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Longevity Studies of Sindbis Virus Infected Aedes Albopictus

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Abstract

The persistent captive life cycle of a virus infected mosquito was examined. The model system used was *Aedes albopictus* and the Alphavirus, Sindbis virus. The adult life span of virus infected *Ae. albopictus* was compared to the life span of virus free *Ae. albopictus*. Intrathoracic inoculation was the method used to infect the experimental group of the adult female *Ae. albopictus* with Sindbis virus. This experimental data demonstrates that this virus does not hamper the mosquito life span. The results from this experiment can be used in future experiments, such as examining the immune system of the mosquito and its responses or protection strategies against the Sindbis virus. This virus serves as a model for more virulent Alphaviruses, such as *Eastern Equine Encephalitis* that can cause serious and fatal diseases. Additionally, *Ae. albopictus* in this experiment can serve as a model for other species of mosquitoes infected with the virus.

Introduction

Ae. albopictus is an important disease vector native to Asia, which has recently become established in the Western Hemisphere. This artificial container—breeding mosquito was first identified in Harris County, Texas, in 1985 (Sprenger D., 1986). Used tires are the primary breeding site of North American *Ae. albopictus* and are the most likely means of introduction to the United States (William A. H., 1987). *Ae. albopictus* is diffusely located in the Mediterranean Basin and according to Romi (2002), and this mosquito was introduced to Italy in 1990 by the import of used tires from United States. *Ae. albopictus* is recognized for its aggressive human-biting habit, and its ability to colonize both tree holes in the forest habitat and human-made containers in the peridomestic environment.

Arthropod-Borne-Viruses referred to as "arboviruses" are perpetuated in the wild through interactions between invertebrate hosts (mosquito) and vertebrate hosts (birds, rodents) and are transmitted to vertebrates by the bite of the infected arthropods, usually mosquitoes and ticks (Brown, D.T., et al., 1986). This efficient virus transmission indicates that it has the potential to serve as both maintenance and an epidemic vector of many arthropod-borne viruses in the Western Hemisphere (William, A. H., 1987).

The term "insect vector" was classically used to describe the passive role of the insect in the transmission of arboviruses to vertebrates. However, it is clear today that active replication of arboviruses in the invertebrate host is essential in the persistence of the virus in nature. The most common method of arbovirus transmission in the wild is a horizontal cycle alternating between vertebrate and invertebrate hosts. Other methods of transmission are vertical transmission, which occurs transovarially from one infected generation to the next, and transtadial transmission where virus is exchanged between a male and a female during copulation (Kow, C.Y., 2001).

Sindbis virus (SIN) is the prototype member of the alphavirus genera, belonging to the family Togaviradae (Griffin, D.E., et al., 1986). Serological surveys suggest that infection is relatively common in certain regions but has different effects on vertebrate and invertebrate hosts. Alphaviruses such as *Easten Equine Encephalitis* (EEE) and *Western Equine Encephalitis* (WEE) can cause serious clinical diseases with high fever,

encephalitis, hemorrhagia, and death in man and domestic animals (Brown, D.T., et al., 1986). However, SIN is among the least virulent of the Alphaviruses, fundamental to its use as a prototype in research. Although protracted disease occurs occasionally, neither fatal infections nor encephalitis have been recognized (Schlesinger, S., 1986). The mosquito vector demonstrates no pathology in response to Alphavirus infection, and the infection persists throughout the life of the arthropod. While the mosquito host is believed to not suffer deleterious effects of the alphavirus infection, tissue-specific pathology has been observed in *Ae. albopictus* following intrathoracic inoculation with the SIN (Bowers, D. F., et al., 2003, in press).

According to Bowers and colleagues (1995), *Ae. albopictus* possesses a wide range of permissive host cells that replicate SIN. *Ae. albopictus* mosquitoes infected by Sindbis virus were examined during the acute phase of infection by Bowers et al., 1995, and these studies were ended at day 18 of post-infection. This infection will now be observed beyond day 18 of post-infection. In order to study the persistent phase of infection in *Ae. albopictus*. The longevity of the virus free mosquitoes was previously determined by Zammito, 2001 (J. Zammito, personal communication). According to Zammito, female mosquitoes lived for 65 days with 80% survival under the insectary conditions at University of North Florida. This study was stopped at 80 days with approximately 65% survivals.

