissection for salivary gland hyperplasia mated with female positive for PCR, but negative by dissection (Table 5).

Two female flies from cage numbers 8 and 11 died after one to two pupae production within the first and third reproduction cycles. Female fly cage number 8 produced one good (D class) pupae in the first cycle and fly cage number 11 produced one good (D class), one high (E high) quality of pupae in the second and third cycles (Table 5).

Six out of 11 female flies were able to produce 6 pupae on the first reproductive cycle. These pupae ranged from average (C class) to high (E class) quality pupae. On the second cycle only two high (E class) pupae were collected. On the third reproduction cycle 3 pupae were produced, one good (D class) and two high (E class) qualities. On the fourth cycle three high (E class) pupae were collected (Table 5).

The best quality and quantity of pupae production was recorded cage number 6, 7

and 10. Fly cage number 6 produced three high (E class), and fly from cage number also produced the same number of pupae. But fly cage number 10 produced one good quality (D class) and two high (E class) pupae were collected.

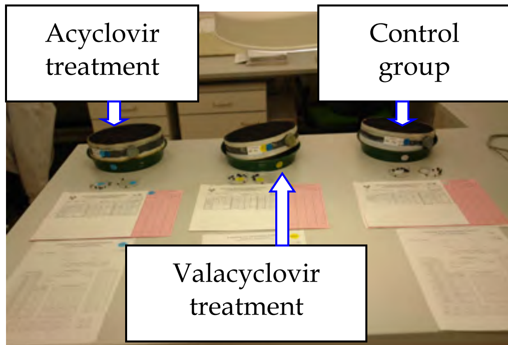
Male cage number 4 mated with female cage number 4 showed positive by PCR and dissection. The female did not produced pupa, but they lived 64 days. Male cage number 5 mated with female cage number 5 showed positive by PCR and dissection. The female did not produced pupa, but they lived 64 days. Female cage number 1 mated with male cage number 1 showed positive by PCR and dissection. The female produced one average (C class).

Table 5. Virus free line four (VFL IV)

							Reproduction cycle			
Cage No	SG male dissection	PCR male	pupae	QPCR	SG female dissection	PCR female	1	2	3	4
1	Negative	Positive	1		Positive	Positive	C			
2	Negative	Positive	0		Negative	Positive				
3	Negative	Positive	1		Negative	Positive	E			
4	Positive	Positive	0	3.33 E+05	Negative	Positive				
5	Positive	Positive	0	7.57 E+05	Negative	Positive				
6	Negative	Positive	3		Negative	Positive	E		E	E
7	Negative	Positive	3		Negative	Positive	E	E		E
8	Negative	Positive	1		Died	Positive	D			
9	Negative	Positive	0		Negative	Positive				
10	Negative	Positive	3		Negative	Positive	D		E	E
11	Negative	Positive	2		Died	Positive		E	D	
			14							

5.3 Anti viral drugs (AVD) treated flies

5.3.1 Preliminary study



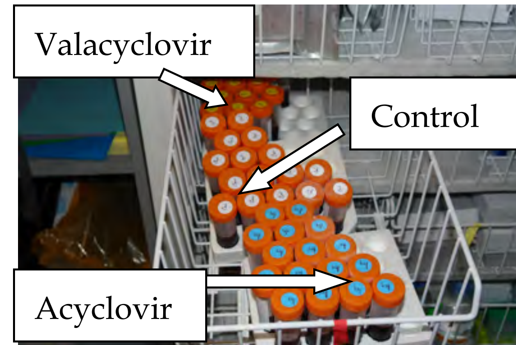


Fig. 6. Preliminary survey cages

Fig. 7. Antiviral drugs

A total number of 90 flies (72 female and 18 male) were treated with acyclovir and valacyclovir (Fig.6 and 7).

Table 6. Preliminary study of Anti viral drugs

Treatment	A >23mg	B 23-29mg	C 29-33mg	D 33-37mg	E 37mg<	Total pupae of different classes
Acyclovir	0	0	6	8	1	15
Valacyclovir	0	1	4	9	8	22
Control	0	0	3	12	12	27
Total	0	1	13	29	21	64

All selected flies were positive for salivary gland virus by PCR. The total pupae production of this experiment in 30 days was 64 (Fig. 6). The average pupae per initial female was 0.465, 0.683, 0.803 acyclovir, valacyclovir and control group, respectively (Table 7).

Table 7. Pupae per initial female per 10 days (PPIF)

Treatment	First cycle	P/F/10days	Second cycle	P/F/10days	Average
Acyclovir	12	0.745	3	0.186	0.465
Valacyclovir	13	0.807	9	0.559	0.683
Control	15	0.892	12	0.714	0.803

In the first cycle acyclovir treated flies produced 12 pupae from 24 female flies. In the second reproduction cycle produced 3 pupae out of which 8 pupae in were in D (good) class, 6 pupae in C (Average) class and 1 pupa in E (high) class.

The valacyclovir treated flies in the first reproduction cycle produced 13 pupae from 24 flies. In the second reproduction cycle produced 9 D (Good) class pupae, 8 E (high) class pupae, 4 C (average) class pupae and 1 B (poor) class pupae.

Control group in the first cycle produced 15 pupae from 24 flies, and in the second reproduction cycle 12 pupae, six each from D (good), E (high) classes and 3 C (average) class pupae (Table 7).

Weekly mortality of the flies shown in Table 8: only 0.48% and 0.615% mortality was recorded in acyclovir and valacyclovir, respectively.

Table 8. Weekly mortality of preliminary survey flies.

Treatment	Week one	Week two	Week three	Week four
Acyclovir	0	0.48	0	0
Valacyclovir	0	0	0	0.61
Control	0	0	0	0

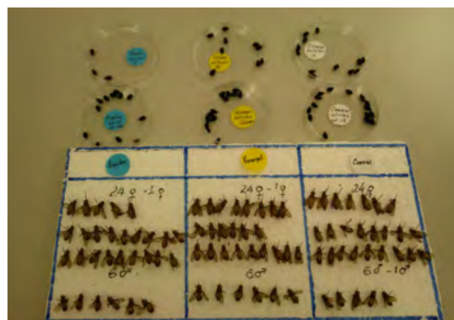


Fig. 8. Preliminary antiviral study

5.3.2 Antiviral treated population

A total of 90 out of 360 flies served as an initial sample for the viral DNA load study. Out of 198 flies 136 pupae were produced in 35 days.

