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Potential of a Software Package and Web Tool (SLDAssay) for Estimating *Leishmania* Parasite Burden in Limiting Dilution Assays

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ABSTRACT

Background and Aim: Several methods exist for assessing parasite load in leishmaniosis. However, limiting dilution assay (LDA) is one of the most reliable tests for quantifying parasite burden in experimental models. Many software programs are available for analyzing LDA results; however, they offer different outputs, which might not be suitable for reporting parasite burden. ELIDA is considered a primary tool for this purpose; nevertheless, this software is inaccessible. Thus, it is essential to identify new software to substitute for this program. Our main objective for this study is to compare the performance of two software platforms, ELIDA and SLDAssay, in assessing parasite burden in the murine leishmaniasis model and determine whether these tools can be used interchangeably.

Methods: In this study, we utilized a dataset derived from our previous research to evaluate the performance of the SLDAssay software. Data analysis focused on comparing correlations and trends between the ELIDA and SLDAssay software programs across various treatment groups.

Results: A robust positive correlation was found between ELIDA and SLDAssay measurements ($R^2 = 0.9367$, $P < 0.0001$). Both software platforms exhibited comparable patterns of infection load across all treatment groups and individual mice, effectively differentiating between therapeutic and control groups.

Conclusion: The results indicate that ELIDA and SLDAssay produce comparable and dependable measurements of *Leishmania Major* infection burdens in murine models. These methodologies can be utilized interchangeably in research on leishmaniasis, providing flexibility in data analysis while maintaining their reliability.

INTRODUCTION

Leishmaniasis is one of the hot topics in pre-clinical research due to the lack of a competent vaccine, and also the drug resistance of this parasite (1, 2). More than 12500 articles about *Leishmania* were found on PubMed from 2015 to 2025, suggesting its significance. One of the most common tests in research laboratories is quantifying parasite load in the lymph node or spleen of animal models. Several methods exist to investigate parasite burden in infected tissues, like qPCR, direct fluorescent microscopy, release of amastigotes, Leishman- Donovan Units, and limiting dilution assay (LDA); however, LDA is still the gold standard for this purpose (3-5).

LDA is extensively employed in the field of infectious disease research .This test is predominantly used to measure the extent and severity of the infection by estimating the frequency of microorganisms within a sample. This method is suitable in situations when accurate counts of the microorganism are complex (6). The obtained sample is divided into subsamples at lower cell counts through the process of dilution. When preparing the dilution series and replicate wells, it is crucial to ensure thorough mixing of each sample before the subsampling process (7, 8). In the

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LDA test, sensitivity for detecting low burdens of *Leishmania* parasites is enhanced by using a large initial count and distributing it into multiple wells for precision in estimating parasite frequency (6, 7). The primary objective of a dilution assay is to select a sufficiently wide range of dilutions to guarantee a transition from positive to negative results as one progresses through the dilution sequence. The dilutions at which the transition occurs contain information on the counts of target entities in the original sample.

Titus et al. introduced the LDA method in the 80s to quantify *Leishmania major* in infected mice (7). Taswell et al introduced the required statistics for analyzing LDA data in the same decade, a software application called ELIDA (9). This software generally operates under the assumption of the single-hit Poisson model equation and the chi-squared statistical method. According to this model, the quantity of biologically active particles present in each culture is distributed according to a Poisson distribution, and the presence of a single biologically active cell is deemed sufficient to elicit a positive response from the culture (9, 10). Taswell has graciously agreed to allow free distribution for those who wish to analyze results of *Leishmania* parasite burden assay.