Adult female *Ae. albopictus* are hematophagous insects requiring the protein found in vertebrate blood to produce eggs. The blood meal provides protein for egg development and only females partake blood (James, A.A., 1991). There are different methods available to infect *Ae. albopictus* mosquitoes with virus. One of the methods is by using live animals during the viremic phase of infection. Animals used are anesthetized or restrained and are the most preferred hosts by *Ae. albopictus* (Gerberg, E. J., 1970). A second method is through an artificial membrane system. The artificial membrane system provides a supply of virus-blood suspension. It consists of a skin-like membrane on which the insect feeds upon (Gerberg, E. J., 1970). A third method is the hanging drop method, by which a blood suspension infected with the virus is delivered fia a hanging drop of suspension on the top of the cage for the insects to feed on (Gerberg, E.J., 1970).

An alternative way to achieve infection is through intrathoracic inoculation (Turell, M.J., 1984). This route assures 100% infection unlike the previously mentioned methods, and although it is not the natural route of inoculation, infection is ensured. Mosquitoes are anesthetized on ice. The thorax of the insect is then injected with a tiny glass needle made specifically for inoculation of virus.

Recognition of arthropod-borne viruses over the last years has regained prominence and attention in the press (Gratz, N.G., 1999). There has been an outbreak of vector-borne diseases such as Malaria, Dengue, and Plague, which have been inactive for a long time in areas where these diseases were thought to be under control. According to Gratz 1999, ecological changes and development projects such as urbanization and deforestation have resulted in the appearance of new diseases and reoccurrence of old ones. Infectious agents are also spread due to increased traveling by people who have introduced the virus to areas where it has been absent. It is important to know how the disease is transmitted in order to prevent appearance of new diseases and reoccurrences of old ones, and also to serve as a model for effective control.

According to Romi, *Ae. albopictus* is considered an underestimated health problem in Italy, to which more attention should be given (Romi, R., 2001). Arboviruses can cause serious problems for eco-tourism. Another example of arboviruses, besides Sindbis virus, is the

West Nile virus. This virus caused Disney World in Florida to be shut down for a day in April 2001. According to Dr. Dame (2001), when Disney World shut down during mosquito control efforts, this sent a strong message to the public that the mosquito control efforts should be taken seriously (New York Academy Science, 2001.)

Materials and Methods

Insectary Conditions

The insectary is a controlled growth room with bio-safety level-2 certification. All mosquito groups were hatched and reared under the same insectary conditions. Thermostatic control temperature, humidifiers and light controls were used to maintain temperature (23 - 26° C), relative humidity (70 – 80%) and photoperiod (16 hours light and 8 hours dark).

Hatching

A small jar was filled with 1% nutrient broth of bacto beef extract in tap water and an egg sheet was submerged into this broth (Difto Laboratories, Detroit, Michigan). Within a few hours, the eggs were hatched and the larvae were observed. A few hours or even overnight were allowed for the eggs to hatch before removing the larvae from the jar.

Rearing

Newly hatched first instar larvae were counted individually and placed into rearing pans. Larvae were reared at equivalent densities, 300 larvae/1500ml. During this stage, larvae were fed three times per week, using 10ml of 2% liver powder per rearing pan. The food was decreased to 5ml of 2% aqueous liver powder, when water was cloudy to prevent surface scum. Pupae were separated by gender and transferred to cages.

Pupae transfer to cages

Pupae were placed in paper cups with tap water. The cups were then transferred into cages. After emergence, adults were supplied with constant access to a carbohydrate source (honey) and water.

Experimental Design

Groups of *Ae. albopictus* mosquitoes were monitored in the insectary during the SVHR inoculation experiment. Mosquito survivals were counted daily and the dead mosquitoes were removed from the cages. The cages were checked and watered three times per week and kept continuously supplied with honey.

