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BIOTIN/AVIDIN SANDWICH ENZYME-LINKED IMMUNOSORBENT ASSAY FOR CULICIDAE MOSQUITO BLOOD MEAL IDENTIFICATION

MARASSÁ A. M. (1), ROSA M. D. B. (2), GOMES A. C. (2), CONSALES C. A. (3)

(1) Adolfo Lutz Institute, São Paulo, Brazil; (2) Department of Epidemiology, School of Public Health, University of São Paulo, São Paulo, Brazil; (3) Pasteur Institute, São Paulo, Brazil.

ABSTRACT: The knowledge of mosquitoes Culicidae host feeding patterns is basic to understand the roles of different species and to indicate their importance in the epidemiology of arthropod-borne diseases. A laboratory assay was developed aiming at standardizing the biotin-avidin sandwich enzyme-linked immunosorbent assay, which was unprecedented for mosquito blood meal identification. The enzyme-linked immunosorbent assay (ELISA) activity was evaluated by the detection of titers on each sample of the 28 blood-fed *Culex quinquefasciatus.* In light of the high sensitivity that the technique permits, by means of small quantities of specific antibodies commercially provided and phosphatase substrate which reinforces additional dilutions, human and rat blood meals were readily identified in all laboratory-raised *Culex quinquefasciatus* tested. The assay was effective to detect human blood meal dilutions up to 1:4,096, which enables the technique to be applied in field studies. Additionally, the present results indicate a significant difference between the detection patterns recorded from human blood meal which corroborate the results of host feeding patterns.

KEY WORDS: biotin/avidin sandwich enzyme-linked immunosorbent assay, Culicidae, blood meal identification.

CONFLICTS OF INTEREST: There is no conflict.

CORRESPONDENCE TO:

ANA MARIA MARASSÁ. Laboratório de Parasitoses Sistêmicas, Instituto Adofo Lutz, São Paulo, Brasil. Phone: +55 11 3068-2891. Email: anamarassa@usp.br.

INTRODUCTION

Blood meals taken by Culicidae mosquitoes constitute an important parameter to determine the extension of hosts which may be included in the feeding patterns in different zoonoses cycles. In singular habitats, the blood feeding behavior may provide information with reference to the mosquitoes adaptation and may contribute to the evaluation of their vectorial capacity in epidemiological studies.

Several mainly serological methods have been used by previous investigators to identify blood meals taken by Culicidae (1, 14, 16). Among them, the ELISA test is considered an alternative due to its high sensitivity and specificity to express host-feeding activity (2, 4, 6, 7, 12).

In addition, the establishment of the sandwich ELISA method provided the detection of very small volumes of blood ingested by several arthropod vectors as well as of mixed feeds on different hosts (8-10, 15).

This study aimed to standardize the biotin-avidin ELISA method, which was unprecedented for the identification of blood meal in Culicidae mosquitoes, in order to characterize the feeding habits of the species which are accounted to spread diseases.

Culex quinquefasciatus was selected to take part in this experiment due to its abundance in residential area and easiness of laboratory rearing, as well as based on its importance as an urban vector and as an experimental model to make use of the technique in endemic and epidemic areas where different zoonoses are transmitted by Culicidae.

MATERIALS AND METHODS

Forty-two laboratory-reared *Culex quinquefasciatus* were included in this study. They were originated from the colony of the Laboratory of Entomology, São Paulo Public Health School, USP, Brazil.

The emerging adults were separated in two groups of females and one of males, consisting of 14 individuals each, which had been kept isolated at room temperature at 23–27°C.

Artificial Xenodiagnosis

A blood sample (5ml) was collected from one human individual and from one *Rattus rattus* for artificial xenodiagnosis which involved 14 females of each group of female *Culex quinquefasciatus* kept in the laboratory.

At 24h interval after feeding, the engorged females and males were killed by freezing and stored at -20°C until the ELISA test was performed.

Preparation of Mosquito Samples

Mosquitoes were individually prepared for the test by grinding with a plastic pestle in 750µl of 0.01M phosphate-buffered saline (PBS), pH 7.4, with 0.1% gelatin and centrifuged at 10,000Xg for 10 min, and the supernatant was used for blood meal analyses.

To standardize the test, positive control samples consisted of a 10µl aliquot of each supernatant from the 14 females, which had fed on the blood sample of each host, and the negative control sample was constituted of a 10µl aliquot of each supernatant from the 14 males.