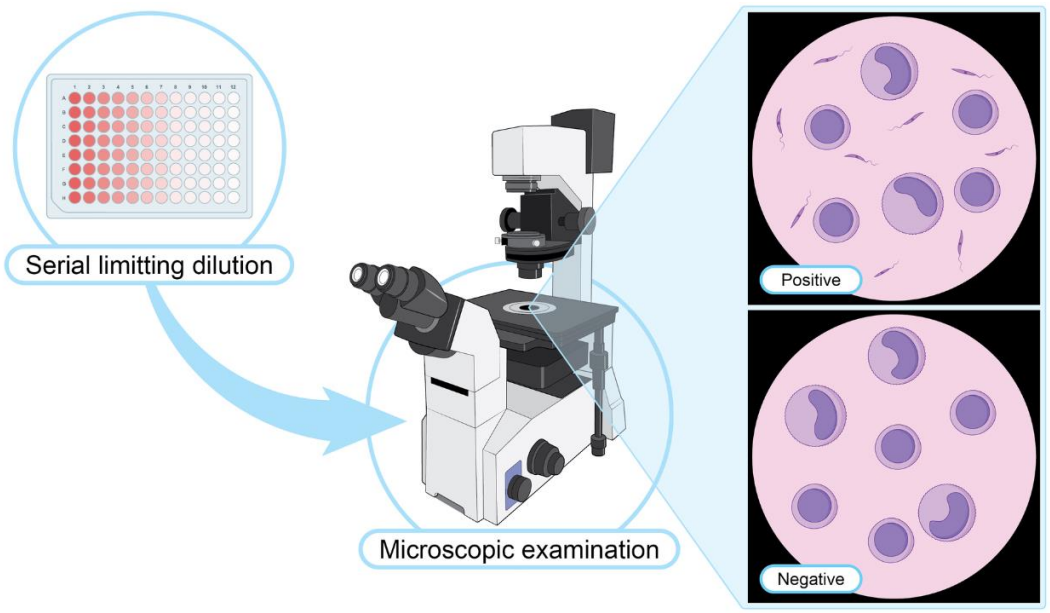
For more than a decade, our team has been utilizing ELIDA and Leishmaniasis LDA (L-LDA), another inaccessible web tool from Imperial College of London http://www.imperial.ac.uk/theoreticalimmunology/llda, to calculate the Leishmania burden in our research. However, in the past years, these software applications have been inaccessible or unavailable to researchers. Therefore, it is imperative to identify and implement new software applications for LDA test analysis.

SLDAssay, a software developed by the University of North Carolina at Chapel Hill, is a new tool for analyzing LDA data sets (11). This web tool utilizes Maximum likelihood estimation (MLE) and minimum chi-square for calculating infection load. Similar to ELIDA, the primary objective of SLDAssay is to determine the infection count of the original sample based on the dilutions at which the transition occurs (11). SLDAssay hasn't yet proven valuable for assessing *Leishmania* parasite burden; therefore, a comparison with ELIDA is necessary to validate this new software. The primary objective of this paper is to assess the correlation between parasite burden obtained from ELIDA and SLDAssay analysis tools. We want to determine whether the analyzed data of the SLDAssay software is comparable to that of the ELIDA. This comparison could be beneficial for validating SLDAssay data in research laboratories. We used the ELIDA data from one of our previous works (12). SLDAssay software was employed to reanalyze the data, which was compared with the parasite burden data obtained from ELIDA to validate the new software.

MATERIALS and METHODS

Study design

This study is based on a re-analysis of raw data obtained from our previously published *in vivo* study (12), and does not include any new *in vivo* experiments. In the original study, twelve female mice were infected with 2×10^5 promastigotes at the base of the tails and divided into three treatment groups: Chitin, Chitosan, and PBS. They were sacrificed twelve weeks post-treatment. Their lymph nodes were collected in order to perform the LDA test following Lima et al.'s protocol with some modifications (13). Briefly, twelve 10-fold dilutions of lymph node cells were prepared using Schneider medium (Himedia, India) supplemented with 12% FBS (Gibco, USA) and 1% penstrep (Gibco, USA), which were done in 96-well cell culture plates. Then, 200 µl of each serial dilution cell suspension was added to eight wells of the plate. These plates were cultured at 26 °C with no CO_2 for 5 days. The test results were read by scoring positive (presence of motile parasites) and negative (absence of motile parasites) wells using an inverted microscope (Nikon Eclipse TE2000, Japan) (Fig.1). The raw data were imported into an Excel file and evaluated by the ELIDA software to assess the parasite load. An example of the raw data is depicted in Fig. 2. The present study used the same raw data to estimate parasite burden using SLDAssay software.