Intrathoracic Inoculations and Infection

Insects were anaesthetized in the cold for 5 to 10 minutes. Mosquitoes were placed on a chill plate under a stereoscopic microscope and inoculated directly into the thorax. This method used compressed air to force the inoculum through a tiny "needle" prepared by drawing out glass borosilicate tubing of known internal diameter to a fine point (Rosen, L., 1974). The outside diameter of the tube is 0.7 to 1.0mm, while the internal diameter has a mean value of 0.469mm+/- 0.002mm. After heating, an alcohol lamp is used to draw the tube to a fine point and jeweler's forceps are used to break off the tip of the tube at the right diameter. This "needle" is connected through plastic tubing to a syringe supported by clamp on a ring stand, which is filled with inoculum and inoculated by pushing down the plunger. Then the mosquito is observed to ensure that the inoculum has invaded the mosquito. Jeweler's forceps are used to dismiss the insect from the tip of the "needle" into a small cage.
Transfer to cages: Survival Following the Intrathoracic Inoculation of Adults.

1. Colony Cage – represented by the ambient control mosquitoes.
2. Mock Infected Cage – represented by Medium Essential Media (MEM) inoculated mosquitoes. This cage was used as a control for the trauma of inoculation.
3. SVHR Infected Cage – represented the SIN inoculated mosquitoes. This cage contained the experimental specimens.

Infection

Survivals were monitored during the whole experiment to provide life span data of the infected mosquitoes as compared to the mock-infected mosquitoes. Examination of mosquito legs provides a rapid and efficient method of determining dissemination status (Turell, M. J. 1984.) To differentiate infected mosquitoes from uninfected, mosquito legs were removed and tested separately in cell culture of Baby Hamster Kidney (BHK-21) vertebrate cells. SIN is cytopathic in vertebrate cell cultures, and if the vertebrate cells show cytopathic effect (CPE), this indicates that the mosquito legs contained SIN. Alternatively, if the vertebrate cells do not show any CPE, the mosquito legs did not contain Sindbis virus (D.F. Bowers, personal communication.)

Results

Intrathoracic inoculation of Sindbis virus in *Ae. albopictus* mosquitoes required many inoculation attempts for training purposes. It is very important for the experimenter to master the intrathoracic inoculation method. If unnecessary pressure is applied during inoculation on the thorax of the mosquito, most of the mosquitoes would die due to the trauma of inoculation and no mosquitoes would be left for experimentation purposes.

Figure 1 shows that intrathoracic inoculation takes practice. Needle sticking and four MEM buffer inoculation attempts were performed for inoculation training purposes. The survival of the needle stick and MEM inoculated mosquitoes in trials 1, 2 and 3 were poor. This was suspected to be due to the trauma of inoculation during the first 24 hours. However, after a lot of practice time, the inoculation techniques improved and 88% survival was observed in trial 4 of MEM inoculated mosquitoes. After the first day, the survival rates remained at greater that 80% for up to two weeks. This 24 hour mortality is due to the trauma of inoculation has been previously described (Moncayo, A.C., 2000).

Figure 2 demonstrates the survival of female *Ae. albopictus* after SVHR inoculation during a 14 day monitoring period. Three groups were monitored: non inoculated group (colony control), MEM mock inoculated group (inoculation control) and SVHR inoculated group (experimental). The non inoculated group demonstrated 100% constant survival rates under ambient control conditions. The mock MEM inoculated group and SVHR inoculated group demonstrated 88% and 60% survival on the first day respectively. After the first day, survival rates remained relatively equivalent. Other than the 24-hour mortality, the survivals of mosquitoes of MEM and SVHR were comparable.

Figure 3 demonstrates the survival of female *Ae. albopictus* after SVHR inoculation in the fall of 2002. Four groups were monitored: Non inoculated group, MEM mock inoculated group, and 2 SVHR groups A and B. The graph was normalized to reflect the results without the 24 hour trauma of inoculation period. The survival of the ambient control, MEM and SVHR groups were comparable during the monitoring period of 32 days. During the first two weeks, the survival rates were above 85%. However during the third week, a decreasing trend in survival was observed. Such declines in the survivals are suspected to be due to the mosquito aging. At week four, SVHR group A and B demonstrated 70

and 85% survival respectively. At the endpoint of the experiment, by day 32, the survival rates in all the cages were comparable and remained above 70%.

