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BIOTIN-AVIDIN SANDWICH ELISA WITH SPECIFIC HUMAN ISOTYPES IgG1 AND IgG4 FOR Culicidae MOSQUITO BLOOD MEAL IDENTIFICATION FROM AN EPIZOOTIC YELLOW FEVER AREA IN BRAZIL

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ABSTRACT: With a view toward investigating the feeding behavior of Culicidae mosquitoes from an area of epizootic yellow fever transmission in the municipalities of Garruchos and Santo Antônio das Missões, Rio Grande do Sul State, Brazil, specimens were collected by aspiration from September 2005 to April 2007. The engorged females were submitted to blood meal identification by enzyme-linked immunosorbent assay (ELISA). A total of 142 blood-engorged samples were examined for human or monkey blood through species-specific IgG. Additional tests for specificity utilizing isotypes IgG1 and IgG4 of human monoclonal antibodies showed that only anti-human IgG1 was effective in recognizing blood meals of human origin. The results indicated a significant difference ($p = 0.027$) in detection patterns in samples of *Haemagogus leucocelaenus* recorded from human blood meals at Santo Antônio das Missões, which suggests some degree of exposure, since it was an area where epizootic outbreaks have been reported.

KEY WORDS: Culicidae, ELISA, feeding habits, isotypes, yellow fever.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Forest canopy mosquitoes of the genera *Haemagogus* and *Sabethes* are known to transmit yellow fever and have recently been involved in major outbreaks of the disease in Brazil (1). Understanding their blood-feeding habits is thus of the utmost importance and permits the estimation of the degree of the human-vector contact significant to epidemiological studies.

In recent years, several investigators have adapted the enzyme-linked immunosorbent assay (ELISA) to identify hosts from which mosquitoes had taken a blood meal (2-6). In addition, the biotin-avidin sandwich ELISA assay has also been included in analyses of mosquito feeding habits and other arthropod-borne diseases (7-10).

This study sought to evaluate species-specific immunoglobulin IgG and human isotypes IgG1 and IgG4 through the use of monoclonal antibodies and exploit the specificity attainable for to identify human blood meals consumed by mosquitoes in an area of epizootic yellow fever transmission in the state of Rio Grande do Sul, Brazil.

MATERIALS AND METHODS

Study Area and Culicidae Sampling

Field studies were conducted at two localities in Rio Grande do Sul state, namely Santo Antônio das Missões (55°21'W, 28°29'S) and Garruchos (55°45'W, 28°17'S), from September 2005 to April 2007. Garruchos, situated on the western side of the Uruguay River, is a rural municipality 13 km from Santo Antônio das Missões.

A large-bore aspirator was utilized to collect resting mosquitoes from vegetation at two sites in Garruchos – one of them located on Cachoeirinha farm and the other in the São José Velho area – while in Santo Antônio das Missões, only one site was selected for investigation (11). Engorged female samples were placed individually in microtubes, transported to the laboratory and stored at –20°C until the ELISA test could be performed. Mosquitoes were identified to the species level in accordance with Consoli and Oliveira (12) and Forattini (13).

Blood Meal Analyses

Blood-fed mosquitoes were identified using the biotin-avidin ELISA by the methodology of Marassá *et al.* (9).

The behavior of feeding on humans or monkeys was investigated using host-specific anti-human IgG (6284-00, Zymed, USA); anti-monkey IgG (617-101-012, Rockland, USA); biotin-conjugated anti-human IgG (6284-40, Zymed, USA); and biotin-conjugated anti-monkey IgG (617-106-012, Rockland, USA). All the tests carried out with extracts that were positive for human or monkey blood, by ELISA, included human IgG1 and IgG4 monoclonal antibodies.

ELISA Assay Optimization

Reagent concentrations were optimized by checkerboard titrations to determine the highest sensitivity and lowest background of mosquito suspensions. Ninety-six-well microplates (Nunc®, Maxisorp, Denmark) were coated with 50 µL/well of anti-human IgG1 (05-3300, Zymed, USA) and anti-human IgG4 (05-3800, Zymed, USA) diluted in PBS at concentrations of 20, 10, 5, 2.5, 1.25 and 0.625 µg/mL and incubated overnight at 4°C. The plates were blocked with PBS/1% gelatin (Difco, Laboratory Inc., USA) and kept covered at room temperature for three hours.

