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Development of a Lepto-IgM EIACR test to diagnose leptospirosis disease in Costa Rican patient samples.

María de los A. Valverde J[1], Bernal León[2], Lizeth Taylor[2] and Kirsten Visona[2].

[1]Instituto Costarricense de Investigación y Enseñanza en Nutrición y Salud, INCIENSA. Tres Ríos, San José, Costa Rica and [2]International Center for Medical Research and Training, Louisiana State University, USA. E-mail: mvalverde@inciensa.sa.cr

Key words: *Leptospira* sp., anti-IgM EIA, serology diagnostic, zoonosis.

Abstract. Leptospirosis is an endemic disease throughout Costa Rica, which could be misdiagnosed because manifestations of this febrile disease may vary from mild flu-like symptoms to severe illness involving vital organs such as liver and lungs. Therefore an early specific diagnosis is important to ensure a favorable clinical outcome. The purpose of this study was to develop a *Leptospira* sp. anti-IgM EIA (Lepto-IgM EIACR) test and to compare it using Lepto-Dipstick IgM (Lepto-DS IgM) and PanBio-EIA IgM with the Microscopy Agglutination test (MAT) as a reference assay. Sera from 736 healthy blood donors were used as negative controls to calculate specificity (97.1%), Confidence Interval 95 (CI (96-98). Cross reactivity was evaluated in 268 patient samples with 6 different diseases. Dengue and measles had the highest cross reactivity (16%) while rubella showed the lowest (3%). To determine the sensitivity of the Lepto- IgM EIACR, 33 samples positive by MAT of 96 paired samples from patients with symptoms related to leptospirosis infection were tested. Lepto-IgM EIACR reached a sensitivity of 90.9% (CI 81-100), while Lepto-DS IgM was 48.5% (CI (31-66). The most frequent serovars detected by MAT in these paired samples were Hebdomadis 14.7%, Hardjo 11.8%, Pomona 8.8% and Icterohaemorrhagiae 5.9%. Furthermore 59 febrile patient samples were tested initially with PanBio-EIA IgM, 21 samples (35%) were positive. When these samples were re-tested by Lepto-IgM EIACR and Lepto-DS IgM, 80.9% and 33% were positive, respectively. The results of the evaluation indicate that Lepto-IgM EIACR test could be a good alternative to detect acute leptospirosis in Costa Rica.

Corresponding author: María de los A. Valverde J. P.O. Box 4 Tres Ríos, Instituto Costarricense de Investigación y Enseñanza en Nutrición y Salud, INCIENSA. San José, Costa Rica. Phone (506) 279-9911, Fax (506) 279-5546. E-mail: mvalverde@inciensa.sa.cr

Desarrollo de una prueba Lepto-IgM EIACR para diagnosticar la enfermedad de leptospirosis en muestras de pacientes de Costa Rica.

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Palabras clave: *Leptospira* sp., anti- IgM, serología diagnóstico, zoonosis.

Resumen. Leptospirosis es una enfermedad febril endémica en Costa Rica que puede ser mal diagnosticada, ya que sus manifestaciones varían desde síntomas similares a gripe, hasta enfermedades severas que afectan órganos vitales como el riñón, hígado o pulmón. Por ello es importante un diagnóstico específico y temprano. El propósito de este estudio fue desarrollar un ELISA anti-IgM (Lepto-IgM EIACR) y compararlo con Lepto-Dipstick IgM (Lepto-DS IgM) y PanBio-EIA IgM utilizando como prueba de referencia la Microaglutinación MAT. Se usó el suero de 736 donadores de sangre como control negativo para determinar la especificidad del ensayo (97,1%, CI (96-98). Pruebas de reacción cruzada fueron analizadas en 268 muestras de pacientes distribuidos en 6 enfermedades. Dengue y Sarampión mostraron los valores más altos de reactividad (16%) y Rubeola el más bajo (3%). La sensibilidad de Lepto- IgM EIACR fue 90,9% (CI (81-100), mientras que Lepto-DS IgM alcanzó un 48,5% (CI (31-66), la cual se calculó a partir de 33 sueros pareados de 96, que fueron enviados para el diagnóstico de Leptospira sp. Las serovariedades más prevalentes detectadas por MAT en estas muestras fueron: Hebdomadis 14,7%, Hardjo 11,8%, Pomona 8,8% e Icterohaemorrhagiae 5,9%. Adicionalmente, de 59 muestras agudas de pacientes febriles que fueron inicialmente analizadas por PanBio-EIA IgM, 21 resultaron positivas, de éstas, Lepto-IgM EIACR y Lepto-DS IgM, detectaron el 80,9% y 33,3% respectivamente. Los resultados de la evaluación indican que Lepto-IgM EIACR podría ser una buena alternativa para detectar leptospirosis aguda en Costa Rica.