Table 1 shows the results of the leg assay analysis, a detection system for the presence of live virus. Six plates were used in the leg assay analysis at day 10 post infection: 2 plates, each containing a mosquito leg from the MEM mock inoculated group, 2 plates each contained a mosquito leg from the SVHR group A, and the last 2 plates each containing a mosquito leg from the SVHR group B. The results were as expected: the MEM mock inoculated mosquito legs tested negative for SVHR, while the SVHR inoculated mosquito legs tested positive for the SVHR. Figure 4 shows a comparison of BHK-21 cell response to uninfected (MEM plate) and infected (SVHR plate) mosquito leg samples. The mock inoculated mosquito legs in the MEM plate tested negative for the SVHR. BHK-21 cells were intact and confluent over the flask substrate. The SVHR inoculated mosquito legs in the SVHR plate tested positive for SVHR. BHK-21 cells were rounded up, clumped together and eventually died.

Table 1. Leg Assay on BHK-21 Cells

Plate Number	MEM	SVHR (A)	SVHR (B)
1	-	+	+
2	-	+	+

Discussion

The mosquito, *Aedes albopictus*, demonstrated persistent infection of the Sindbis virus at day 10 post inoculation. While the mosquito host demonstrates a persistent infection throughout the life of the arthropod (Schlesinger, S., 1986) pathology has been documented (D.F. Bowers et. al., 2003 in press). Arboviruses can cause serious clinical diseases with high fever, encephalitis, hemorrhagia, and death in man and domestic animals (Brown, D.T., et al., 1986). However, Sindbis virus is among the least virulent of the arboviruses and therefore it is used in the laboratory to perform research. In a previous experiment, Schiefer and Smith (1974) conducted research on the susceptibility of 8 different mosquito species: *Culex taeniorhynchus, C. salinarius, Ae. aegypti, An. quadrimaculatus, An. stephensi, Aedes taeniorhynchus, Ae. triseriatus and Armigeres subalbatus* to Sindbis virus infection. *Ae. aegypti* and *C. salinarius* showed highest infectivity and persistence of virus throughout the life of the arthropod (Schiefer, B.A., 1974). *Ae. albopictus* which is similarly related to the *Ae. aegypti also* demonstrated high infection rates in response to SIN at day 10 in this experiment.

This research demonstrates that SIN does not hamper the life span of *Aedes albopictus* at day 32 post infection. According to Moncayo (2000), when mosquito species such as *Coquillettidia perturbans, Ae. albopictus* and *Anopheles quadrimaculatus* were infected with EEE, the life span of *Co. pertubans* was significantly reduced as compared to the uninfected *Co. perturbans,* while the life span of *Ae. albopictus* and *An. quadrimaculatus* was not affected when compared to the uninfected species respectively. Moncayo's experiment supports our data where SVHR, like EEE, does not adversely affect the longevity of *Ae. albopictus*. The *Ae. albopictus* mosquito is used as a host for the SVHR virus in the insectary since SVHR is the least virulent of the Alphaviruses and not a human pathogen. However, *Ae. albopictus* is the natural host of the EEE virus in nature.

Intrathoracic inoculation is a challenging technique and takes practice. The deaths occurring during the first 24 hours were suspected to be due to the trauma of inoculation. Other than the 24-hour mortality, the survival rates were comparable for the control and the experimental groups. At day 16 post infection, all the mosquito groups expressed a $\geq$ 85% survival rates. A generalized decrease in the mosquito survivorship is observed between day 16 and 21st. At day 32, the end point of the experiment, all the mosquito groups expressed $\geq$ 70% survival rates. The normal aging process was a possible factor in the deaths of *Ae. albopictus* mosquitoes. Survival of mosquitoes such as *Ae. aegypti,* which are closely related to *Ae. albopictus,* are age dependent. Results performed from non-linear analysis demonstrated greater survivorships for the younger females than the older females in Puerto Rico, but demonstrated no differences in the survivorships of females with same age in Thailand (Harrington, L.C., 2001). The experiment must be repeated again for accuracy. In this experiment it is important for the experimenter to master the intrathoracic inoculation technique.

