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RESEARCH ARTICLE

Synthetic Peptides Derived from Ribosomal Proteins of *Leishmania spp.* in Mucocutaneous Leishmaniasis: Diagnostic Usefulness

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Abstract: ***Background*:** The serological diagnostic methods currently available for mucocutaneous leishmaniasis (MCL) lack specificity when complete parasites are used; however, such specificity increases when protein fractions are used. Ribosomal proteins have been reported to induce antibodies in animal and humans infected with the parasite, making them a worth candidate to assess its diagnosis potential.

***Objective*:** This study was thus aimed at evaluating synthetic peptides derived from *Leishmania braziliensis* ribosomal proteins S25 and S5 as antigen candidates for diagnosing MCL by ELISA

***Methods*:** It was used 8 and 13 peptides derived from ribosomal proteins 25 and S5 respectively as antigens in order to detect IgG antibodies by ELISA in people with active MCL, Chagas disease (CH) and autoimmune disease (AID).

***Results*:** 4 of these 21 peptides (P4, P6, P19 and P21) had the greatest sensitivity (21.7%, 13.04%, 20% and 20%, respectively) as well as having 95%, 100%, 100% and 82.5% specificity, respectively.

***Conclusion*:** The study revealed the limited usefulness of the peptides being studied as a diagnostic tool in the conditions used here, because its low sensitivity, but it is worth highlighting that the use of peptides as antigen in the serodiagnosis of MCL may overcome the cross reaction presented with other antigens, thus avoiding false positives.

Keywords: Mucocutaneous leishmaniasis, diagnosis, synthetic peptide, ribosomal protein, parasite, *Leishmania braziliensis*.

1. INTRODUCTION

Leishmania spp. transmission is restricted to the world's tropical and subtropical areas which are inhabited by the parasite concomitantly with its insect vector to which it has become adapted. The parasite thus subsists by passing from vector to vertebrates (animals and humans) and vice versa when such insects ingest blood [1, 2].

The diagnosis of mucocutaneous leishmaniasis (MCL) is mainly based on the detection of signs and symptoms associated with its clinical presentation, supported by confirmatory laboratory tests. The parasitological diagnosis for MCL, lacks sensitivity (15%) due to an exacerbated inflammatory reaction and few parasites in the lesions [3], therefore, serological diagnosis becomes important because it has greater sensitivity (70%-80%) [4, 5]. However, as the last technique uses whole parasites as antigen, cross-reactions occur, as evidenced by the large amount of false positives due to the antibodies produced in these patients by other infectious entities sharing antigenic characteristics with *Leishmania* [6, 7].

To avoid cross-reaction in serological tests, different methods have thus been developed which involve using fractions of these parasites or specific proteins from genus *Leishmania* in an attempt to increase specificity for MCL diagnosis. For example, Soto, M. *et al.* 1996, obtained promising results when studying ribosomal proteins in immunodiagnostic tests for canine visceral leishmaniasis caused by

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Leishmania infantum, especially when fractions are selected instead of the complete protein [8].

In *Leishmania*, highly conserved proteins have been identified as immunogenic in animal and humans infected with the parasite [9]. Among these, heat shock proteins (HSP), nucleosomal proteins and Ribosomal proteins are released from the cell as a multiprotein complex, favouring their recognition by the immune system. Those proteins have been reported as immunogenic in several infectious diseases or autoimmune diseases and they have been named as "Pan-antigens" [9]. *Leishmania* Ribosomal proteins and its ability to induce antibodies in its host makes them a worth candidate to assess its diagnosis potential.

The aim of this study was to evaluate 2 ribosomal proteins, S25 and S5 from *Leishmania braziliensis*, which were identified as potential B epitopes by bioinformatics tools in previous studies (unpublished data), taking synthetic peptides derived from their sequences, as antigens for the serological diagnosis of MCL (by ELISA).

2. MATERIALS AND METHODS

2.1. Antigens

This study involved the evaluation of 2 ribosomal proteins (S25 and S5) from *Leishmania braziliensis*, selecting 8 (P1-P8) and 13 (P9-P21) synthetic peptides derived from their sequences, respectively to be asses its diagnostic usefulness. The sequences to be evaluated came from 15- to 19-aminoacid-long peptides covering the whole linear form of the proteins being studied, as follows:

2.1.1. Protein S25

P1: MPPKAGQTKKAKM EAA, *P2*: KGAKKTTKKW SKGQSREA, *P3*: LQ NAVMFDKETYDKL, *P4*: RSEVP-KYKLITPSI, *P5*: SDRLKIAVSIAADGL, *P6*: KQLCREK-LIRL VSCS, *P7*: VSCSSKTRVYTRIVQ, *P8*: AAPAET AAAAPASE.