Optimization of the Sandwich Assay

Reagent concentrations were optimized by chequerboard titrations to determine the highest sensitivity and lowest background of mosquito suspensions.

ELISA was conducted using ninety-six well microplates (Nunc®, Maxisorp, Denmark) covered with 50µl/well of each anti-IgG (H+L) host-specific [anti-human IgG 6284-00 Zymed, USA; anti-rat IgG 6295-00, Zymed, USA], diluted in PBS at concentrations of 20µg/ml, 10µg/ml, 5µg/ml, 2.5µg/ml, 1.25µg/ml and 0.625µg/ml and incubated overnight at 4°C. Plates were blocked with PBS/1% gelatin and kept covered at room temperature for 3h.

Each plate was washed five times with PBS-0.05%Tween20 (P-1379, Sigma, USA); then, the competitive reaction was performed in two consecutive wells by addition of 50µl/well of the PBS/0.1% gelatin and 50µl/well of each positive control in each plate. Those samples were diluted from 1:50 to 1:3,200. After 18h at 4°C, biotin conjugated anti-human IgG (6284-40, Zymed,USA) and biotin-conjugated anti-rat IgG (B7139, Sigma,USA) were added.

After 1h at room temperature, 50µl/well of avidin-alkaline phosphatase conjugate (A7294, Sigma,USA) in PBS/1% gelatin was distributed. After 1h, the enzymatic reaction was obtained by addition of p-nitrophenyl phosphate (Sigma Chemical) in diethanolamine buffer. Absorbance was measured by spectrophotometry (Multiskan ® EX) at λ405nm.

ELISA Activity

After the primary antibody concentrations had been established, ELISA activity was evaluated by detecting titers on each sample of the 28 blood-fed *Culex quinquefasciatus*.

The individual blood meal was double diluted with ELISA buffer (PBS/0.1% gelatin) and the positive and negative controls for each host were included in each plate.

To titer *Culex quinquefasciatus* samples containing human blood, 5 $\log_2$ (1:32) was considered as the initial dilution and to titer samples containing *Rattus rattus* blood, 4 $\log_2$ (1:16) was used.

Titers were expressed as $\log_2$ of the reciprocal of the highest dilution at which the absorbance was equivalent to three times the background values.

Variables related to the host samples were analyzed separately and the means were log transformed. Student's t test (PROG SPSS 10.0 for Windows) was used to determine whether there were significant differences in the mean of human and rat samples ($p<0.001$).

RESULTS

As shown in Figures 1 and 2, chequerboard titrations were done with the objective to determine the highest sensitivity and lowest background of mosquito suspensions including the two hosts by the addition of the positive controls in two consecutive wells, and the optimal concentration of anti-human IgG and anti-rat IgG to coat plates was 1.25µg/ml.

Based on what had been settled down for blood-fed specimens of *Lutzomyia* (*Lutzomyia*) *longipalpis* (8, 10), the concentrations of 1µg/ml of biotin anti-human IgG, 2µg/ml of biotin anti-rat IgG and 1:40,000 dilution of avidin were incorporated. This protocol for ELISA had the objective to minimize the consumption of reagents without interfering with the test sensitivity.

According to Figures 1 and 2, the absorbance values which allowed the cutoff point to optimize the sandwich assay were 0.350 for human blood meal sample and 0.240 for rat blood meal sample.

The next step was to verify the avidin-biotin ELISA test activity by the titration of the samples which were bound for the detection of the smallest blood meal dilution from mosquitoes fed on human and rat blood.

The ELISA detected human blood meals in all samples that were tested (Figure 3) and the mean value was $10.36\log_2$; 1.39∂; $1.94\surd$. The absorbance value for cutoff was 0.237.

For each plate, positive and negative controls were included and the mean values were determined as $11\log_2$ and $1\log_2$.

According to what is observed in Figure 3, the mean value for human blood meal dilutions was 1:2,048; nevertheless, the assay detected blood meal dilutions up to 1:4,096 ($12\log_2$).

Results of the dispersion of the 14 samples of females that had been fed on rat blood are also represented in Figure 3 and the mean value was $5.5\log_2$; 0.76∂; $0.58\surd$. The absorbance value for cutoff was 0.226.

Positive and negative controls were also included in each plate and the mean values were determined as $6\log_2$ and $1\log_2$.

