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Inter-test reliability of the anti-RESA indices based on ELISA tests using eluates from whole blood spots dried on filter paper

E. C. DUARTE[1,2], T. W. GYORKOS[3]*, L. PANG[4], S. ÁVILA[5] AND C. J. F. FONTES[2,6]

[1] *Fundação Nacional de Saúde – FUNASA-MT, Ministry of Health, Brazil*
[2] *TROPICA-Núcleo de estudos em doenças infecciosas e tropicais de MT, Brazil*
[3] *Department of Epidemiology and Biostatistics – McGill University and Division of Clinical Epidemiology, Montreal General Hospital, Montreal, Quebec, Canada*
[4] *Walter Reed Army Institute, Thailand – USA*
[5] *Instituto de Medicina Tropical de São Paulo, Universidade de São Paulo, Brazil*
[6] *Universidade Federal de Mato Grosso – Faculdade de Medicina, Brazil*

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SUMMARY

The ring-infected erythrocyte surface antigen (RESA), is one of the falciparum malaria vaccine candidates rarely studied in Brazil. Fieldwork logistics to conduct serology studies is simplified when eluates from whole blood dried on filter paper can be used. Therefore, this study aimed to assess the inter-test reliability for the anti-RESA ELISA-based indices using eluates from filter paper and from serum samples. The study population consisted of 210 individuals (Brazil) from whom matched samples were collected. Anti-RESA ELISA-based index means ($\pm$S.D.) were 15·29% ($\pm$28·13%) for filter paper and 11·79% ($\pm$23·67%) for serum samples. The intra-class correlation coefficient was estimated to be 82·38%, indicating high test reliability. However, there was a significant tendency for filter paper test results to have higher values than serum sample test results ($P < 0{\cdot}001$). Explanations for this finding may be the presence of haemoglobin in the eluates from filter paper, which may interfere with ELISA testing.

INTRODUCTION

The ring-infected erythrocyte surface antigen (RESA or Pf155), an antigen deposited on the erythrocyte membrane immediately after *P. falciparum* invasion, is one of the malaria vaccine candidates directed against *P. falciparum* asexual blood stages [1–4]. This antigen disappears after the parasite matures [5]. Several reasons can be highlighted to explain why RESA is especially important. It has been demonstrated that anti-RESA antibody production increases with age and cumulative previous exposure [6, 7]. Moreover, Berzins et al. have demonstrated that antibody to RESA inhibits merozoite invasion *in vitro* [8]. Immunization trials, although not completely satisfactory, show that fragments of RESA partially immunized monkeys against *P. falciparum* malaria; a polypeptide homologous to RESA partially protected mice against *P. chabaudi* malaria; and monkeys passively immunized with human anti-RESA antibodies self-cured [9–11]. In addition, Perlmann et al. have demonstrated the absence of antigenic diversity of RESA among several isolates of *P. falciparum* [4, 12].

Selected epidemiological data from human studies have identified associations between levels of anti-RESA and protection against malaria morbidity and

* Author for correspondence: Division of Clinical Epidemiology, Montreal General Hospital, 1650 Cedar Avenue, Montreal, Quebec, Canada H3G 1A4.

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mortality *in vivo*. Petersen et al. studying 118 adult Liberians over a 1-year period, observed that high RESA-responders (titres $\geqslant 250$) presented lower parasite densities during study follow-up compared to low RESA-responders (titres $\leqslant 50$), suggesting that high levels of antibodies to RESA may play a role in protection against malaria [13]. Astagneau et al. demonstrated that antibodies to defined RESA epitopes (measured by ELISA-based optical densities) were a reliable tool for predicting the level of protection against malaria (clinical criteria) among individuals living in Madagascar during a *P. falciparum* epidemic ($n = 271$) [14]. Similarly, in another study, anti-RESA antibody levels differed in a consistent pattern between pregnant women with no initial *P. falciparum* parasitemia and pregnant women with initial *P. falciparum* parasitemia [15]. Modiano et al. studied the prevalence and levels of antibodies against RESA in three ethnic groups living under intense malaria transmission in Sudan [16]. They observed an increased response to RESA with increased age concomitant with a decreased prevalence of parasitemia.

In Brazil, there have been few studies involving RESA measurements [17–19]. These researchers studied the prevalence of RESA among patients infected with *P. falciparum* malaria and correlations between anti-RESA antibodies and season, parasitemia level and number of malaria episodes in populations from the Brazilian Amazon region [17–19].

Fieldwork logistics to conduct serology tests are difficult and time-consuming when venous blood needs to be collected. Whole blood dried on filter paper is a very convenient method for blood sample collection and storage in field epidemiological studies; however, its reliability compared to sera samples has not yet been tested.