2.1.2. Protein S5

P9: MSSKTPKLFNKWSFE, *P10*: GLQTSELAL RDHIST, *P11*: TAAYVPHTSGRWQKR, *P12*: RF HKVRM PIVERLAN, *P13*: GLMFKGRGNGRKL QA, *P14*: ARLLK HTLEIIHLLT, *P15*: DENPLQ VVVDAVSKG, *P16*: VSKGAPREDSTRVGSGG VV, *P17*: RRQAVDVSPMRR VNE, *P18*: AIYQM CKGAREAAFR, *P19*: NLKSMPE-CLADEIVN, *P20:* VNASKGSSNSYAIKKKD, *P21*: YAIKKK DEVERVAKANR.

These peptides were synthesized in *Curauma Nucleus Biotechnology Center Of* Pontifical Catholic University Of Valparaíso. Chile.

2.1.3. Soluble Leishmania Antigen (SLA)

SLA was made by taking promastigotes from parasites (Leishmania braziliensis (MHOM/CO/2011/UA3320), Leishmania panamensis (MHOM/CO/98/UA1702), Leishmania guyanensis (MHOM/CO/84/CL-007) or Leishmania infantum (MCAN/ES/96/BCN150) in exponential growth phase. These strains were kindly donated by Sara Robledo from Antioquia University. They were washed 3 times with saline solution, followed by 10 freezing-thawing cycles (-80ºC and 37ºC, respectively) and then sonicated. The vials were centrifuged at 10,000 rpm for 10 minutes and the supernatants were stored at -20ºC until use. A Bradford assay (Thermo Scientific) was used for determining protein concentration.

2.2. Sera Samples

Sera were obtained from several sera-banks for this study. The samples from patients having a confirmed diagnosis of leishmaniasis were obtained from the Colombian Instituto Nacional de Salud (INS) (n:10: 5MCL and 5 VL) and the Brazilian Gonçalo Moniz Research Centre, FIOCRUZ (CPqGM-FIOCRUZ) (n: 40: 20MCL and 20 CL). Twenty-five samples from MCL patients were evaluated, twenty suffering CL and five having visceral leishmaniasis (VL). Twenty sera were also obtained from patients having a confirmed diagnosis of Chagas' disease (CH) (attending the Universidad Javeriana's Infectious Diseases' Research Group in Bogota, Colombia), as well as twenty sera from patients having a confirmed diagnosis of an autoimmune disease, particularly Systemic Lupus Erythematosus (AID) (attending the Universidad del Rosario's Centre for Studying Autoimmune Diseases (CREA) in Bogota, Colombia). Twenty samples from individuals living in non-endemic areas for leishmaniasis were used as negative controls; all patients and controls voluntarily agreed to participate in the study by signing an informed consent form.

2.3. Conventional ELISA

The technique described by Souza *et al*., 2013, was used for the ELISA test. Briefly, 100µl/well SLA at a 10µg/ml concentration in PBS were added to in Maxisorp (NUNC) ELISA plates overnight at 4ºC. The plates were washed 4 times with PBS-0.05% Tween and blocked with 1% BSA in PBS-Tween for 2 hours at room temperature (RT). The plates were then washed again and 100µl patients' sera added (diluted 1:100 in 0.25% BSA PBS-Tween), followed by incubation at 37ºC for 1 hour. The plates were washed 5 times and 100µl alkaline phosphatase-labelled secondary antibody (Sigma-Aldrich Anti-Human IgG produced in goat) diluted 1:2,500 in PBS-Tween-BSA were added; the plates were then incubated for 1 hour at 37ºC. The plates were then washed 5 times with PBS-Tween and revealed by adding 100µl para-nitrophenyl phosphate (pNPP) substrate for 30 minutes at RT. The reaction was stopped with 50µl 3M NaOH and read at 405nm wavelength.

2.4. ELISA Ribosomal Peptide Assay (Peptide-ELISA)

This test protocol was carried out as described above, with small variations. Specifically, the peptides were added at 50µg/ml concentration and the sera were incubated for 2 hours at 37ºC.

2.5. Analysing the Data

The results were given as reactivity index (RI), calculated as each sample's optical density divided by the cut-off point (average of healthy controls + 2 standard deviations). Values above 1 were taken as positive.