Fig. 9. Population antiviral drug treatment

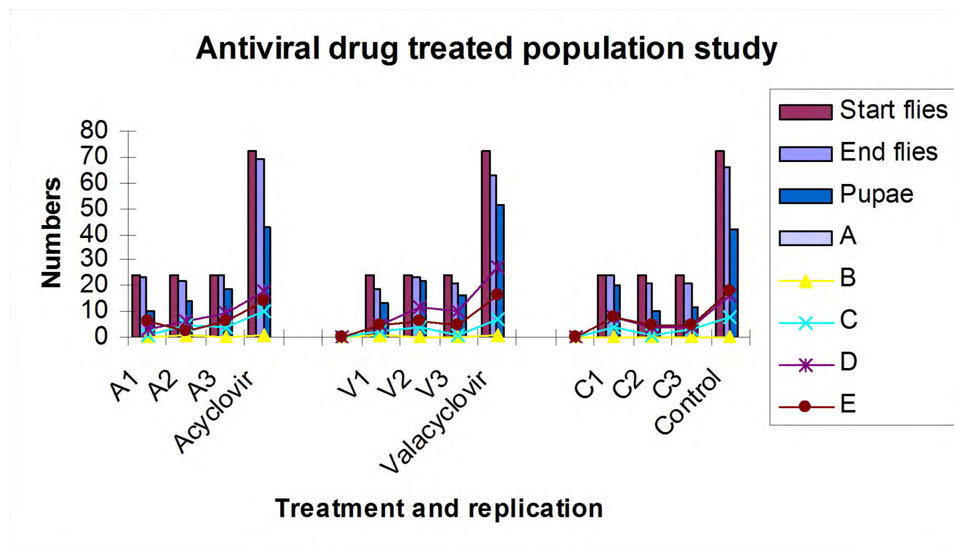


Fig. 10. Population sample survival and pupae production

The acyclovir treatment possessed 72 flies of which after one month 23, 22, and 24 flies remained in replications 1, 2 and 3, respectively. The total pupae production was 43. All pupae were categorized 10, 14, and 19 in replication 1, 2 and 3, respectively. The feeding response showed that most of the flies fed in average 89.7%, which is better that valacyclovir and control groups. There was significant

difference P< .005 at P=.05 (Annex 1).

There was no pupa in A class, but there was one pupa of B class in the second replication. The total number of C class pupae was 10 and there were 1, 5, 4, in replication 1, 2, 3 respectively. Class D had 18 pupae 3, 6, 9, in replications 1,2,3 respectively. 14 high (D) class pupae were distributed, 6 in the first replication, 2 in second replication and 6 in the third replication (Table 9).

Table 9. Antiviral drug treated population flies.

Treatment	Start flies	End flies	Pupae	A	B	C	D	E	Feeding Response	Quality Factor	Average QF
Acyclovir	72	69	43	0	1	10	18	14	89.7	3.43	1.143333
Valacyclovir	72	63	51	0	1	7	27	16	85.1	3.9	1.3
Control	72	66	42	0	0	8	16	18	84.5	3.48	1.16

In valacyclovir treated flies study 72 flies were observed and there were 5, 1, and 3 flies mortality in replications 1, 2 and 3 respectively. The total pupae production in one month was 51 in all three replications in which 13, 22 and 16 pupae in replication 1, 2 and 3 respectively. The quality factor for the replication one, two and three was 1.2, 1.43, and 1.27 with the average feeding response of 85.1 %. There was no significant difference P>.072 (Annex 1) at P=.05

There was no A class pupae but, there was one B class pupa in the first replication. There were 7 pupae in class C, 2 pupae in first replication, 4 pupae in second replication and 1 pupa in third replication. The total number of D class was 27 and there were 5, 12 and 10, in replication 1, 2, 3 respectively. The E class had 16 pupae 5, 6, 5, in replication 1, 2, 3 respectively (Table 9).

A total of 72 flies were observed in the control group and all flies fed in average of 84.5%. The quality factor for the replication one, two and three was 1.34, 1.03 and 1.11.

The total pupae production was 42 in all three replications in which 20, 10 and 12 pupae in replication 1, 2 and 3 respectively. There was no pupa in A and B classes, there were 8 pupae of class C, in first replication 4, in second 1 and in the third replication three, there were 16 pupae in class D, in which the first replication had 8 pupae, the second 4 pupae and the third replication four pupae.

The total number of E class pupae was 18 and there were 8, 5, 5, in replication 1, 2, 3 respectively. The E class had 3 pupae and all pupae were distributed one pupa in each replication (Table 9).

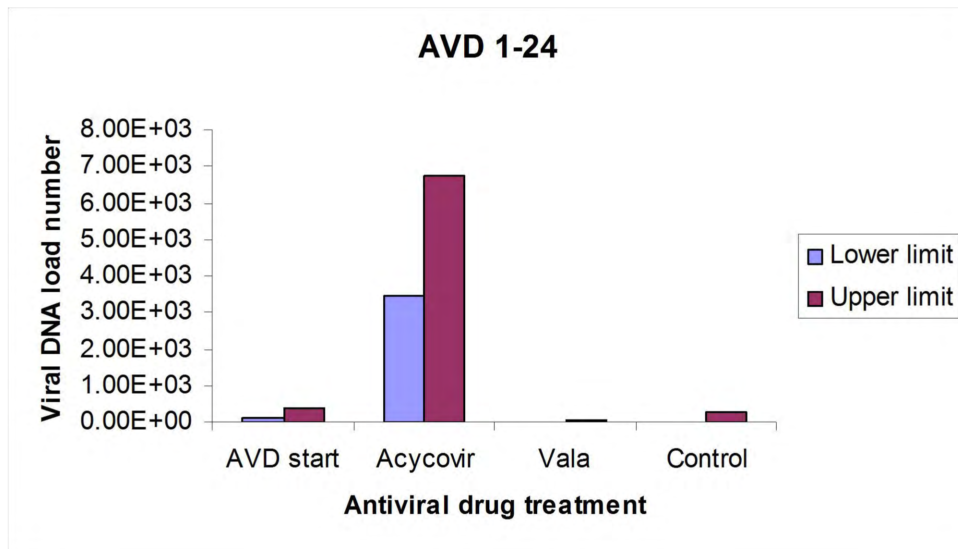


Fig. 11. Population viral load study 1-24 days

The initial viral DNA load of flies 1-24 was with minimum 1.24E +02 and the maximum 3.92 E + 02, Acyclovir treated flies 3.45 E + 03 and 6.76 E +03, Valayclovir, 1.32E +01 and 5.59E + 01 and control 1.01E +01 and 2.78 E + 02 (Fig.11).