Each plate was washed five times with PBS 0.05% Tween-20 (P-1379, Sigma, USA); then the competitive reaction was carried out in two consecutive wells by the addition of 50 µL/well of PBS/0.1% gelatin and 50 µL/well of positive control pool on each plate. The pool of 14 females that had fed on a human for artificial xenodiagnosis was diluted from 1:2 to 1:64. After 18 hours at 4°C, biotin-conjugated anti-human IgG1 (05-3340, Zymed, USA) and biotin-conjugated anti-human IgG4 (05-3840, Zymed, USA) were added. After one hour at room temperature, 50 µL/well of avidin-alkaline phosphatase conjugate (A7294, Sigma, USA) in PBS/0.1% gelatin was added in each well. After another hour, the enzymatic reaction was obtained by adding p-nitrophenyl phosphatase (Sigma Chemical, USA) to diethanolamine buffer. Absorbance was measured by spectrophotometry (Multiskan® EX, Thermo Scientific, USA) at a wavelength of 405 nm.

Data Analyses

The chi-squared (χ^2) test for proportions was calculated with Excel® (Microsoft, USA) for Windows XP to ascertain whether frequencies of human and monkey samples differed significantly ($p < 0.05$).

RESULTS

The ELISA system for human and monkey blood meals was evaluated for specificity by testing a total of 142 blood-engorged specimens of Culicidae mosquitoes collected at the two municipalities. According to Tables 1, 2 and 3, polyclonal anti-human IgG and anti-monkey IgG cross-reacted and could not provide the higher specificity needed for the detection of human or monkey blood meals.

Table 1. Distribution by purified antibody (IgG) of Culicidae samples from Santo Antônio das Missões, RS, collected between September 2005 and April 2007

	Species								
Purified antibody	*Ae. crinifer*	*Ae. serratus*	*Ae. scapularis*	*Cx. (Cx.)* sp.	*Cx. (Mel.)* spp.	*Hg. leucocelaenus*	*Sa. albiprivus*	*Sa. quasicyaneus*	*Ps. ferox*
Anti-human IgG	2/2	3/4	8/8	1/1	2/2	17/17	6/6	1/1	4/4
Anti-monkey IgG	2/2	2/4	3/8	1/1	2/2	8/17	2/6	1/1	3/4
Anti-human IgG1	1/2	3/4	8/8	0/1	0/2	17/17	6/6	1/1	3/4
Total	2	4	8	1	2	17	6	1	4
Total positive (human)	1	3	8	0	0	17	6	1	3
Total positive (monkey)	1	1	0	1	2	0	0	0	1

Ae.: *Aedes*; *Cx.* (*Cx.*): *Culex (Culex*); *Cx.* (*Mel.)*: *Culex* (*Melanoconion)*; *Hg.*: *Haemagogus*; *Sa. quasicyaneus*: *Sabethes quasicyaneus; Ps.*: *Psorophora*.

Table 2. Distribution by purified antibody (IgG) of Culicidae samples from Cachoeirinha, Garruchos, RS, collected between September 2005 and March 2007

	Species								
Purified antibody	*Ae. crinifer*	*Ae. serratus*	*Ae. scapularis*	*Hg. leucocelaenus*	*Sa. albiprivus*	*Sa. chloropterus*	*Sa. quasicyaneus*	*Ps. albigenu*	*Ps. ferox*
Anti-human IgG	1/1	8/8	18/18	11/11	5/5	1/1	1/1	4/4	12/12
Anti-monkey IgG	1/1	6/8	11/18	7/11	2/5	0/1	1/1	3/4	8/12
Anti-human IgG1	0/1	8/8	18/18	11/11	5/5	1/1	1/1	4/4	11/12
Total	1	8	18	11	5	1	1	4	12
Total positive (human)	0	8	18	11	5	1	1	4	11
Total positive (monkey)	1	0	0	0	0	0	0	0	1

Ae.: *Aedes*; *Hg.*: *Haemagogus*; *Sa. quasicyaneus*: *Sabethes quasicyaneus; Ps.*: *Psorophora*.

Table 3. Distribution by purified antibody (IgG) of Culicidae samples from São José Velho, Garruchos, RS, collected between September 2005 and March 2007

	Species					
Purified antibody	*Ae. crinifer*	*Ae. scapularis*	*Cx. (Cx.) spp.*	*Hg. leucocelaenus*	*Sa. albiprivus*	*Ps. ferox*
Anti-human IgG	1/1	17/17	3/4	7/7	5/5	2/2
Anti-monkey	1/1	17/17	4/4	6/7	5/5	1/2
Anti-human IgG1	1/1	14/17	0/4	5/7	4/5	2/2
Total	1	17	4	7	5	2
Total positive (human)	1	14	0	5	4	2
Total positive (monkey)	0	3	4	2	1	0

Ae.: *Aedes*; *Cx.* (*Cx.*): *Culex (Culex*); *Hg.*: *Haemagogus*; *Sa.*: *Sabethes; Ps.*: *Psorophora*.

Additional tests for specificity were assayed with monoclonal antibodies, anti-human IgG1 and IgG4. Checkerboard titrations were undertaken to determine the highest specificity and lowest background of mosquito suspensions and the results are shown in Figures 1 and 2.

