Identification and Validation of Loa loa Microfilaria-Specific Biomarkers: a Rational Design Approach Using Proteomics and Novel Immunoassays

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ABSTRACT Immunoassays are currently needed to quantify Loa loa microfilariae (mf). To address this need, we have conducted proteomic and bioinformatic analyses of proteins present in the urine of a Loa mf-infected patient and used this information to identify putative biomarkers produced by L. loa mf. In total, 70 of the 15,444 described putative L. loa proteins were identified. Of these 70, 18 were L. loa mf specific, and 2 of these 18 (LOAG_16297 and LOAG_17808) were biologically immunogenic. We developed novel reverse luciferase immunoprecipitation system (LIPS) immunoassays to quantify these 2 proteins in individual plasma samples. Levels of these 2 proteins in microfilaremic L. loa-infected patients were positively correlated to mf densities in the corresponding blood samples (r = 0.71 and P < 0.0001 for LOAG_16297 and r = 0.61 and P = 0.0002 for LOAG_17808). For LOAG_16297, the levels in plasma were significantly higher in Loa-infected (geometric mean [GM], 0.045 μg/ml) than in uninfected (P < 0.0001), Wuchereria bancrofti-infected (P = 0.0005), and Onchocerca volvulus-infected (P < 0.0001) individuals, whereas for LOAG_17808 protein, they were not significantly different between Loa-infected (GM, 0.123 μg/ml) and uninfected (P = 0.06) and W. bancrofti-infected (P = 0.32) individuals. Moreover, only LOAG_16297 showed clear discriminative ability between L. loa and the other potentially coendemic filariae. Indeed, the specificity of the LOAG_16297 reverse LIPS assay was 96% (with a sensitivity of 77%). Thus, LOAG_16297 is a very promising biomarker that will be exploited in a quantitative point-of-care immunoassay for determination of L. loa mf densities.

IMPORTANCE Loa loa, the causative agent of loiasis, is a parasitic nematode transmitted to humans by the tabanid Chrysops fly. Some individuals infected with L. loa microfilariae (mf) in high densities are known to experience post-ivermectin severe adverse events (SAEs [encephalopathy, coma, or death]). Thus, ivermectin-based mass drug administration (MDA) programs for onchocerciasis and for lymphatic filariasis control have been interrupted in parts of Africa where these filarial infections coexist with L. loa. To allow for implementation of MDA for onchocerciasis and lymphatic filariasis, tools that can accurately identify people at risk of developing post-ivermectin SAEs are needed. Our study, using host-based proteomics in combination with novel immunoassays, identified a single Loa-specific antigen (LOAG_16297) that can be used as a biomarker for the prediction of L. loa mf levels in the blood of infected patients. Therefore, the use of such biomarker could be important in the point-of-care assessment of L. loa mf densities.

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TABLE 1 Details of *L. loa* mf-specific proteins identified in urine of patients