			Responding group									
	1	2	3	4	5	6	7	8	9	10	11	12
A	+	+	+	-	-	-	-	-	-	-	-	-
B	+	+	+	-	-	-	-	-	-	-	-	-
C	+	+	+	+	+	-	-	-	-	-	-	-
D	+	+	+	-	-	-	-	-	-	-	-	-
E	+	+	+	+	-	-	-	-	-	-	-	-
F	+	+	+	+	-	-	-	-	-	-	-	-
G	+	+	+	-	-	-	-	-	-	-	-	-
H	+	+	+	-	-	-	-	-	-	-	-	-

FIGURE 1. LDA test report. For performing and reporting the LDA test, the total number of lymph node cells was counted and serially diluted 10-fold across a 96-well plate. The plate was then incubated at 26 °C with no CO_2. Five days post-incubation, plates were observed with an inverted microscope for the presence of motile parasites and reported as positive or negative for each well.

		Start Dilution Factor	1\10	cell count	Total in 2ml	Result (SAS)
		Input Numeric of dilution	0.10			
Group		Input Reverse of DF*10	200.00			
Chitin-2	Dilutions (10 fold)	Positive wells	cell count	15	15000000	
1	0.1	8	300000.00			
2	0.01	8	30000.00			
3	0.001	8	3000.00			
4	0.0001	3	300.00			
5	0.00001	1	30.00			
6	0.000001	0	3.00			
7	0.0000001	0	0.30			
8	0.00000001	0	0.03			
9	0.000000001	0	0.00			
10	1E-10	0	0.00			
11	1E-11	0	0.00			
12	1E-12	0	0.00			1.26E+06

FIGURE 2. LDA test result. After performing LDA test, we entered the cell counts and positive responses of each dilution into an Excel data sheet to analyze the data using ELIDA and SLDAssay.

SLDA program and data analysis

We used the SLDAssay to analyze the data collected from our previous LDA tests. This section is written for readers wishing to use the web tool. SLDAssay is implemented as a web tool through the Shiny R package at https://iupm.shinyapps.io/sldassay/ (11). No software downloads or other actions are necessary to use this web tool. Researchers can input their data into the website's table, which consists of three columns representing the number of cells per well, the number of replicates, and the count of positive wells. Rows could be added or removed based on the number of dilution levels and the experiment. Additionally, the user can specify the number of Monte Carlo samples, the desired confidence level for the confidence interval (CI), and whether to provide the MLEs (maximum likelihood estimates) as IUPM (infectious units per million) or as the probability that a cell is infected. Fig. 3 illustrates the use of the web tool for the example assay shown in Fig. 2.