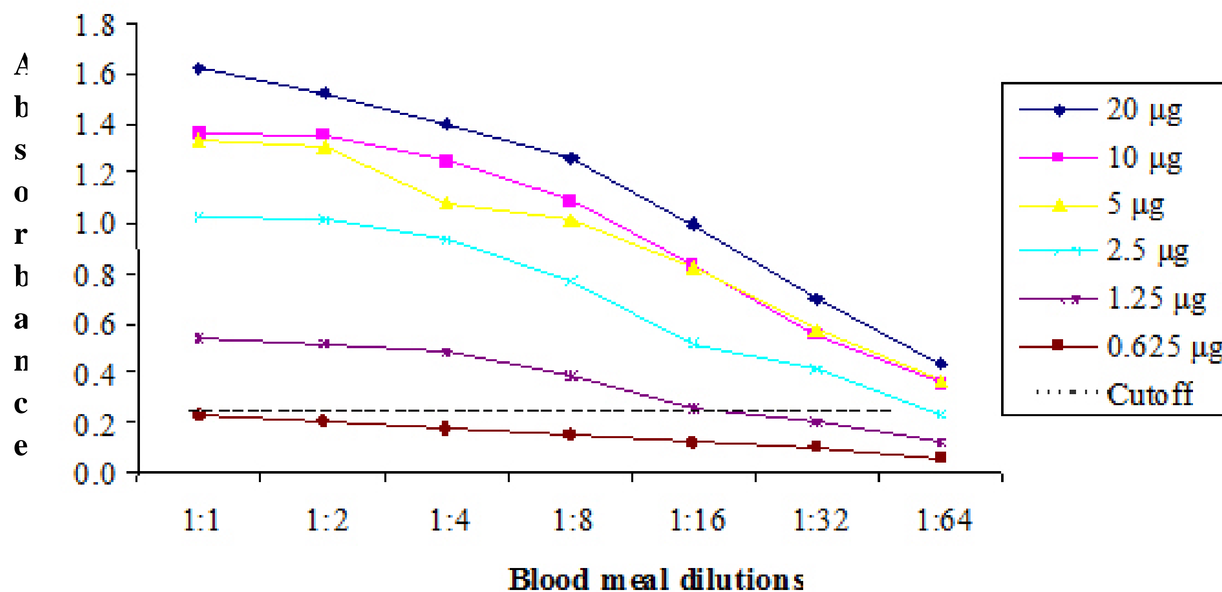

Figure 1. ELISA optimization with monoclonal anti-human IgG1.

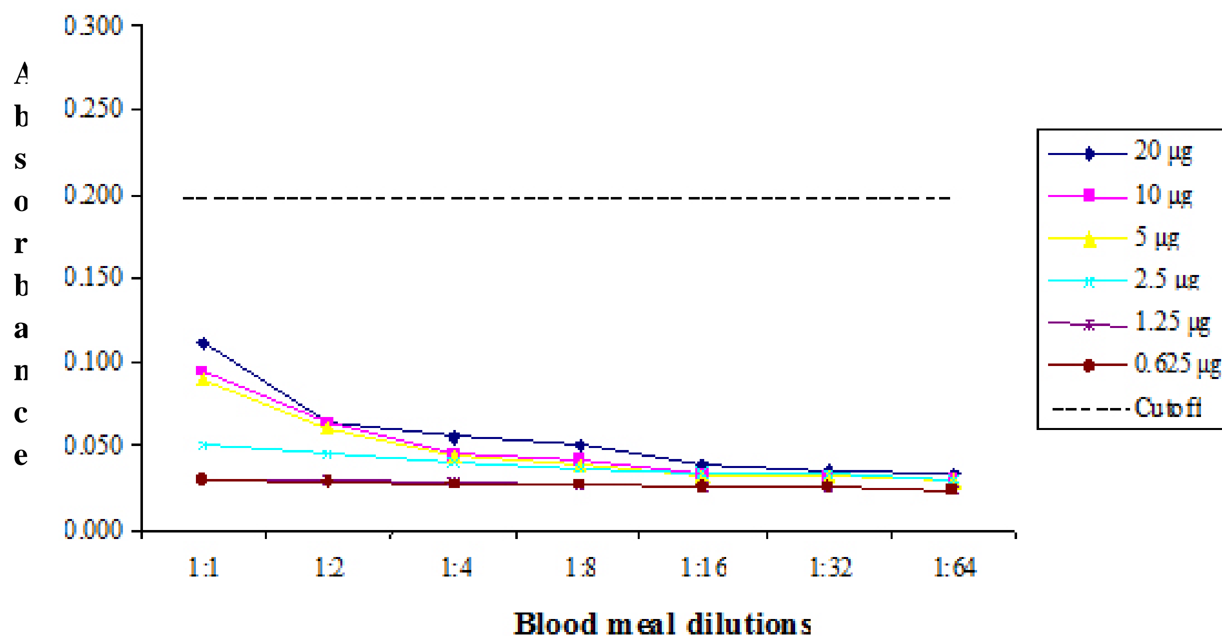

Figure 2. ELISA optimization with monoclonal anti-human IgG4.