| Protein ID no. | Description | Peptide count | Mol mass (kDa) | FKPM | Homology to human |
|---------------|-------------|---------------|----------------|------|------------------|
| LOAG_00073    | Heat shock protein 90 | 2 | 69.4 | 3,841.1 | 0E+00 | 99 | Yes |
| LOAG_01395    | WD repeats and SOFI domain-containing protein | 2 | 51.0 | 101.3 | 5E−174 | 97 | Yes |
| LOAG_01611    | Hypothetical protein | 2 | 64.5 | 29.8 | 1E−85 | 74 | Yes |
| LOAG_02628    | Low-density lipoprotein receptor repeat class B-containing protein | 2 | 196.0 | 33.7 | 4E−129 | 86 | Yes |
| LOAG_03988    | Hypothetical protein | 2 | 54.0 | 152.3 | 2E−08 | 92 | Yes |
| LOAG_04876    | Peptidase M16 inactive domain-containing protein | 2 | 47.4 | 40.7 | 2E−43 | 93 | Yes |
| LOAG_05583    | U1/U6 small nuclear ribonucleoprotein hPrp4 | 2 | 56.6 | 43.4 | 2E−157 | 99 | Yes |
| LOAG_05701    | 14-3-3-like protein 2 | 2 | 28.3 | 2,392.0 | 1E−147 | 94 | Yes |
| LOAG_05915    | Hypothetical protein | 2 | 104.3 | 14.7 | 2E−02 | 33 | No |
| LOAG_06631    | Troponin | 2 | 171.1 | 8.8 | 1E−05 | 13 | Yes |
| LOAG_09325    | Hypothetical protein | 2 | 33.0 | 68.3 | 1E−13 | 27 | Yes |
| LOAG_10011    | Hypothetical protein | 2 | 13.6 | 3,257.3 | 7E−66 | 98 | Yes |
| LOAG_16297    | Hypothetical protein | 2 | 143.0 | 0.4 | 5E−04 | 67 | No |
| LOAG_17249    | Pyruvate kinase | 2 | 59.0 | 609.7 | 0E+00 | 99 | Yes |
| LOAG_17808    | PWWP domain-containing protein | 2 | 69.8 | 13.9 | 9E−04 | 5 | No |
| LOAG_18456    | Cullin-associated NEDD8-disassociated protein 1 | 2 | 124.1 | 45.5 | 0E+00 | 98 | Yes |
| LOAG_18552    | Hypothetical protein | 2 | 106.5 | 2.1 | 1E−03 | 44 | No |
| LOAG_19057    | Hypothetical protein | 3 | 30.2 | 89.2 | 5E−128 | 77 | Yes |

Proteins that do not share significant sequence homology to human proteins are highlighted in bold. FKPM represents the relative mRNA expression level obtained using transcriptome sequencing (RNA-seq) (22).

We postulated that certain *L. loa* parasite antigens secreted or excreted into the human bloodstream might not be fully reabsorbed following filtering by the renal glomeruli and could thereby be concentrated in the urine of *L. loa*-infected individuals. Studies have shown that urine is a sample source of high importance for biomarker discovery because it is easily available, can be collected noninvasively in large quantities (20, 21), and, from a protein point of view, is much less complex than human serum or plasma. In the present study, utilizing a nontargeted (shotgun) nanobore reversed-phase liquid chromatography tandem mass spectrometry (RPLC-MS/MS) proteomic approach, we attempted to identify *L. loa* mf proteins present in the urine of *L. loa*-infected patients that could be used as the basis of quantitative immunoassays for the detection of *L. loa* mf-specific biomarkers in either plasma/serum or in urine.

RESULTS

Specificity of identified and selected proteins. Mass spectrometry analyses of urine samples from an *L. loa*-infected individual resulted in the identification of spectra matching those of 70 *L. loa* proteins, of which 18 proteins were detectable by at least 2 unique peptides and not present in normal uninfected urine (Table 1). All 18 proteins were identified to be *L. loa* mf proteins. Their corresponding mRNA expression (22) ranged from 2.07 to 3,841.10 fragments per kilobase per million (FPKM). Eight (44.4%) of the 18 *L. loa* urine-specific proteins were annotated as “hypothetical” proteins with unknown function (Table 1).

Further filtering the data for proteins with little or no sequence homology with human proteins shortlisted four *L. loa* proteins: LOAG_05915, LOAG_16297, LOAG_17808, and LOAG_18552 (Table 1). These four proteins were then assessed for having homologues in the other filaria sequenced to date—*B. malayi*, *Wuchereria bancrofti*, and *Onchocerca volvulus* (Table 2). As can be seen, LOAG_05915, LOAG_17808, and LOAG_18552 share significant sequence homology with *W. bancrofti*, *B. malayi*, and *O. volvulus* proteins, while LOAG_16297, a small 14-kDa hypothetical
protein, showed no homology to proteins of any of these filarial parasites nor to any nematode for which genomic sequences are available.