Therefore, the present study assessed the inter-test reliability of the ELISA-based anti-RESA antibody indices using eluates from whole blood spots dried on filter paper and using serum samples.

METHODS

Data for this study come from matched samples obtained at baseline from a cohort study. The study was carried out in Leonislândia (Travessão II), a rural area of Peixoto de Azevedo City, in the Amazon region of Mato Grosso – Brazil. The majority of Leonislândia's population is made up of migrants who were born in malaria non-endemic states, but who subsequently lived for various lengths of time in malaria endemic regions of Brazil. The level of malaria transmission in Leonislândia can be considered hypo-endemic and is relatively stable.

The eligible population included all residents of all ages, living in Travessão II – Leonislândia (Peixoto de Azevedo municipality – MT, Brazil), at the time of the first study visit for the cohort study (September, 1996). This population included 258 individuals. The final sample used for the study included 210 (81·4%) individuals for whom matched samples were available.

Data collection procedures during fieldwork

During the fieldwork, for each eligible individual, blood samples were collected in Vacutainers® and 2–3 drops of blood were collected on filter paper (INLAB, Brazil; type 10) for serology. After air-drying, the filter paper samples were covered with aluminium foil and stored in a dry box. At the end of each field visit, the filter papers were put into plastic bags and stored in a freezer (-5 to -10 °C). Serum samples were separated in Peixoto de Azevedo City within 24 h of collection and kept at -70 °C or less. Serum samples and whole blood samples collected on filter paper were used to determine anti-RESA ELISA-based indices.

Lab test procedures for anti-RESA ELISA-based examination

RESA peptide

The synthetic peptide from RESA was [(Lauroyl-CTG$(EENV)_4$-$OH)_2$] (where EENV is the letter code for the tetrapeptide GluGluAsnVal), which was obtained from Bachem Feinchemikalien AG (Bubendorf-Switzerland).

Sera samples

Blood samples were transported to the research lab in Peixoto de Azevedo, where sera were extracted by centrifugation and one aliquot from each subject was sent to São Paulo (University of São Paulo) for analysis. The presence of antibodies against RESA was determined by ELISA. Briefly, flat-bottom, 96-well micro-ELISA plates (NUNC™, Denmark) were coated with 100 μl/well of the synthetic RESA peptide

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(2·5 μg/ml in phosphate-buffered saline-PBS) and incubated overnight at 4 °C. Plates were spray-washed six times with PBS plus Tween 20 (0·05%, PBST), and then blocked (200 μl/well) with 5% non-fat powdered milk in PBST and incubated for 2 h at 37 °C. After spray-wash, sera samples were added (100 μl/well) at a dilution of 1:50 in 2·5% non-fat powdered milk in PBS, incubated for 1 h at 37 °C and, then, spray-washed. Bound antibodies were detected by incubations for 1 h at 37 °C with peroxidase-conjugated goat antibodies to human IgG (100 μl/well). After washing, the enzymatic reaction was developed with 0·013 M 30% hydrogen peroxide and 0·0022 M (OPD) in 0·05 M citrate-phosphate buffer, pH 5·0 as the substrate (100 μl/well), for 30 min at 37 °C in the dark. The reaction was stopped by adding 2N sulphuric acid (50 μl/well). The concentration of antigen chosen (2·5 μg/well RESA peptide in PBS), the serum dilution and the incubation times were defined by a pre-testing routine. Optical densities (OD) were read at 420 nm. All samples were tested in duplicate with a positive control (pool of high-titered samples) and a negative control (sample from never-exposed individual). In addition, one column of wells was kept as reference (without serum) for background assessment.

Filter paper samples

When samples were from whole blood dried on filter paper, samples were processed in Cuiabá-MT by the research team (National Health Foundation) and eluates were used for the ELISA examination. To adjust the quantity of filter paper impregnated with blood that should be used in the eluate to be comparable to sera at a 1:50 dilution, specimens from an independent sample of individuals from a similar malaria-endemic region in the north of Mato Grosso State were used (Duarte et al. 2001). Briefly, different numbers of small circles of filter paper completely impregnated with blood were compared with their matched individual serum. The best eluate 'dilution' was chosen from that comparison giving the highest Pearson's correlation coefficient. This identified five circles of filter paper eluates as the best correspondent to sera at a dilution of 1:50 ($r = 0{\cdot}95$, $n = 73$). Mean ($\pm$S.D.) OD from ELISA based on filter paper and sera samples were 0·884 ($\pm$0·720) OD and 0·704 ($\pm$0·636) OD, respectively. The filter paper-based ELISA technique was exactly the same as the sera-based, except that after incubation for 1 h at 37 °C, the enzymatic reaction was stopped by adding sodium dodecyl-sulphate (SDS, 50 μl/well) and the optical densities (OD) were read at 405 nm using 2,2′-azino-di-[3-ethyl-benzthiazoline sulphonate] (ABTS, Sigma) as substrate.

