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Seroconversion of sentinel chickens as a biomarker for monitoring exposure to visceral Leishmaniasis

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***Leishmania infantum chagasi* causes visceral leishmaniasis (VL); it is transmitted by the sand fly *Lutzomyia longipalpis* that injects saliva and parasites into the host's skin during a blood meal. Chickens represent an important blood source for sand flies and their presence in the endemic area is often cited as a risk factor for VL transmission. However, the role of chickens in VL epidemiology has not been well defined. Here, we tested if chicken antibodies against *Lu. longipalpis* salivary gland sonicate (SGS) could be used as markers of exposure to sand fly bites. All naturally exposed chickens in a VL endemic area in Brazil developed anti-SGS IgY antibodies. Interestingly, *Lu. longipalpis* recombinant salivary proteins rLJM17 and rLJM11 were also able to detect anti-SGS IgY antibodies. Taken together, these results show that chickens can be used to monitor the presence of *Lu. longipalpis* in the peri-domiciliary area in VL endemic regions, when used as sentinel animals.**

Visceral leishmaniais (VL), caused by *Leishmania infantum chagasi (L. i. chagasi)*, is a serious public health problem in several parts of the world[1]. The disease is spreading in Brazil[2], emphasizing the need for better control methods and strategies. The sand fly *Lutzomyia longipalpis* (*Lu. longipalpis*) is geographically dispersed in Brazil and is considered the main vector for VL; it fulfills the requirements of vectorial competence and is adapted to domestic conditions[3,4].

Chickens are not susceptible to *Leishmania* infection[5] and do not naturally develop anti-*Leishmania* antibodies[6], however chicken blood is frequently observed in the digestive material of sand flies[7]. Therefore, an interesting and alternative approach to evaluate chicken exposure to sand fly bites is the investigation of the presence of antibodies to sand fly saliva. Indeed, the use of sentinel animals is instrumental in identifying and monitoring areas with a high density of insect sites. Chickens have been used as sentinel animals for viral diseases transmitted by insect bites through the monitoring of anti-viral antibody responses[8–11].

Chickens are the most frequently kept animals in the vicinity of human houses in endemic areas for VL[12,13] and sand flies are recurrently captured near chicken houses[14]. The presence of chicken houses near a human domicile is a known risk factor for human VL[15,16]. Moreover, studies of vectorial competence conducted by our group revealed infection in 90% of *Lu. intermedia* sand flies artificially fed on chicken blood spiked with *Leishmania braziliensis*[17]. Recently, we demonstrated the possibility of evaluating anti-saliva antibody responses using recombinant proteins, circumventing the limitation of obtaining large quantities of sand fly salivary glands for large scale studies[18]. Although probing human immune responses to sand fly saliva may be a useful epidemiological marker of exposure, obtaining blood samples from the human population in the endemic areas may face cultural resistance. Evaluating the anti-sand fly saliva response of animals living in the peridomicile, on the other hand, may be an easier and more advantageous approach. Herein, we investigate the possibility of using chickens as sentinel animals to identify areas of intense exposure to the vector. Moreover, we also report the possibility of using recombinant *Lu. longipalpis* salivary proteins as surrogates for sand-fly saliva.

Results

Sera from chickens immunized against *Lu. longipalpis* salivary gland sonicate (SGS) were used as a positive control for anti-SGS IgY antibodies. Chickens naturally exposed to sand fly bites developed significant anti-SGS

