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Short Communication

Chicken eggs as a surveillance tool for malaria and leishmaniasis vector presence

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Abstract

Introduction: Malaria and leishmaniases are transmitted by vectors during blood-feeding. Vector-infected animals develop antibodies against the vector's saliva. This study evaluated IgY antibody detection in the chicken eggs exposed to bites from *Migonemyia migonei*, *Lutzomyia longipalpis* and *Anopheles aquasalis*. **Methods:** We used ELISA to quantify the antibody levels in the sera and exposed chicken eggs. **Results:** High IgY levels were observed following immunization; furthermore, higher reactivity was observed in the eggs and species-specific immune response was observed post final immunization. **Conclusions:** Chicken eggs can be used as sentinels to surveil vector saliva antibodies.

Keywords: *Lutzomyia longipalpis*. *Migonemyia migonei*. *Anopheles aquasalis*. Sentinel surveillance. Chicken. Salivary proteins.

Malaria and leishmaniases are vector-borne diseases caused by protozoans. Adult Phlebotomine and Anopheline females are vectors of *Leishmania* and *Plasmodium*, respectively[1]. These parasites are transmitted to vertebrate hosts during blood feeding[2]. Furthermore, saliva proteins are introduced into the hosts, inducing immunogenic responses such as anti-homeostatic and vasodilatory effects on the skin[2,3].

Antibodies developed against the hematophagous arthropods saliva were used as exposure markers for different vector species. Several studies report that humans and other vertebrates can develop antibodies against salivary components of different hematophagous vectors, such as mosquitoes and sand flies (e.g Andrade et al. 2009, Souza et al. 2010)[4,5]. In endemic areas, several animals, including chickens (*Gallus gallus*), serve as blood meal sources for sandflies and anopheline mosquitoes. Since chickens suffer frequent exposure to insect bites, they serve as ideal subjects for serological analyses of antibodies developed against the hematophagous arthropod saliva[6,7]. Egg yolks facilitate efficient and non-invasive sampling of antibodies. This study assessed the viability of using eggs as a tool to monitor exposure to leishmaniases and malaria vectors by detecting IgY antibodies in the eggs of chickens exposed to bites from *Migonemyia migonei*, *Lutzomyia longipalpis* and *Anopheles aquasalis*. Although chickens are important epidemiological survey tools, no studies have examined the use of chickens in detecting antibodies against sand fly saliva in an area endemic to cutaneous leishmaniasis.

The study protocol was approved by the Animal Ethics Committee of the Federal University of Amazonas (035/2013-CEUA/UFAM). We performed an experimental study using 12 chickens divided into four groups of three each. One group

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was exposed to bites from the sand fly species *Lutzomyia longipalpis*, a vector of visceral leishmaniasis in Brazil. A second group was exposed to bites from the phlebotomine species *Migonemyia migonei*, a vector of cutaneous leishmaniases. Both sand fly species are permissive vectors for several *Leishmania* species[8]. A third group of chickens was exposed to bites from *Anopheles aquasalis*, a vector of *Plasmodium vivax* and *P. falciparum*[9]. A fourth group was not exposed to any vector bites and served as the negative control. All vector specimens were obtained from lab colonies maintained in insectaries at Oswaldo Cruz Foundation-Leônidas & Maria Deane Institute (FIOCRUZ/ILMD) facilities.

Chickens were administered four immunizations in total and immunized once every 15 days. Before immunization, blood sample from each chicken was collected to detect antibody production. During each immunization, chickens in the experimental groups were exposed to bites from 30 insects of a given species. One egg and 0.5 mL of blood was collected from every chicken after each vector repast, and a total of 48 eggs were collected for immunological testing. Antibodies were extracted from eggs and blood samples and analyzed using ELISA. Chicken egg antibodies were extracted using the precipitation method with Polyethylene Glycol[10]. The process entails removing lipids from the egg yolk and precipitating total IgY from the supernatant. The quality of the final preparation was analyzed by SDS-PAGE.