The sensitivity, specificity, positive predictive value and negative predictive value were determined for each peptide analysed for diagnosing MCL.

3. RESULTS

This study evaluated the usefulness of *Leishmania braziliensis*-derived ribosomal peptides as antigen candidates for the immune-detection of anti-MCL antibodies for diagnostic purposes. Leishmaniasis patients' sera were initially evaluated with SLA as antigen for establishing its reactogenicity to the whole parasite (conventional diagnosis test). *Leishmania braziliensis* SLA had 84% sensitivity for detecting MCL and 75% for CL (Figure **1**). Table **1** summarises the results for the other *Leishmania* species used here.

In spite this method detects as positive individuals having leishmaniasis, such test has low specificity, having cross-reactivity with sera from individuals suffering CH and, to a lesser extent, AID (Figure **2**). There were no significant differences between the CL group and the MCL group, indicating that anti-leishmania antibody titres were similar in both clinical presentations of the disease.

Figure **3** A (S5) and B (S25) show that each protein-derived peptide (initially evaluated with 4 samples from MCL patients) was positive when the ELISA method was used for screening these peptides as antigens.

The P3, P4, P5, P6 and P7 sequences from the S25 protein-derived peptides had the highest RI, as did P12, P19 and P21 from the S5 protein. These peptides were preselected for being evaluated with a larger sample size. Figure **4** shows the S25 protein-derived peptides which were preselected.

Anti-*Leishmania* antibody detection was low when using each of these 5 peptides, since none detected more than 30% of the samples used as being positive; however, peptides 4 and 6 showed the best results and displayed the highest reactivity indices (RI), compared to the other peptide sequences.

Figure **5** lists the preselected S5 protein-derived peptides. As above (S25 protein), the three S5 peptides evaluated did not detect more than 30% of the samples used here. S5 protein-derived P12 displayed the highest sensitivity. Bearing the foregoing results in mind, the cross-reactions of P4, P6 and P12 with sera from individuals having a confirmed diagnosis of CH or AID were then evaluated (Figure **6**). Except for a few samples when P4 was used as antigen, both

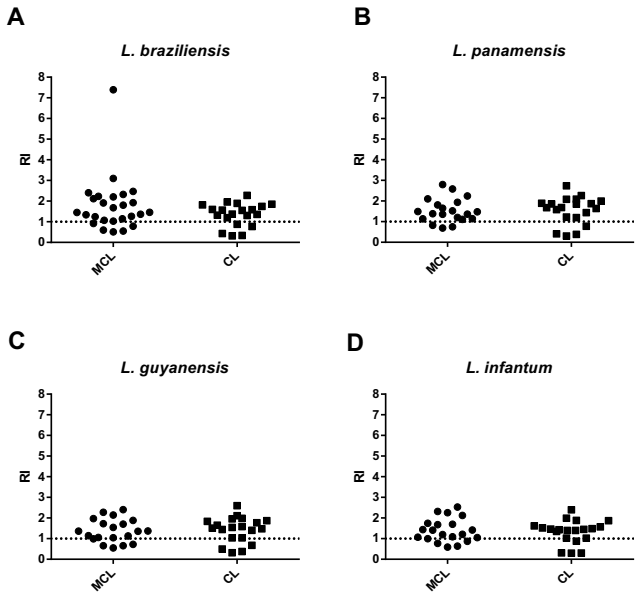

Figure 1. SLA-ELISA of leishmaniasis patients using lisate parasite from a) *Leishmania braziliensis;* b)*Leishmania panamensis;* c)*Leishmania guyanensis;* d) *Leishmania infantum*
MCL: Mucocutaneous leishmaniasis patients, CL: Cutaneous Leishmaniasis patients.

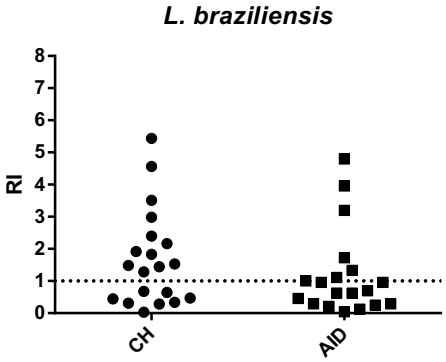

Figure 2. SLA-ELISA with sera from patients with Chagas Disease (CH) and Autoimmune diseases (AID).

Table 1. SLA- ELISA of leishmaniasis sera paients.