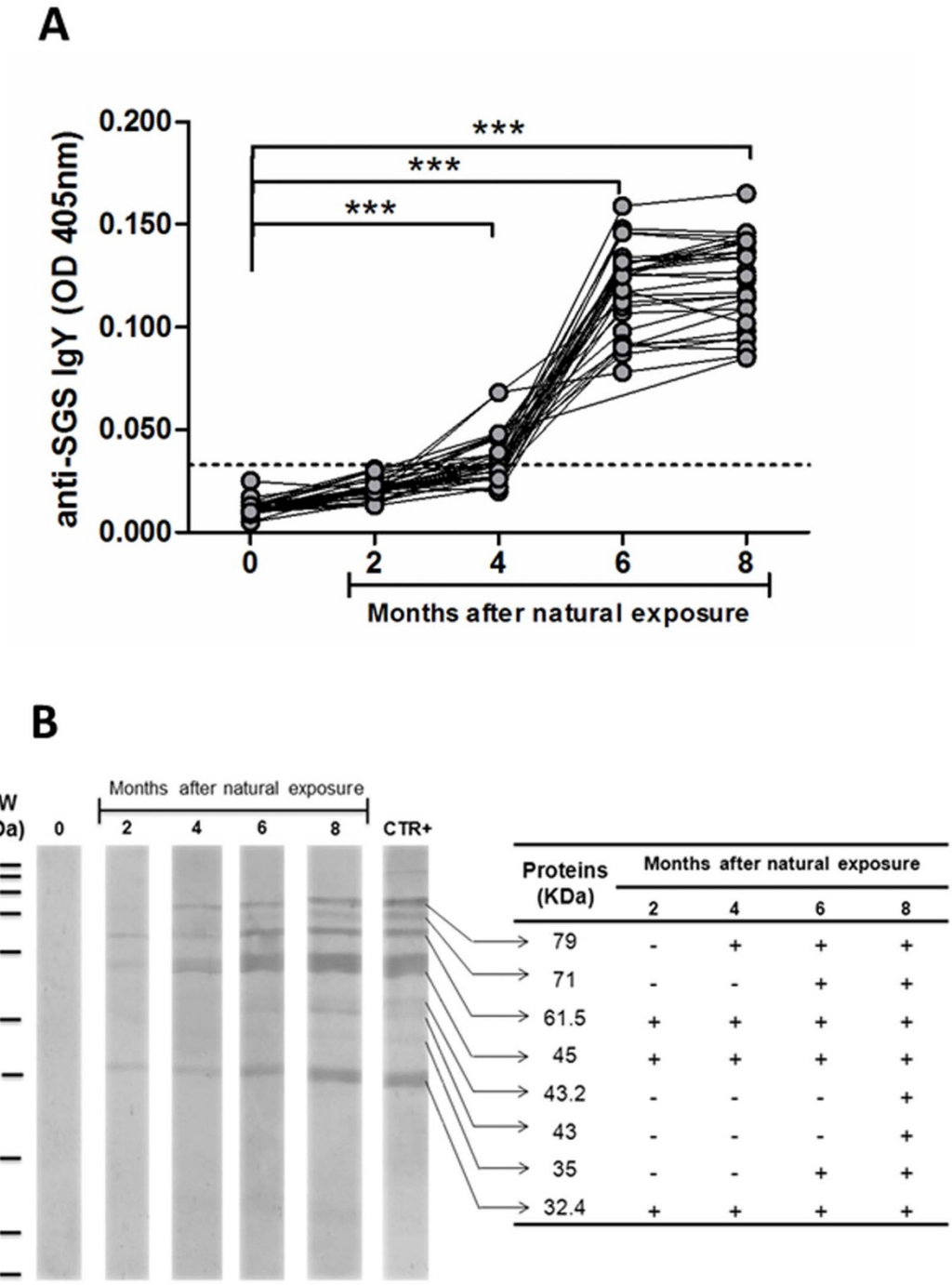

Figure 1 | Anti-SGS antibody response of chickens naturally exposed to sand fly bites in an VL endemic area. Chickens (n = 40) were naturally exposed to sand fly bites for 8 months and their sera were obtained prior to exposure and every 2 months thereafter. (A) ELISAs were used to evaluate the chicken anti-*Lu. longipalpis* SGS IgY antibody production. Each point represents the mean of the duplicate values for the same chicken serum with a standard deviation lower than 20%. The cut-off value (dotted line) was established from ROC curves by comparison of the reactivity values from chicken serum exposed and not exposed to sand flies bites. The data for the antibody levels at different times were compared using the Kruskal-Wallis test with Dunn's post test for multiple comparisons.***, $p<0.0001$. (B) Western blot was used to screen *Lu. longipalpis* SGS proteins recognized with a pool (n = 5) of sera from chickens naturally exposed to sand fly bites. The numbers at the top of each line indicate the months of exposure of the chickens to sand fly bites. Sera from chickens experimentally immunized were used as positive controls (CTR+). Molecular weight markers are represented in kDa (left). The table on the right indicates the molecular weight of protein recognized by these sera every two months during 8 months. +, proteins recognized by the sera; -, proteins not recognized by the sera.