**Immunogenicities of the four selected *L. loa* proteins.** The immunogenicities of the protein antigens were assessed using hyperimmune rabbit antisera in a standard luciferase immunoprecipitation system (LIPS) assay. As shown in Fig. 1, there was minimal reactivity with the respective prebleed sera and robust reactivity with the hyperimmune sera (and their purified IgG) from two of the four fusion proteins, LOAG_17808 and LOAG_16297. In addition, the LOAG_17808 fusion protein was also recognized by purified IgG antibodies raised against *L. loa* somatic mf antigen (Fig. 1).

To evaluate the reactivity of these proteins in humans, sera/plasma from *L. loa*-infected patients and uninfected control subjects were used and compared to the reactivity to *L. loa* SXP-1, a previously described *L. loa* antigen (23). As expected, healthy-control samples had very low signals, with median anti-LOAG_16297, anti-LOAG_17808, and anti-SXP-1 antibody titers of 236, 97, and 746 light units (LU), respectively (Fig. 2). For *L. loa*-infected patients, the median values were 6 times higher for LOAG_16297 (1,423 LU), 148 times higher for LOAG_17808 (674,990 LU) than LOAG_16297 (1,423 LU), 148 times higher for LOAG_17808 than LOAG_16297. In addition, the LOAG_17808 fusion protein was also recognized by purified IgG antibodies raised against *L. loa* somatic mf antigen (Fig. 1).

**Specific Biomarker of Loa loa Microfilarial Infection**

**TABLE 2** Specificity of the four downselected *L. loa* mf proteins

| Protein ID (accession no.) | Immunogenic peptide sequence | *B. malayi* | *W. bancrofti* | *O. volvulus* |
|---------------------------|-----------------------------|------------|----------------|--------------|
| LOAG_05915 (EFO22569.1)   | CMRDKYRDTENE CLDDEKEQYKNKL | 76         | 65             | 0            |
| LOAG_18552 (EJD74082.1)   | CEEKQNREKAPNGD CEEKLEKPKSKKPNP | No hit      | 42             | 0            |
| LOAG_17808 (EJD74956.1)   | CEGENKDGKRRMDKSP CRPFDDERNSTYDKGN CVETRKYENR CKDSUTGVRNDESYFKQ | 91         | No hit         | No hit       |
| LOAG_16297 (EFO12236.1)   | No hit                      | No hit      | No hit         | No hit       |
considering microcopy to be the “gold standard” for *L. loa* mf quantification, the LOAG_16297 antigen LIPS assay had a sensitivity of 76.9% (95% confidence interval [95% CI], 56.3 to 91.0%), a specificity of 96.0% (95% CI, 79.6 to 99.9%), a positive predictive value (PPV) of 95.2% (95% CI, 76.2 to 99.9%), and a negative predictive value (NPV) of 80% (95% CI, 61.4 to 92.2%). For the LOAG_17808 competitive LIPS assay, the sensitivity, specificity, PPV, and NPV were 80.7 (95% CI, 60.6 to 93.5%), 37.5 (95% CI, 18.8 to 59.4%), 58.3% (95% CI, 40.8 to 74.5%), and 64.3% (95% CI, 35.1 to 87.2%).

**Correlation between the amount of LOAG_16297 antigen and the number of microfilariae.** To evaluate if the levels of antigen circulating in the plasma of *Loa*-infected individuals with a range of mf counts were correlated with the density of mf, Spearman’s rank correlation was performed between the plasma concentrations of the LOAG_16297 and LOAG_17808 proteins and the corresponding counts of *L. loa* mf as determined by microscopy (Fig. 5). As can be seen, there were significant positive correlations for LOAG_16297 (*r* = 0.71, *P* < 0.0001) (Fig. 5A) and for LOAG_17808 (*r* = 0.61, *P* = 0.0002) (Fig. 5B).

**DISCUSSION**

*L. loa* infection has recently gained prominence because of the SAEs occurring after ivermectin administration in some individuals harboring high *L. loa* mf densities (4, 5). Most of the currently available tools and methods (11, 13, 23, 24) that are being used to quantify *L. loa* mf are impractical for POC field-testing. Developing a quantitative immunoassay for *L. loa* mf that could be used for field screening to identify individuals at high risk of SAE would be of great benefit to MDA programs.