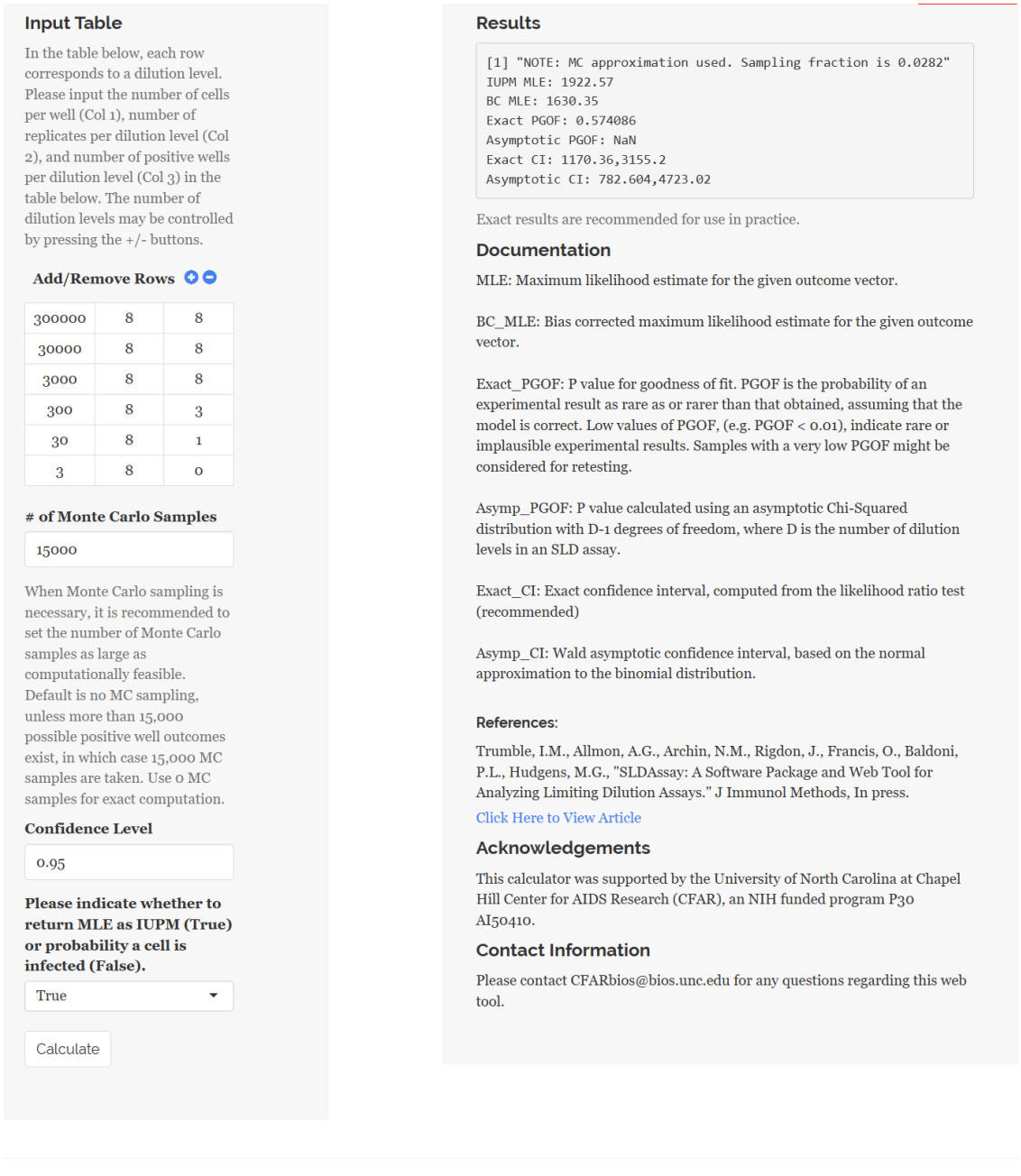


FIGURE 3. Utilizing SLDAssay to obtain parasite load. Following the LDA test and the acquisition of initial results, data regarding the quantity of cells at each dilution, the number of replicates for each dilution, and the count of wells testing positive for motile parasites were documented in the table on the left side. A confidence level of 0.95 was established, and the number of Monte Carlo iterations was fixed at 15,000. Ultimately, the parasite burden was determined in terms of IUPM units, which is reported as 1922.57 IUPM.

Statistical analysis for comparing SLDAssay with ELIDA

All statistical analyses were conducted using SPSS (version 29.0) and GraphPad Prism (version 10.0). One-way ANOVA and Pearson's correlation analyses were utilized to compare the outcomes of the SLDAssay with those of the ELIDA method. A p-value of < 0.05 was deemed statistically significant. Data are presented as mean ± standard deviation (SD).

RESULTS

Evaluation of the effect of treatments on the parasite load by SLDAssay

L. major leads to a persistent infection in susceptible BALB/c mice, characterized by advancing skin lesions and the spread of the parasite to internal organs, causing visceralization (14, 15). In this study, we found that the parasite burden in the lymph nodes of the treated groups was significantly lower than in the control group, and the parasite load in mice receiving chitin was lower than in the chitosan receiving group. Fig. 4 illustrates the *L. major* burden in the lymph nodes of twelve mice in three study groups, which was obtained by analyzing LDA results with SLDAssay and ELIDA software. The control group with no therapy has the highest load ($2.87\times10^5 \pm 2.9\times10^5$ IUPM and $7.37\times10^7 \pm 7.11\times10^7$ parasite / lymph node). Meanwhile, chitin ($2.20\times10^3 \pm 1.97\times10^3$ IUPM and $1.31\times10^6 \pm 1.73\times10^6$ parasite / lymph node), and chitosan ($7.32\times10^3 \pm 8.6\times10^3$ IUPM and $7.49\times10^6 \pm 1.15\times10^7$ parasite / lymph node) treatment groups had lower parasite count. Both data sets suggest the same trend of treatment effectiveness and reduction in infection.