The absorbance value that determined the cutoff point to optimize the immunoenzymatic capture technique against IgG1 mAb was 0.207 (Figure 1). By contrast, absorbance values detected by ELISA against IgG4 mAb did not exceed the cutoff value of 0.200 to identify human blood meal samples (Figure 2).

The optimum concentration of anti-human IgG1 mAb for coating the plates was 5 µg/mL and according to what had been previously established for blood-fed specimens of *Culex quinquefasciatus*, dilution of biotin-anti-human IgG1 mAb and avidin was incorporated (8).

Serum dilutions from the primates *Cebus* spp. and *Alouatta* spp. were tested against IgG1 mAb, and were not detected by ELISA. The absorbance values for both *Cebus* spp. and *Alouatta* spp. were less than 0.030 while the mean absorbance cutoff for human blood meal samples was 0.207.

During processing of field-collected mosquitoes, samples investigated for human or monkey blood meal sources comprised specimens of: *Aedes crinifer*, *Aedes serratus*, *Aedes scapularis*, *Culex* (*Culex*) spp., *Culex* (*Melanoconion*) spp., *Haemagogus leucocelaenus*, *Psorophora ferox*, *Psorophora albigenu*, *Sabethes albiprivus*, *Sabethes chloropterus* and *Sabethes quasicyaneus*.

At Santo Antônio das Missões, *Haemagogus leucocelaenus*, *Aedes scapularis* and *Sabethes albiprivus* were the species that most frequently fed on humans (Table 1). At Cachoeirinha, Garruchos, the results indicated that *Aedes scapularis* and *Haemagogus leucocelaenus* had also consumed human blood (Table 2) whereas in the area of São José (Table 3) the frequency was not so evident and no difference was observed between these two sites ($p < 0.05$).

The present data reveal a significant difference only among samples of *Haemagogus leucocelaenus* that had fed on human and monkey blood at Santo Antônio das Missões ($p = 0.027$).

DISCUSSION

Knowledge of mosquito host-feeding patterns is an important indicator for the estimation of species vector potential in disease transmission cycles, and is of great value in the establishment of control measures.

Currently, methods for the identification of insect blood meals have included ELISA, which has been employed to determine host preference and has proven useful for field investigations (2-6). Among these techniques, the use of avidin-biotin system in

ELISA has also been applied to determine sand fly and mosquito blood meal sources (7-10).

Further, sensitivity and specificity are conditions that must be taken into consideration when detecting small amounts of blood consumed by these insects. Thus, monoclonal antibodies offer the advantage of accuracy and have been successfully used in numerous investigations (14-18).

In the present study, the limitations of anti-human IgG and anti-monkey IgG, which revealed cross-reaction with human and monkey blood meals employed in the identification process, were overcome when responses to mAb isotypes were observed.

This was the first report in which anti-human IgG1 and IgG4 were evaluated to identify mosquito blood meals; and our results indicated that only anti-human IgG1 was effective in recognizing blood meals of human origin. Since IgG1 levels were higher (60-70%) than those of IgG4 (2-6%) in human sera and given the small amounts of blood consumed by mosquitoes – ascertained as being 3.46 mg for *Anopheles quadrimaculatus* and 2.75 mg for *Aedes aegypti* – these factors could explain the non-detection of IgG4 (19, 20).

The fact that serum samples from the non-human primates *Cebus* sp. and *Alouatta* sp. presented negative absorbance values indicates that human blood meals can be accurately identified, which reinforces the concept that the number and properties of isotypes vary greatly among species, a fact that could be exploited by means of monoclonals in any application (21). Furthermore, the most important improvement of the technique was the ability to detect of human blood to estimate the exposure to *Haemagogus leucocelaenus* in areas where the yellow fever virus has been isolated.

Subsequent tests that incorporated IgG1 to evaluate field-collected mosquitoes showed a significant difference among samples of *Haemagogus leucocelaenus,* from Santo Antônio, that had fed on human blood. This behavior may suggest human-vector contact and since the yellow fever virus was isolated in monkeys from the two municipalities of Rio Grande do Sul, the detection patterns recorded for samples of *Haemagogus leucocelaenus* emphasize the risk of yellow fever transmission and highlight the need for careful monitoring in the area (1, 22).

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