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First Approach to an Ultramicro Enzyme-Linked Immunosorbent Assay for the Detection of Igg Antibodies Associated to Human Leptospirosis

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ABSTRACT

Background: *At present, leptospirosis is one of the most widespread zoonosis in the world. Both the microbiological diagnosis and the detection of previous exposition to leptospiras is challenging with the current laboratory tests, because they are consumers of high volumes of samples and time, except rapid tests used for screening.*

Objective: *To evaluate an Ultra micro enzyme-linked immune sorbent assay (UMELISA) for the detection of IgG antibodies associated to human leptospirosis, proposed for the Faculty of Medical Sciences of Pinar del Rio.*

Methods: *The UMELISA was evaluated according to the proposed instructions of supplier with a panel of positive and negative human sera to leptospirosis. Different sera dilutions and blockage were assayed. Qualitative performing parameters were calculated, using Micro agglutination test with 2 mercapto-ethanol serum treatment as reference.*

Results: *The test permitted the differentiation of positive and negative sera, but it did not reach good values of sensitivity (95.45%) and specificity (63.15%); therefore it was carried out a new standardization. It was determined 1:21 as optimal serum dilution instead of 1:3 proposed originally, being proven the cutoff value indicated by the authors for a histogram of frequency of the response of the UMELISA to positive and negative sera confirmed by Micro agglutination test. The blockage of the plates was introduced, obtaining a good precision of the assay for one and three batches of leptospiral antigen. The sensitivity, specificity, positive and negative predictive values were 90.78%, 89.73%, 65.34% and 97.76%, respectively.*

Conclusion: *The proposed UMELISA could be used for the detection of IgG antibodies associated with human leptospirosis according to the modifications described in this study.*

Keywords: *leptospirosis; UMELISA; diagnosis; Cuba*

INTRODUCTION

Leptospirosis is one of the most widespread zoonosis in the world today. Its microbiological diagnosis is based mainly on serological methods, among which we can mention the Micro agglutination of Serogroups with Live Antigens (MAT) used as international reference test, Indirect Hemagglutination, enzyme-linked immune sorbent assays (ELISA) and rapid tests[1, 2, 3].

The ultramicro ELISA (UMELISA) began to be developed in Cuba from the 1980s onwards for the diagnosis of various infectious and non-infectious diseases [4, 5, 6]. They have the advantages of testing large quantities of samples, offer reliable results given the high sensitivity values when detecting low concentrations of antigens or antibodies; they are of rapid standardization and automation, the reagents used are easily kept for long periods and required in minimal quantities on the test.

UMELISA has never been used for the determination of antibodies against leptospiral infection, so this study aimed to standardize and evaluate an UMELISA test, using a genus-specific antigen, for the detection of immunoglobulin G (IgG) levels associated to human leptospirosis, designed at the Faculty of

Medical Sciences of Pinar del Rio and based on existing UMELISA packages for the diagnosis of hepatitis C virus (HCV) and human immunodeficiency virus (HIV).

MATERIALS AND METHODS

Samples

Four hundred seventeen sera samples from the National Reference Laboratory of the Tropical Medicine Institute "Pedro Kourí" (IPK), conserved to -20°C, were used to carry out this research. Sera were grouped according to their origin and use as follows:

Group 1: 122 sera from patients with leptospirosis confirmed by MAT

Group 2: 79 sera from patients with suspicion of leptospirosis and non-reactive by MAT

Group 3: 177 sera from blood donors

Group 4: 10 sera from syphilis patients, 4 sera from HIV seropositive patients, 6 sera reactive to *Borreliaburgdorferi* infection, 9 sera from hepatitis B patients and 10 sera from patients with hepatitis C.

Laboratory Tests

MAT: It was performed as recommended in the Guidelines for the Control and Prevention of Leptospirosis from the World Health Organization. Sera dilution started in 1:10, as only modification, and we usedan antigen panel composed by the main pathogenic leptospira serogroups circulating in Cuba (Icterohaemorrhagiae, Canicola, Pomona, Ballum, Pyrogenes, Sejroe, Tarassovi and Hebdomadis).Sera were pre-treated with 2-mercapto-ethanol (2ME) for the final evaluation [1, 7].