IgY antibodies (Fig. 1a). Anti-SGS IgY antibodies were detected in 26% of chickens, after four months of exposure (Fig. 1a). At the 6-month time point, all naturally exposed animals had significantly elevated anti-SGS IgY responses and remained positive at eight months of exposure with levels up to 2.7 times higher compared to the cut-off value (Fig. 1a).

In order to identify the most immunogenic components of *Lu. longipalpis* SGS for chickens, sera were pooled from five chickens

presenting the highest optical density (OD) values, as judged by ELISA (Fig. 1a). These selected sera were evaluated by Western blot before and at various time points after exposure (Fig. 1b). Bands of molecular weight 61.5, 45 and 32.4 kDa were faintly recognized at the first time-point examined (2 months of exposure; Fig. 1b). Of note, none of the sera evaluated were positive by ELISA at this same period (Fig. 1a). Progressively increased recognition of salivary proteins was detected using sera obtained at later time points. An increase in the intensity and in the number of recognized bands was also detected, with addition of proteins of 79 kDa (four months of exposure), 71 and 35 kDa (six months of exposure) and 43.2 and 43 kDa (eight months of exposure; Fig. 1b).

Next, we investigated the specificity of the humoral IgY response to *Lu. longipalpis* bites by analyzing the possible cross-reactivity with serum from chickens exposed to triatomine (Fig. 2) or to *Aedes aegypti* bites (Fig. 2). Only chickens exposed to triatomine bites exhibited reactivity to two salivary antigens (61 and 79 kDa) whereas no reactivity was observed with sera from animals exposed to *Ae. aegypti* bites (Fig. 2).

ELISA using two Yellow-related proteins, the *Lu. longipalpis* salivary recombinant LJM11 or LJM17, detected anti-saliva IgY antibodies in sera from chickens exposed for eight months (Fig. 3a–c). Seroconversion to *Lu. Longipalpis* SGS was confirmed using these recombinant antigens (Fig. 3a). rLJM11 yielded positive results with 100% of sera tested after eight months of exposure (Fig. 3b) whereas rLJM17 generated positive results in 86.6% of sera tested (Fig. 3c). Additionally, rLJM11 and rLJM17 proteins exhibited 6.6% and 23.3% positive results with sera obtained before sand fly exposure, respectively (Fig. 3b–c). In agreement with these findings, the median OD value after exposure increased 18.14 times for SGS, 14.5 times for rLJM11 and 10 times for rLJM17, compared with median OD values before exposure (Table 1). Receiver operating characteristic (ROC) curve analyses showed similar performances between rLJM11 (AUC: 0.96, $p<0.0001$, Likelihood ratio: 14) and SGS antigens (AUC: 1.00, $p<0.0001$) (Table 1). On the other hand, the other tested antigen, rLJM17, had a lower performance (AUC: 0.81, $p<0.0001$, Likelihood ratio: 3.25) (Table 1). Results obtained with SGS correlated with those obtained with rLJM11 (Spearman rank of $r = 0.422$ and $p<0.01$; Fig. 3d), but not with rLJM17 (Spearman rank of $r = -0.327$; data not shown) .