	Sensitivity		Specificity	PPV	NPV
	MCL	CL		MCL	
L. braziliensis	84% (21/25)	75% (15/20)	52.5%	55.8%	69.4%
L. panamensis	85% (17/20)	80% (16/20)	ND	ND	ND
L. guyanensis	75% (15/20)	80% (16/20)	ND	ND	ND
L. infantum	75% (15/20)	80% (16/20)	ND	ND	ND

MCL: Mucocutaneous leishmaniasis CL: Cutaneous leishmaniasis ND: No determinated because of limited CH sera volume; PPV: positive predicted value; NPV: Negative Predicted Value.

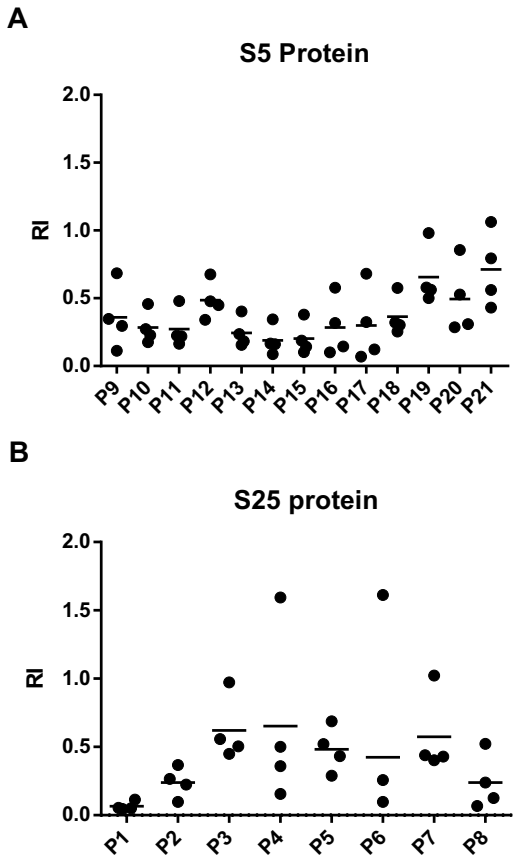

Figure 3. Peptide-ELISA with peptides derived from **a**) S5 protein and **b**) S25 protein.

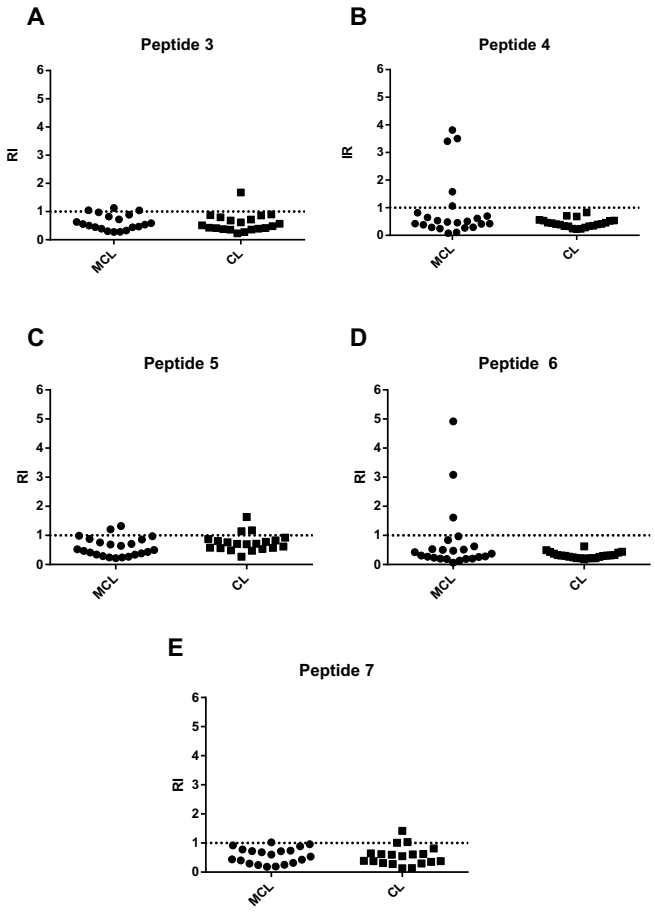

Figure 4. Protein S5 Peptide-ELISA with leishmaniais patients **a**) peptide 3; **b**) peptide 4; **c**) peptide 5; **d**) peptide 6; **e**) peptide 7 as antigen.