For the titration ELISA, dilutions of 1/100 to 1/12800 were used. 1 µL of the sample was placed in 200 µL of PBS / BSA block buffer; furthermore 1 µL of this solution was diluted with 100 µL of PBS / BSA and the procedure was repeated until a dilution of 1/12800 was achieved. In the cross-reactivity test, ELISA was performed using the salivary gland sonicate of *Migonemyia migonei* as an antigen to test the egg and blood samples of chickens exposed to bites from *Lutzomyia longipalpis* and *Anopheles aquasalis*, and to test negative control chicken samples. This procedure was repeated with samples exposed to the other hematophages.

In order to detect IgY antibodies using ELISA, antigen lysates were procured from 20 salivary glands taken from the three-day old female species of *Migonemyia migonei*, *Lutzomyia longipalpis* and *Anopheles aquasalis*. The purified IgYs were used as the positive control and samples collected before vector repast were used as the negative control. An indirect ELISA was performed: 0.1mL/well salivary gland extract from *M. migonei*, *L. longipalpis* and *A. aquasalis* (equivalent to 1 pair of salivary glands/mL) was used to coat ELISA plates, according to Barral et al. (2000)[11]. Plates were incubated overnight at 4ºC. Furthermore, the plates were incubated with sera from chickens and IgY from eggs after three washes with PBS-0.05% Tween. The plates were blocked for 1 hour at 37ºC in PBS-0.05% Tween and 5% non-fat milk. Furthermore, the plates were incubated with antibody anti-IgY for 1 hour at 37ºC, after washing. Post the fourth washing cycle, the reaction was developed for 5 minutes using a chromogenic solution of tetramethylbenzedine 1M (TMB-Sigma) and hydrogen peroxide (H_2O_2). Optical density (O.D) was determined at 450 nm.

Antibodies were produced in the chicken eggs exposed to all three insect-vector species, and antibodies were detected as early as the first immunization (**Figure 1**). This data demonstrates the high immunogenic capacity of salivary gland antigens.

Results from the pre-immunization samples wherein no antibodies were present are represented in **Figure 1** as "day $0 \leq 0$," and the response to sand-fly salivary gland antigens at day fifteen is represented as "day $1 \geq 1$." Our study suggests that antibody levels increased significantly with each successive immunization. By the fourth immunization, antibody levels were comparable with the positive control. Post insect blood-feeding, chickens in all groups exhibited increased sera and IgY reactivity; however, post last immunization, all groups exhibited higher reactivity in IgY antibodies extracted from eggs. These results indicate that eggs can be used to obtain IgY antibodies against the saliva of sand flies and anophelines, and that eggs can be used as a marker in endemic areas with high exposure to hematophagous bites.

No plateau was observed in the titration tests. Furthermore, the graph declined soon after the first dilution (**Figure 2**). Although high reactivity was observed in the initial dilution, our results suggest that antibody concentration was not high. However, since phlebotomine and anopheles blood-feeding occurs daily and at different times, it is likely that sera produced under natural conditions has a higher concentration of antibodies than that examined in this study.

A low cross-reactivity was observed among samples exposed to different insect species (**Figure 3**) as compared to the O.D of 1.5 demonstrated in **Figure 1**. This indicates that performing specific detection tests for each species is a possibility. Higher reactivity can be detected between *M. migonei* and *L. longipalpis*, than between *Anopheles aquasalis* and the other species tested. Such results are likely due to phylogenetic proximity as the sand fly genera likely share similar protein structures.

Antibody responses against salivary proteins of *Anopheles aquasalis*, *Lutzomyia longipalpis* and *Migonemyia migonei* were studied using two groups of chickens: one group exposed to insect bites and an unexposed negative control / repast group. The first group developed antibodies against salivary gland antigens from sandflies and mosquitoes, and the second group confirmed that no antibodies were present prior to immunization.

Post first immunization, high levels of IgY antibodies (O.D >1) were found in all sera and egg samples. These values were much higher than those found by Soares et al. (2013), who immunized chickens with sonicated salivary glands of *Lutzomyia longipalpis*, and reported an O.D. lower than 0.3. The higher antibody levels observed in the current study may indicate that inoculation by bite elicits a more intense immune response[12].