Positive and negative controls

The positive sera-control pool was the same for both filter paper and sera-based ELISA. The mean ($\pm$S.D.) ODs for negative and positive controls using filter-paper samples were 0·104 ($\pm$0·107) and 1·336 ($\pm$0·449), respectively ($n = 9$ plates). For the serum-based ELISA tests, they were 0·063 ($\pm$0·012) and 1·536 ($\pm$0·286), respectively ($n = 14$ plates). There was no statistically significant difference in negative control means or positive control means between the two ELISA sets (Kruskal–Wallis $\leqslant 1{\cdot}75$; $P \geqslant 0{\cdot}18$).

ELISA-based index calculation

Final OD was given by the mean OD of each sample minus the mean OD of the background in each plate (negative values were set equal to 0). Moreover, to avoid assay-by-assay variation, results were expressed in terms of ELISA arbitrary units (ELISA index) calculated as follows [20]: ELISA index (%) = [(Sample OD − Negative control OD)/(Positive control OD − Negative control OD)] × 100.

Data analysis and statistical procedures

Data were entered in Epi-info v. 6.0, and analysed using the software STATA Statistical Software: release 5.0 [21–22].

The inter-test reliability for the anti-RESA indices based on ELISA using eluates from whole blood spots dried on filter paper and from serum samples was determined using the intra-class correlation coefficient based on a mixed two-way analysis of variance model [23]. Moreover, the ability of ELISA anti-RESA indices based on filter paper to predict the ELISA anti-RESA indices based on serum samples was assessed through linear regression analysis. Results were used for regression-based adjustment of anti-RESA indices using eluates from filter paper samples.

Ethical considerations

This study received ethical approval from the Brazilian Ministry of Health – National Health Foun-

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dation and the Montreal General Hospital Ethics Review Committee and written informed consent was obtained from each individual, following acceptance of the study.

RESULTS

Description of study population

The study population consisted of 258 individuals, of whom 57·6% were male and 50·5% had more than 20 years of age (Table 1). The average age was 25·3 years, from 1–64 years old. The study population reported a mean time living in mining environments of approximately 5 years (56·3 months), and 39·5% had lived in these areas for some time during their lives. Moreover, only a small proportion of individuals (5·2%) had never lived or lived for 1 year or less in other types of endemic environments.

Table 1. *Description of study population, Leonislândia – Peixoto de Azevedo (MT-Brazil), September 1996 (N = 258)*

Variable	Categories	No (%)
Gender	% Male	121 (57·6)
Age (years)	< 5	16 (7·6)
	5–20	88 (41·9)
	20	106 (50·5)
Months lived in mining environments	0	127 (60·5)
	1–95	24 (11·4)
	⩾ 96	59 (28·1)
Months lived in endemic environments (except mining)	0–12	11 (5·2)
	13–48	52 (24·8)
	49–131	91 (43·3)
	⩾ 132	56 (26·7)
Anti-RESA ELISA-based index*		
Filter paper samples	0%	72 (34·3)
	> 0%–⩽ 50%	121 (57·6)
	> 50%–⩽ 100%	10 (4·8)
	> 100%	7 (3·3)
Sera samples	0%	108 (51·4)
	> 0%–⩽ 50%	84 (40·0)
	> 50%–⩽ 100%	15 (7·1)
	> 100%	3 (1·4)

* ELISA index = [(sample OD-negative control OD)/(positive control OD-negative control OD)] × 100; OD = optical density. Crude categorical agreement between filter paper and sera for results < = 50% and > 50% = 97·6%; 15/17 (88%) filter paper and 15/18 (83%) sera had ELISA index values > 50%.

Reliability of anti-RESA indices based on ELISA tests

For the eligible 258 individuals, 210 (81·4%) matched samples were obtained. Based on these matched samples it was possible to assess the inter-test reliability for anti-RESA ELISA-based indices using eluates from dried drops of blood on filter paper and serum samples.

Anti-RESA ELISA-based index means (±standard deviations) were 15·29% (±28·13%) for the filter paper samples and 11·79% (±23·67%) for the serum samples. Medians (Q1–Q3) were 5·16% (0–15·12%) for filter paper data and 0% (0–11·25%) for serum data.

The results of the two-way analysis of variance (ANOVA) are presented in Table 2. Based on these results, the variances between individuals, within individuals and the error variance were estimated: 561·42, 5·58 and 114·46, respectively. The intra-class correlation coefficient was estimated to be 82·38% indicating high reliability of the anti-RESA ELISA-based test.