CH and AID sera were negative with peptides P6, P12 and P19, indicating low cross-reaction in individuals suffering conditions other than leishmaniasis (when these peptides were used for detecting antibodies). Table **2** summarises the results for the peptides derived from both proteins.

It was thus seen that the selected peptides' sensitivity was lower than the conventional ELISA; however, their specificity was greater since, unlike the SLA technique, none of the CH sera reacted with them.

4. DISCUSSION

The indirect ELISA technique is widely used in detecting antibodies directed against infectious agents, for diagnostic purposes. This technique has proved more sensitive for diagnosing leishmaniasis compared to the indirect immunofluorescence (IFI) method as it detects antibodies directed against both the parasite surface and intracellular proteins when parasite lysates (SLA) is used as antigen.

The present study revealed that such technique's sensitivity and specificity, in the conditions used here, were similar to that reported in the pertinent literature [4, 5, 10-12].

Comparing SLA results for one species with those for another revealed that *Leishmania braziliensis* and *Leishmania panamensis* detected more MCL samples as being positive than *Leishmania guyanensis* and *Leishmania infantum*.

Table 2. Peptide-ELISA.

Peptides	Sensitivity		Specificity
S25 Protein	**MCL**	**CL**	
P3	15%	5%	ND
P4	21.7%	0%	95%
P5	8.6%	15%	ND
P6	13.04%	0%	100%
P7	5%	15%	ND
S5 Protein			
P12	5%	20%	100%
P19	20%	15%	100%
P21	20%	5%	82.5%

MCL: Mucocutaneous leishmaniasis; CL: Cutaneous leishmaniasis; ND: No determinated (no preselected peptides)

This was probably due to the former being the main species having tropism for the mucosa, thereby indicating that ELISA sensitivity could be higher when the

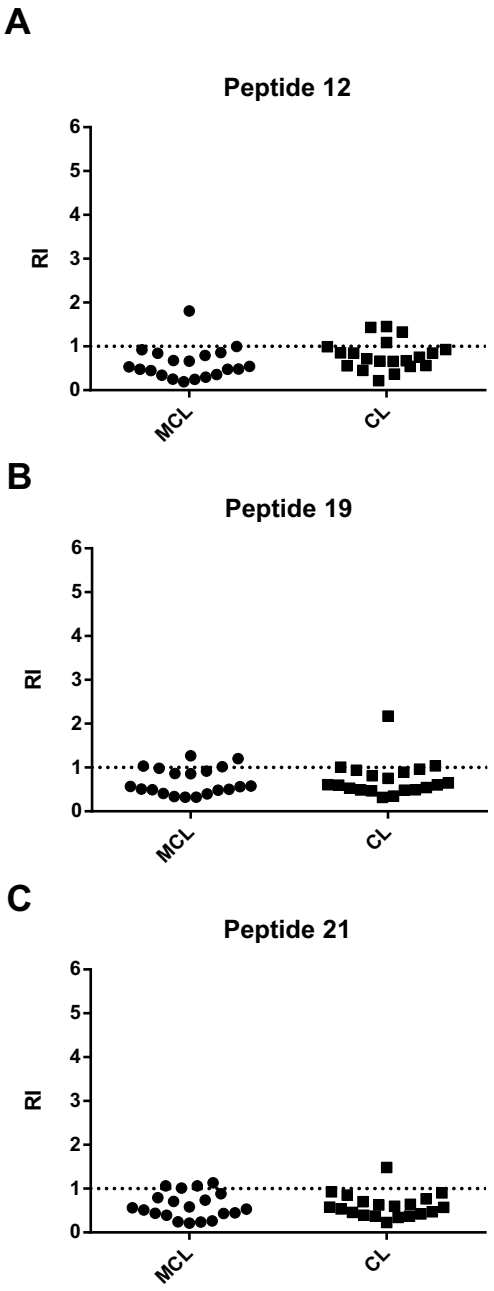

Figure 5. Protein S25 Peptide-ELISA with leishmaniais patients **a**) peptide 12; **b**) peptide 19; **c**) peptide 21 as antigen.
MCL: Mucocutaneous leishmaniasis patients, CL: Cutaneous Leishmaniasis patients.

infecting specie is used as antigen (even considering the high degree of conservation of antigenic proteins from the genus *Leishmania*).

In spite of this, a high cross-reaction occurred with sera from individuals suffering Chagas' disease, as reported in the literature [7, 10]; this is due to the taxonomic closeness between both parasites, thereby leading to diagnosis by ELISA with low specificity. For that reason, some researchers have done a screening for intracellular, highly conserved *Leishmania*-specific immunodominant proteins, such as heat shock proteins (HSP) or ribosomal proteins, in an attempt to reduce the amount of false positives (i.e. instead of using antigens from a whole parasite) [8, 9, 13-18].