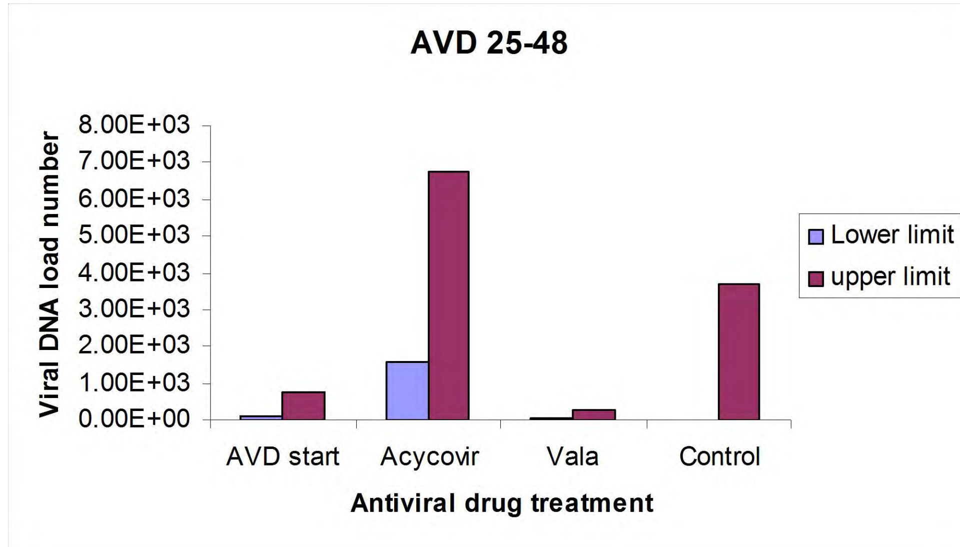


Fig. 12. Population study 25-48 days

Samples 25-48, the initial viral DNA load 9.77E + 01 and 7.37 E +02, acyclovir treated 1.58 E + 03 and 6.73 E + 03, valacyclovir treated 5.57 E + 01 and 2.81E +02 and control group showed 1.73E + 00 and 3.71E + 03 (Fig.12).

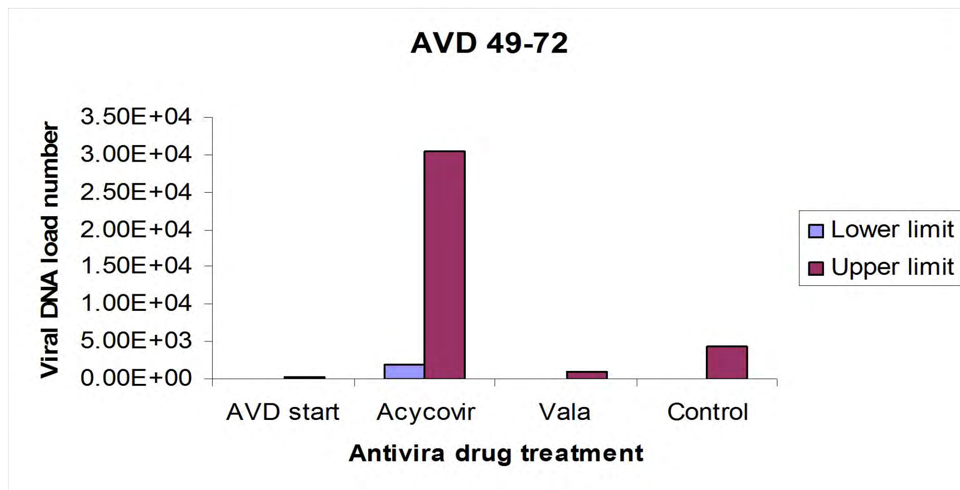


Fig. 13. Population study 49-72 days

Sample 49-72 initial viral DNA load was 4.88 E + 01, and 3.18E + 02, in acyclovir treated 1.92 E + 03 and 3.05 E +04, valacyclovir treated flies 6.93 E + 01 and 1.05E +03 and control group 1.42 E + 01 and 4.32 E +03 (Fig 13).

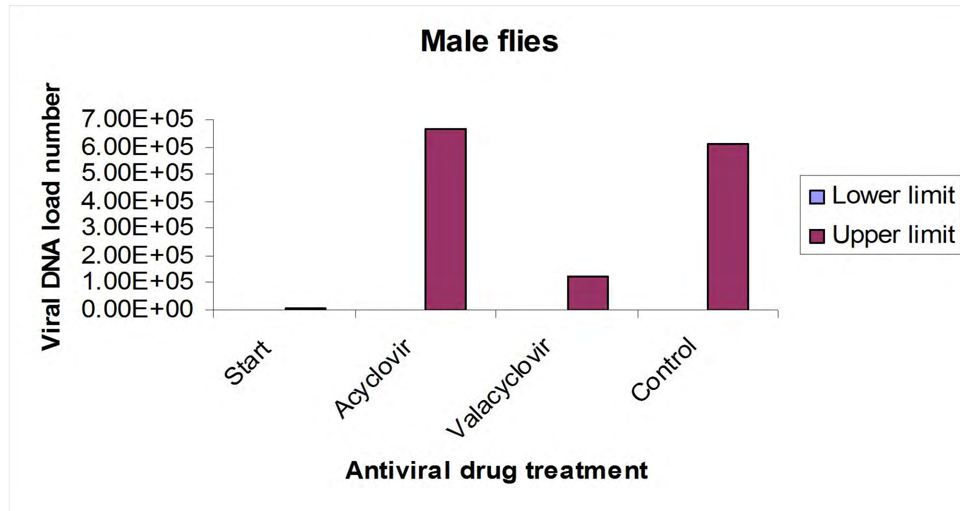


Fig. 14. Population male flies

Viral DNA load of population male flies from previous non treated initial flies showed 2.87E + 02 and 4.35 E + 03, acyclovir treated 4.71 E +01 and 6.66 E + 05, valacyclovir 6.58 E + 00 and 1.22 E +05 and control group 8.18 E + 01 and 6.11E +05 (Fig.14).

5.3.3 Antiviral treated individuals

A total number of 216 female flies were taken from 432 (1:1) male to female flies. The total production of pupae was 99 and 191 flies survived in one month (Fig.15). The dissection result showed 13.85%, 26.87% and 25.42 in acyclovir, valacyclovir and control groups, respectively (Table 10).