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INTRODUCTION

Leptospires are members of the Spirochaetales order, according to Dario (1) and Lucchesi et al. (2), there are 17 genomospecies, 21 serogroups and more than 300 serovars; additionally to non-pathogenic strains (3-5).

Leptospirosis is a zoonosis with worldwide distribution affecting mainly rural and urban populations in tropical areas, although many cases, both in humans and animals are reported in Europe every year (4-7). According to reports from the Ministry of Health, leptospirosis is endemic throughout Costa Rica.

Clinical manifestations of this acute febrile disease may vary from mild flu-like symptoms to severe illness with renal failure and hemorrhagic forms involving vital organs such as kidneys, liver and lungs. Weil´s disease, can occur in some patients and could have a fatal outcome (4, 8). However, leptospirosis is often misdiagnosed as

dengue, malaria, meningitis, viral hepatitis, encephalitis or influenza (9) and therefore an early specific diagnosis of leptospirosis is important to ensure a favorable clinical outcome. At the present time the definitive diagnostic test is the recovery of leptospires from clinical specimens by culture. However, this procedure is very complex and requires several weeks of culture with low sensitivity (10). The laboratory diagnosis most commonly used is the increase in sera antibodies titers in paired samples against leptospira to determine seroconversion. The microscopy agglutination test (MAT) is commonly used for this purpose as a reference method (11). However, MAT is an inadequate assay for rapid diagnosis since it requires the testing of paired sera and the sensitivity has to be determined by cell culture and the specificity is related to the serovars included in the panel. Furthermore, MAT is a laborious technique and therefore only done in few reference laboratories (12). The enzyme- linked immunoabsorbent assays (ELISA) is a useful alternative with a high sensitivity to detect specific IgM antibodies as a sign of current or recent leptospirosis which also gives a more objective interpretation of results than other methods (13).

Other commercially available antibody tests to detect specific IgM anti-Leptospira are also commonly used, Lepto-DS IgM (86.8% sensibility and 92.7% specificity in collected serum between 10 and 30 days of onset) (3) and PanBio-EIA IgM (100% sensibility and 94% specificity according to the commercial specification sheet).

The purpose of this study was to develop an EIA test to detect IgM antibodies against *Leptospira sp.* in human serum samples from Costa Rica to diagnose current or recent leptospirosis (Lepto-IgM EIACR) and compare it with other available reagent kits, MAT, PanBio EIA IgM and Lepto-DS IgM.

MATERIALS AND METHODS

Study population

A total of 736 sera from healthy blood donors from the National Blood Bank in Costa Rica, screened negative by the Lepto-DS IgM (3) and MAT tests (14), were used as negative controls to establish the specificity of the assay under development, Lepto-IgM EIACR. A group of 268 samples were used to determine cross reactivity from patients with other diagnostic disease markers, as follows: IgM anti-HAV (n = 50), cytomegalovirus antibodies IgG with titers ≥ 1:12800 (n = 40), dengue antibodies IgM (n = 57), measles antibodies IgM (n = 32), rubella antibodies IgM (n = 36) and syphilis antibodies (n = 53).