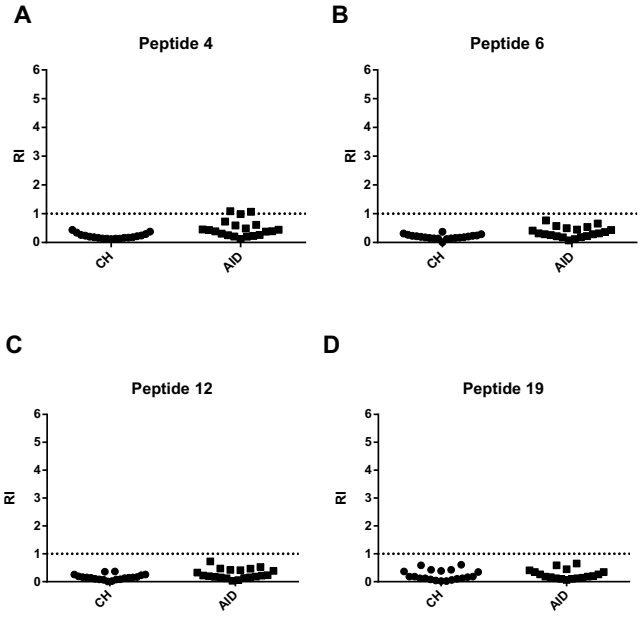

Figure 6. Peptide-ELISA with sera from patients with Chagas Disease (CH) and Autoimmune diseases (AID) **a**) peptide 4; **b**) peptide 6; **c**) peptide 12; **d**) peptide 19 as antigen.

The advantage of using highly conserved proteins, such as ribosomal proteins, is that their structure is almost identical amongst *Leishmania* species thereby allowing antibodies to be detected against it, regardless of the species causing the disease; however, their main disadvantage lies in the fact that other non-*Leishmania* species of microorganisms can also induce them.

Different linear and conformational B-epitopes can be found throughout a protein's folded structure. Epitope mapping is a methodology used for identifying each linear sequence acting as epitope in order to make detecting antibodies more specific. In that sense, intracellular protein-derived peptides have been studied, in which immunogenic determinants have been identified, so using epitope mapping methods, these immunodominant peptides have shown to be useful for diagnosing leishmaniasis in humans and canines,[19, 20] as well as in Chagas' disease [21, 22] and diseases caused by other infectious agents (such technique having up to 100% sensitivity and specificity)[23, 24].

The peptides evaluated in the present study covered the whole proteins being studied; however, the ELISA ribosomal peptide assay's ability to diagnose leishmaniasis was low, maybe due to the selected sequence not necessarily coinciding with the epitope. Taking into account that epitope mapping methodology used here allows the detection of linear epitopes only, this protein's antigenicity may also have resulted from protein folding (conformational epitope) and it not being a linear epitope. However, when the preselected peptides were evaluated using more samples (even though

ELISA sensitivity for these continued to be low), all the samples proved positive, indicating that some individuals developed antibodies against these sequences. This led to supposing that there could be low concentrations of antibodies against this sequence in the other individuals, or that only some of those affected actually develop these antibodies following infection. Either way, a peptide having sufficient sensitivity to be used in a serological diagnosis method could not be selected in the conditions used here.

On the other hand, none of the CH patients' samples reacted with the 3 preselected peptides when evaluating cross-reaction and only P4 reacted against some AID sera, thereby highlighting that these peptides could have had high specificity.

CONCLUSION

ELISA continues being a widely-used diagnostic method due to its relative ease of use and low cost-effectiveness, thereby enabling its accessibility in remote places where complex equipment is not available such in places where diseases like leishmaniasis are endemic and where early diagnosis is required for timely treatment, thereby avoiding irreversible consequences in those affected or the unnecessary administration in patients with different affections. Even though having low sensitivity, Peptide-ELISA had a higher specificity than the SLA-ELISA, showing therefore that detecting anti-*Leishmania* antibodies using synthetic peptides as antigens, may be extremely useful because it may avoid false positives, therefore making this technique more accurate in the diagnosis of MCL. Finally, we conclude that Peptide-ELISA could provide a suitable complement for the difficult clinical diagnosis of MCL.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

This work was approved by Ethic committee belonging to Science Faculty of National University of Colombia by the agreement 05 on August 21, 2012.