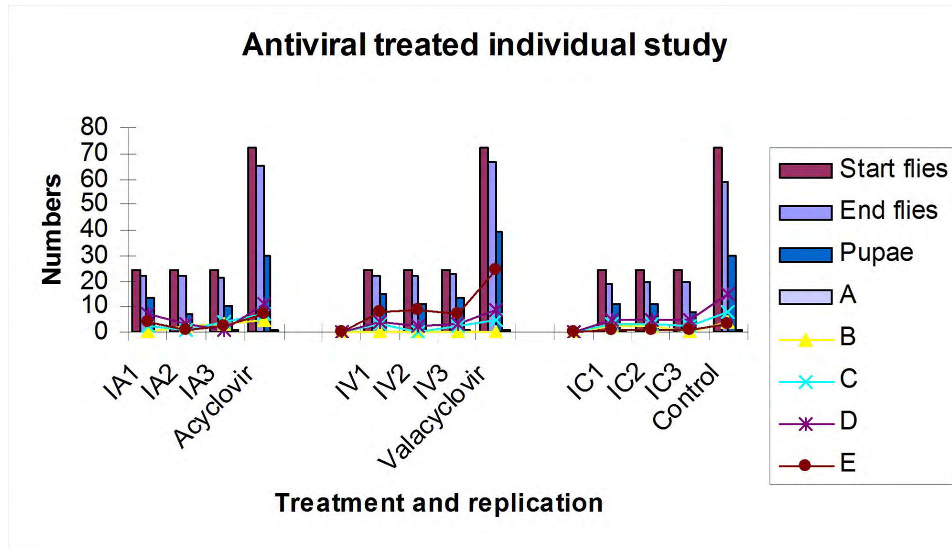


Fig. 15. Individual treatment survival and pupae production

Acyclovir treated 72 female flies with blue label on individual cages (Fig.16) they remained 65 flies in 30 days and 22, 22, and 21 flies in replication 1, 2 and 3, respectively. The total pupae production was 30 from three replications of acyclovir and 13, 7 and 10 pupae were collected in replication 1, 2 and 3, respectively (Fig.15). There was significant difference P<.005 (Annex 2).

Fig. 16. Individual antiviral drug treatment

One pupa in A class in the first replication, 5 pupae in class B in which replication 2 had 2 pupae and replication three had three pupae. The total number of C class pupae was 7, and 2, 1, 4, in replication 1, 2, 3 respectively. The D class had 11 pupae 7, 3, 1, in 1, 2, 3 replication respectively. There were 7 pupae in E class and 4

of them in first replication, 1 pupa in second replication and 2 pupae in their replication. It was observed that 7 flies were died and the quality factor for the replication one, two and three was 1.15, 0.87, and 1.0 (Table 10)

Table 10. Antiviral drug treated individual flies.

Treatment	Start flies	End flies	Pupae	A	B	C	D	E	**Salivary Gland Hyperplasia**	**Quality Factor**	Quality factor Average
Acyclovir	72	65	30	1	5	7	11	7	**9**	**3.02**	**1.006667**
Valacyclovir	72	67	39	1	0	5	9	24	**18**	**3.38**	**1.126667**
Control	72	59	30	1	4	8	15	3	**15**	**3.02**	**1.006667**

72 valacyclovir treated flies with yellow label were observed (Fig.16) and there was only 2, 2, and 1 fly mortality in replications 1, 2 and 3 respectively. The quality factor for the replication one, two and three was 1.21, 1.07, and 1.0. 39 pupae were produced in one month and 15, 11 and 13 pupae in replication 1, 2 and 3, respectively (Table 10). There was no significant difference $P > .217$ (Annex 3).

The total collected from valacyclovir treated flies was 39 pupae and the distribution was as follow, there was one pupa from replication three, and there were 5 pupae in class C, in first replication 3 pupae and in the third replication two pupae. There were 9 pupae in D class 4, 2, 3, in replication 1, 2, 3 respectively. The E class had 24 pupae 8, 9, 7, in which replication 1, 2, 3 respectively But, there was no pupa in class B (Table 10).

In control group with white label (Fig.16) of the experiment, 72 flies were observed and there was no mortality in all replications. The quality factor for the replication one, two and three was 1.05, 1.02 and 1.1 respectively (Table 10). There was significant difference $P<.000$ (Annex 4).

30 pupae were produced from three replications of the control group. In replication one 11 pupae, replication two 11 pupae and in replication three 8 pupae were recorded. From these pupae there was only one A class pupa in first replication. Four pupae of class B were equally distributed in the first and second

replications. 8 pupae were in class C, from the first replication 3 pupae, second 3 pupae and from the third replication were two pupae. The total number of D class pupae was 15 and there were 5, 5, 5, in replication 1, 2, 3 respectively. The E class had 3 pupae and one pupa in each replication (Table 10).

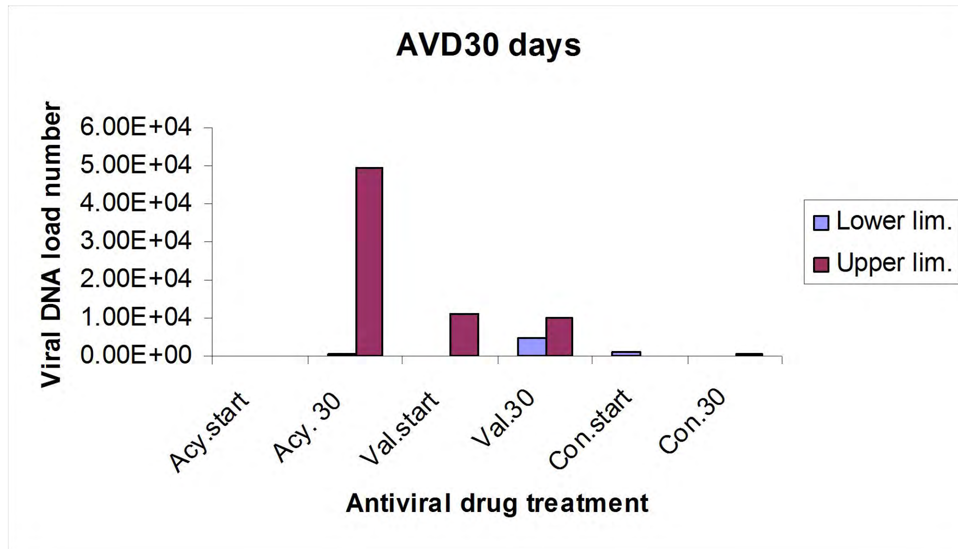


Fig. 17. Individual study 30 days

The initial viral DNA load from 30 days experiment in acyclovir start group varied between 2.57 E + 01 and 8.01 E + 01, acyclovir treated group 2.52 E + 03 and 4.93 E + 04. In the valacycovir start the initial viral DNA load was observed between 2.46 E + 02 and 1.09 E + 04, and the valacyclovir 30 days treated flies showed the variation between 4.99 E + 03 and 1.01 E + 04. In the Control start the initial viral DNA load varied between 1.94 E + 02 and 9.79 E + 02, and control 30 days showed the variation between 1.40 E + 02, 2.99 E+ 02 (Fig. 17)