The present results indicate a significant difference in samples of *Culex quinquefasciatus* which had fed on human and rat blood ($U=1$; $z=-4.57$; $p<0.001$).

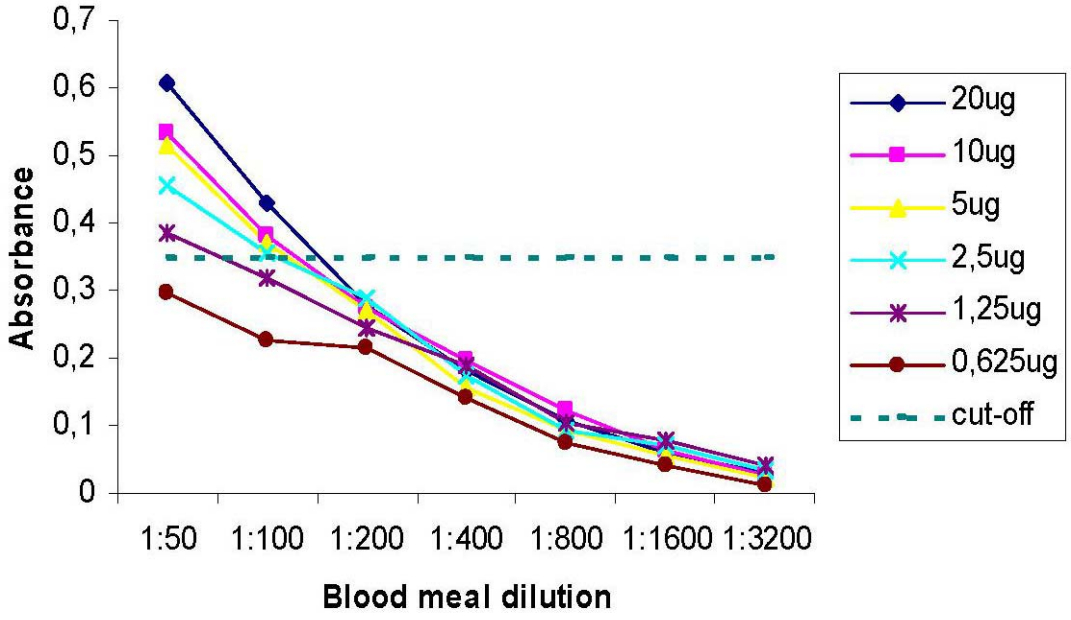

Figure 1. Optimization of the biotin/avidin ELISA assay for identifying human blood meal dilutions for *Culex quinquefasciatus*.

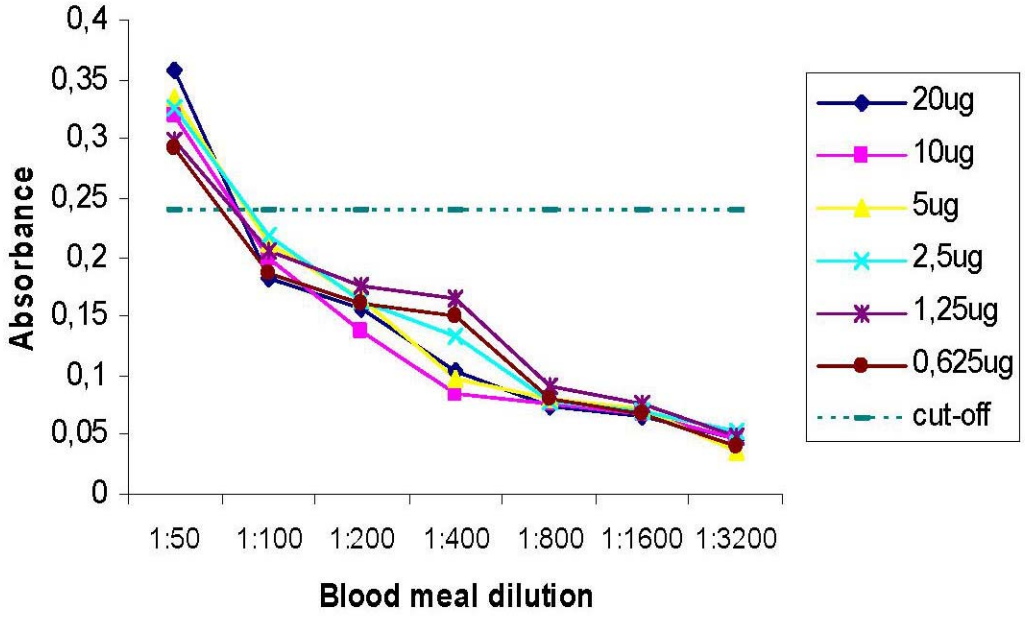

Figure 2. Optimization of the biotin/avidin ELISA assay for identifying rat blood meal dilutions for *Culex quinquefasciatus*.