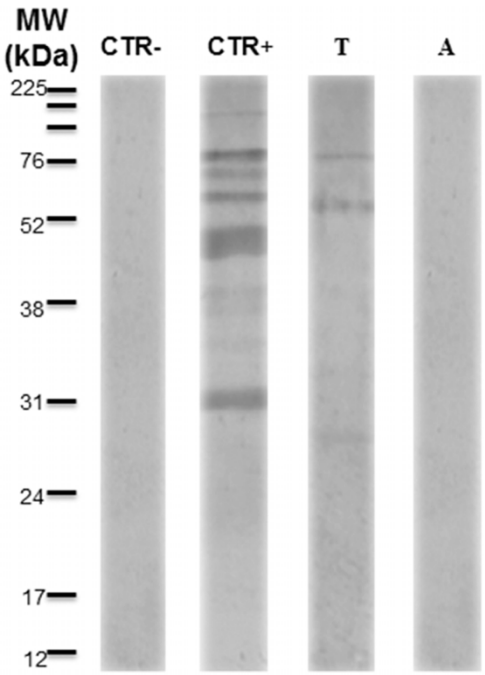

Figure 2 | Recognition of *Lu. longipalpis* SGS among chickens exposed to other hematophagous arthropod vectors. Pool of sera (n = 3) from chickens exposed to triatomines (*Triatoma dimidiata, Dipetalogaster maximus, Rhodnius robustus, Rhodnius pallescens or Rhodnius prolixus*) (T) and *Ae. aegipty* (A) bites were tested for cross-reactivity with total *Lu. longipalpis* sandfly saliva. Sera of chickens from a non-endemic area were used as negative controls (CTR-) and sera from chickens experimentally immunized against *Lu. longipalpis* SGS were used as positive controls (CTR+).

Discussion

We have demonstrated that *Lu. longipalpis* saliva is immunogenic for chickens in a VL endemic area. All naturally exposed chickens developed anti-SGS IgY antibodies in a remarkably homogenous response, considering that they are outbred animals and that exposure in a natural setting is expected to lead to immunization with different doses. This situation probably results from the continuous exposure to sand fly bites, even if not in uniform numbers.

Interestingly, although we observed low OD values after two months of natural exposure, this period was sufficient to induce anti-SGS IgY against three salivary gland proteins from *Lu. longipalpis*. These three proteins (32.4, 45 and 61.5 kDa) have been described as the most abundantly secreted salivary gland proteins of *Lu. longipalpis*[19,20]. Among these, the 45 kDa (*L. longipalpis* LJM17), a Yellow-related protein, is one of the most immunogenic proteins in *Lu. longipalpis* saliva[20]. Indeed, this protein is also the most frequently recognized protein with sera from humans[21], dogs and foxes exposed to sand fly saliva[22,23]. Another protein related to the Yellow- family such as *Lu. longipalpis* LJM11 was also recognized by sera from humans and dogs (43.2 KDa) or humans only (43 KDa)[23]. Here, we have also shown that these proteins are antigenic in chickens.

The 79 kDa apirase precursor, a member of the 5′-nucleotidase family that inhibits platelet aggregation, was recognized by sera from chickens exposed to triatomine bites, indicating the need to choose carefully the salivary antigens for similar types of studies. The observed natural seroconversion of chickens to *Lu. longipalpis* SGS reported here is unlikely to be due to exposure to other insects. Sera from chickens exposed to *Ae. aegypti* did not recognize salivary proteins whereas those exposed to triatomine bugs showed limited cross-reactivity. The most immunogenic salivary protein present in *Lu. longipalpis*, the 45 kDa Yellow-related protein (LJM17) from Diptera, is not found in the salivary glands of *Ae. aegypti*[24]. Additionally, none of the other salivary proteins was recognized by *Ae. aegypti*-exposed chickens. Similar results were observed by Schwarz et al. with sera from chickens exposed to *Triatoma. infestans* and tested against *Aedes, Culex, Anopheles* and *Lutzomyia* saliva[25]. Furthermore, sera from chickens kept in non-endemic areas (negative control), did not cross-react despite being exposed to other insect bites.

Synthetic salivary components have been used as immunological markers of exposure to arthropod bites[18,23,26]. Using recombinant products is advantageous as it overcomes the limitation of collecting sand-fly saliva or salivary gland extracts. Recombinant proteins can be produced on a large scale, avoiding both the natural and insect-colony variations observed in the profile and concentration of sand fly salivary proteins[27–29]. There are a limited number of *Lu. longipalpis* salivary proteins recognized by humans naturally exposed to sand fly bites[21]. In this context, the recombinant salivary proteins LJM17 and LJM11 are effective and sensitive enough to estimate the levels of exposure to *Lu. longipalpis* sand flies in humans and dogs[18,23]. In this report, only rLJM11 was capable of reproducing the serological results obtained with whole *Lu. longipalpis* SGS. Nonetheless, this result opens the possibility of monitoring exposure to *Lu. longipalpis* bites in chickens reared close to human houses. This monitoring