However, there was a statistically significant tendency of the filter-based test to yield higher values than the serum sample-based tests ($P < 0{\cdot}001$). Therefore, linear regressions were also performed to assess how well the anti-RESA ELISA indices based on filter paper predicted the results based on serum samples (Table 3).

It was noted that results from ELISA tests using filter paper explained a large amount of the variance of the serum-based results ($R^2 = 71\%$). As expected, the regression intercept ($ß_0 = 0{\cdot}94$; 95%CI: −1·03, 2·91) was not significantly different from zero (model 1), indicating that the model without the intercept is appropriate (model 2). However, the slope of the regression line (regression coefficient for the independent variable: anti-RESA indices from ELISA tests using filter paper samples) was statistically different from one ($\beta_{x_1} = 0{\cdot}72$; 95%CI: 0·67, 0·78). These results indicate that for each unit increase on the anti-RESA ELISA-based index using filter paper samples, an average increase of only 0·72 in the test index using serum samples is expected. This confirms a slight overestimation of anti-RESA ELISA indices based on filter paper compared to test results based on serum samples.

According to these results, anti-RESA ELISA filter paper-based indices should be corrected using the

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Table 2. *Two-way analysis of variance (ANOVA) comparing the anti-RESA ELISA-based indices* derived from the use of eluates from dried drops of blood on filter paper and serum samples (MT-Brazil), September 1996 (N = 210)*†

Source of variance	Sum of squares‡	Degrees of freedom	Mean square	*F*-test
Between individual	258597·36	209	1237·31	10·81
Within individual (ELISA tests)	1286·98	1	1286·98	11·24
Residual (error)	23922·67	209	114·46	
Total	283807·02	419		

† 210 matched samples obtained from an eligible population of 258.
* ELISA index = [(sample OD-negative control OD)/(positive control OD-negative control OD)] × 100; OD = optical density;
‡ ANOVA overall mean = 13·54%.

Table 3. *Linear regression coefficients for the association between anti-RESA ELISA-based indices* derived from the use of eluates from dried drops of blood on filter paper (independent variable: X_1) and serum samples (dependent variable: Y), MT-Brazil, September 1996 (N = 210)*†

	Coefficient β	SE (β)	95%CI(β)	R^2
Model 1‡				
β_0 (Intercept)	0·94	1·003	−1·03, 2·91	0·711
β_{x_1}	0·71	0·031	0·65, 0·77	
Model 2§				
β_{x_1}	0·72	0·028	0·67, 0·78	0·709

* ELISA index = [(sample OD-Negative control OD)/(Positive control OD-Negative control OD)] × 100; OD = optical density;
† 210 matched samples obtained from an eligible population of 258.
‡ Overall *F*-test = 510·52, $P < 0·001$, $r = 0·84$;
§ Model fitted without intercept ($\beta_0 = 0$). Overall *F*-test = 689·38, $P < 0·001$, $r = 0·88$.

corresponding linear regression equation with no intercept ($\hat{Y} = 0·7236*X_1$) to estimate the unknown serum-based results.

DISCUSSION

Whole blood dried on filter paper is a very convenient method for blood sample collection and storage in field epidemiological studies. Several studies have demonstrated the reliability and validity of test results based on blood samples collected on filter paper for detecting antibodies against different antigens by ELISA or other techniques [24–33]. Moreover, Vai et al. demonstrated that IgG concentration from serum samples collected on filter paper was fairly stable when stored for 6 months at 4 °C and measured subsequently by ELISA [34].

In the present study, results confirm the high inter-test reliability for the anti-RESA indices based on ELISA using eluates from whole blood spots dried on filter paper and from serum samples (ICC = 82·28%). However, a small but statistically significant overestimation of optical density indices from filter paper samples was identified when compared to results based on serum samples. Three possible explanations for this finding may be considered. First may be the presence of haemoglobin in the eluates from whole blood dried on filter paper which may interfere with ELISA results; secondly, small different procedures used in the ELISA carried out for the two sets of exams; or finally, some unknown systematic technical or measurement error.

It is not possible to know if these results are specific to our study, and more specifically related to procedures and samples used in this study, or if it is a common finding when comparing these two techniques. In the latter case, the overestimation of optical density indices from filter paper samples compared to results based on serum samples may be considered an intrinsic characteristic of the filter-paper ELISA-based tests, and generalization of some correction is advised. Given these considerations, it seems prudent that researchers intending to use filter paper eluates for epidemiological research of this kind should perform a validation study to establish the relationship between filter paper eluates and sera under their specific field and laboratory conditions.

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