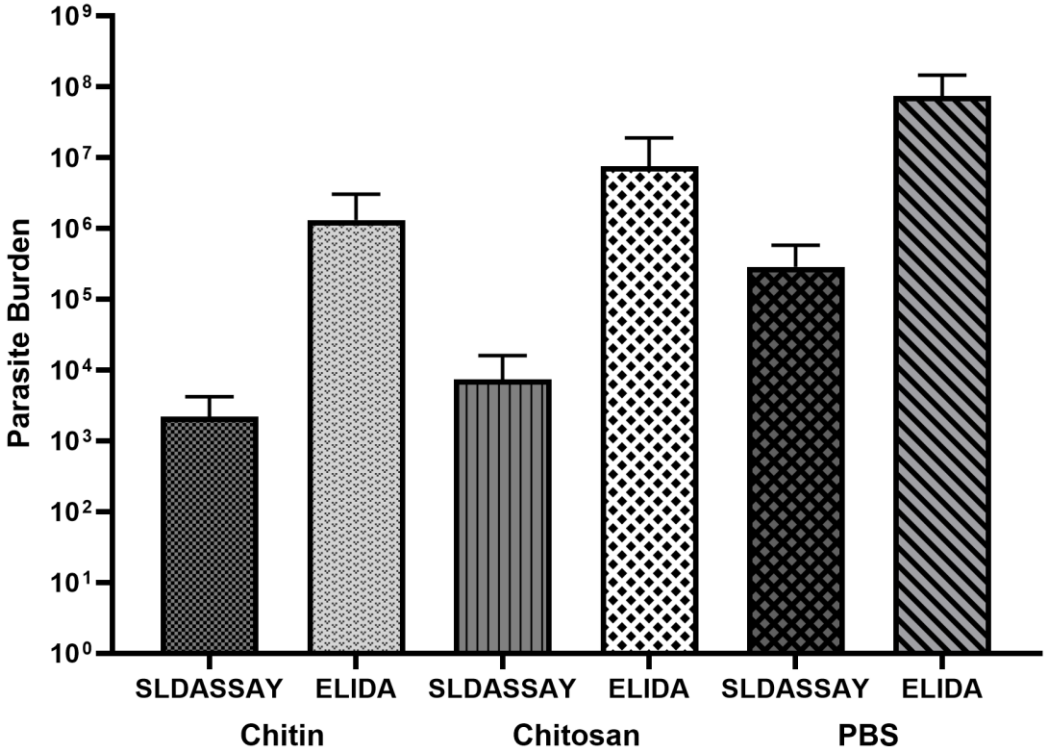


FIGURE 4. Parasite burden assay via SLDAssay and ELIDA. The effect of treatments on the multiplication of *L. major* was quantified by limiting dilution assay. Each column represents the mean parasite load among four mice. The parasite burden determination with SLDAssay for chitin, chitosan, and control groups was $2.20\times10^3 \pm 1.97\times10^3$, $7.32\times10^3 \pm 8.6\times10^3$, $2.87\times10^5 \pm 2.9\times10^5$ IUPM, respectively. Parasite load quantification with ELIDA for chitin, chitosan, and control groups was $1.31\times10^6 \pm 1.73\times10^6$, $7.49\times10^6 \pm 1.15\times10^7$, $7.37\times10^7 \pm 7.11\times10^7$ parasite / lymph node, respectively. The standard deviation is represented by the bars.

Correlation of two Data sets

Upon analyzing the infection load across the different groups using two distinct software tools, we observed a potential correlation between the results obtained from each platform (Fig. 5). Linear regression analysis and the computation of the correlation coefficient are commonly employed to assess the outcomes of two measurement methods for the same data sets (16). We observed a positive correlation between ELIDA and SLDAssay in determining parasite load with a correlation coefficient of 0.9689 ($P < 0.0001$). The coefficient of determination (R^2) for the linear model was 0.936 ($P < 0.0001$) (Fig. 6). This finding suggests

concordance among the data obtained from two distinct software programs. These results suggest that ELIDA and SLDAssay may be used interchangeably for assessing infection loads.

