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REPRODUCIBILITY OF ALKALINE ANTIGENS OF *Leishmania major*-LIKE AND *Leishmania (V.) braziliensis* EVALUATED BY IgG-ELISA. COMPARISON OF ANTIGENS ADDED OF A PROTEIN INHIBITOR (PMSF) OR NOT

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SUMMARY

This paper deals with the analysis of 10 batches of *L.major*-like and *L.(V.) braziliensis* antigens added or not of a proteases inhibitor evaluated by means of an IgG-ELISA on three consecutive days using positive standard sera from patients with diagnosis of American Leishmaniasis previously tested for the presence of IgG antibodies by means of ELISA. The statistical analysis showed that for *L. (V.) braziliensis* the PMSF-containing antigen did not show any difference among batches or days of testing; the *L.(V.) braziliensis* antigen without PMSF showed statistical significance for differences among batches and a two-way ANOVA showed significant differences between antigens. *L.major*-like antigen prepared with or without PMSF showed differences among batches; all 3 days of testing displayed differences for the PMSF antigen but only for days 1 and 2 for the antigen without inhibitor. A two-way ANOVA showed differences among batches of the antigens but not for antigens with and without the protein inhibitor. According to the statistical analysis the *L.major*-like antigen added or not of PMSF has shown that it is the choice antigen for mucocutaneous leishmaniasis serology.

KEYWORDS: Reproducibility of serological tests; Alkaline antigens; *Leishmania major*-like; *Leishmania (V.) braziliensis;* IgG-ELISA; Protein inhibitor (PMSF).

INTRODUCTION

IgG-ELISA is the choice serological test for the diagnosis of American Leishmaniasis because of its positive predictive value[9]. Other diagnostic tests have proven to be useful such as the immunofluorescence test[6], the direct agglutination test[11] and the dot-ELISA[8] however the IgG-ELISA and its variation the dot-ELISA have shown a set of diagnostic indices that give a sufficiently high positive predictive value both on the standardization stage as well as field conditions[8,9,10] to serve as a subsidiary tool on the diagnosis of American Leishmaniasis. On a previous paper CELESTE at al., 1997[2] compared the performance of one batch each of *L.major*-like and *L.(V.) braziliensis* antigen using or not a protease inhibitor and verified that the agreement of positive standard serum titer was the same regardless of the method used to prepare the antigen. It remained to be investigated if different batches of each antigen displayed reproducible results. In this paper we investigated this hypothesis by preparing 10 batches of each antigen and testing them with standard positive sera on three consecutive days and analyzing the results by means of analysis of variance.

MATERIALS AND METHODS

Sera

Fourteen positive standard sera were drawn from patients with a clinical diagnosis of mucocutaneous leishmaniasis and further diagnosis by means of a Montenegro skin test[12] and ulcer imprint or biopsy[3]. Sera were drawn from male patients (86.0%) and female (13.8%), 7 to 83 years of age. On previous assays sera had tested positive for anti-*Leishmania* IgG class antibodies by a *L.major*-like enzyme-linked immunoassay (ELISA)[9]. The positive standard sera were drawn from in-patients at the Department of Dermatology, University of São Paulo Medical School. Four sera previously found to be negative for anti-*Leishmania* antibodies and anti-*Trypanosoma-cruzi* antibodies by IgG-immunofluorescence and IgG-Elisa were used as negative controls.

All sera had been previously diluted in an equal volume of glycerin pH 7.0 and kept frozen at -20°C until tested.

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Antigens

Detailed description for antigen preparations were given elsewhere[1]. Briefly, promastigotes of *L. major*-like (MHOM/BR/71/49) and *L.(V.) braziliensis* (MHOM/BR/75/M2903) were used as antigen for IgG-ELISA tests. For both species, $2x10^6$ cells/ml were grown either in LIT (liver infusion tryptose) culture medium[4] for 7 days at 25°C for *L. major*-like and in Schneider's Drosophila medium supplemented with 20% fetal calf serum, for 5 days at 25°C[1] for *L.(V.) braziliensis*. Both species cells were harvested at the log growth stage and cells from 240 ml of culture were pooled and processed each time[1].