IgY antibody levels were high relative to the low exposure level of 30 insects per chicken. This result corroborates results obtained by Schwarz et al. (2009b), where antibody responses against *Triatoma infestans* salivary gland antigens in guinea pigs, pigs and chickens were detected under conditions of high and low exposure. Schwarz et al. identified the major salivary gland antigens and assessed their potential as exposure markers. They discovered that two salivary proteins (14 and 21 kDa from

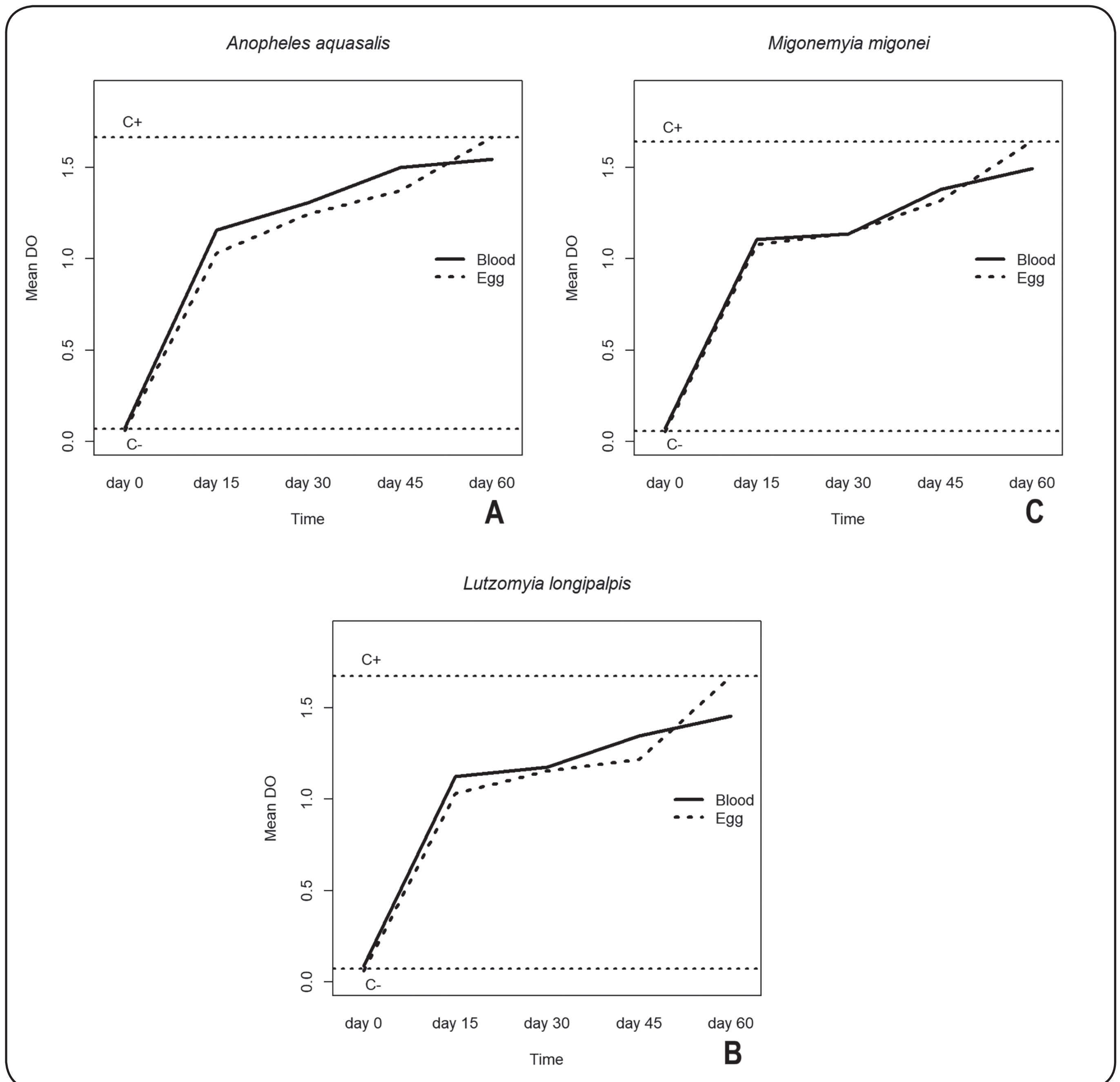

FIGURE 1: Production of antibodies against salivary gland proteins in insect vector species. Production of antibodies against salivary gland proteins from (A) *A. aquasalis,* (B) *L. longipalpis* and (C) *M. migonei*, in chicken eggs. Time in days; Y-axis response: mean optical density. **C-:** Negative control; **C+:** Positive control; **DO:** Optical Density.

the salivary glands of *T. infestans*) are capable of inducing IgY production in chickens and IgG production in guinea pigs, even during low exposure to triatomines[7].

