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Research Article



# The Efficiency of the Polytetrafluoroethylene Membrane in Hematophagy of Aedes Aegypti in the Laboratory

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## Abstract

Neglected Diseases, by definition, are morbidities that affect a large number of people in a situation of economic vulnerability and do not have effective treatments or those that, during their therapeutic protocol, pose a high risk of side effects. Examples include Chagas disease, Malaria, and Dengue. Significant challenges affect the research of these diseases, as there is a need for Laboratory Animal Science to advance the study of these diseases by implementing methodologies that replace animals, apply reduction and refinement in the use of animals, and improve the quality of life of the individuals involved in pre-clinical trials at the reference centers for studying these diseases. The objective of our work is to modernize and refine the food maintenance methods for the invertebrate hosts that transmit these diseases, specifically Aedes aegypti. For example, replacing the use of pig membranes (PM) with synthetic membranes and suggesting a new protocol to replace the "Paste Technique," which involves immobilizing the animal for blood feeding. Our methodology was based on the use of Polytetrafluoroethylene Membranes (PTFE) and the evaluation of success in maintaining, reproducing, and sustaining the invertebrate vectors in insectaries. Our results demonstrated that the PTFE met the criteria for bug engorgement time, mosquito weight, egg-laying number, and A. aegypti female longevity, which were similar to those of pig membranes. Thus, by replacing the use of animals and the practicality of acquiring and maintaining this type of PTFE, it becomes an efficient alternative methodology for the hematophagy of mosquitoes in the laboratory.

**Keywords:** Neglected Disease, Alternative Membranes, Aedes aegypti, Hematophagy

## Introduction

Neglected diseases are a group of endemic tropical diseases, found especially among socially vulnerable populations in Africa, Asia, and Latin America. Together, these diseases cause 500.000 to 1 million deaths annually. Among them, diseases caused by Leishmania sp., Malaria, and Arboviruses [1].

Diseases caused by viruses transmitted by blood-sucking arthropods are called arboviruses. According to the World Health Organization, it is estimated that annually 80 million people are infected by viruses transmitted by mosquitoes. An average of 550.000 people are hospitalized, and approximately 20.000 progress to death due to systemic complications of the diseases. Among those existing, the one that most affects the Brazilian population is dengue, transmitted by the mosquito species Aedes aegypti, which can be classical or hemorrhagic [1,2].

Life cycle of the mosquito A. aegypti, showing the stages of egg, larva, pupa, and adult (Figure 1). The biological cycle of the mos-

quito is based on the laying of eggs by females in containers with standing water, where the eggs later hatch, which can take from hours to 3 days, giving rise to larvae that have 4 stages (L1, L2, L3, and L4, with an average duration of 5 to 7 days). Then, pupae emerge, lasting from 2 to 3 days, and subsequently, adult mosquitoes, both male and female [3].

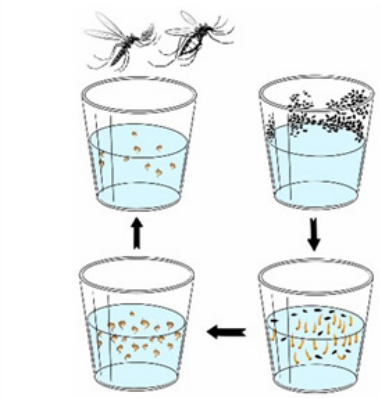

**Figure 1:** Life cycle of A. aegypti. The engorged females carry out their oviposition in clean water (A), which can take from hours to 3 days, giving rise to larvae (B) that have 4 stages (L1, L2, L3, and L4, with an average duration of 5 to 7 days). Then, pupae emerge (C), lasting from 2 to 3 days, and subsequently, adult mosquitoes, both male and female (D).

The study of neglected diseases becomes important in the discovery of new biological mechanisms, host-parasite interactions and the discovery of new drugs. The maintenance of insects in the laboratory is a vital practice in preclinical trials. Vectors must be able to develop their entire life cycle for the reproducibility and reliability of study results.