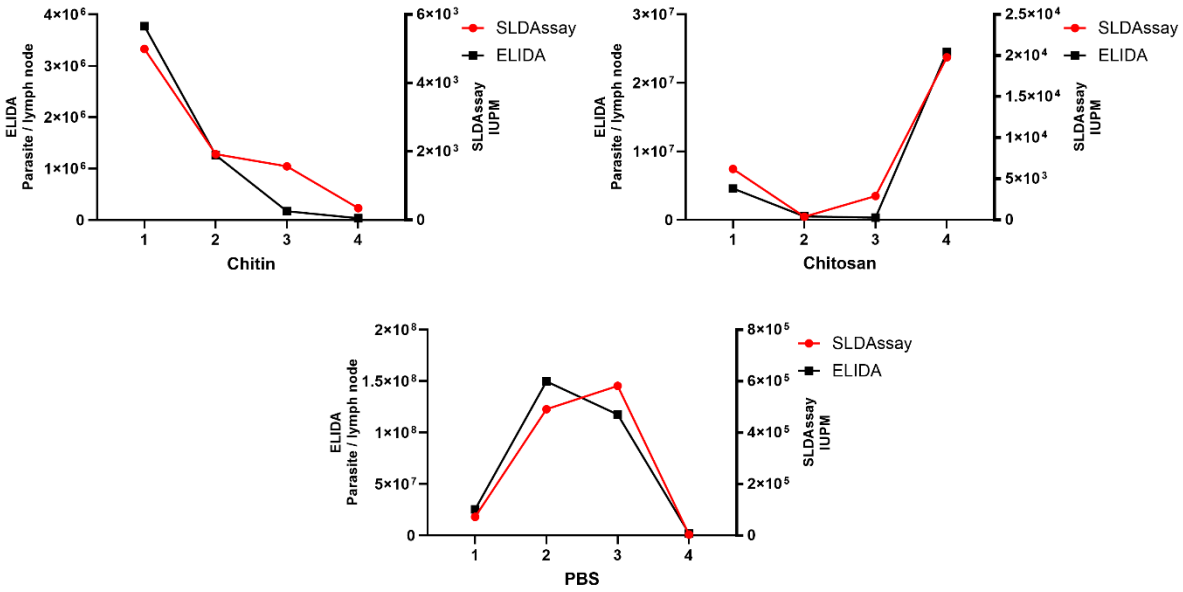


FIGURE 5. Parasite burden across treatment groups via *ELIDA and SLDAssay*. The line graphs illustrate the parasite burden obtained from ELIDA (black square, left y-axis) and SLDAssay (red circles, right y-axis) for each individual mouse within study groups. Both assays exhibit similar trends of infection load across the treatment groups, suggesting consistent and parallel measurements.

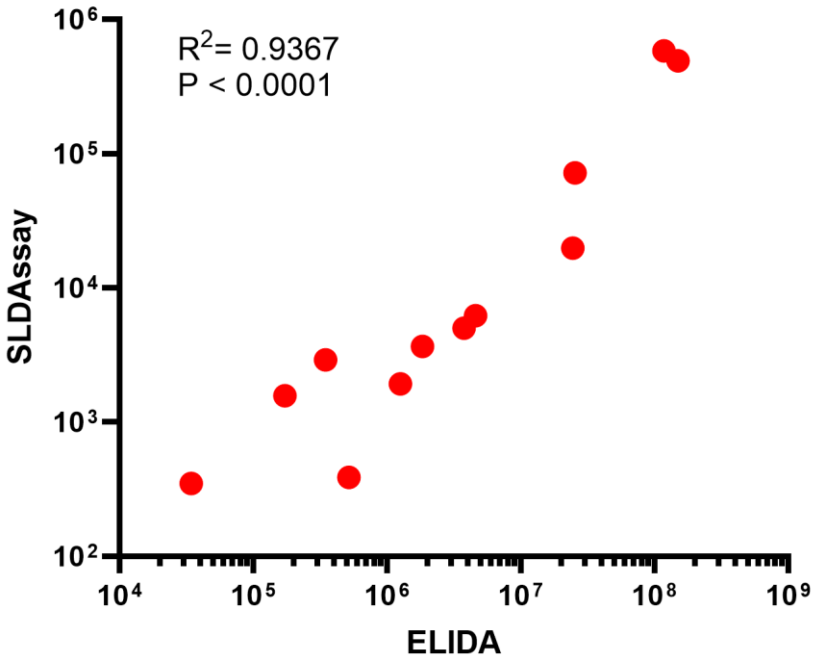


FIGURE 6. Correlation between ELIDA and SLDAssay LDA measurements. This scatter plot (logarithmic scale) shows the relationship between ELIDA and SLDAssay results, indicating a strong positive correlation with an R^2 value of 0.9367 and p-value of < 0.0001.