Two groups of samples were used to evaluate sensitivity for Lepto-IgM EIACR. The first group of 96 paired sera with an inclusion criteria with less than 30 days after onset of symptoms in the first samples and more than 15 days of interval between the samples. These patients with symptoms of leptospirosis, referred for diagnostic purposes to the Costa Rican Institute for Research and Training in Health and Nutrition (INCIENSA), from different regions of the country (years 1999 to 2001), were analyzed by Lepto-IgM EIACR and Lepto-DS IgM in parallel using MAT test as the reference assay. The second group, 59 samples collected with less than 30 days after onset of illness and prescreened by PanBio-EIA IgM (11) were re-tested using Lepto-IgM EIACR and Lepto-DS IgM.

MAT was performed using the following antigens: serogroup/serovar (strain in parenthesis) Australis/Australis (Ballico), Autumnalis/Autumnalis (Akiyami A), Ballum/Castellonis (Castellon 3), Bataviae/Bataviae (Swart), Canicola/Canicola (Hond Utrecht IV), Grippotyphosa/Grippotyphosa (Moskva V), Hebdomadis/Hebdomadis (Hebdomadis), Icterohaemorrhagiae/Icterohaemorrhagiae (RGA,

KA), Icterohaemorrhagiae/Copenhageni (M20), Pomona/Pomona (Pomona), Pyrogenes/ Pyrogenes (Salinem), Sejroe/Hardjo (Hardjoprajitno), Sejroe/Sejroe (M84), Tarassovi/Tarassovi (Perepelitsin). Description of the method in brief: all wells of a microtiter plate were filled with 50 μL PBS pH 7.2. Then, another 40 μL PBS and 10 μL of serum were added to the wells of column 2 (dilution was now 1:10). Dilution was done by pipetting 50 μL from these wells to the wells of the next column. The final 50 μL were discarded. This was followed by the addition of 50 μL of leptospira culture to all wells. The dilution in column 2 was 1:20, until 1:20480 in column 12. The plates were mixed thoroughly in a microshaker and incubated 2 hours at 30°C. As an alternative, incubation overnight at room temperature was considered (14). The cut-off value was agglutination in a 1:100 dilution and paired samples with a two-fold rise of agglutination was considered as a positive result for *Leptospira* sp. infection.

Leptospira IgM ELISA PanBio test (PanBio-EIA IgM) (Brisbane, Australia) was performed according to the manufacturer's instructions.

Lepto-Dipstick IgM (Lepto-DS IgM) test was obtained from Organon Teknika Ltd. (Boxtel, The Netherlands) and processed following the manufacturer's protocols.

Lepto-IgM EIACR, Immulon 2 microtiter plates (Dynatech Laboratories, Chantilly, USA), were coated with 100 μL of *L. interrogans* serovar Copenhageni antigen, strain Wijnberg (absorbance ≥ 0.456) obtained from Royal Tropical Institute (Amsterdam, The Netherlands), diluted 1:4 in 0.1 M carbonate buffer, pH 9.6 (Sigma, St. Louis, USA). The plates were incubated at 4°C for 24 h in a humid chamber, washed, post-coated with 5% sucrose for 24 h, washed and dried to be stored at 4°C until use.

Negative and positive control samples, were diluted 1:400 in a sample diluent containing 0.15M PBS (pH 7.3), 0.5% Tween 20 (Fisher, N. Jersey, USA), 5% bovine albumin fraction V (Sigma, St. Louis, USA) and 0.1 M EDTA (Fisher, N. Jersey, USA). One hundred microliters of each pre-diluted sample were added to the pre-coated microplate wells and incubated at 37°C for 1 hr. The plate was washed three times with PBS-0.5% Tween 20; the same washing step was repeated after each incubation period. Next 100 μL of anti-human IgM biotin labeled (μ chain specific–biotin antibody produced in goat, Sigma, St. Louis, USA) in a dilution of 1:10000 were added to each well and incubated at 37°C for 1 h. After the washing step, 100 μL of extrAvidin-alkaline phosphatase diluted 1: 50000 (Sigma, St. Louis, USA) were added followed by incubation at 37°C for 30 min and washed. The color reaction was developed using 100 μL of p-nytrophenyl phosphate-diethanolamine (DEA) solution (Sigma St. Louis, USA), pH 9.6. After one-hour incubation in the dark at room temperature, the Optical Density (OD) was measured in an EIA Multi-Well Reader II (Sigma, St. Louis, USA) at 405 nm, using 630 nm as reference.