Various artificial methods for feeding mosquitoes have been developed and used to maintain these insects in the laboratory, some simple, others more complex [4,5]. Normally, these feeders can have one or more blood reservoirs for feeding the insect colonies. We emphasize that for mosquito feeding, the blood used must meet certain parameters, such as: a) females must ingest a sufficient amount of blood for digestion and vitellogenesis; b) artificial feeding must provide for the production of large batches of eggs laid by them, similar to females fed on natural host sources; c) it should not alter the behavior of the mosquitoes [6]. To maintain the proper temperature of the blood in the reservoirs of artificial feeders, certain procedures are necessary, which may vary according to the technique used for this purpose [7].

Most of the time, blood feeding is performed using physically restrained mice using the "Paste Technique" and not anesthetized, since the presence of anesthetic metabolites in the bloodstream influences the appetite and transmission of the vector to the host, compromising the reliability and reproducibility of the test. This management is falling into disuse because the well-being of the animal is seriously compromised. However, it is the best form of hematophagy for female A. aegypti [8]. The ideal would be to standardize with more modern methodologies. In relation to mosquitoes, the solution would be to standardize insectariums with the Hemotek® apparatus (Hemotek, United Kingdon). This would achieve the maintenance of vectors, the quality of research and the replacement of the use of animals. However, its high acquisition cost makes it unfeasible [9].

Porcine membranes are currently widely used (PM). This membrane comes from the intestines of pigs and is acquired informally from slaughterhouses without sanitary control. In addition, it has the disadvantage of being kept in refrigerators inside the laboratory and undergoing an entire preparation process so that it can be used to feed mosquitoes. This method, to date, is the most efficient in achieving hematophagy parameters for A. aegypti, and it also replaces the use of laboratory animals [7].

Alternative methodologies that are being studied include examples of synthetic materials for artificial feeding of mosquitoes [8,9]. Polytetrafluoroethylene membranes (PTFE) are a product composed of a thin film of collagen, a delicate material, thus allowing the rapid removal of the net from a product without damaging the finished products. The transparent cellophane membrane (TCM) is ideal for efficient packaging, protecting and preserving the quality of your products. These products have the advantages of being standardized, low cost and accessible to any laboratory. The critical point is to test whether the female mosquito A. aegypti can use its mouthparts to pierce the material and carry out its complete hematophagy [10].

The objective of our study is to compare the effectiveness of engorgement during blood feeding between PM and artificial membranes, such as PTFE. The parameters evaluated will be: a) Number of females that performed the hematophagy; b) Typification of the evolutionary form of the mosquito in its cycle performing the hematophagy; c) Total time of each evolutionary stage performing the meal; d) Survival and mortality of insects and of each evolutionary stage in each group; e) Each female that completes the blood meal

will be counted, separated and confined for oviposition. f) The eggs obtained will be placed to dry at room temperature and then counted, identified and stored in a humid chamber for later hatching and evaluation of viability.

## Materials and Methods

a) Aedes aegypti females: The mosquitoes were reared in an insectary in a controlled environment with a temperature of 27°C ± 2°C, humidity of 70± 5% and a photoperiod of 12 hours of light and 12 hours of dark. During this rearing, approximately 2.000 eggs hatched in 2,5 liters of dechlorinated water in 6 white plastic polypropylene basins, covered with a fine fabric screen on the lid for the development of the larvae. Thus, they were fed every three days or when necessary, with fish food in flakes (TetraMin®, Brazil), 1 to 2 grams or according to the needs of the larval stages. The pupae were transferred to plastic cups with the aid of a Pasteur pipette, placed inside the cardboard cages (25 x 18 cm) and glass cages (30 x 30 x 30 cm) for the emergence of the adults. The insects, male and female, will be fed with a 10% sucrose solution. Prepared as follows: 25 grams of crystal sugar were added to a glass beaker, weighed on a semi-analytical balance, then 250 ml of dechlorinated water were added. Around 170 females were separated and confined in groups of 30 females for each cage, totaling 5 cages (transparent glass 15 x 15 x 15 cm), with the females fasting from sugar for 48 hours. To perform the tests, females aged between 6 and 7 days were tested. The 30 excess fasting females were kept replacing dead females in the test cages [11].