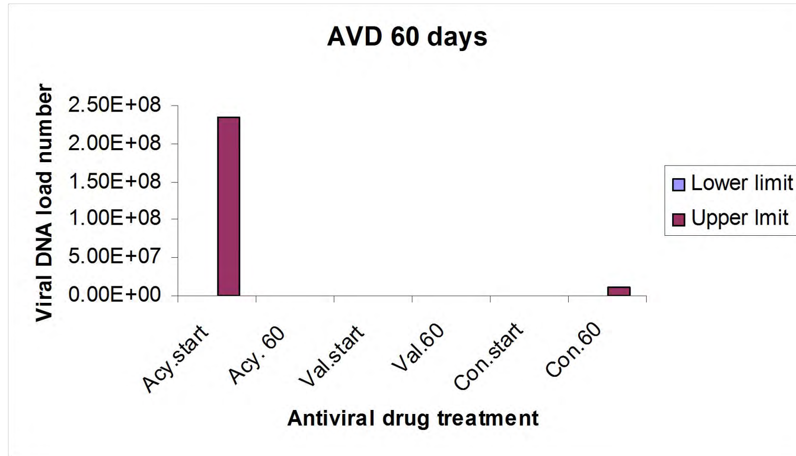


Fig. 18. Individual study 60 days

The initial viral load of acyclovir start for 60 days varied within the range of 1.30 E + 03 and 2.35E +08 and the 60 days acyclovir treated flies showed the variation between 7.82E + 02 and 4.93 + 04. In the valacyclovir start the viral DNA load was between 3.33E +01 and 1.27E + 03, and the 60 days valacyclovir treated flies showed between 3.94 E +03 and 9.58E + 03, control 6.93 E + 01 and 3.35 E + 02. The control group showed the range of the viral DNA load between 6.93 E + 01 and 4.48 E + 02 and control group after 60 days 4.56E + 03 and 1.00 E +07 (Fig.17).

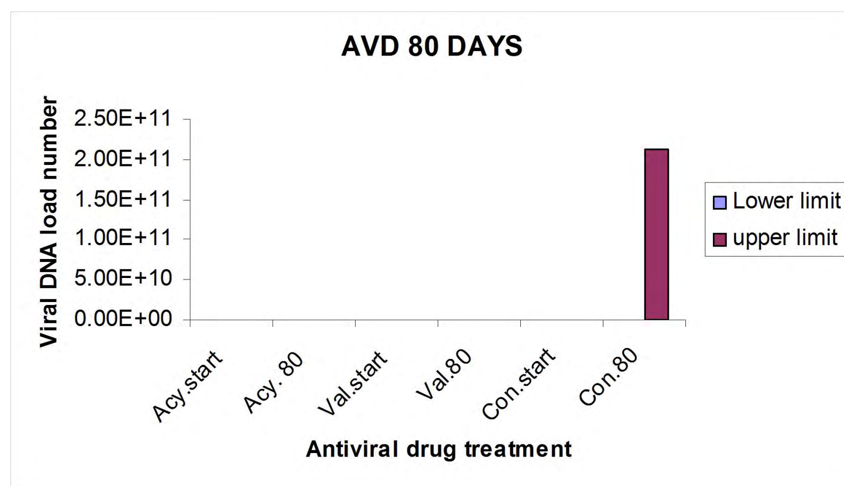


Fig. 19. Individual flies study of 80 days

The initial viral load of acyclovir start flies showed variation between 2.57E +01 and 8.24E +01 and acyclovir treated flies viral DNA load was between 3.41 E + 03 and 1.49 E+ 04.

In the valacyclovir start the viral DNA load was between 9.96 E + 00 and 3.96 E + 01, and 80 days valacyclovir treated flies showed the variation between 4.91 E + 03 and 1.07 E + 04. In control start the initial viral DNA load was between 1.04 E + 02 and 3.25 E + 02, and control after 80 days ranged between 2.20 E + 03 and 2.12 E +11 (Fig.19)

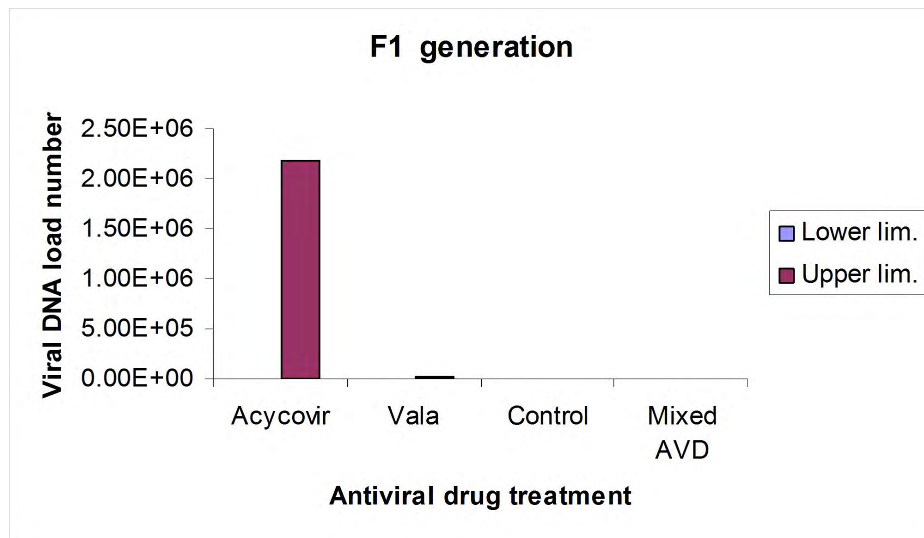


Fig. 20. Individual F1 generation

F1 generation of antiviral treated and non treated 72 flies showed that acyclovir treated flies with the range between 4.77 E + 01 and 2.19 E+06, valacyclovir between 1.37 E +03 and 2.52 E +04, control 6.57 E + 02 and 1.81 E + 03 and the mixed samples (six from each samples) ranged between 8.03E + 01 and 2.33 E + 02 (Fig.20).

6.0 Discussions

Tsetse SGHV has been studied extensively in the past decades. The present experiments mainly focused on the preventive ways of the SGHV through virus free line establishment and antiviral drug treatment.