**FIG 2** Immunogenicity of LOAG_16297, LOAG_17808, and SXP-1 in humans. The levels of IgG specific to LOAG_16297 (A), LOAG_17808 (B), and SXP-1 (C) were assessed by LIPS assay, and light units (LU) were compared between *Loa*-infected subjects and uninfected controls. The horizontal red solid line represents the median level for each group, and the horizontal black dotted line indicates the threshold of sensitivity/specificity of the assay determined by ROC analysis. Each individual is represented by a single dot, with closed circles used for the *Loa*-infected individuals and open circles for the uninfected individuals.

**FIG 3** Principle of the antigen LIPS assay and relationship between the percentage of protein inhibition and amount of protein. (A) Schematic of the general steps involved in competitive LIPS antigen detection in which the *Renilla* luciferase (Ruc) fusion constructs of the antigen of interest are incubated with serum containing unfused antigen. These antigens are then immobilized on agarose beads containing antigen-specific IgG. After washing, the amount of specific antigen present is determined by the inhibition of the Ruc fusion construct by the unfused antigen after addition of luciferase substrate. Panel B shows the percentage of inhibition as a function of spiked recombinant protein in human AB serum for LOAG_16297 (blue) and LOAG_17808 (red).
Thus, we have identified 18 proteins present only in *L. loa* mf-infected urine by using a high-throughput RPLC-MS/MS proteomic approach. We then developed antigen-based competitive LIPS assays for the 2 (LOAG_16297 and LOAG_17808) that were immunogenic and highly (and/or relatively) specific to *L. loa* mf. One of these, LOAG_16297 showed excellent diagnostic performance and has great promise for a potential field use as a POC diagnostic tool.

The presence of parasite proteins in urine should be a surrogate for their availability in the circulation and, therefore, should provide an accurate source for biomarker discovery useful for disease diagnosis (25). In our study, only 18 were found exclusively in the urine of the *Loa*-infected patient compared to urine from uninfected individuals, suggesting some promiscuity in this proteomic approach. This number of urine-identified proteins was relatively low considering the total number of putative *L. loa* proteins (15,444) (22). That is certainly due to the fact that there are many fewer proteins in urine than in the plasma (26), the majority of proteins found in the blood being reabsorbed through the renal glomeruli. We also cannot exclude the role played by variability among the analytes in the urine (27), factors related to the MS/MS instrument itself (28), or the fact that so many *Loa* proteins had similar human sequences. Therefore, it would be more advantageous to perform proteomics of urine samples from multiple *Loa*-infected individuals. Nevertheless, the data gleaned from one urine sample from an infected individual allowed us to identify potential biomarker candidates and then validate the most important ones.

Most of the identified *Loa*-specific urine proteins (14/18) have orthologues in humans. Among the four *Loa* mf proteins unrelated to human proteins, only two, LOAG_16297 and LOAG_17808, could be studied closely as they were immunogenic in rabbits. In addition, antibodies raised against *L. loa* crude somatic mf antigen recognized LOAG_17807 and LOAG_16297 (to a lesser degree), suggesting that they make up a significant fraction of the mf-specific antigen mix. Furthermore, antibodies to both LOAG_16297 and LOAG_17808 were present in *L. loa*-infected patients. Although not the main purpose of the present study, these antigens show promise in an antibody-based immunoassay with sensitivities and specificities similar to or close to what has been observed with *L. loa* SXP-1 (23, 29) antibody profiling for *L. loa* diagnosis.