Ten antigens batches were prepared for each parasite species: one followed the preparation described in GUIMARÃES et al.[7] and another consisting of an alkaline extract to which 1 ul% of a 0.574M solution of phenylmethane sulphonyl fluoride (PMSF) (Sigma Chemical Co.) was added prior to cell disruption[13].

Enzyme immunoassays

Wells of U-shapped microtiter plastic plates (Hemobag, Brazil) were filled with 100 ul of *L.major*-like or *L.(V.) braziliensis* antigen at a protein concentration of 5ug/ml in carbonate-bicarbonate buffer (0.06 M, pH 9.6) and left overnight at 4°C. For use, plates were drained and washed 3 times with PBST (NaCl 0.15 M; phosphates 0.01 M; pH 7.2; Tween-20 0.05%). Sera were diluted twofold (starting dilution 1/40) with PBST, and 100 ul of the dilution was added to each well. Plates were incubated at 37°C for 60 minutes and washed 3 x 5 minutes in PBST; wells were filled with 100 ul of an anti-IgG conjugate (gamma-chain specific) (Biolab, Brazil) diluted in PBST. The optimum dilution of conjugate and antigen had been determined previously by block titration in order to ensure maximum reactivity. Incubation proceeded for 60 minutes at room temperature followed by washings as described and 100 ul of substrate (5.2 mM 5-aminosalicylic acid, and 1.5 mM H_2O_2) was added to each well[14]. Reaction was stopped by adding 25 ul of 1M NaOH to each well and the contents were read at a spectrophotometer using a 450nm wavelength with the chromogen solution as blank.

Controls for IgG-ELISA consisted of four standard negative sera, conjugate and antigen and a positive result considered when its absorbance was greater than the average plus 2 standard deviations of the four negative sera.

Reproducibility test

The study on test reproducibility was made on consecutive days: on day 1 six sera were tested with each of the ten batches of *L.major*-like or *L.(V.) braziliensis* antigen with or without addition of PMSF. On day 2 five sera assayed on day 1 were re-tested and one more was added; on day 3, five sera tested on days 1 and 2 were re-tested and one more. The procedure was repeated until all sera were tested 3 times. This procedure was envisaged because it was not possible to titrate all sera simultaneously due to technical limitations regarding manual handling of all steps.

Statistical analysis

Individual serum titers were transformed into log(x+1) for use in statistical analysis by Statgraphics 5.1. Mean, standard deviation and standard error of mean were calculated for each batch of each antigen, for each day of test and each serum tested. Individual 95% confidence intervals (95% CI) for mean were calculated based on pooled standard deviations. Results were submitted to analysis of variance (ANOVA)[5] with a critical p=0.05 and the "F" statistic[15] was used to ascertain if differences were found among batches.

RESULTS

L.(V.) braziliensis PMSF antigen

Log(x+1) of mean, standard deviation and mean standard error for each antigen batch are shown on table 1. The "F" statistic for the contrast among batches for tests conducted on 3 consecutive days was 1.31 (p=0.229).

TABLE 1

Mean, standard deviation and standard error of mean for PMSF *L.(V.) braziliensis* antigen batches.

Batch	Mean	Standard Deviation	Standard Error of Mean
1	2.2871[1]	0.2485	0.0262
2	2.2609	0.2976	0.0314
3	2.2413	0.3154	0.0332
4	2.2976	0.3069	0.0324
5	2.2776	0.2958	0.0312
6	2.3206	0.2460	0.0259
7	2.2374	0.2796	0.0295
8	2.1853	0.3640	0.0384
9	2.2778	0.3110	0.0328
10	2.2419	0.3466	0.0365

[1] log (x+1)

On day 1 the "F" statistic for 10 batches of PMSF-added antigen was 1.37 (p=0.205), for day 2 was F=0.51 (p=0.865) and for day 3 was F=0.98 (p=0.462).

A two-way analysis of variance searching for differences among batches of *L.(V.) braziliensis* antigen resulted on a "F" statistic of 1.48 (p=0.150) whereas the ANOVA for the difference between antigens prepared with or without protease inhibitor resulted on a "F" statistic of 34.62 (p<0.0001).