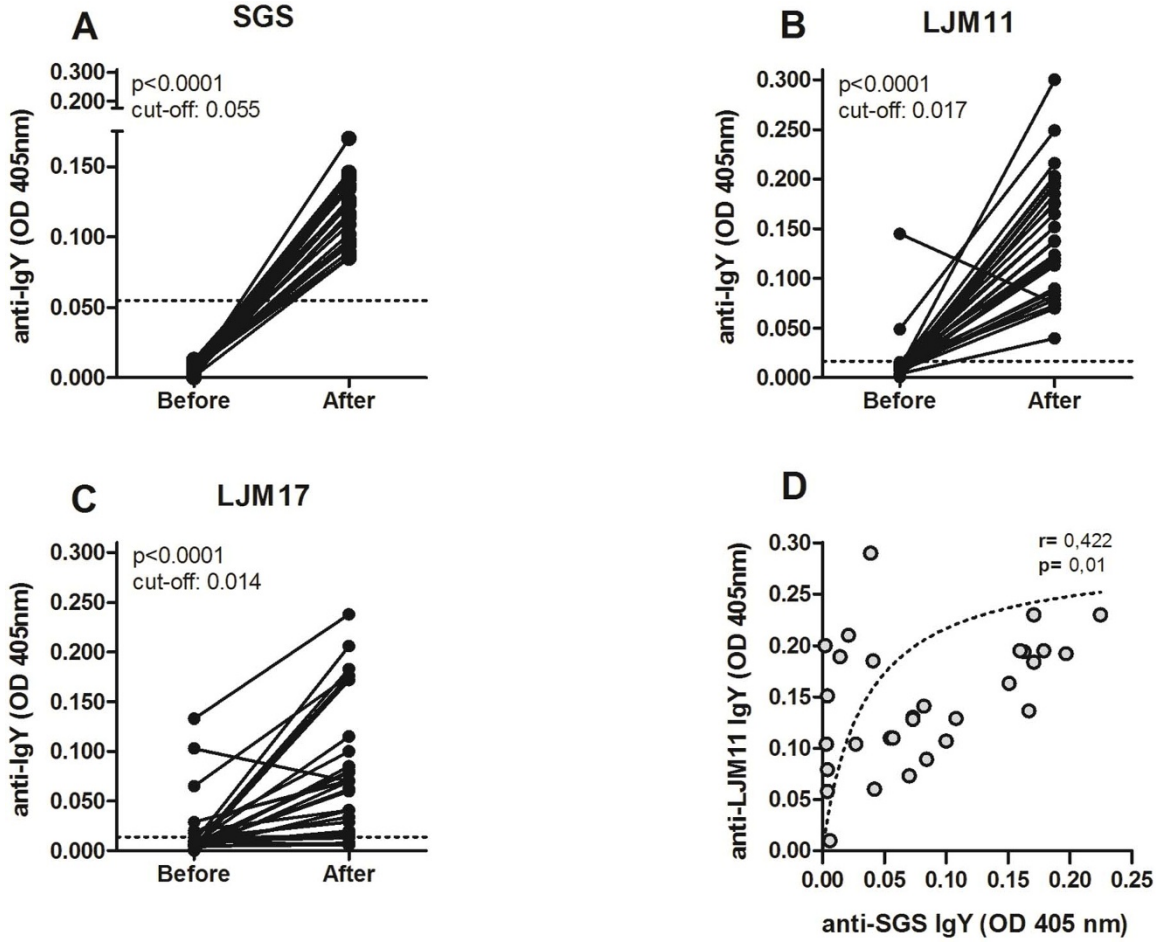

Figure 3 | Seroconversion of chickens naturally exposed to sand fly bites to *Lu. longipalpis* saliva recombinant proteins. (A) Chicken sera (n = 30) were tested by ELISA for the presence of anti-*Lu. longipalpis* SGS (A) or anti-recombinant protein IgY antibodies (B and C) before and after eight months of natural exposure to sand fly bites in an endemic area of VL. The data show the mean and standard error of the mean of duplicate values with a standard deviation lower than 20%. The cut-off value for samples is indicated (dotted line). Cut-off was calculated by comparison of the reactivity values from each group with exposed chicken and unexposed chicken sera tested in Receiver Operating Characteristic (ROC) analysis. The antibody levels at different times were compared using the Wilcoxon signed rank paired test. (B) Sera from chickens naturally exposed to sand fly bites (n = 40) were tested by ELISA, and the IgY antibody levels (OD) against *Lu. longipalpis* SGS or rLJM11 salivary protein were correlated. Data were analyzed using the Spearman test.

could be used as a tool for detecting areas of sand fly exposure in endemic regions which, ultimately, may help in directing control efforts against VL.