In general, antibody-based assays often are unrelated to parasite burden, and a correlation between antibody level and parasite density is likely to be difficult (if not impossible). In addition, specific antibodies cannot distinguish between previous and new infections and often persist indefinitely after treatment or exposure (29). Antigen-based assays are then the sine qua non for diagnosis of infectious diseases. Thus, we developed methods for rapidly testing the validity of such assays using a single antibody specific for the protein in question and a mammalian-expressed recombinant protein that could be used without purification. Such an assay, termed reverse (or competitive) LIPS, relies on the ability of the antigen(s) in serum (or other biological samples) to inhibit a fixed concentration of the same protein that is luciferase fused (Fig. 3A). In addition to its simplicity, the competitive LIPS assay could identify *L. loa*-infected patients and quantify the *L. loa* mf level rapidly and with good accuracy. Only 45 to 60 min of total processing time (including preparation and wash times) per 94 plasma samples is needed compared to hours for enzyme-linked immunosorbent assays (ELISAs). Moreover, ELISAs for antigen

### TABLE 3 Performance of *L. loa* mf-specific proteins on clinical samples using a LIPS competitive assay

| LIPS assay type | Status | No. of samples: | % sensitivity (95% CI) | % specificity (95% CI) | PPV (95% CI) | NPV (95% CI) |
|----------------|--------|-----|---------------------|------------------------|-------------|------------|
| LOAG_16297     | Positive | 20 | 76.9 (56.3–91.0) | 96.0 (79.6–99.9) | 95.2 (76.2–99.9) | 80.0 (61.4–92.3) |
|                | Negative | 6  | 24                   |                        |             |            |
| LOAG_17808     | Positive | 21 | 80.7 (60.6–93.5) | 37.5 (18.8–59.4) | 58.3 (40.8–74.5) | 64.3 (35.1–87.2) |
|                | Negative | 5  | 9                    |                        |             |            |

*PPV, positive predictive value; NPV, negative predictive value. The 95% confidence interval (95% CI) is indicated for each parameter.*
often require at least two different antibodies (e.g., directed against different epitopes of the same protein), a requirement that often cannot be satisfied. The presumed increased sensitivity of the LIPS-based system is likely due to the assay performance in solution, our ability to detect many more conformational epitopes than a standard solid-phase ELISA (23), and the fact that highly purified recombinant antigen is not needed.

In the present study, we have shown that both circulating LOAG_16297 and LOAG_17808 antigens can be detected in some plasma of Loa-infected individuals. Interestingly and more in line with its potential utility as a POC method to identify those at risk for SAEs following ivermectin, there was a significant positive relationship between the amounts of LOAG_16297 and LOAG_17808 proteins detected in Loa-infected samples and the mf levels in blood of the same samples. Furthermore, the assay to detect LOAG_16297 described herein showed the best correlation to mf data and had the best specificity, PPV and NPV (Fig. 5 and Table 3). The lower specificity of the assay for LOAG_17808 is not surprising since the protein shares 91% sequence identity to a PWWP domain protein of W. bancrofti (and other filariae).

There are currently no commercial products available using LIPS-based tests. In addition, the development of a LIPS assay for a POC use may be difficult (if not impossible) mainly because of the high cost of the LIPS platform. Nevertheless, the use of LIPS has allowed us to validate a single identified antigen (LOAG_16297) as being quantitative and specific. With a potential candidate biomarker in hand, we hypothesize that generation of monoclonal antibodies for use in a cheaper standard antigen capture or lateral-flow immunoassay can be configured for POC testing. The use of monoclonal antibodies will increase the affinity of the searched antigens to their specific antibodies and likely improve the performance of the assay.

In summary, we have used an untargeted protein profiling approach (RPLC-MS/MS) to discover 18 new putative biomarkers of L. loa mf infection in the urine of a microfilaremic patient with L. loa. Among them, one immunogenic and highly L. loa mf-specific protein, LOAG_16297, can be detected in plasma/serum in a competitive LIPS assay format, with the amounts being detected correlating well with the quality of L. loa mf found in the peripheral blood. Therefore, the use of LOAG_16297 as a biomarker could be important in a POC assessment tool to be used as the basis of an effective TNT strategy.

**MATERIALS AND METHODS**

**Study population and samples.** Samples were collected from subjects as part of registered protocols approved by the Institutional Review Boards of the National Institute of Allergy and Infectious Diseases for the filaria-infected patients (NCT00001345) and for healthy donors (NCT0090662). Written informed consent was obtained from all subjects.