Cut off was defined as the mean value plus three Standard Deviation (SD) of negative control samples. The cut off value was established using a procedure in two-steps. After the first calculation of the 3 SD the values outside the area of 3 SD was excluded and the cut off value re-calculated. The test was considered valid when the positive/negative ratio was ≤ 5-fold.

To evaluate the repeatability value of the Lepto-IgM EIACR, Coefficient Variation (CV) was calculated from three samples: 1 negative, 1 borderline and 1 positive with 23 repetitions of each.

Data analysis

Unequal variances were established according to the Levene test, F 706.9, p = 0.0000. Therefore, the two-sample t Test

for independent samples with unequal variances (Satterthwaite 's method) was used to compare the OD mean values between the *Leptospira* sp. negative and positive controls, = 0.01.

The sensitivity, specificity, predictive positive (PPV) and negative (PNV) values were calculated. The 95 confidence intervals (CI 95) were estimated using binomial proportion for each one (15). The Kappa statistic was used to determinate the concordance between two tests in which > 0.75 denotes excellent concordance, 0.4 ≤ ≤ 0.75 show a good concordance and 0 ≤ < 0.4 indicates a poor concordance. The McNemar statistic was also used to measure the discordance between MAT assay and Lepto-IgM EIACR test, $p < 0.05$.

In addition, chi square statistic (= 0.05), was used to evaluate the relationship between cross-reactivity by Lepto-IgM EIACR in other diseases different from leptospirosis. These analyses were done using the software JMP, a business unit of SAS Copyright© 1989-2001 SAS Institute Inc and STATISTICA for Windows software (16).

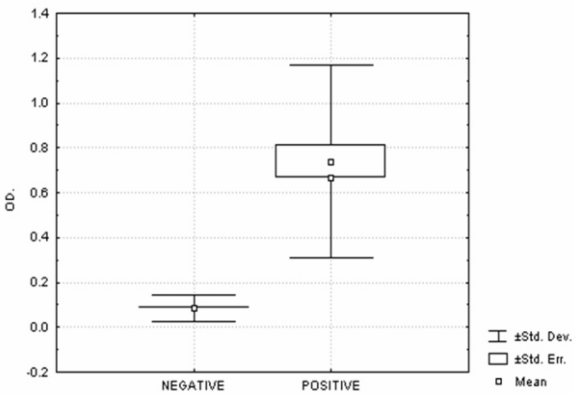

Fig. 1. Distribution of negative and positive samples used to calculate the specificity, sensitivity and the cut off of the Lepto-IgM EIACR. The OD mean value of the 736 negative blood donors was 0.086, CI (0.081-0.090) 0.060 Standard Deviation (std. Dev), 0.0022 Standard Error (Std. Err). The minimum and maximum value was 0.003 and 0.331 respectively (after recalculation of the cut off). The 33 positive control samples had a mean of 0.756, CI (0.607-0.905) 0.42 SD, 0.0731 Std.Err. The minimum OD value was 0.155 and the maximum OD value was 1.543. The sensitivity of the Lepto-IgM EIACR was 91 (CI 81-100).

RESULTS

Of the 736 selected negative samples, 21 samples (2.9%) were positive with the lepto-IgM EIACR test, using the defined cut off criterion with a specificity of 97.1% (CI 96-98). The cut off value was 0.266 OD and the range for the positive samples were 0.270 to 0.512 with a OD mean value of 0.372. There was a significant statistical difference between the OD mean value of the positive group and the OD mean of the negative group, T test 10.43, 39.11 freedom degree (fd) $p = 0.000$. The spread of negative and positive control samples is shown in Fig. 1.