Despite the high number of sand flies present in the area and the likelihood of exposure to *Leishmania* parasites, none of the exposed chickens developed anti-*Leishmania* antibodies (data not shown), confirming previous reports[5]. Reports suggest that chickens may represent a risk factor for human VL as has been reported[15] because they may maintain a high population of phlebotomines near human residences and may attract *Leishmania* reservoirs such as foxes[16]. On the other hand, chickens may still be useful as sentinel animals for VL since they are able to indicate exposure to the vector, as demonstrated in the present report. Additionally, chickens are not natural hosts for *Leishmania* and, therefore, they do not increase the availability of parasites for sand fly infection when used as sentinel animals. The limited mobility of chickens, compared to other animals such as dogs is also another advantage when considering them as sentinel animals. Seroconversion in chickens may be indicative of the locality where they have been exposed as they do not usually move long distances from their houses. Taken together, our data suggest that recombinant

Table 1 | Different parameters to salivary antigen evaluation

Antigens		Range of OD			ROC curves							
		Min	Max	Median	cut-off	AUC	Sens.	IC	Spec.	IC	p	Likelihood ratio
SGS	**Before**	0.001	0.029	0.007	0.055	1.00	1.00	(0.87–1.00)	1.00	(0.87–1.00)	≤0.0001	—
	After	0.029	0.238	0.127								
LJM11	**Before**	0.003	0.154	0.01	0.017	0.96	0.93	(0.78–0.99)	0.93	(0.78–0.99)	≤0.0001	14
	After	0.019	0.249	0.145								
LJM17	**Before**	0.002	0.133	0.007	0.014	0.81	0.75	(0.57–0.89)	0.77	(0.56–0.91)	≤0.0001	3.25
	After	0.008	0.206	0.070								

LJM11 may be used to monitor the exposure of chickens to phlebotomine bites, in areas endemic for VL.

Methods

Salivary gland sonicate. Salivary glands were obtained from adult sand flies after insect colonization as previously described[30]. Briefly, *Lu. longipalpis* sand flies (Cavunge strain captured at Cavunge in Bahia, northeastern Brazil) were reared at the Centro de Pesquisas Gonçalo Moniz (Salvador, Bahia, Brazil) using fermented food mixture. Adult sand flies were offered cotton containing 10% sucrose solution. Salivary glands from 3 to 10 day old adult female flies were dissected and stored in groups of 20 pairs in 20 µl Hepes 10 mM pH 7.0, NaCl 0.15 M in 1.5 ml. Salivary glands were kept at −75°C until needed, when they were disrupted by ultrasonication within 1.5 ml conical tubes. Tubes were centrifuged at 10,000 g for 2 min and the resultant supernatant (salivary gland sonicate - SGS) was used for the studies.

Chicken immunization with salivary gland sonicate (SGS). Three unexposed chickens (*Gallus gallus*) (25 weeks old) were obtained from a commercial breeder. Immunization with *Lu. longipalpis* salivary gland sonicate (SGS) consisted of three injections of 50 µg of laboratory-reared *Lu. longipalpis* SGS. For the first dose, SGS was resuspended in 500 µl of pH 7.4 phosphate-buffered saline (PBS) mixed with an equal volume of complete Freund's adjuvant (SIGMA). The mixture was thoroughly emulsified and was administered intramuscularly into the chicken's pectoral muscle. For the second and third immunizations, the same dose of SGS was emulsified in incomplete Freund's adjuvant (SIGMA). Injections were performed at 15 day intervals. Blood was collected from the wing vein immediately before each immunization and 15 days after the third immunization. All experimental procedures were approved and conducted according to the Brazilian Committee on the Ethics of Animal Experiments of the FIOCRUZ (Permit Number: 028/2011).