L.(V.) braziliensis antigen without addition of PMSF

Log(x+1) of mean, standard deviation and mean standard error for each antigen batch are shown on table 2. The "F" statistic for the contrast among batches for tests conducted on 3 consecutive days was 2.88 (p=0.003).

TABLE 2
Mean, standard deviation and standard error of mean for *L.(V.) braziliensis* antigen batches without addition of PMSF.

Batch	Mean	Standard Deviation	Standard Error of Mean
1	2.2672[1]	0.2513	0.0375
2	2.2082	0.3345	0.0499
3	2.1949	0.3218	0.0480
4	2.3007	0.2902	0.0433
5	2.2215	0.3401	0.0507
6	2.2478	0.2932	0.0437
7	2.0105	0.3977	0.0593
8	2.0105	0.3977	0.0593
9	2.2486	0.3591	0.0535
10	2.1169	0.4047	0.0603

[1] log (x+1)

For day 1 the "F" statistic for 10 batches of antigen without addition of PMSF was 1.44 (p=0.176), for day 2 F was 1.05 (p=0.408) and for day 3, F was 1.15 (p=0.332), respectively.

L.major-like PMSF antigen

Log(x+1) of mean, standard deviation and mean standard error for each PMSF antigen batch are shown on table 3. The "F" statistic for the contrast among batches for tests conducted on 3 consecutive days was 10.07 (p<0.0001).

TABLE 3
Mean, standard deviation and standard error of mean for PMSF *L.major*-like antigen batches.

Batch	Mean	Standard Deviation	Standard Error of Mean
1	1.8988[1]	0.3155	0.0360
2	1.8166	0.4293	0.0489
3	2.1185	0.2727	0.0311
4	2.1491	0.2565	0.0292
5	2.0755	0.2538	0.0289
6	2.1453	0.2667	0.0304
7	2.0844	0.3331	0.0380
8	1.8850	0.3961	0.0451
9	2.1146	0.2991	0.0341
10	2.0612	0.3339	0.0381

[1] log (x+1)

For day 1 the "F" statistic for 10 batches of PMSF-added antigen was 4.79 (p<0.0001), for day 2 F was 4.14 (p<0.0001) and for day 3 F was 3.25 (p=0.001).

A two-way analysis of variance searching for differences among batches of *L.major*-like antigen resulted on a "F" statistic of 11.00 and a p<0.0001 whereas the ANOVA for the difference between antigens prepared with or without protease inhibitor resulted on a "F" statistic of 1.75 (p=0.187).

L.major-like antigen without addition of PMSF

Log(x+1) of mean, standard deviation and mean standard error for each antigen batch are shown on table 2. The "F" statistic for the contrast among batches for tests conducted on 3 consecutive days was 6.16 (p<0.0001).

TABLE 4
Mean, standard deviation and standard error of mean for *L.major*-like antigen batches without addition of PMSF.

Batch	Mean	Standard Deviation	Standard Error of Mean
1	1.8093[1]	0.3166	0.0535
2	2.0116	0.2273	0.0503
3	2.1219	0.2352	0.0398
4	2.1646	0.2418	0.0409
5	2.0197	0.2408	0.0407
6	2.1220	0.2567	0.0434
7	2.1731	0.2264	0.0383
8	1.9690	0.2869	0.0485
9	2.0201	0.2896	0.0490
10	2.1048	0.2162	0.0366

[1] log (x+1)

For day 1 the "F" statistic for 10 batches of antigen without addition of PMSF was 4.89 (p<0.0001), for day 2 was 2.14 (p=0.033) and for day 3 was 0.75 (p=0.661), respectively.

DISCUSSION

The performance of leishmania alkaline antigens differed according to the species used and whether the antigen was prepared with or without PMSF. For *L.(V.) braziliensis* the addition of PMSF resulted on a stable reagent where no differences were found among the 10 batches (p=0.229) or tests on consecutive days (p=205, 0.865 and p=0.462, respectively). For the antigen prepared without protein inhibitor the statistical analysis showed that differences were detected among the contrast "10 batches" (p=0.003): batch 8 had a mean and 95%CI lower than the individual mean and confidence interval of the other batches, but not for the contrast "days 1, 2 or 3" (p=0.176, p=408 and p=332, respectively). The significance seems also to be related to differences on antigen preparation due to addition of PMSF as shown by the result of a two-way analysis of variance for the difference between antigens (F statistic of 34.62 and p<0.0001) while no significance was found for the analysis of differences among batches.