b) Membranes preparation: Two types of membranes were used: the porcine membrane, to desalt 200 grams of the membrane, it was weighed on a semi-analytical scale. A rigorous wash in running water until all apparent salt was removed. Place it in a plastic bowl, spreading the sauce with 5 liters of water for 4 days in the refrigerator. Change the water twice a day. Cut the membrane into 6 mm pieces and store in 15 mL Falcon tubes, keeping frozen in the freezer -10oC, with a shelf life of 6 months [7]. The polytetrafluoroethylene membrane was immersed separately in a glass container with warm water (37°C to 39 °C, for approximately 30 minutes, after which they were washed in running water). The procedures were repeated 3 times to remove fat and minimize the smell. After this was done, they were stored separately in plastic jars with a little water and closed to prevent drying out [11].

c) Membrane efficiency tests: The tests aimed at evaluating the efficiency of each membrane were carried out by performing three tests with the same type of membrane. In these tests, we typified: i) Engorged females (fully fed) and ii) Unfed females fed (insignificant blood in the abdomen). Only the engorged females (of each membrane) were confined in an acrylic petri plate (60 x 15 mm) lined with filter paper divided into quadrants, moistened with 0,5 ml of dechlorinated water and a small cotton ball soaked in a 10% sugar solution to feed the female during confinement for 60 minutes, for laying, counting eggs and observing the longevity of the females and puppies.

d) Statistical analysis: The values between both items studied were analyzed through the mean value and its standard deviation (±SD). Statistically, we used the Nonparametric Mann Whitney and the Student T tests.

## Results

During the tests with both types of membrane, we observed the hematophagy of the females and recorded the engorgement time of each insect. The PTFE membrane presented similar mean time values to the PM membrane (PTFE: 2,42±0,8 and PM: 2,53±0,9 minutes) (Figure 2). Through hematophagy, we estimated the body weight of each female of A. aegypti, visualizing the difference in sucrose and blood intake between the two types of membrane.

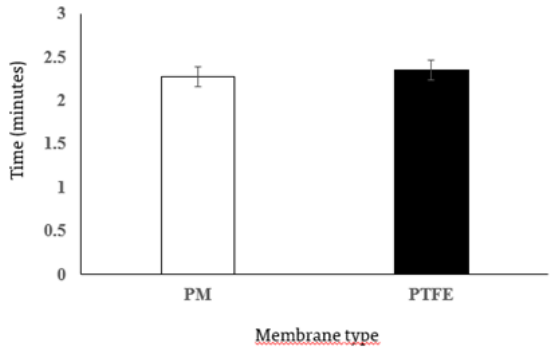


**Figure 2:** Hematophagy time: Time taken for A. aegypti females to engorgement with blood showed a similar period between the use of the porcine membrane (PM, white bar) and the one made of polytetrafluoroethylene (PTFE, black bar). No statistical significance was calculated in the Mann Whitney or Student's T test. Mean values were calculated relative to the standard deviation of triplicate assays. (±SD).

We measured the body weight of females groups on two types of sucrose diet and after ingesting blood. We observed an increase in body weight of around 57% for both females that used PM and PTFE between the sucrose and blood diets. The average weight values of A. aegypti females using PTFE in sucrose regime was 0.016±0,001 grams and after hematophagy it was 0.029±0,001 grams. When

using PM the average weight of females under sucrose offer was 0.016±0,001 grams increasing to 0.028±0,001 grams when engorged with blood (Table 1). Blood ingestion similarly increased the body weight of females using both PM and PTFE, respectively.

**Table 1:** Average body weight of female mosquitoes: We determined the individual weight values of female mosquitoes by 08 groups in the laboratory. Initially, the diet consisted of sucrose and we weighed them before hematophagy. After engorgement of each female, we weighed them again to determine the average weight of a female and the weight gain between diets.

| Groups | Weigth Body | | | |
|---|---|---|---|---|
| | PM | | PTFE | |
| | Sucrose | Blood | Sucrose | Blood |
| 01 | 0,016±0,001 | 0,029±0,001 | 0,015±0,001 | 0,030±0,001 |
| 02 | 0,016±0,001 | 0,028±0,001 | 0,018±0,001 | 0,029±0,001 |
| 03 | 0,018±0,001 | 0,029±0,001 | 0,017±0,001 | 0,029±0,001 |
| 04 | 0,015±0,001 | 0,028±0,001 | 0,015±0,001 | 0,028±0,001 |
| 05 | 0,016±0,001 | 0,029±0,001 | 0,015±0,001 | 0,029±0,001 |
| 06 | 0,017±0,001 | 0,028±0,001 | 0,016±0,001 | 0,030±0,001 |
| 07 | 0,017±0,001 | 0,029±0,001 | 0,017±0,001 | 0,028±0,001 |
| 08 | 0,018±0,001 | 0,028±0,001 | 0,016±0,001 | 0,029±0,001 |
| Median values | 0,016 | 0,028 | 0,016 | 0,029 |
| ±SD | 0,001 | 0,001 | 0,001 | 0,001 |