Temperatures in the insectary were maintained between 23-26° C (73-76° F) to provide the best growth environment for the mosquito population. Great temperature changes affect the population dynamics of *Ae. albopictus* by reducing reproductivity and increasing mortality rates. *Ae. albopictus* in high temperatures have a higher rate of population growth; however, these populations attain low peak densities of adults. On the other hand, *Ae. albopictus* in low summer temperatures experience slower and steadier population growth with higher peak densities of adults (Alto, B.W., 2001).

Conclusion

This study indicates that Sindbis virus does not shorten the life span of *Ae. albopictus,* even though the virus lives and replicates in the body of the mosquito. Since Sindbis virus does not demonstrate a detectable effect in the life span of the *Ae. albopictus*, this virus does not have a significant effect on the mosquito. It is suggested that the invertebrate *Ae. albopictus* and Sindbis virus interact in harmony (Bowers, D. F., 1995). The survival study is still in progress. If the SVHR infected mosquito lives longer, it can be used in future research as a model host for the other alphaviruses such as EEE and WEE, which cause serious and fatal diseases in humans and domestic animals. It can also be employed in future research to determine any changes in the behavior of the insect host.

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Figure 1. The graph in this figure demonstrate the survivals of mock injected *Ae. albopictus*. The x-axis represents days post inoculation and the y-axis represents the percent survival of the mosquito after the mock injection. Fifty mosquitoes from 5 different cages: 1 needle sticking mosquito cage and 4 MEM inoculated mosquito cages were used for inoculation training purposes. The experiment was monitored for 14 days. The survival rates of the needle stick and MEM inoculated mosquitoes in trials 1, 2 and 3 were poor. This was suspected to be due to the trauma of inoculation during the first 24 hours. Trial 4 reveals better survival rates of MEM inoculated mosquitoes. In trial 4, there was only a 12 % death in the first 24 hours, and after the first day, the survival rates remained relatively equivalent for up to two weeks.