L.major-like antigens showed very similar behavior whether prepared with or without addition of the protein inhibitor. Differences were found for the contrast "batches" (p<0.0001) of the PMSF *L.major*-like antigen as batch 2 had a mean and 95% CI lower than the other nine batches and for days of testing: days 1 and 2 had a p<0.0001 and day 3 had a p=0.001; batch 2 consistently gave lower mean and 95%CI than any other batch. The *L.major*-like antigen displayed a significant difference when the contrast among batches was analysed (p<0.0001); in this case batch 1 displayed a lower mean and 95%CI than the other batches whereas no significance was found

for day 3 of testing for this antigen while days 1 and 2 displayed a $p<0.0001$ and 0.033, respectively . Such findings were corroborated by a significant two-way analysis of variance for the contrast "differences between batches of *L.major*-like added or not of PMSF" ($p<0.0001$) but not for the analysis of a two-way contrast between antigen preparation ($p=0.187$). This finding corroborates a previous conclusion by CELESTE et al., 1997[2] that the agreement on titer of positive standard sera when using an alkaline *L.major*-like antigen added or not of PMSF was 100% whereas the agreement between *L.(V.) braziliensis* antigen in the same conditions was 96.5%. The property of the alkaline *L.major*-like antigen to display the same titer of positive standard sera, the similarity of behavior between antigens and the ease and amount of yield when cultivating the parasite make it the choice antigen for American Leishmaniasis' serology.

In this paper the specificity of the IgG-ELISA employing the different antigens was not investigated because this was not the scope of the research and the sera consisted of positive and negative standards previously tested for the presence of anti-*Leishmania* antibodies by IgG-ELISA as mentioned in M&M. However, on a previous work GUIMARÃES et al.[8] have shown the IgG-ELISA *L.major*-like antigen to cross-react with Chagas' Disease and visceral leishmaniasis sera although with different range in titer. The solution to cross reactivity will come from Molecular Biology techniques although for Public Health purposes the endemic areas for American and Visceral Leishmaniasis do not overlap and American Leishmaniasis and Chagas' Disease can be distinguished, among other features, by seroepidemiological data.

RESUMO

Reprodutibilidade de antígenos alcalinos de *Leishmania major*-like e *Leishmania (V.) braziliensis* avaliados por IgG-ELISA. Comparação entre antígenos adicionados ou não de PMSF.

Neste trabalho estudou-se o comportamento de 10 partidas de antígenos alcalinos de *L.major*-like e *L.(V.) braziliensis* adicionados ou não de um inibidor de proteases (PMSF) avaliados em tres dias consecutivos por meio de ELISA-IgG empregando soros padrão positivo de pacientes com diagnóstico de leishmaniose mucocutânea previamente testados para a presença de anticorpos anti-leishmania por ELISA. A análise estatística mostrou que para o antígeno de *L.(V.) braziliensis* adicionado de PMSF não houve diferença significante entre as partidas ou dias de teste. Para o antígeno sem PMSF houve diferença entre as partidas mas não entre os dias de teste. Uma ANOVA bi-caudal mostrou diferenças entre os antígenos com e sem PMSF. Os antígeno de *L.major*-like preparados com e sem adição de PMSF mostraram diferenças significativas entre as partidas; os tres dias de teste foram significativamente diferentes para o antígeno preparado com PMSF mas somente os dias 1 e 2 o foram com o antígeno sem adição de inibidor. A análise bi-caudal mostrou diferenças entre as partidas sucessivas dos dois antígenos mas não entre os antígenos preparados com e sem inibidor de proteína. Este comportamento do antígeno de *L.major*-like fazem-no o antígeno de escolha para a sorologia da leishmaniose mucocutânea.

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