The results of the repeatability evaluation had a CV for the negative, borderline and positive samples of 0.13 (0.098/0.074), 0.069 (0.059/0.85) and 0.066 (0.089/1.361), respectively.

Table I, illustrates the cross reactivity of Lepto-IgM EIACR with different diagnostic markers from other febrile diseases with Dengue and Measles showing the highest reactivity (15.8%, 15.6%, respectively) and Rubella the lowest (2.7%). No statistical difference between disease and cross reaction was established; likelihood ratio 5.34 fd, $p = 0.37$.

The results of 96 paired samples analyzed by MAT, Lepto-DS IgM and Lepto-IgM EIACR, are shown in Table II. The highest rate of positivity was found using the assay under development, Lepto-IgM EIACR 41 samples (42.7%) followed by MAT with 33

TABLE I
SAMPLE REACTIVITY BY LEPTO-IgM EIACR TEST AND DIAGNOSTIC MARKERS OF OTHER DISEASES

Diagnostic Marker	Lepto-IgM EIACR Reactivity		
	Total	No	(%)
Anti-Dengue IgM	57	9	(15.8)
Anti-Syphilis	53	6	(11.3)
Anti-HAV IgM	50	5	(10)
Anti-HCMV ≥ 1:12.800	40	4	(10)
Anti-Rubella IgM	36	1	(2.7)
Anti-Measles IgM	32	5	(15.6)
Total	268	30	(11.2)

TABLE II
DISTRIBUTION OF 96 PAIRED SAMPLES FROM PATIENTS WITH SYMPTOMS RELATED TO LEPTOSPIROSIS USING THREE DIAGNOSTIC METHODS

Category	MAT	Lepto-DS IgM	Lepto-IgM EIACR
Positive by one assay	2	0	5
Pos. by MAT and Lepto-DS IgM	1	1	-
Pos. by MAT and Lepto-IgMEIACR	15	-	15
Pos. by Lepto-DS IgM and Lepto-IgM EIACR	-	6	6
Pos. by all assays	15	15	15
Total Positives	33	22	41
Negative by any assay	11	22	3
Neg. by all assays	52	52	52
Total negatives	63*	74	55

*Seven samples had a decrease in titer between the first and the second sample, when compared to the results of Lepto-DS IgM and Lepto- IgM EIACR 1 was positive by both and 2 additional only by Lepto- IgM EIACR.

samples (34.3%) and Lepto-DS IgM 22 samples (22.9%). The PNV and PPV calculated in this population appeares in Table III, the Kappa value was 69.4 and the McNemar test was not significant = 0.05.

The most commonly serovars detected in this group were Hebdomadis 14.7%, Hardjo 11.8%, Pomona 8.8% and Icterohaemorrhagiae 5.9%.

The results of 59 samples from febrile patients using PanBio IgM, Lepto-IgM EIACR and Lepto DS IgM had the following positive rates of 21 samples (35.6%), 26 samples (44.1%) and 9 sera (15.3%), respectively. Fig. 2, illustrates the distribution of positive and negative samples screened by PanBio IgM retested by Lepto-IgM EIACR and Lepto-DS IgM. The positive samples correlated better between PanBio IgM and Lepto-IgM EIACR with 4 discordant samples, where as for the negative group a higher correlation was estab-

TABLE III
RESULTS OF SENSITIVITY, SPECIFICITY, PREDICTIVE NEGATIVE VALUE AND PREDICTIVE POSITIVE VALUE FOR LEPTO-IGM EIACR AND LEPTO-DS IGM IN 96 PAIRED SAMPLES BY MAT (REFERENCE METHOD)

MAT Category	Lepto-IgMEIACR*				Lepto-DS IgM**			
	Positive		Negative		Positive		Negative	
	No.	(%)	No.	(%)	No.	(%)	No.	(%)
Positive (total 33)	30	(91)	3	(9)	16	(48)	17	(52)
Negative (total 63)	11	(17.5)	52	(82.5)	6	(9.5)	57	(90.5)

*Sensitivity: 91% CI (81-100), specificity: 83% CI (77-92), PNV: 95% CI (89-100), PPV: 73% CI (60-87).
**Sensitivity: 73% CI (54-91), specificity: 77% CI (67-87), PNV: 90% CI (83-98), PPV: 48% CI (31-66).