UMELISA: At first, it was performed according to the protocol proposed by the supplier (Immnosassay Center, Cuba) and using the reagents on the UMELISA kits for HCV and HIV. Briefly, as follow:

Coating of Plaques with Leptospiral Antigen

Forty microliters of well-homogenized antigen (cell crude obtained from a *L. biflexa* reference strain) was deposited in the wells of the strips and allowed to dry in an oven at 50°C.

Incubation of Samples and Controls

Ten microliters of positive and negative controls and from each sera diluted 1:3 in work-solution R2 (20% sheep serum) previously diluted 1:4 in R1 (Tris-Tween 20), were added to the wells and the reaction strips were incubated for 40 minutes at 37°C in humid chamber.

Positive control sera of the UMELISA kits were replaced by a human serum positive for leptospirosis. Negative controls from the kits were tested by MAT in order to determine the serological negativity to leptospirosis.

Washing Plates

Four washes were performed with R1 wash-solution and distilled water (1+25) using ultramicro analytic system (SUMA)washing machine.

Conjugate

Ten microliters of alkaline phosphatase-labeled anti-human IgG conjugate was added, incubating the reaction strips for 40 minutes at 37°C in a humid chamber.

Washing Plates

Four washes were repeated as above.

Substrate

Ten microliters of substrate (4-methylumbeferyl phosphate) diluted 1:10 with its buffered solution were added, and the reaction was incubated for a maximum of 30 minutes at 20-25°C in a humid chamber, protected from light.

Reading

The reading, validation, interpretation and printing of results were performed automatically by the SUMA reader with the UMELISA HCV program, modifying the cut-off value of the same by 0.380 (suggested by Faculty of Medical Sciences of Pinar del Rio).

All sera negative by UMELISA and positive by MAT were treated with 2-mercaptoethanol and retested by MAT, in order to know whether the antibodies detected were IgG or IgM.

Determination of Cut-Off Level: It was done using frequency histograms of the fluorescence values obtained in each test.

Determination of the Work-Dilution of Serum Samples: 81 negative and positive sera with different degrees of MAT reactivity were selected. Dilutions 1:11, 1:21 and 1:41 were tested.

Blocking of the plates: Two variants were assessed; the first one, the concentration of the reagent R2 for the dilution of sera was increased to 10%, and the second one blocking directly the

wells with bovine serum albumin fraction V (BSA) 0, 1% and sucrose 5%, after drying the antigen on the strips.

Accuracy of the System: Intra- and inter-assay precisions were determined using four serum samples and controls in six replicates, in three consecutive assays for three days and using the same batch of leptospira antigen. Accuracy was also determined using different batches of antigen in one day.

Lastly the UMELISA was evaluated using the different groups of sera mentioned above.

RESULTS

When evaluating the 1:3 serum dilution proposed by the supplier in the UMELISA, it was obtained a specificity of 63.15% (12/19) and a sensitivity of 95.45% (21/22) (see the frequency histogram of fluorescence values for negative and positive samples in Figure).As these results were not satisfactory, other serum dilutions (1:11, 1:21 and 1:41) were tested, and the frequency histograms with the new fluorescence values are depicted in Figure and the sensitivity, specificity and UMELISA-MAT coincidence values are shown in table 1.