Table 2: Life cycle of A. aegypti in the laboratory. We induced the reproduction of the mosquito life cycle in the laboratory through artificial feeding, comparing two different membranes: PM and PTFE. We calculated the number of engorged females, oviposition, larval and pulp stages, and sexing of adult mosquitoes.

| Life stages | Membranes | |
|---|---|---|
| | PM | PTFE |
| Engorged females | 9,7±1,5 | 12,7±4,6 |
| Females/ovoposition | 8,0±1,7 | 10,7±4,6 |
| Eggs total | 346,3±145,7 | 428,7±334,8 |
| Larvae | 235,7±128,8 | 330,7±236,7 |
| Pupae | 232,3±127,2 | 328,0±237,2 |
| Adults | 229,7±127,2 | 325,0±235,5 |
| Adults males | 120,3±63,8 | 172,7±128,2 |
| Adults females | 110,3±63,1 | 152,3±107,4 |

Comparing the types of membrane used for hematophagy of 30 females per experiment of A. aegypti, a similar life cycle was observed. During the use of PTFE, we observed an average of 12,7±4,6 engorged females, while the use of PM demonstrated values around 9,7±1,5 females. Of these females, 10,7±4,6 laid eggs on PTFE and 8,0±1,7 on PM. Resulting in a total number of eggs laid of 428,7±334,8 when PTFE was used and 346,3±145,7 at the PM use. Regarding the evolutionary stages, we counted the average number of larvae, pupae and adults. For the PTFE membrane, the values were 330,7±236,7; 328,0±237,8 and 325,0±235,5 respectively, while when using PM, the values were 235,7±128,8; 232.3±127,2 and 229.7±127,2 adults. Regarding the sexing of this life cycle, we estimated an average of 172.7±128,2 male mosquitoes and 152.3±107,4 females when using PTFE and 120,3±60,3 males and 110,3±60,1 females when using PM (Table 2).

Regarding oviposition after engorgement of females, we counted the number of eggs of each female of A. aegypti in the tests. On average during the use of the PTFE membrane, the average number of eggs was 37,6±1,8 eggs per female and during the use of the PM membrane, the average number of eggs was 41,8±13,5 eggs. We emphasize that the variability of individual oviposition was higher when the PM membrane was used (Figure 3). In relation to lifespan of each female using the PTFE membrane when compared to the PM membrane, we observed similar times. The longevity of the females that were offered the PTFE membrane was 13,7±2,5 days, while when the PM membrane was used it was 14,3±3,7 days of life (Figure 4).

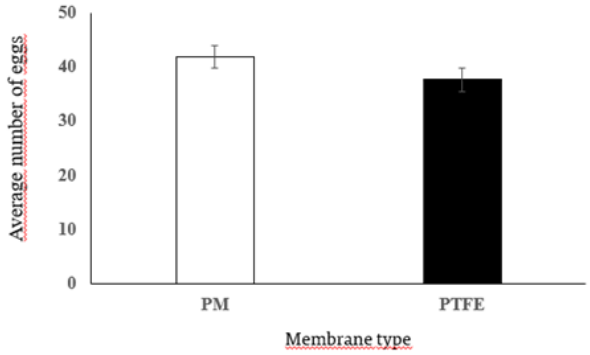


**Figure 3:** Average egg values: After oviposition, we counted the number of eggs that each female laid in her life cycle. The average number of eggs of the females that used PM (white bar) was similar to the females that used PTFE (black bar). No statistical significance was calculated in the Mann Whitney or Student's T test. Mean values were calculated relative to the standard deviation of triplicate assays. (±SD).