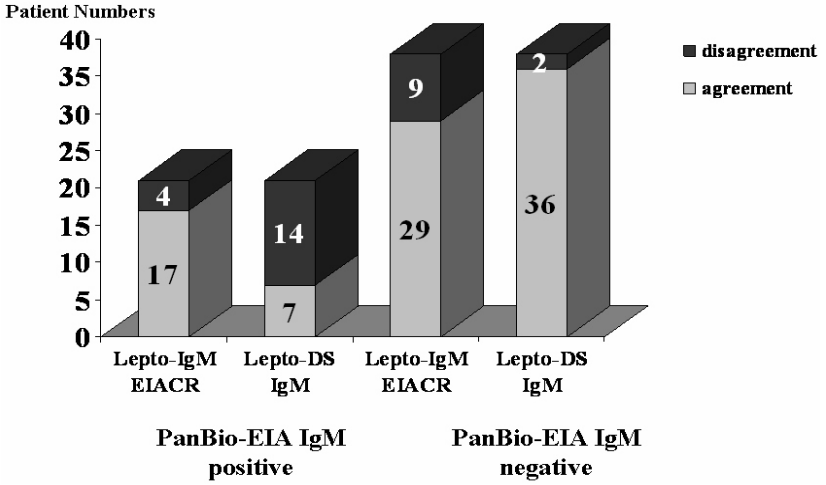

Fig. 2. Results of 59 acute febril patient samples secreened by PanBio-IgM test, compared with Lepto-IgM EIACR and Lepto-DS IgM tests.

lished between PanBio-EIA IgM and Lepto-DS IgM with only 2 discordant samples. The Kappa values between PanBio-EIA IgM and Lepto-IgM EIACR, Lepto-DS IgM were 54% and 32%. The McNemar test significance were $p = 0.26$ and $p = 0.004$, respectively.

DISCUSSION

There is an urgent need to improve diagnostic tests to determine the acute state of leptospirosis to support the selection of an appropriated treatment of these patients. A diagnostic assay that requires a long period of cell culture or paired samples (MAT), will delay the period for treatment intervention; in spite of this, MAT is still chosen as reference assay (12, 13, 18, 22). Leptospirosis culture was not done since at the time of this study in Costa Rica there were no institutions performing it. Therefore, a test based on the detection of specific IgM antibodies would be a better choice since it only requires one sample and the IgM antibodies are present at the time of clinical onset of disease.

In this study some of the paired sera had more than 14 days after onset of disease based on the medical clinical criteria in the first sample. The sensitivity of the Lepto-IgM EIACR test, in samples with less than 14 days of onset was 77% and the spec-

ificity was 89%, compared with 41% and 97% of the Lepto-DS IgM respectively.

The clinical manifestations of leptospirosis are similar to other febrile diseases and therefore the specificity of any diagnostic assay is very important. In this study samples from other febrile diseases were analyzed and a low cross reactivity was shown with the assay under development (Lepto-IgM EIACR, table 1). Similar studies revealed that PanBio-EIA IgM, reacted with brucella 21%, CMV 14% (11) while in Lepto-DS IgM most of the reacting cross samples were from patient with HIV, Hanta virus and toxoplasma infection (3). These different may represent either the population immunity status or the antigen circulation degree for each country.

In clinical samples referred for diagnostic purposes and tested by the different assays included in this study, the highest rate of positivity was determined using the Lepto-IgM EIACR test, established to have an acceptable level of sensitivity 91% and specificity 97.1% in the blood donors and 83.0%, in the paired samples (Table III, Fig. 1).