The exact taxonomic status of the SGHV is unclear. The preliminary results of the entomology unit of the IAEA showed that the SGHV genome size is around 185 kbp. It is close to that of 137 Kbp estimated for house fly salivary gland hyperplasia virus genome. But, there is some gap between this estimate of the SGHV and genome size compared to the 20-34 Kbp (Odino *et al*., 1986)

According to 2005 annual report of the Entomology unit of IAEA/FAO laboratory hyperthrophied salivary glands viral PCR product originated from *G. pallidipes* Tororo (Uganda) and *G. pallidipes* Arbaminch (Ethiopia) were sequenced and compared by NTI vector 9 Software. The alignment of the sequences showed that the Ethiopia and Tororo strains have more similarity and fewer differences in the nucleotide sequence.

The alignment of the sequences showed that all samples from *G. pallidipes* Tororo strain were identical and all the samples from *G. pallidipes* Arbaminch strain were also identical, but different from those samples from the *G. pallidipes* Tororo strain unit. All sequences from *G. pallidipes* Tororo strain Arbaminch have four nucleotides changes compared to *G. pallidipes* Tororo strain, i.e, A instead of G position 86, T instead of G position 107, T instead of C position 137 and A instead of G position 245.

Salivary gland hyperplasia of *G. pallidipes* caused by virus has been shown to be 1-5.5% in both *G. pallidipes* Arbaminch and *G. pallidipes* Tororo strains. The prevalence rate of this enlargement in the virus free line was 1.96% female and 3.91% male flies. The female SGH prevalence rate in the experiment was lower than the dissection of 2003 and male flies infection was compared to 3.09%.

The prevalence rate of SGH infection in *G. pallidipes* was higher by PCR than in dissection. This had been demonstrated in virus free line establishment. Out of 920 flies, only 50 (32 male and 18 female) flies were selected as salivary gland hyperplasia virus negative flies. The remaining 870 flies, which accounted 94.56 % were positive.

In this experimental work there are some evidences on the horizontal transmission of the SGHV. Fifty flies including the dead two flies were negative by PCR. After certain time, out of 32 virus free flies (16 males and 16 females), four flies (3 males and 1 female) become positive for salivary gland hyperplasia by dissection. These could be due to the presence of the salivary gland hyperplasia virus in the rearing facility. The type of transmission most likely could be horizontal. This is the good demonstration of horizontal virus transmission due to contaminated rearing facility, equipment and handling. So, the horizontal transmission of the virus has high risk in the establishment of the virus free colony.

As stated by Jaenson (1978) that the transovarian and transovum transmission of the virus and its important role plays in the interstadial and inte-generation passage of the particles. But it was not mentioned about the horizontal transmission.

The more important point arising from the virus free line establishment is that from 90-100% infected tsetse colony, 1-5% of virus free flies can be selected and if they will not get the appropriate handling and care, the virus introduction may occur and contaminate the fly population at any time.

The mortality of VFL I was equal to VFLIII but, more than VFLII, VFLIV. This result showed that the occurrence of equal mortality in other virus free lines could be due to the environmental factors rather than the virus.

Tsetse flies positive for SGHV by PCR and dissection were able to produce pupae

and live as mush as the negative flies do. This observation is contradictory with the observation of Jaenson (1978) that the virus could induce sterility 70-80% male and female tsetse flies. At the same time, it needs a series of experiments on the level of SGHV which may cause enlargement of the glands and testicular degeneration and ovarian abnormalities which was stated by Sang *et al.,* (1997).

It is more likely that the SGHV transmission in the laboratory could depend on both horizontal and vertical transmissions. The results of this study revealed that the feeding response was 90-100% which is more acceptable. How ever, the mortality of three dead flies of VFL I was due to failure to feed the offered blood meals for 10 minutes.

Jaenson (1978) work about sterility seems to be challenged based on this research work. As the positive male and female flies for SGHV flies produced good qualities of pupae with 95% emergence rate, which was more than 5-10% of the emergence rate in the main rearing facility.

Therefore, the cause for sterility in both sexes of the flies has to be checked by applying different quantity of viral DNA. This could be the injection of high, medium, low quantity of SGHV using different strains and time. Such experiment allows us to find appropriate dose of viral DNA, which causes salivary gland enlargement and sterility.

The long survival of virus free line negative and positive flies in the colony of the experimental flies showed that, the flies can be affected by tsetse virus up to 95% or more, but they can survive and produce good quality of pupae.

In virus free line one, three male flies were positive for Salivary gland hyperplasia by dissection and PCR. These positive male flies were mate with negative female. Only one of the mated male from cage number three produced two high (E class) pupae and the other from cage number 1 and 6 were not able to produce pupa. The absence of pupa in infected flies can be attributed partly to the presence of the

virus in the insect body. The replication and the assembly of virions in cells provide a stress on the affected organ as the cell macromolecular synthesis machinery is switched over the synthesis of viral protein.

The replication of the SGHV in the body of the insect could have an impact on the nutritive requirements and on the cell development could lead to lack of larval development.

This result of the observation may vary from the statements of Jaenson (1986) which said that, the lifespan of both sexes of infected tsetse was significantly shorter than those of healthy tsetse.

The 2003 annual report of the Entomology unit of IAEA/FAO laboratory stated about collapse of the *G. pallidipes* Arba Minch (Ethiopia) strain a few years after successful expansion in the unit was nevertheless attributed to the very high infection rate observed in the colony.

Antiviral drug study on tsetse flies against salivary gland hyperplasia virus is THE first in its kind and this work had been started in Entomology unit of IAEA/FAO laboratory, the effect of this antiviral drugs treatment had been demonstrated in human medicine against dsDNA virus. In future, equivalence trials have to be used increasingly in different insects.

Based on the main objective of this thesis work, one of the two crucial aspects of experiments was feeding of antiviral treated drugs at the dose of 0.3mg/ml. The test in preliminary survey of antiviral drugs with DFFBB on *G. pallidipes* Tororo strain found to be non-toxic at the concentrations of 0.3mg/ml. Following the good result of this preliminary survey large scales experiments on population and individual tsetse flies were designed.

The results revealed the difference and similarity between the two treatment regimens on the SGHV, and the impact of valacyclovir in protecting salivary gland enlargements, as compared with acyclovir treated flies enlargements, Moreover,

the high detection accuracy of QPCR demonstrated that the antiviral drug treatment demonstrated the good effect of the Valacyclovir in terms of pupae production and survival and this result may be considered slightly provocative because valacyclovir treated flies produced more pupae and survived well as compared with acyclovir treated flies. Furthermore, acyclovir treated flies gave less pupae in comparison to valacyclovir treated and control groups, but survived more.