The cross-reactivity analysis showed that specific IgY antibodies develop against salivary proteins from malaria-transmitting mosquitoes. These results corroborate the findings of Armiyanti et al. (2015), who used salivary gland extracts from *Anopheles sundaicus* to detect the IgG antibodies production in serum samples from individuals living in malaria-endemic areas. Armiyanti et al. identified sixteen proteins that induce the production of IgG antibodies in hosts: these varied from 22 to 144 kDa, with protein bands of 46, 41, 33 and 31 kDa being the most immunogenic. This demonstrates that antibodies can be developed against the salivary proteins of specific species[13].

A variety of antigens were present in the vector saliva examined in this study. Identifying the antigen candidates for the most effective immunological markers can help design a laboratory test or rapid immunoassay for detecting exposure to the relevant vectors, as previously demonstrated by Kostalova et al. (2015) and Sima et al. (2016)[14,15].

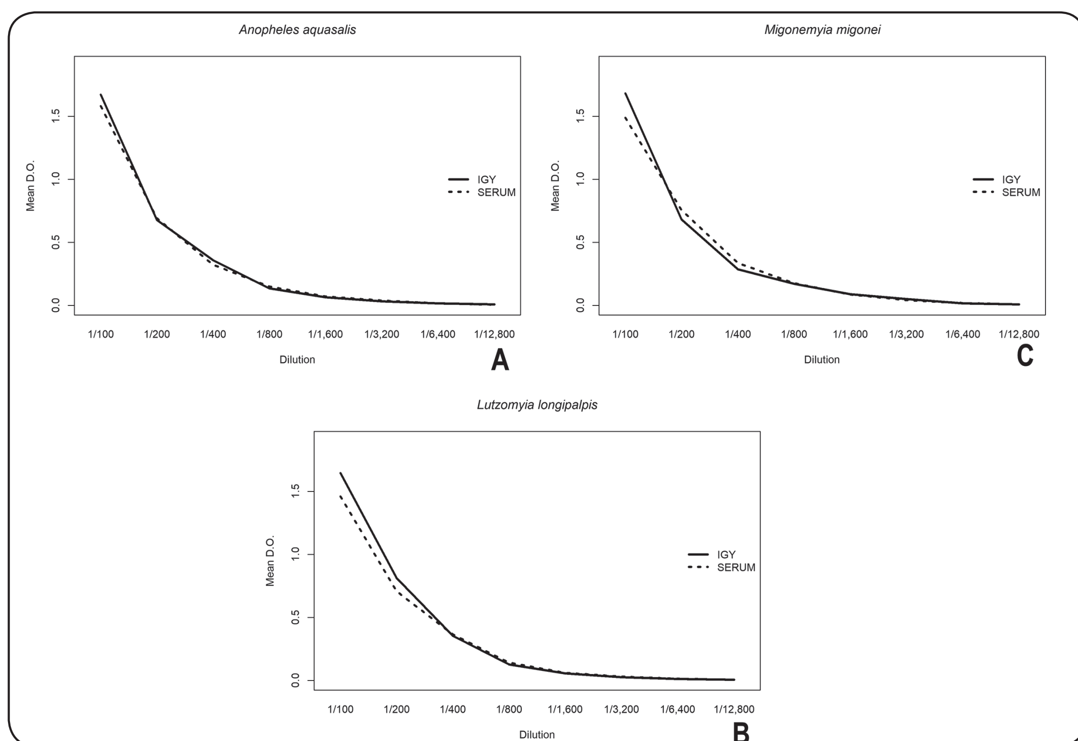

FIGURE 2: Titration ELISA. Serial dilution titration ELISA of IgY antibodies produced in eggs after chickens are infected by (A) *Anopheles aquasalis*, (B) *Lutzomyia longipalpis* and (C) *Migonemyia migonei*. Samples: blood (serum) and eggs (IgY) of chickens. **O.D:** Optical Density.