HUMAN AND ANIMAL RIGHTS

No animals were used and all the human were used in accordance with the ethical standards of the committee responsible for human experimentation (institutional and national), and with the Helsinki Declaration of 1975, as revised in 2008 (http://www.wma.net/en/20activities/10ethics/10helsinki/).

CONSENT FOR PUBLICATION

All the individuals included here agree to participate by a written consent.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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REFERENCES

[1] WHO | New Technical Report Series on the Control of Leishmaniasis. http://www.who.int/neglected_diseases/integrated_media/integrated_media_2010_leishmaniasis/en/.

[2] Leishmaniasis - Pan American Health Organization - Organización Panamericana de la Salud. http://new.paho.org/hq/index.php?option=com_content&task=blogcategory&id=3835&Itemid=4098(=en.

[3] Goto, H.; Lindoso, J.A. Current diagnosis and treatment of cutaneous and mucocutaneous leishmaniasis. *Expert Rev. Anti Infect. Ther.,* **2010**, *8*(4), 419-433.

[4] Barroso-Freitas, A.P.; Passos, S.R.; Mouta-Confort, E.; Madeira, M.F.; Schubach, A.O.; Santos, G.P.; Nascimento, L.D.; Marzochi, M.C.; Marzochi, K.B. Accuracy of an ELISA and indirect immunofluorescence for the laboratory diagnosis of American tegumentary leishmaniasis. *Transact. Royal Soc. Trop. Med. Hyg.,* **2009**, *103*(4), 383-389.

[5] Romero, G.A.; de la Gloria Orge Orge, M.; de Farias Guerra, M.V.; Paes, M.G.; de Oliveira Macedo, V.; de Carvalho, E.M. Antibody response in patients with cutaneous leishmaniasis infected by *Leishmania (Viannia) braziliensis* or *Leishmania (Viannia) guyanensis* in Brazil. *Acta Trop.,* **2005**, *93*(1), 49-56.

[6] Vargas-Duarte, J.J. Evaluacion por western blot, inmunofluorescencia indirecta y ELISA de Perros Infectados con *Leishmania (Leishmania) infantum*. *Revista de Salud Pública,* **2009**, *11*(4), 641-652.

[7] Malchiodi, E.L.; Chiaramonte, M.G.; Taranto, N.J.; Zwirner, N.W.; Margni, R.A. Cross-reactivity studies and differential serodiagnosis of human infections caused by *Trypanosoma cruzi* and *Leishmania* spp; use of immunoblotting and ELISA with a purified antigen (Ag163B6). *Clin. Exp. Immunol.,* **1994**, *97*(3), 417-423.

[8] Soto, M.; Requena, J.M.; Quijada, L.; Alonso, C. Specific serodiagnosis of human leishmaniasis with recombinant *Leishmania* P2 acidic ribosomal proteins. *Clin. Diag. Lab. Immunol.,* **1996**, *3*(4), 387-391.

[9] Requena, J.M.; Alonso, C.; Soto, M. Evolutionarily conserved proteins as prominent immunogens during *Leishmania* infections. *Parasitol. Today,* **2000**, *16*(6), 246-250.

[10] Celeste, B.J.; Angel, S.O.; Castro, L.G.; Gidlund, M.; Goto, H. *Leishmania infantum* heat shock protein 83 for the serodiagnosis of tegumentary leishmaniasis. *Braz. J. Med. Biol. Res.,* **2004**, *37*(11), 1591-1593.

[11] Guimaraes, M.C.; Celeste, B.J.; Franco, E.L.; Cuce, L.C.; Belda Jr., W. Evaluation of serological diagnostic indices for mucocutaneous leishmaniasis: Immunofluorescence tests and enzyme-linked immunoassays for IgG, IgM and IgA antibodies. *Bull World Health Org.,* **1989**, *67*(6), 643-648.

[12] de Carvalho, C.A.; Partata, A.K.; Hiramoto, R.M.; Borborema, S.E.; Meireles, L.R.; Nascimento, N.; de Andrade Jr., H.F. A simple immune complex dissociation ELISA for leishmaniasis: Standardization of the assay in experimental models and preliminary results in canine and human samples. *Acta Tropica,* **2013**, *125*(2), 128-136.

[13] Montoya, Y.; Leon, C.; Talledo, M.; Nolasco, O.; Padilla, C.; Munoz-Najar, U.; Barker, D.C. Recombinant antigens for specific and sensitive serodiagnosis of Latin American tegumentary leishmaniasis. *Transact. Royal Soc. Trop. Med. Hyg.,* **1997**, *91*(6), 674-676.