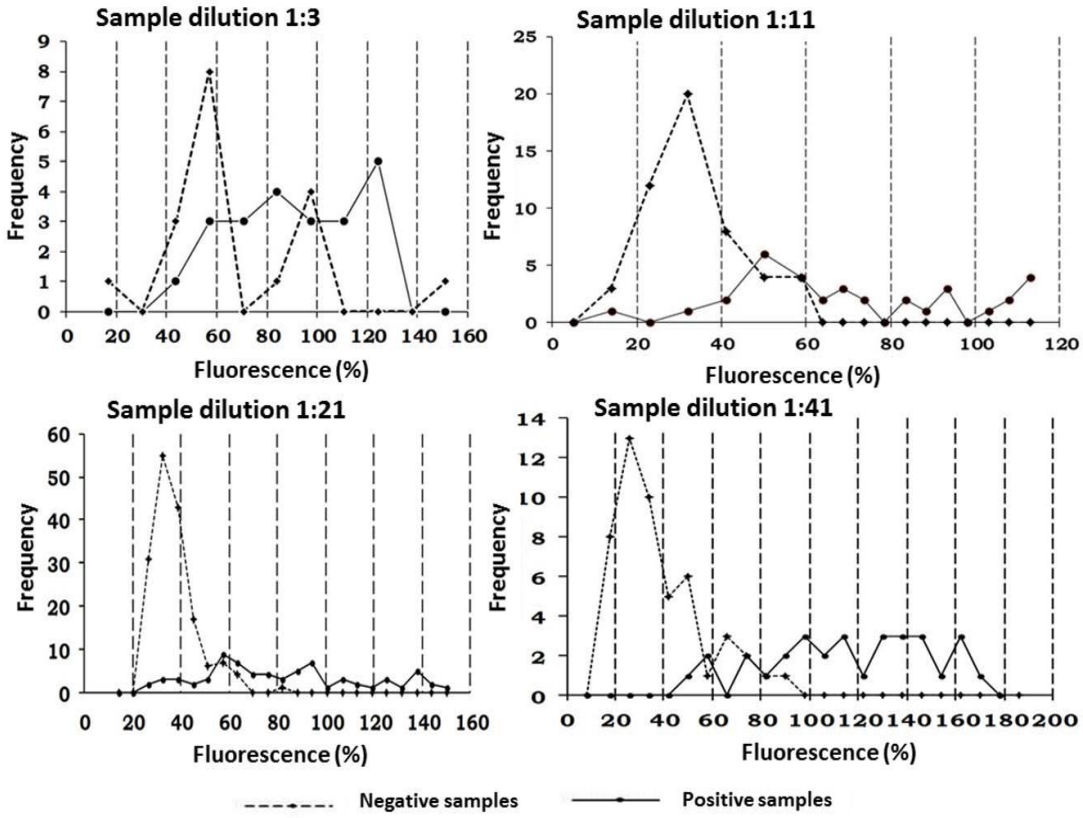

Figure. *Histograms of fluorescence values for different negative and positive sample dilutions obtained by UMELISA*

Table1. *Sensitivity, specificity and coincidence values of UMELISA versus MAT using different serum dilutions*

Serum dilution	Sensitivity (%)	Specificity (%)	Coincidence (%)
1:3	95.4	63.2	80.5
1:11	90.6	87.8	88.9
1:21	100	85.1	91.2
1:41	100	89.1	93.4

The cut-off fluorescence value for these dilutions corresponded to the value fixed by the supplier (91.25%).

During this evaluation, the blockage of wells was necessary and the use of 0.1% BSA with 5% sucrose reduced the target fluorescence values to 7-9%. The accuracy of the assay was determined for the same and different batches of the leptospira antigen using the 1:21 serum dilution. The results obtained when studying a batch are shown in Table 2. As can be observed, the coefficients of variation obtained when evaluating the intra-assay precision were inferior to10; only in the case of the negative control were higher values. The coefficients of inter-assay variation were kept below 15.

Table2. *Precision of UMELISA using the same batch of leptospiral antigen*

Sample	Intra-assay									Inter-assay		
	Day 1			Day 2			Day 3					
	F	SD	CV	F	SD	CV	F	SD	CV	F	SD	CV
1	40.16	2.76	6.95	44.18	3.00	6.8	34.68	2.01	5.80	41.78	3.86	9.24
2	32.84	1.91	5.36	35.21	2.91	8.28	32.00	1.65	5.17	34.06	2.70	7.94
3	99.68	7.64	7.66	92.05	5.05	6.06	94.03	6.35	6.75	95.26	7.02	7.37
4	78.47	6.29	8.02	84.57	3.18	3.76	76.21	4.41	5.78	79.95	5.79	7.26
Negative control	10.02	1.17	11.67	8.58	1.21	14.11	9.49	1.02	10.79	10.07	1.26	12.56
Positive control	123.99	9.90	7.98	137.60	6.44	4.68	105.66	6.19	5.89	123.39	17.14	13.89

F: fluorescence value; SD: standard deviation; CV: coefficient of variation

When analyzing the accuracy of the test using different batches of antigen [differing in the optical density (OD) of each one], variation coefficients of less than 20% were obtained for each of the sera tested as shown in table 3.