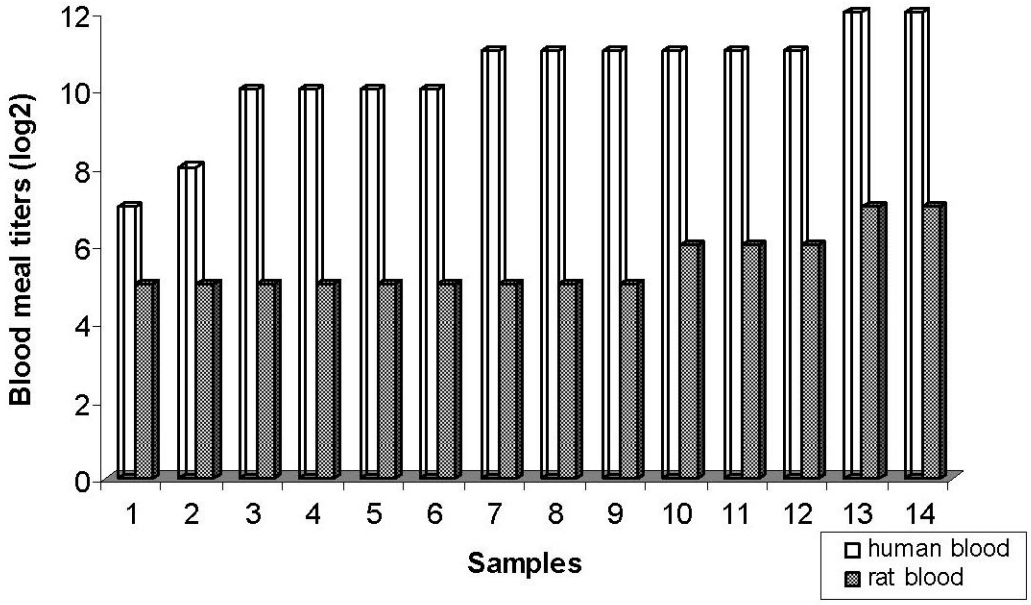

Figure 3. Titers distribution for identified human and rat blood meals of *Culex quinquefasciatus*.

DISCUSSION

The need to further lean data on the activity and frequency at which Culicidae mosquitoes take their blood meals are important to better understand the host-vector relationship, representing an advance in the dynamics of their interrelationship with men, animals and the environment.

The ELISA has fulfilled those conditions and has been used in many ecological investigations (5, 11, 12, 15). In regard to the high sensitivity and specificity, the choice of the avidin-biotin ELISA method described in this study also offers the advantage, based on sensitivity, to detect small amounts of blood taken by phlebotomine sandflies (8, 9, 10) and has been widely applied in other studies (3, 17).

In relation to the samples that were utilized in this study, it was demonstrated that with small concentrations of anti-IgG (1.25µg/ml), anti-IgG biotin-conjugated (1µg/ml for human samples and 2µg/ml for rat samples) and with the avidin dilution of 1:40,000, the test was effective to determine and quantify each one of the samples from this investigation.

Those results are due to the phosphatase substrate (13) which provides additional dilutions and also offers the option of carrying out several tests.

In the second phase of this evaluation, which involved the quantification of samples, the dilution of 1:2,048 that corresponds to $11\log_2$ was the most frequent at the titers detection for human blood meals of *Culex quinquefasciatus* samples (Figure 3). The assay also detected blood meal dilutions up to 1:4,096 of *Culex quinquefasciatus*, which correspond to $12\log_2$, and the difference between titers of human and rat blood samples was patent.

Although, there was a difference in the ELISA method that was used in this investigation, Beier *et al*. (5) could also detect human blood meal dilutions up to 1:3,200 for frozen *Anopheles stephensi* samples.

In this study, the difference between the detection patterns recorded from human blood meal of *Culex quinquefasciatus* corroborate the host feeding results that were obtained by several authors (4, 11, 14, 18), even considering the heterogeneity of samples that are captured in the field.

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