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Brief Definitive Report

B-cell epitopes of antigenic proteins in *Leishmania infantum*: an *in silico* analysis

L. M. ASSIS,[1,2] J. R. SOUSA,[3] N. F. S. PINTO,[4] A. A. SILVA,[5] A. F. M. VAZ,[6] P. P. ANDRADE,[7] E. M. CARVALHO[8,9] & M. A. DE MELO[6]

[1]Graduate Program in Medicine and Health, Professor Edgard Santos University Hospital Complex, Federal University of Bahia (Universidade Federal da Bahia-UFBA), Salvador, Brazil, [2]Academic Nursing Unit, Federal University of Campina Grande (Universidade Federal de Campina Grande)-UFCG, Cajazeiras, Brazil, [3]Santa Maria College (Faculdade Santa Maria-FSM), Cajazeiras, Brazil, [4]Graduate Program in Veterinary Medicine, Veterinary Medicine Academic Unit, Federal University of Campina Grande-UFCG, Patos, Brazil, [5]Academic Unit of Exact and Natural Sciences, Federal University of Campina Grande, Cajazeiras, Brazil, [6]Academic Unit of Veterinary Medicine, Federal University of Campina Grande-UFCG, Patos, Brazil, [7]Department of Genetics, Federal University of Pernambuco-UFPE, Recife, Brazil, [8]Graduate Program in Medicine and Health, Immunology Service, Professor Edgard Santos University Hospital Complex, Federal University of Bahia, Salvador, Brazil, [9]National Institute of Science and Technology of Tropical Diseases (Instituto Nacional de Ciência e Tecnologia de Doenças Tropicais) - INCT DT(CNPq/MCT), Salvador, Brazil

SUMMARY

Serodiagnosis of visceral leishmaniasis is often hindered by cross-reactions to other parasitic diseases. Identifying specific B-cell epitopes in proteins is therefore important for immunodiagnostics, as well as for disease control by vaccines. This study aimed to identify linear and conformational B-cell epitopes and to evaluate the secondary structure of antigen proteins in Leishmania infantum *using* in silico *analysis. Linear epitopes were predicted using the Immune Epitope Database and Analysis Resource (IEDB), BepiPred and BcePred programs. The conformational B-cell epitopes were identified using the CBTOPE server. The combination of the predictions using IEDB, BepiPred and BcePred generated 148 linear epitopes from the calpain-like cysteine peptidase (CP), thiol-dependent reductase 1 (TDR1) and HSP70 proteins. In total, 164 conformational epitopes were predicted, mostly located in the linear epitope region. The predicted epitopes are located in α helix and random coil regions in the thiol-dependent reductase 1 and HSP70 proteins. New linear and conformational B-cell epitopes of* L. infantum *proteins were identified* in silico*, and the prediction using various programs ensures greater accuracy of the results, as suggested by confirmation of previously identified HSP70 epitopes.*

Correspondence: Marcia Almeida de Melo, Academic Unit of Veterinary Medicine, Federal University of Campina Grande-UFCG, Av. Universitária, s/n, Bairro Santa Cecília, Patos, Paraíba CEP: 58. 708-110, Brazil (e-mail: marcia.melo@pq.cnpq.br).
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INTRODUCTION

Identifying antigenic/immunogenic regions in protein antigens is important for the diagnosis of infectious diseases as well as for vaccine development. By contrast, the production and use of native antigens for diagnostic tests and vaccine assays are frequently hindered by variation in species and lead to high costs for purification, especially in producing vaccines (1). An alternative to resolve these limitations is the use of synthetic peptides made from epitopes identified with computational tools for predicting antigenic and/or immunogenic peptides (2–4).

The first method for predicting linear epitopes was based on the properties of the amino acids (5), assuming that hydrophilic regions of the proteins are predominantly located on the surface and thus are potentially antigenic. Subsequently, Parker *et al.* (6) used the modified hydrophilic scale and observed improved epitope prediction compared with the method of Hopp and Woods (5). Other studies predicting B-cell epitopes have been published based on the physicochemical properties of amino acids, such as flexibility (7) and solvent accessibility (8). Kolaskar and Tongaonkar (9) used the antigenicity parameter to predict antigenic sites in proteins and observed approximately 75% accuracy. Subsequently, Pellequer *et al.* (10) found a correlation between antigenic sites and the prediction of turns in the protein. A comparison of 12 scales

applied to 85 linear epitopes identified in 14 proteins demonstrated that most of the scales had 50–62% correct predictions (11); the B-turn scale had the best performance, correctly predicting 70% of the known epitopes (10). BcePred (12) combines seven different physicochemical scales of the existing scalar prediction methods that can be used individually or in combination and has one of the best accuracy rates presently reported.