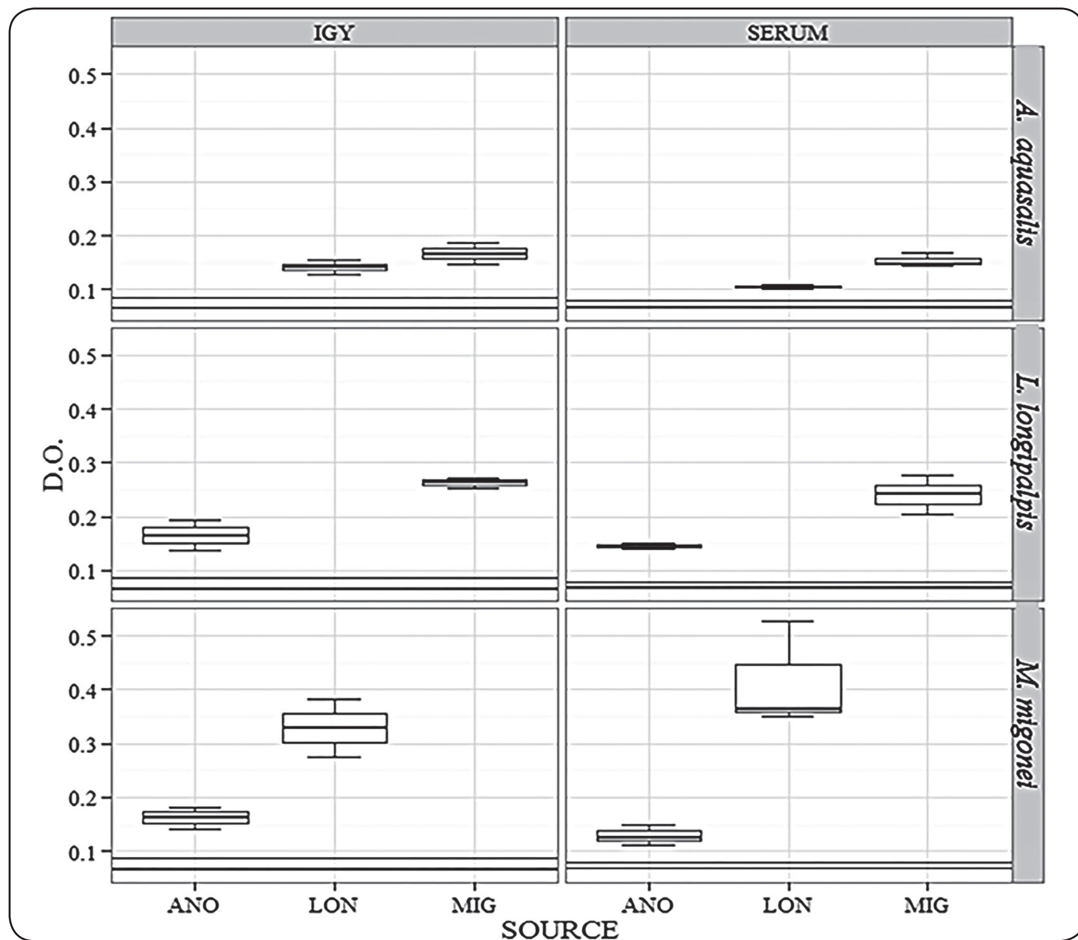

FIGURE 3: Cross reactivity analysis ELISA between the saliva of phlebotomine species *Lutzomyia longipalpis* and *Anopheles aquasalis*. **D.O:** Optical density; **ANO:** *Anopheles aquasalis*, **LON:** *Lutzomyia longipalpis*; **MIG:** *Migonemyia migonei*; **IgY:** egg sample; **Serum:** blood sample.

Further research is required to identify these antigens and validate their use in immunoassays. It is recommended to test the described method in the current study on samples collected from the field.

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Availability of data and materials

The datasets used during the current study are available from the corresponding author on request.

Conflict of Interests

The authors declare that they have no competing interests.

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