Urines from one microfilaremic (17,000 mf/ml) L. loa-infected patient assessed at the NIH Clinical Center and one normal North American donor (who had never traveled outside the United States) were used for the profiling of specific L. loa mf proteins by RPLC-MS/MS.

Plasma samples used to validate the utility of potential biomarkers were from L. loa-infected individuals (n = 31 [26 microfilaremic and 5 microfilarematic]). Samples used as controls included those from subjects with W. bancrofti infection (mf; n = 15) from India and the Cook Islands (both nonendemic for L. loa), subjects with O. volvulus infection (mf; n = 15) from Ecuador (nonendemic for L. loa), and those from North America who had no history of exposure to filariae or other helminths and who had never traveled outside North America (n = 31). The parasitological diagnosis of all infections was made based on the demonstration of mf in the blood (for W. bancrofti and L. loa) or in the skin (for O. volvulus) using standard techniques (11, 30) or by finding adult parasites in the tissues (e.g., the eye for L. loa).

**Sample preparation prior to mass spectrometric analysis.** Urine samples were processed according to a workflow adapted from Nagaraj et al. (31). Briefly, urine samples were centrifuged for 15 min at 4°C and the supernatant was concentrated using a spin filter with a molecular mass cutoff of 3 kDa. Proteins were precipitated by acetone precipitation and subsequently treated in 10 mM Tris-HCl at 95°C for 5 min. The samples were then reduced, alkylated, and double digested with Lys-C in combination with trypsin overnight at 37°C. Tryptic peptides were further desalted, lyophilized, reconstituted in 25% acetonitrile with 0.1% formic acid, and further fractionated using strong cation exchange (SCX) chromatography. The SCX fractions of the urine samples were pooled into 32 fractions, lyophilized, and reconstituted in 0.1% trifluoroacetic acid (TFA) to be analyzed by liquid chromatography-mass spectrometry (LC-MS).

**Nanobore RPLC-MS/MS.** Nanobore reversed-phase liquid chromatography-tandem mass spectrometry (RPLC-MS/MS) was performed using an Agilent 1200 nanoflow LC system coupled online with an LTQ Orbitrap Velos mass spectrometer. The RPLC column (75-μm inside diameter [i.d.] by 10 cm) was slurry packed in-house with 5-μm, 300-Å pore-size C18 stationary phase into fused silica capillaries with a flame-pulled tip. The mass spectrometer was operated in a data-dependent mode in which each full MS scan was followed by twenty MS/MS scans, wherein the 20 most abundant molecular ions were dynamically selected for
collision-induced dissociation (CID) using a normalized collision energy of 35%.

Protein identification and quantification. The RPLC-MS/MS data were searched using SEQUEST through Bioworks interface against an L. loa database downloaded from the Broad Institute (version 2.2). Dynamic modifications of methionine oxidation as well as fixed modifications of carbamidomethyl cysteine were also included in the database search. Only tryptic peptides with up to two missed cleavage sites meeting the following specific SEQUEST scoring criterion were considered legitimate identifications: delta correlation (ΔCn) of ≥0.1 and charge-state-dependent cross-correlation (Xcorr) of 1.9 for [M+H]+, ≥2.2 for [M+2H]2+, and ≥3.5 for [M+3H]3+.

Transcriptomics data. mRNA expression levels (putative proteins) of the mf state of L. loa were obtained using RNAseq as part of the L. loa genome project previously described (22).

Protein/peptide selection for immunosassays. L. loa mf proteins identified only in the infected urine (absent in the uninfected urine) were downselected for immunosassays based on comparison of sequence homologies against human proteins and those of L. loa and other related filarial species (B. malayi, O. volvulus, and W. bancrofti) or any other relevant nematode for which genome is available.

Proteins that showed no or little homology to non-Loa sequences were selected for identification of immunogenic peptides using Protein (LaserGene Suite). Among these, we chose the 2 peptides that were potentially the most immunogenic and Loa specific (i.e., with no significant hit to human or other filarial nematodes) per protein. These peptides were synthesized by the NIAID Peptide Facility as unconjugated free peptides and conjugated to keyhole limpet hemocyanin (KLH), the latter used to produce specific polyclonal antibodies in rabbits.