Table3. Precision of UMELISA using different batches of leptospiral antigen

Sample	Batch 1			*Batch 2*			Batch 3			*Inter-batch*		
	F	SD	CV	F	SD	CV	F	SD	CV	F	SD	CV
1	39.35	3.17	8.07	38.51	1.96	5.11	34.68	2.01	5.80	37.52	3.10	8.28
2	85.41	2.66	3.12	86.00	5.35	6.23	94.03	6.35	6.75	87.73	5.27	6.00
3	108.30	11.82	10.91	78.32	5.92	7.56	76.21	4.41	5.78	87.61	16.87	19.25
4	38.08	1.13	2.97	48.08	3.34	6.94	32.00	1.65	5.17	39.99	7.19	18.25
Negative control	10.47	1.90	18.20	9.66	1.68	17.47	9.49	1.02	10.79	9.73	1.43	14.79
Positive control	115.87	8.25	7.12	129.4	5.97	4.61	105.66	6.19	5.89	123.39	17.14	13.89

F: fluorescence value; SD: standard deviation; CV: coefficient of variation

Finally, the modified UMELISA system was evaluated using the panel of sera at 1:21 dilution. The sensitivity of the UMELISA was 57.7%, 57 negative sera were obtained by UMELISA being positive for the MAT, so they were treated with 2ME to eliminate the seropositivity given by IgM in the MAT. Once the MAT was performed with the treated sera, 35 false positive results and 7 false negatives were obtained for a specificity of 89.73% and a sensitivity of 90.78%. The coincidence between both methods was 89.93%. The prevalence or relative frequency was 18.22%. The predictive value for a positive test was 66.34%, whereas for a negative test 97.76%.

Table 4 shows the results obtained by the UMELISA system and MAT-2ME on the different groups of sera.

Table4. *Positivity by UMELISA and MAT-2MEon the serum panel*

Group	N. samples	UMELISA		MAT-2ME	
		Positive	%	Positive	%
1	122	65	53.3	72	59.1
2	79	14	17.7	0	0
3	177	14	7.9	1	0.6
4 (syphilis)	10	4	40.0	0	0
4 (HIV)	4	2	50.0	1	25.0
4 (Lyme)	6	0	0	0	0
4 (Hep B)	9	0	0	0	0
4 (Hep C)	10	4	25.0	2	20.0

DISCUSSION

The laboratory diagnosis of leptospirosis is mainly based on serological tests, which are also used in sero prevalence studies and evaluation of vaccine candidates, for which determination of IgG is important [1, 8, 9].

UMELISAs are often applied in field studies because of their economic characteristics, the possibility of analyzing several samples at the same time, their possibilities of automation and for requiring non-highly qualified personnel [10].

The working dilution of the samples depends mainly on the sensitivity of the system used. UMELISAs are very sensitive systems, therefore, high dilutions of the samples are used [4, 5, 7]. This was verified by evaluating the 1:3 dilution of human sera and false positive results

were obtained. This result could be due to the high degree of non-specificity and diversity of antibodies present in the sera, which are not eliminated with this dilution, being able to obtain nonspecific reactions with certain epitopes of the antigen fixed in the ultra micro-titration plate. False negative results could be caused by the high concentration of proteins in the samples, which can form a barrier that prevents the diffusion of the specific antibodies. The results obtained reaffirm that reported by De Savigni and Voller in 1990, who suggest the use of high dilutions of the sample in order to eliminate the saturation phenomena and interference [11]. Another drawback for the work with this dilution was the low specificity obtained, as well as the high degree of overlap of the fluorescence values of positive and negative sera in the frequency histogram.

On the other hand, there are studies with indirect UMELISA systems where low serum dilutions as 1:20 and 1:40 have been used, using an excess of reagents, which allowed to reach a high sensitivity in these procedures without affecting the specificity of the assay [10].

Based on it and considering that the sensitivity and specificity of the test can be affected by the serum working dilution, the cut-off level, the purity of the fixed antigen and the conjugate dilution (these latter two were invariable in this study), 1:11, 1:21 and 1:41 serum dilutions were evaluated. From the frequency histograms, it was possible to verify the cut-off value given for the dilutions tested, which ensured the achievement of acceptable sensitivity and specificity values for 1:21 and 1:41 dilutions. Taking into account these values and the concordances between this test and the gold standard, they were selected as possible working dilutions, but due to operability reasons of the ERIZO pipette of the SUMA system in which it is recommended to perform a 1:20 dilution of the samples, it was decided to optimize the 1:21 dilution.