Figure 1. Survival of Mock Injected *Ae. albopictus*

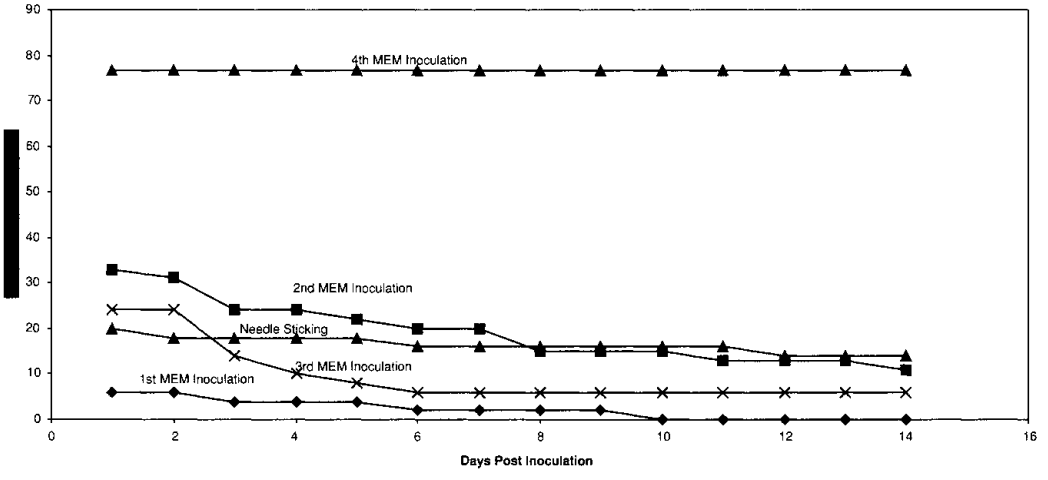


Figure 2. This figure demonstrates the survival rate of female *Ae. albopictus* after SVHR inoculation during a 14 day monitoring period. The x-axis represents days post inoculation and the y-axis represents the percent survival of the mosquito after the inoculations. Thirty mosquitoes were used for the non-inoculated group (colony control), MEM mock inoculated group (inoculation control) and SVHR inoculated group respectively. The non-inoculated group demonstrated 100% constant survival rates under ambient control conditions. The mock MEM inoculated group and SVHR inoculated group demonstrated 88 % and 60% survival on the first day post-infection, respectively. After the first day, survival rates remained constant. Other than the 24-hour mortality, the survivals of mosquitoes of MEM and SVHR were comparable.

Figure 2. Survival of *Ae. albopictus* after SVHR Infection

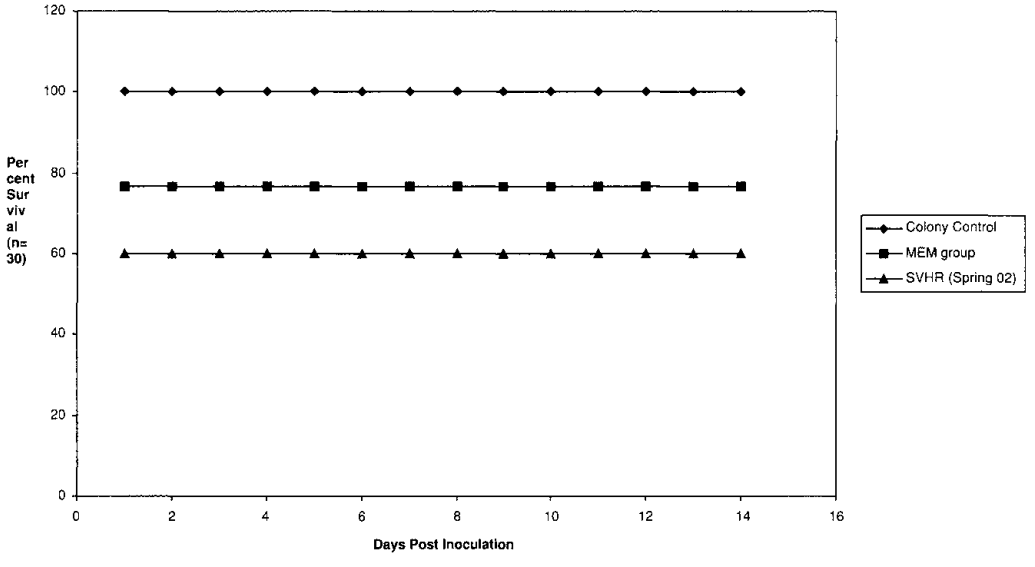


Figure 3. This figure demonstrates the survival rate of female *Ae. albopictus* after SVHR inoculation. Four groups containing 42 mosquitoes each were monitored and represented: the non-inoculated group, MEM mock inoculated group, and 2 SVHR groups A and B. The graph was normalized to 100% reflecting the results without the 24-hour trauma of inoculation period. The survival rates of the ambient control, MEM and SVHR groups were comparable during the monitoring period of 32 days. During the first two weeks, the survival rates were above 85%. However during the third week, a trend in decreasing survival is observed. Such declines in survival are suspected to be due to the mosquito aging. At week four, SVHR group A and B demonstrated 70 and 85% survivals, respectively. At day 32 post-inoculation, the survival rates in all the cages were comparable and remained above 70%. At day 10 of the experiment, a mosquito leg assay on BHK-21 cells was performed to test for SVHR infection.