Langley (1977) demonstrated that the first pregnancy cycle takes 18 days under the laboratory conditions while subsequent cycles take 9 to 10 days. Whereas, the subsequent cycle of the antiviral drug experimental female pregnancy cycle of treated flies was longer 3±2 days. This longevity of feeding interval could be due to the unequal distribution of feeding interval in a week and number of feeding days. The impact of the antiviral drugs in general and the dose in particular will not be underestimated. Such kind of long pregnancy cycle affects the colony size directly and the cost of the rearing facility indirectly.

However, the question of what makes Valacyclovir more effective than Acyclovir and the difference between two treatments and how this can be demonstrated remains open. Indeed, a significant difference between acyclovir and control groups does not necessarily indicate the equivalence of the groups.

Valacyclovir treated female flies produced high quality of pupae in comparison to acyclovir and it is believed that this result provide useful alternative information, which may be preferred by some scientists.

It was well observed that a three replication of each treatment in the experiment performed well and reduces the error on experiment basis, provided that well organized coordinating and monitored structures are established.

Finally, in addition to the virus free line, antiviral drug treatments should also be

considered as an important factor in protecting the SGHV transmission in tsetse colony.

The presence of the salivary gland hyperplasia virus in the F1 generation is another important indication of the trans-ovarial transmission of the virus and as the results obtained. The level of viral DNA load in F1 antiviral treated flies showed that acyclovir between 4.77 E + 01 and 2.19 E+06, valacyclovir between 1.37 E +03 and 1.84 E +04, control 6.57 E + 02 and 1.81 E + 03 and the mixed samples (six samples from each treatment including control group) ranged between 8.03E + 01 and 3.18 E + 02.

Jaenson (1986) has demonstrated the nature of transmission of *G. palldipes* virus to off-spring, and determined that the females with enlarged salivary glands provided only infected progeny and the above observations on F1 generation supports this evidence.

Therefore, the off-spring of antiviral treated flies gave us the opportunity to observe that the applied dose of treatment don't protect the possible transmission of parent-to-offspring transmission of the SGHV in tsetse flies.

7.0 Conclusions and Recommendations

The main source of SGHV estimated to be the infected tsetse flies from wild flies in nature or rearing facility including all tools, equipments of the rearing facility including personnel.

A much higher rate of virus infection is observed by PCR and QPCR in tsetse colonies compared to the dissection of laboratory populations of *G. pallidipes,* Arbaminch and Tororo strains.

The dissection result for salivary gland hyperplasia, PCR for salivary gland hyperplasia virus and QPCR for viral DNA load status, gave us the clue to be more reliable on PCR and QPCR rather than on dissection.

The high specificity of the primers could be the main reasons for the reliability of PCR and QPCR. The failure to detect the infection by dissection according to the symptom could be due to the low level of virus load in the fly. It is obvious that the enlargement of the gland may start to occur as the SGHV level starts to increase in quantity.

However, the absence of salivary gland hyperplasia during dissection didn't indicate the negativity of the fly for SGHV. The only confirmation about the presence or absences of the virus in the insect body was the application of virus specific primers. At the same time the presence of viral DNA in both sexes and of different ages including newly emerged flies (day 0) showed 95-100% SGHV. This finding indicates that, most of the colonies in the laboratory are infected and they are not showing the salivary gland enlargement.

The experiment on virus free line establishment showed that the flies negative for salivary gland hyperplasia by PCR become positive for SGH dissection after 64 days of the experiment, this result demonstrated that, any fly screened by PCR as virus free insect can be contaminated horizontally if they are handled in the same insectory with infected flies and utilizes the infected fly utilities.

The study about the transmission of SGHV under laboratory conditions and especially the role of contaminated rearing facility and its equipments showed that the horizontal transmission of the virus to the flies may occur at any time and contaminate some of the experimental flies.

G. pallidipes laboratory colony has been shown to carry SGHV in high number of flies which varied between 90-95%, this was confirmed using virus specific PCR primers.

This longevity of feeding interval could be due to the unequal distribution of feeding interval in a week and number of feeding days. The impact of the antiviral drugs in general and the dose in particular will not be underestimated. But, such kind of long pregnancy cycle affects the colony size directly and the cost of the rearing facility indirectly.

At the same the study on F1 generations demonstrated that the transovarial transmission of the disease can not be underestimated even parents their parents will be treated with antiviral drugs. It is important to select carefully virus free tsetse flies from infected tsetse colony.

Even though decontaminating the infected colony and continuing the activity in the previously infected facility is the first recommendation. But, this could be the temporary solution and the hazard of disease may occur at any time accidental outbreak in the rearing facilities.

The preliminary study on antiviral drug treatment demonstrated the effect of antiviral drugs as an inhibitor of the dsDNA virus. In both experiments of the AVD, there were successful results. Acyclovir treated flies showed the least result, but with good survival of the experimental flies. On the other hand, valacyclovir treated flies showed good results comparing to the Acyclovir treated flies.

Therefore, application of valacyclovir as antiviral agent against the SGHV showed

encouraging result as viral DNA replication interfering agent. But, this result has to be considered as a good clue for further detail investigation, where it needs more detailed experiments.

These two antiviral drugs, which designed to fight dsDNA virus as most of the antiviral drugs they may have a common problem. However, over the long run, the SGHV may evolve to acquire resistance to the drugs. This means that no antiviral drug will ever be a permanent solution. In fact, the structure of an antiviral compound will have to be tweaked as its target virus changes. This is the nature of the antiviral drugs in general and acyclovir and valacyclovir in particular. However, one of the two antiviral drugs is found to be promising to be the good solution against SGHV.

Finally, mortality of tsetse flies in any rearing facility should be investigated in multidisciplinary approach to have the clear picture on the case and to protect risk of new virus strain introduction and the hazard of mixed virus.

7.1 Recommendations

Based on the results and observations of virus free line establishment and antivirus drug treatment experiments, I would like to suggest:

7.1.1 Transmission study

The impact of sterilized equipments on the tsetse SGHV transmission in the colony using sterilized equipments will give us the opportunity to know if the designed sterilization temperature is safe or not.

7.1.2 Virus free line establishment pilot project application

High percent of SGHV prevalence is suspected to cause an expected problem in the colony. There fore the development of new strategy of the virus free colony

establishment is crucial. For this reason a pilot project is important.