During the development of the tests, an increase in the fluorescence value of the blank was observed, above the quality limits of the system, this could be due to the presence of free spaces in the walls of the plate wells, which were being occupied by the conjugate. In order to eliminate the emitted background, two blocking strategies were drawn up, the first one was to increase the concentration of sheep serum used in the dilution buffer from 5 to 10%, treating the proteins present in the sheep serum could exert a blocking action when deposited on the walls of the well. For this, it was taken into consideration there were no antibodies against leptospires in this serum. The results were not satisfactory since it was not possible to decrease the level of fluorescence, so it was necessary to increase the block using 0.1% BSA and 5% sucrose as a second strategy. With the latter, satisfactory results were obtained by reducing the fluorescence level.

The goal of any laboratory test is to provide highly repeatable and reliable results. Multiple steps and opportunities for error are introduced in this kind of test, contributing to the complexity of the antigen-antibody reaction and the enzyme-substrate reaction [10].

The coefficient of variation was calculated for one and three batches of antigen. As could be seen for the samples and the positive control, the coefficient of variation was inferior to 10%, as reported in the literature for intra-assay precision, and for the negative control it took values above 10%; however, the inter-assay coefficient of variation in all cases was less than 15%. When different batches of antigen were compared, the coefficient took values less than 15% and in samples 3 and 4 they were less than 20%. The antigen supplied for the test was obtained after a boiling process from cellcrude; afterwards it was subjected to a final processing where the concentration of antigen for the coating was fixed in terms of a range of optic density, being the interest of the supplier to carry out the evaluation with these predetermined characteristics. The antigen supplied was not referred in terms of quantitative mass that supported the performance of a study to determine the optimal concentration of coating from the elaboration of an antigen saturation curve. Taking into account that a protein determination is not carried out by a more exact method and the optic density is not a fixed value, the slight but important variations obtained in the assay are considerably justified.

Results of the modified UMELISA with the 417 sera were compared with the reference serological test; this technique detects IgM and IgG antibodies, so it was necessary to apply the 2-ME variant for the specific determination of agglutination produced by IgG. When the sera were not treated with this chemical compound, the sensitivity and specificity of the system were lower than when they were treated, since in this

way the seropositivity given by IgM was eliminated, and acceptable sensitivity and specificity values were achieved. The modified UMELISA system was useful in determining positivity for leptospirosis with good discrimination between positive and negative results.

The existence of serological positivity in blood donors when using an anti-human IgG conjugate can be explained taking into account that this test exclusively detects IgG antibodies in the serum of these people, as a result of previous exposure to this zoonosis widely diffused in tropical climates and with diverse hosts. In addition, the various clinical forms of this disease must be taken into account, which can range from a simple flu state to other clinical conditions that lead to the death of the patient, without forgetting that in some cases the subclinical form is observed, which makes basal levels of antibodies against infection can be detected in apparently healthy individuals. It should also be taken into account that the production of IgG is inconsistent, being in many cases impossible to establish its time of appearance, sometimes it appears as early as IgM, apparently depending on the leptospiral strain [1, 2, 3]. In order to know the cross reactivity of this test, sera from individuals with other diseases were evaluated, finding reactivity in the groups of patients with syphilis, HIV and hepatitis C, some of them confirmed by MAT-2ME, a similar result to the one found by other authors [12, 13].

As usual in serological tests, the UMELISA presented unspecific reactions, showing once again the need for antigen purification and quantification, or failing that, the introduction of a variation to the assay, in addition to verifying the optimal concentration of the conjugate.

CONCLUSION

The results found in this study demonstrate that the UMELISA evaluated is a useful alternative for the detection of IgG antibodies levels associated with leptospirosis, although it is recommended to continue working for its improvement, as well as its standardization for the detection of IgM antibodies, a marker of the acute phase of the disease.

The introduction of the UMELISA test, to search for the immune response against leptospira infection in the national microbiology network, would be a valuable alternative due to its advantages and easy technology transfer; the conditions for working with the SUMA equipment have been created and the human resources have been trained to carry it out. It is also an inexpensive technology used by other countries.

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