Generation of rabbit polyclonal antibodies. KLH-conjugated peptides were used to raise polyclonal antisera in rabbits using standard protocols as previously described (32). In addition, polyclonal antisera were raised against a somatic extract of L. loa mf using the same standardized protocols. After assessment of the reactivity of each of the antisera to its appropriate free peptide by enzyme-linked immunosorbent assay (ELISA), the IgG was purified from the sera using protein A/G (Pierce, Rockford, IL) columns. These purified IgG antibodies were used as capture antibodies in the luciferase immunoprecipitation system (LIPS) assay for antigen detection (see below).

Fusion proteins and COS-1 cell transfection. Fusion proteins were made for each of the in silico selected proteins by cloning the full-length gene expressing the protein of interest into a FLAG-epitope-tagged mammalian RevTulla reniformis luciferase (Ruc)-containing expression vector, pREN2 (33). Extracts (lysates) containing the light-emitting Ruc-antigen fusions were prepared from 100-mm2 dishes of 48-h-transfected COS-1 cells as previously described (33, 34) and frozen until use for LIPS.

LIPS-based antibody and antigen detection systems. For evaluation of antibody titers, a standard LIPS antibody-based assay was used (23, 34, 35). Briefly, 100 µl of the assay master mix (20 mM Tris [pH 7.5], 150 mM NaCl, 5 mM MgCl2, 1% Triton X-100), 1 µl of undiluted plasma/serum, and 2 × 105 light units (LU) of the Ruc-antigen fusion protein were added to each well of a 96-well polystyrene plate. This plate was then incubated for 10 min at room temperature. Next, 7 µl of a 30% suspension of Ultralink protein A/G beads (Pierce, Rockford, IL) in phosphate-buffered saline (PBS) was added to the bottom of a 96-well high-throughput-screening filter plate (Millipore, Bedford, MA). The 100-µl antigen-antibody reaction mixture from each microtiter well of the 96-well polystyrene plate was then transferred to the well of the filter plate, which was further incubated for 10 to 15 min at room temperature. The filter plate containing the mixture was then applied to a vacuum manifold. The retained protein A/G beads were washed with the assay master mix and with PBS (pH 7.4), the plate was blotted, and the LU were measured in a Berthold LB 960 Centro microplate luminometer, using a coelenterazine substrate mixture (Promega, Madison, WI).

For quantification of antigens, the original LIPS antibody-testing for-mat was modified for use in a competitive LIPS assay (Fig. 3A). Having first coupled the purified antigen-specific IgG to Ultralink beads (Pierce, Rockford, IL), 5 µl of a 50% suspension (in PBS) of these beads (specific IgG-Ultralink beads) was added to the bottom of a 96-well filter plate. Glycine-treated plasma/serum (36) diluted 1/5 was added to the beads for 30 min at room temperature. Then, an optimized number of specific LU of Ruc-antigen fusions was added in each well and the mixture was incubated for 10 min at room temperature. Specific IgG-Ultralink beads were washed with the assay master mix and then with PBS. The plate was blotted, and LU were measured with a Berthold LB 960 Centro microplate luminometer. The percentage of inhibition was calculated for each sample, and the quantity of specific protein in each sample was estimated by using a standard curve designed using known concentrations of each protein in 1/5 diluted human AB serum (Fig. 3B).

All samples were run in duplicate. All LU data presented were corrected for background by subtracting the LU values of beads incubated with Ruc-antigens but no serum.

Statistical analysis. Figures and statistical analyses, including specificity and sensitivity calculations (ROC analysis) and correlations (Spearman’s rank), were performed using Prism 6.0 (GraphPad Software, Inc., San Diego, CA). Fischer’s exact test was used to compare the percentages of positivity between groups, and the nonparametric Mann-Whitney test was used to estimate differences in amounts of antigen between two groups. All differences were considered significant at the P < 0.05 level.

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