Figure 3. Survival of Female *Ae. albopictus* Post SVHR Inoculation (n=42)

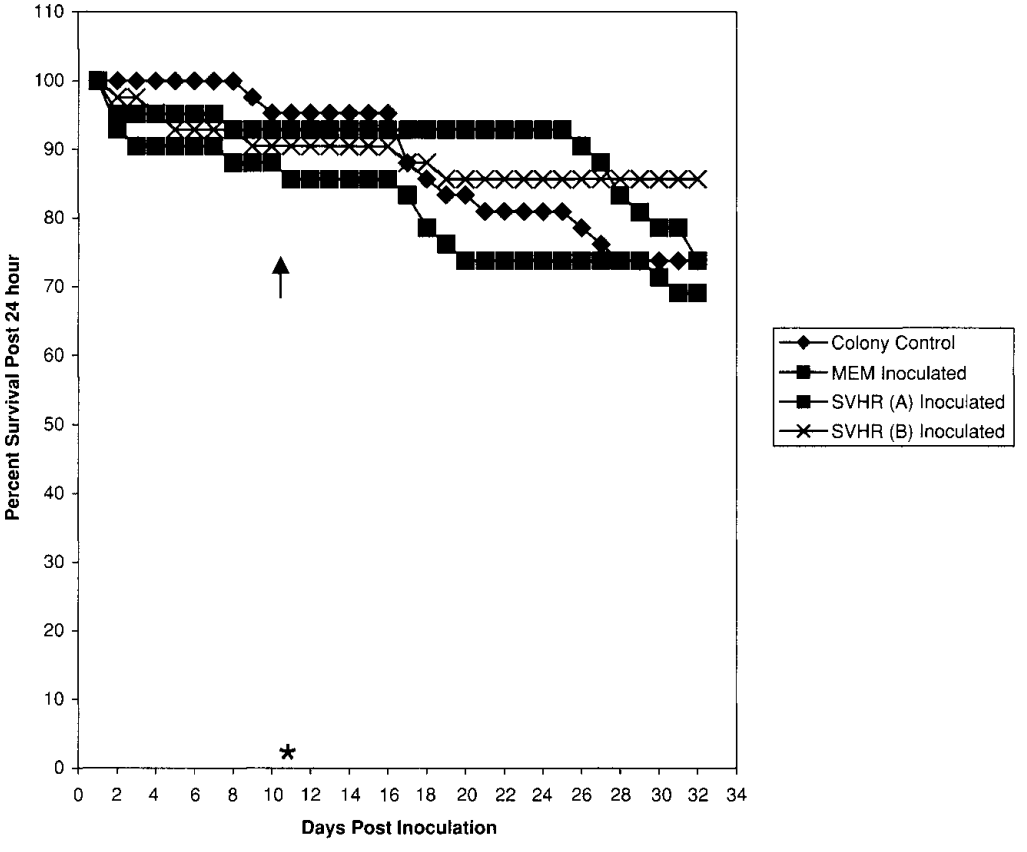
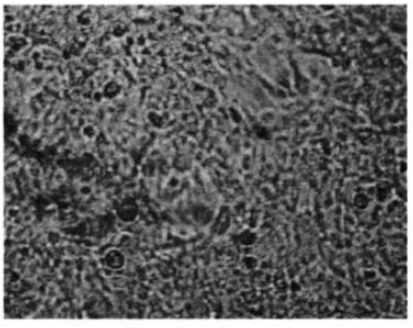
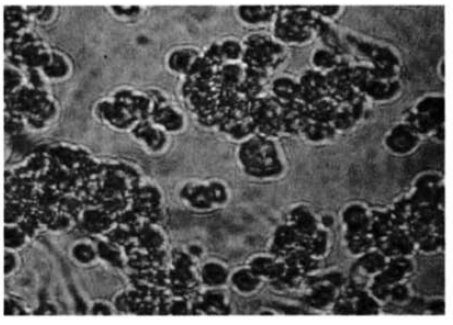


Figure 4. The light micrographs demonstrate a comparison of the effects of mosquito leg assay in the BHK-21 eukaryotic cell cultures. The MEM infected plate and the SVHR infected plate are demonstrated. Legs from mock-inoculated mosquitoes are shown in the MEM plate, and tested negative for SVHR. BHK-21 cells were intact and confluent over the flask substrate. Legs from SVHR inoculated mosquitoes are shown in the SVHR plate, and tested positive for SVHR. BHK-21 cells were rounded up, clumped together and eventually died.



MEM Plate



SVHR Plate