7.1.3 Antiviral drugs treatment

The antiviral treatment of infected colony is one of the solutions to maintain the fly population in qualitative and quantitative ways. Antiviral drugs as a virus life cycle blocker have to be studied more detailed.

7.1.4 To study level of sterility causing by SGHV

It had been stated in different publications, that SGHV can cause sterility. But in this experiment, the results showed that the theory about sterility requires more detailed study. Therefore, study on sterility needs to be specified by injecting different levels of virus load in to the third instar larvae and emerged flies. This will assist to find the level of virus which causes sterility on both sexes of tsetse flies.

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Annex 1: ANOVA antiviral treated population samples

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Acyclovir	Between Groups	8E+008	59	13776428.00	5.407	.005
	Within Groups	22930792	9	2547865.741		
	Total	8E+008	68			
Valacyclovir	Between Groups	1132755	56	20227.771	3.201	.072
	Within Groups	37912.657	6	6318.776		
	Total	1170668	62			
Control	Between Groups	27419474	57	481043.396	92.957	.000
	Within Groups	41399.089	8	5174.886		
	Total	27460873	65			

Annex 2: ANOVA acyclovir (treatment 1) individual samples

ANOVA

Treatment1

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4E+009	54	77919596.09	9.458	.000
Within Groups	82385183	10	8238518.333		
Total	4E+009	64			

Annex 3: ANOVA valacyclovir (treatment 2) individual samples

ANOVA

Treatment2

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	2E+008	63	2409001.493	2.786	.217
Within Groups	2594100	3	864700.000		
Total	2E+008	66			

Annex 4: ANOVA control individual samples

ANOVA

Treatment3

	Sum of Squares	df	Mean Square	F	Sig.
Between Groups	4E+022	53	8.336E+020	8E+010	.000
Within Groups	5E+010	5	1.001E+010		
Total	4E+022	58			

Annex 5: Pupae and survival of Virus free line

ANOVA

		Sum of Squares	df	Mean Square	F	Sig.
Pupae	Between Groups	44.083	1	44.083	6.961	.119
	Within Groups	12.667	2	6.333		
	Total	56.750	3			
Survived	Between Groups	14.083	1	14.083	42.250	.023
	Within Groups	.667	2	.333		
	Total	14.750	3			
VFL	Between Groups	3.000	1	3.000	3.000	.225
	Within Groups	2.000	2	1.000		
	Total	5.000	3			

Annex 6: Salivary gland hyperplasia prevalence (2003)

Age (Days)	Dissected male Flies	Dissected female flies	SGH of Female	SGH of Males	Enlarged SG of female %	Enlarged SG of Male %
Emerged	100	100	3	1	3	1
10	100	100	9	0	9	0
20	100	100	9	2	9	2
30	100	100	5	6	5	6
40	100	100	3	5	3	5
50	100	100	2	4	2	4
60	100	100	4	4	4	4
70	100	100	3	0	3	0
80	100	100	3	4	3	4
90	100	100	5	6	5	6
100	100	100	3	2	3	2
Total	**1100**	**1100**	**49**	**34**	**4.45%**	**3.09%**

Annex 7: Sequence of salivary gland hypertrophy virus DNA fragment

```
1 ATAAAATTATTGAATGTATAATAAGTAAAATGTTTTCACATCAAAACAAGTTTAATAATTTATTATATTCGATTGGCGGTTACTATGGATTTACTGCAA

100 TTATTTTTATAGCGATAATAATTGTTATTACGATTGCAATTTATATAATAATGTATTATAGAGAGAAATTGAAATGGAAAAATAAATTTTATCGATACA

200 GTAGTTTAATAAAAAAATACACAATTAATATGGCAAGAGGCATGAGTGATACACAAGTTGAATATTTAAAAAGTGTCTTTCAATATAATGATGAGCTAA

300 TTGATACACAGCATCTTAATATATCAAAATACACTGGCAGTGAAAAATGGTATTTACACGCGGAAGTATTCCATCATCTAACCAAGATATTAACGTTAT

400 TACTTGGACGCACCCTATCACCTAAAGATAAATATTATAAAGATAAAGAAATATGGAAAATGATTTTATTCAGTATAATAACAATAACATCATTTTTAC

                                                                                  GpSGHV2F
500 CCGAAGAAGTCATACCAAATGTTTCCCATGGGGCAAAAATTTGGTATTCATTTTCAATAACTTATCCATTATTACTTGTCAGCGCCACGTACATGCATT

                                                                GpSGHV1F
600 TACATTTATTTAAAAAACACAATCAGGATTTGGCGCGTAATTTAACTAAATACGCTTCAGCATATTATTCCGAACATACCGAACCTACATGTATCAAGA

700 GCATTGGATGGAGAAGAGATGGATCTAACGCTATTATGATCAGCATTGTTTACATTGGAGCACATTTGTATATGAAAGACTTTGATCCCAATTGGCCAA

800 GTCAAATATACATTAGAAATTTTATAAAAGCCGATTATGTCATGGAAGGAGAGGGCTTCTACCACGACGGATCATTTGTTACCCATACCTCATTACCAG

                                                                          GpSGHV2R
900 GATACGGATATCCAACATCGGCATATAATGATTTTATTTTAATAGCGACTTTTTATGAAAATTGGGATGCTGTGAATGCGATAAATAATATATTAAAAA

                                                 GpSGHV1R
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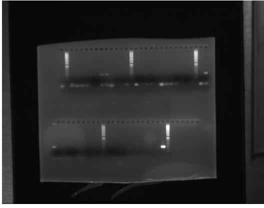
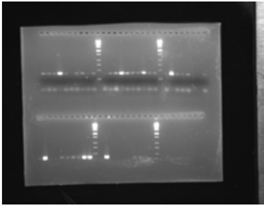
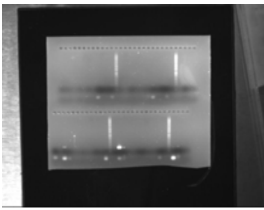
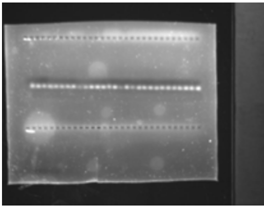
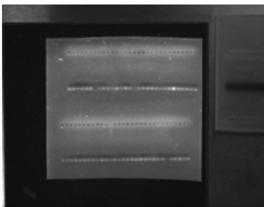
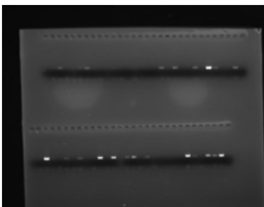

Annex 8: PCR product