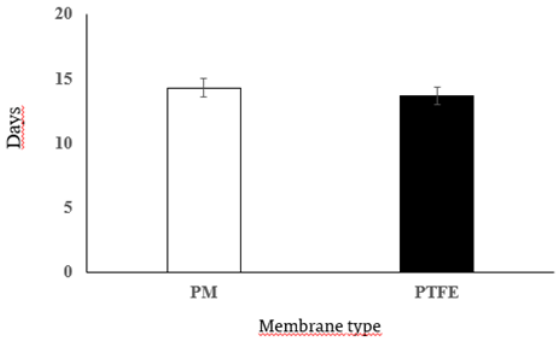


**Figure 4:** Female longevity: We estimated the lifespan of A. aegypti females in the laboratory after completing their life cycle and compared the days between females that were offered blood by PM (white bar) and PTFE (black bar). No statistical significance was calculated in the Mann Whitney or Student's T test. Mean values were calculated relative to the standard deviation of triplicate assays. (±SD).

## Discussion

The process of hematophagy in mosquitoes is not recent, it dates back to around 145 to 65 million years ago, in the Jurassic and Cretaceous periods [12,13], with an approximate date of 90-100 million years. Fossil of a female mosquito classified in the genus Burmaculex and species antiquus found in amber, with its morphology well preserved, including containing blood in the abdomen, in the city of Myanmar (ancient Byrmania) in 1999 [14]. This eating habit, during evolution, allowed these insects to carry several pathogens to humans and animals. Neglected diseases that affect a large number of people around the world stand out. Among the neglected diseases that have the mosquito as a vector, we highlight leishmaniasis, malaria and arboviruses [1].

The mosquito A. aegypti originates from the Old World, proba-

bly from the Ethiopian region. It was first identified and described in Egypt. This species has accompanied human migration throughout the world, settling wherever anthropogenic changes have favored its development and proliferation [15,16]. In Brazil, the great importance of A. aegypti lies in the fact that it is considered the main vector of urban yellow fever [15], dengue [16], chikungunya [17] and zika [18].

The study of these diseases in research institutions requires the formation of insect colonies maintained in laboratories that allow the reproduction of several species on a large scale. For this to be possible, some requirements are necessary for the success of insect production. It is essential to have controlled environments, with photoperiod, temperature and humidity appropriate to the needs of each species, associated with a structured environment for insect management [3,19].

Currently, for ethical and animal welfare reasons, the use of animals to feed these insects in laboratories is being replaced. Artificial methods then emerging, which vary in quality and mainly in cost. The use of these techniques offers advantages over traditional methods used to date, as it reduces the risk of pathogen transmission and increases the safety of insectariums. In addition, it is possible to standardize the food source and make it available at any time [19].

Several devices have been developed, are being studied and are being used to feed mosquito colonies in the laboratory. Basically, these devices consist of a reservoir for storing blood, which is surrounded by thin films, called membranes, which can be of artificial or natural origin, and which allow the penetration of the proboscis of female mosquitoes, to feed on the concentrated blood cells. The way to attract females to these artificial devices is by heating this blood confined in the reservoir [20-23].

In our study, we used porcine membrane as a standard for feeding A. aegypti mosquitoes kept in the laboratory. However, as previously described, this membrane is not ideal. It is of dubious origin, lacks sanitary safety and is inconvenient to be maintained and processed within a research laboratory. Therefore, the search for an alternative membrane that presents the same or superior performance as PM membrane.

The object of study of this work was the use of the polytetrafluoroethylene membrane for blood feeding of mosquitoes. Our results demonstrate that its performance was similar to PM. The engorgement time of A. aegypti females was the same when compared to PM. Similar parameters of the life cycle of mosquitoes in the laboratory. They demonstrated a weight gain of 57% due to blood ingestion and a blood volume also similar when PM was used. Other important factors were oviposition and longevity. The average number of eggs laid by females fed with PTFE was the same as that of females fed with PM, as was their longevity.

These results are in agreement with *Phasomkusolsil, et al*. (2013), who discuss the use of artificial blood reservoirs, covered by membranes, which allow mosquitoes to feed, is increasingly improved, effective and safe. Artificial feeding facilitates controlled experimentation, reducing the risks associated with the transmission of pathogens during mosquito feeding [24].

## Conclusions

Our data demonstrates the efficiency of PTFE membrane when compared with the commonly used PM, which has the advantages of replacing animal use, being aseptically safe, low cost, easy accessibility and hematophagous feeding ensured by A. aegypti mosquitoes in the laboratory.

## Ethical Considerations

There is no need for approval in ethics committees.

## Declaration of Conflicting Interest

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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