Sentinel chicken serum samples. Chickens (*Gallus gallus*) (25 weeks old) were obtained from a commercial breeder and were used as sentinel chickens in a VL endemic area (Cavunge). Forty naive chickens were pooled into five groups (n = 8), and each group was randomly distributed in five different houses. Chickens were maintained for eight months and serum samples were obtained bimonthly from each chicken, by blood collection from the wing vein. Plasma was stored at −20°C until use.

Lu. longipalpis recombinant proteins. Lu. longipalpis recombinant proteins (LJM11, LJM17, LJL143, LJM19 and LJM111) were obtained as previously described[23].

Analysis of IgY anti-saliva antibodies by ELISA. IgY antibody detection was performed by ELISA following a protocol adapted from Barral et al.[31] ELISA 96-well plates were coated with 0.1 ml/well of *Lu. longipalpis* SGS (equivalent to 5 pairs of salivary glands/ml; approximately 5 µg protein/ml) or with 1 µg recombinant protein/ml in carbonate buffer ($NaHCO_3$ 0.45 M, Na_2CO_3 0.02 M, pH 9.6). Plates were incubated overnight at 4°C. After three washes with PBS-0.05% Tween, the plates were blocked for 1 hour at 37°C with PBS-0.05% Tween plus 5% non-fat milk. Sera were diluted 1 : 100 with PBS-0.05% Tween and incubated overnight at 4°C. After five washes, the wells were incubated with anti-IgY alkaline-phosphatase-conjugated antibody (SIGMA, St. Louis, MO), at 1 : 5,000 dilution for one hour at 37°C. Following another washing cycle, the reaction was developed for 30 minutes with a chromogenic solution of p-nitrophenylphosphate in sodium carbonate buffer pH 9.6 with 1 mg/ml of $MgCl_2$ and read at an optical density of 405 nm (OD). The concentrations of saliva or recombinant proteins used were determined in a dose- response experiment to evaluate an optimal signal without loss of specificity (data not shown). Cut-off was determined by the mean plus three standard deviations of the OD with chicken sera from a non-endemic area following Receiver-Operating Characteristic (ROC) analysis.

Analysis of IgY anti-saliva antibodies by Western blot. Anti-SGS Western blot was adapted from the method previously described for other species[23]. Salivary glands (40 pairs approximately equivalent to 40 mg total protein) were run on a 4–20% Tris-glycine gel (Invitrogen). After transfer, nitrocellulose membrane was blocked with 3% (w/v) nonfat dry milk in Tris-buffered saline-0.05% Tween (TBS-T), pH 8.0, overnight at 4°C. After washing with TBS-T, pH 8.0, the membrane was placed on a mini-protean II multiscreen apparatus (Bio-Rad, Hercules, CA), and different lanes were incubated with chicken serum (1 : 10) for 2 h at room temperature. After washing with TBS-T, pH 8.0, three times for 5 min, the membrane was incubated with anti-chicken IgY alkaline phosphatase-conjugated antibody (1 : 1,000; Sigma, St. Louis, MO) for 1 h at room temperature. Membranes were developed by addition of Western Blue stabilized substrate for alkaline phosphatase (Promega), and the reaction was stopped by washing the membrane with deionized water.

Statistical analysis. Data regarding anti-saliva IgY antibody levels before and after seroconversion were compared by Wilcoxon signed rank paired test. Kruskal-Wallis with Dunn's post test was used for multiple comparisons. Receiver operating characteristic (ROC) curves were used for determining cut-off values and for comparing the performance of the recombinant proteins in relation to SGS. Correlation between values of SGS and those of LJM11 were performed by Spearman test. All tests were performed using GraphPad software Prism 5.0 (GraphPad Prism Inc., San Diego, CA).

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Author contributions

All authors conceived and designed the experiments. B.R.S., A.P.A.S. and J.C.M. performed the experiments. B.R.S., A.P.A.S., D.B.P. analyzed the data and wrote the paper. B.R.S., A.P.A.S., D.B.P., C.I.O., M.B.N. and A.B. authors participated in critical discussion of the manuscript and approved the final, submitted version of the manuscript.

Additional information

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