Variability in screening test sensitivity has been observed in different studies. Effler et al, in agreement with our study, obtained similar low sensitivities (31%) using Lepto-DS IgM in patients confirmed by MAT (17). However, other studies showed a better sensitivity of Lepto-DS IgM with 93.2% detected as positive in the paired sera, 52.7% in the acute phase samples, 83.6% in the convalescent phase-sera (18), and 86.8% in patients previously confirmed to have infection with pathogenic leptospire (3).

MAT has been reported as the golden standard assay by many investigators (13, 19, 20). It is interesting to notice that there are many different criteria to consider a positive sample by this test and both single and paired samples have been used (3, 17, 19, 21, 22). However, the use of single samples is mostly inappropriate, even if the samples have high titers, since following acute infection the titer may be extremely high (≥ 25600;) and it can take months or even years to fall to low levels (23). In this study, of 33 positives samples by MAT, only 3 were not detected by Lepto-IgM EIACR (Table III), this disagreement could be due to the early antibiotic treatment which may, suppress the antibody production (24), though this explanation could not be confirmed in these particular samples. In the other hand, of 63 negative samples by MAT, 11 were positives by Lepto-IgM EIACR, this discrepancy could be due to either cross reaction, unknown strain or local serovars no considered in the panel.

There is little information available regarding leptospirosis prevalence or serovars in Costa Rica. A clinical epidemiological investigation in humans and reservoirs in Yucatan, Mexico reported 7% of positive samples from 206 healthy blood donors by MAT. In this study two samples of 738 healthy blood donors were excluded, one was positive by Lepto- DS IgM and other had high titers by MAT (1:1600 Castellonis, 1:400 Icterohaemorrhagiae), both were positive by Lepto-IgM EIACR which represents (0.13%) of reactive samples in this population. The most reactive serovars in the Mexico research were Shermani (53%) followed by Canicola (33%), Pyrogenes (20%), Pomona (13%) and Icterohaemorrhagiae (6%) (25). In the present investigation Shermani was not within the 14 serovars used. During an outbreak in Nicaragua in 1995 serovars that had a high prevalence and were not included in this study were: Mankarso (33.3%), Bratislava (15.2%) and Alexi (12.1%). The animals evaluated in this outbreak, that had the highest prevalence were porcine and canine reacting with; Alexi (12.1%, 1.4%), Bratislava (8.7%, 12.3%) y Shermani (26.1%, 7.3%), respectively; these serovars were not

used to analyze the human samples (21). In El Salvador 17.5% were reactive out of 984 human sera and the main serovars determined were included in this study, except Shermani (20). While in the Bolivar State, Venezuela Icterohaemorrhagiae, Copenhageni (21.3%), Autummalis and Australis (12.8%) were the most frequent serovars identified all included in this study (26).

Considering the prevalence of different serovars detected by MAT in Central America and Yucatan, Mexico and based on the fact, that some of these serovars were excluded from the reference MAT assay used in this study, it could be speculated that the "none reactive" samples by MAT, could be due to the limited diversity of serovars included. Recently a new serovar of *L. santarosai* serogroup Shermani and possible another new subspecie of *L. interrogans* as well as *L santarosai* serogroup Shermani were isolated in Costa Rica (27), supporting the idea that some of the positive samples by the Lepto-IgM EIACR could react with this isolation, unfortunately there were not enough sera to evaluate these new isolations, that now are included in the panel. Another possibility is cross reaction with Dengue which is spread in the country and the test showed false positives in sample of patients with this disease (Table I).

In conclusion, the Lepto-IgM EIACR test seems to be a good alternative to detect anti IgM antibodies against leptospire, when compared to Lepto-DS IgM and PanBio IgM. More studies are necessary to clarify the impact of leptospirosis in Costa Rica; however, the need for more sensitive and specific diagnostic assays is relevant and urgent.

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