[14] Abass, E.; Bollig, N.; Reinhard, K.; Camara, B.; Mansour, D.; Visekruna, A.; Lohoff, M.; Steinhoff, U. rKLO8, a novel *Leishmania donovani* - derived recombinant immunodominant protein for sensitive detection of visceral leishmaniasis in Sudan. *PLoS Negl. Trop. Dis.,* **2013**, *7*(7), e2322.

[15] Souza, A.P.; Soto, M.; Costa, J.M.; Boaventura, V.S.; de Oliveira, C.I.; Cristal, J.R.; Barral-Netto, M.; Barral, A. Towards a more precise serological diagnosis of human tegumentary leishmaniasis using Leishmania recombinant proteins. *PloS One,* **2013**, *8*(6), e66110.

[16] Kaur, J.; Kaur, S. ELISA and western blotting for the detection of Hsp70 and Hsp83 antigens of *Leishmania donovani*. *J. Parasit. Dis.,* **2013**, *37*(1), 68-73.

[17] Lakhal, S.; Mekki, S.; Ben-Abda, I.; Mousli, M.; Amri, F.; Aoun, K.; Bouratbine, A. Evaluation of an enzyme-linked immunosorbent assay based on crude Leishmania histone proteins for serodiagnosis of human infantile visceral leishmaniasis. *Clin. Vaccine Immunol.,* **2012**, *19*(9), 1487-1491.

[18] Celeste, B.J.; Arroyo Sanchez, M.C.; Ramos-Sanchez, E.M.; Castro, L.G.; Lima Costa, F.A.; Goto, H. Recombinant *Leishmania infantum* heat shock protein 83 for the serodiagnosis of cutaneous, mucosal, and visceral leishmaniases. *Am. J. Trop. Med. Hyg.,* **2014**, *90*(5), 860-865.

[19] Faria, A.R.; Costa, M.M.; Giusta, M.S.; Grimaldi Jr., G.; Penido, M.L.; Gazzinelli, R.T.; Andrade, H.M. High-throughput analysis of synthetic peptides for the immunodiagnosis of canine visceral leishmaniasis. *PLoS, Negl. Trop. Dis.,* **2011**, *5*(9), e1310.

[20] Costa, M.M.; Penido, M.; dos Santos, M.S.; Doro, D.; de Freitas, E.; Michalick, M.S.; Grimaldi, G.; Gazzinelli, R.T.; Fernandes, A.P. Improved canine and human visceral leishmaniasis immunodiagnosis using combinations of synthetic peptides in enzyme-linked immunosorbent assay. *PLoS, Negl. Trop. Dis.,* **2012**, *6*(5), e1622.

[21] Bottino, C.G.; Gomes, L.P.; Pereira, J.B.; Coura, J.R.; Provance Jr., D.W.; De-Simone, S.G. Chagas disease-specific antigens: Characterization of epitopes in CRA/FRA by synthetic peptide mapping and evaluation by ELISA-peptide assay. *BMC Infect. Dis.,* **2013**, *13*, 568.

[22] Bhattacharyya, T.; Falconar, A.K.; Luquetti, A.O.; Costales, J.A.; Grijalva, M.J.; Lewis, M.D.; Messenger, L.A.; Tran, T.T.; Ramirez, J.D.; Guhl, F.; Carrasco, H.J.; Diosque, P.; Garcia, L.; Litvinov, S.V.; Miles, M.A. Development of peptide-based lineage-specific serology for chronic chagas disease: Geographical and clinical distribution of epitope recognition. *PLoS Negl. Trop. Dis.,* **2014**, *8*(5), e2892.

[23] Metz, G.E.; Lorenzon, E.N.; Serena, M.S.; Corva, S.G.; Panei, C.J.; Diaz, S.; Cilli, E.M.; Echeverria, M.G. Development of a peptide ELISA for the diagnosis of Equine arteritis virus. *J. Virol. Methods,* **2014**, *205c*, 3-6.

[24] Intapan, P.M.; Sanpool, O.; Janwan, P.; Laummaunwai, P.; Morakote, N.; Kong, Y.; Maleewong, W. Evaluation of IgG4 subclass antibody detection by peptide-based ELISA for the diagnosis of human paragonimiasis heterotrema. *Kor. J. Parasitol.,* **2013**, *51*(6), 763-766.