DISCUSSION

Limiting dilution assays have been employed in numerous biological and scientific settings for over a hundred years, most notably for quantifying bacteria (17), immunocompetent cells (18) or stem cells (19). Despite over a century of methodological development in LDA

techniques, the sophisticated statistical methods required for accurate analysis have often been inaccessible to immunologists due to a lack of user- friendly software. In this study, we investigated SLDAssay for calculating parasite burden and compared it with the most common tool, ELIDA. Our findings suggest that this web-based tool may serve as a suitable alternative to ELIDA, enabling interchangeable use in relevant applications. The SLDAssay provides researchers access to optimal LDA statistical techniques, without the need to install software or undertake any programming.

We also recognized that SLDAssay is more sensitive in the case of fold changes among the study groups; but the overall trend of changes remains consistent across both software programs (Fig. 4). The parasite burden assessed using the SLDAssay revealed that the chitin and chitosan groups demonstrated a reduction of 130-fold and 39-fold, respectively, when compared to the control group. Additionally, the results obtained from the ELIDA showed reductions of 56-fold and 10-fold for the same groups, respectively. However, both software applications demonstrate the immunotherapeutic effects of chitin and chitosan against *Leishmania major* infection. We should keep in mind that when calculating *Leishmania* parasite burden, the absolute value of results is not the main consideration; instead, the trend and comparison between groups are the primary purpose of the method. Thus, it seems that in the absence of access to the ELIDA software, SLDAssay is an alternative for parasite burden evaluations. Nevertheless, determining which program can provide a more accurate distinction requires further studies, additional laboratory evidence, a larger sample size, comparison with other common techniques, and expert opinions.

The main focus of SLDAssay is on the improvement of the data measurement obtained from serial limiting dilution assays, including the quantitative virus outgrowth assay (QVOA) utilized in HIV cure studies, to evaluate the efficacy of anti- latency treatments (11). This software computes the maximum likelihood estimate (MLE) for the counts of target cells, along with corresponding exact and asymptotic confidence intervals. Exact and asymptotic goodness of fit p-values, and a bias- corrected (BC) MLE are also provided. One significant advantage of SLDAssay over other applications is that the software's R package is available for offline use. In the event of a server shutdown for the web tool, the offline version remains accessible. Developers of SLDAssay claimed that there is no other publicly available software that currently implements the BC MLE or the exact methods for LDA tests. Nevertheless, we found that another online tool (IUPMStats available at http://silicianolab.johnshopkins.edu) (20), which was developed before the SLDAssay, can assist as a calculator to estimate infection frequencies from limiting dilution assays of *Leishmania* parasite burden and give similar results based on IUPM (data not shown).

Several software applications have been developed for LDA data analysis; for instance, L-Calc (by STEMCELL Technologies) and ELDA (developed by The Walter and Eliza Hall Institute of Medical Research) (21). Yifang Hu and Gordon K. Smyth, developers of ELDA, claim that it is applicable in all common LDA situations as a valuable resource for stem cell, cancer, and immunological research (21). However, not all of these programs are suitable for immunoparasitological assays, as they report outputs in different formats, which are not directly appropriate for expressing *Leishmania* parasite burden. Although manual conversion to IUPM is possible, this extra step can be time-consuming and may lead to misinterpretation. Our experience with these tools supports the adoption of IUPM as a more intuitive and standardized reporting format, facilitating clarity and comparability across studies.

Future investigations will require collaboration between biostatisticians and immunoparasitologists to refine and standardize these tools, as well as to develop consensus guidelines for quantifying parasite burden among researchers working in the field of *Leishmania* infections. This will enable the comparison of results from various studies conducted globally.

CONCLUSION

Our findings suggest that SLDAssay may serve as a promising alternative to ELIDA for the detection of parasite load of *Leishmania* infection in murine models. The consistent trend and robust correlation with ELIDA, in addition to demonstrating greater sensitivity in detecting fold changes among the study groups, underscore the reliability of this software. Progress toward more standardized methodologies and accessible, sustainable tools plays a critical role in ensuring reproducibility in immunoparasitological research. Future investigations should focus on refining these tools and developing consensus guidelines for quantifying parasite burden.

AUTHOR CONTRIBUTIONS

Author 1 and Author 3 developed the theory and planned the research. Author 3 supervised the project. Three authors provided critical feedback and assisted in writing the manuscript.

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CONFLICT OF INTEREST

The authors declare no conflict of interest.

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