In the last decade, more sophisticated methods based on machine-learning models were developed in an attempt to improve epitope prediction accuracy (13). The BepiPred program (14) is based on combining the hidden Markov model with two scalar methods: Parkers hydrophilicity scale (6) and Levitts secondary structure scale (15). The program demonstrated that the performance of the scales combined is better than the performance of only a single scale. Currently, the BepiPred method is incorporated into the IEDB program (Immune Epitope Database and Analysis Resource) (16), which is a technique that has been increasingly used for epitope prediction. On the other hand, the ABCpred predictive tool uses an artificial neural network and, when applied, yields 66% maximum accuracy (17). New *in silico* methodologies for predicting B-cell epitopes were also developed using supported vector machine (SVM) models, a class of supervised machine-learning methods used for classification and regression. Tools employing these methodologies include BCPred (18), COBEpro (19) and, more recently, sequence-based methods – CBTope (20) and BEST (21). The CBTope predictor was proposed for predicting conformational epitopes and uses multiple propensities with 86·59% accuracy.

The first step in designing synthetic peptides for application to vaccines and disease immunodiagnostics is to identify potential epitopes. Several studies have been performed using synthetic peptides that mimic epitopes for diagnosing infectious and parasitic diseases (22). Combining methods and analysis of results can reduce the number of potentially useful epitopes and consequently optimize the resources and effort in identifying effectively useful epitopes *in vitro* and *in vivo*. This study describes and analyses the prediction of B-cell epitopes on antigenic proteins of *Leishmania infantum* using bioinformatics tools.

MATERIALS AND METHODS

The genes corresponding to the proteins analysed in this study were identified by immunoscreening of a complementary DNA (cDNA) library from *Leishmania chagasi*, employing human serum with visceral leishmaniasis. Partial nucleotide sequences were obtained by sequencing the corresponding cDNA. Three *L. infantum* proteins were analysed as follows: putative calpain-like cysteine peptidase (CP) (GI: 146090707), thiol-dependent reductase 1 (TDR1) (GI: 146097469) and 70 kDa – HSP70 heat-shock protein (GI: 758136·1) with 6168, 450 and 653 amino acids (aa), respectively. The HSP70 protein was used as a control for predicting the presence of experimentally identified immunodominant epitopes (23).

For determining linear epitopes, the *L. infantum* protein sequences were submitted to the IEDB (24), BepiPred (14) and BcePred web servers (12). The methods of predicting B-cell epitopes that are common for the IEDB and BcePred programs used in this study were based on the following scales: hydrophilicity (6), flexibility (7) and solvent accessibility (8). The linear B-cell epitopes that were partially or entirely common to scalar hydrophilicity, flexibility and accessibility methods of the IEDB and BcePred were compared with the BepiPred results. The linear epitopes common to the three programs were selected to determine the frequency of amino acid residues. The CBTope program was used to predict conformational B-cell epitopes (20). The default settings were applied to all the tools used. The secondary structure of the proteins was predicted using the PHD server (http://npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=/NPSA/npsa_phd.html).

RESULTS AND DISCUSSION

The IEDB, BcePred and BepiPred programs identified 130, 103 and 437 linear epitopes, respectively, obtained from the three proteins CP, TDR1 and HSP70. Using the IEDB, BepiPred and BcePred programs, 357 linear epitopes were predicted for the CP protein, of which 130 were partially or entirely common to the three programs (Table 3). Of the 19 epitopes of the TDR1 protein predicted using the three methods, only four were common to all programs. Of the 37 epitopes predicted for HSP70, 14 were common to all three programs (Table 1).

Some of the predicted epitopes were either previously described as B-cell epitopes or contained in antigenic regions of *L. infantum* HSP70 and supported the use of our *in silico* strategy for the identification of B-cell epitopes. Indeed, the Ph5 epitope (aa 241–261) has all the amino acid residues of the H17 epitope (FFTEEFKRKNKGKNLASSHR) identified as species-specific by (23). The Ph9 and Ph10 epitopes have the partial sequence of amino acid residues of the H35 and H36 epitopes that were also immunogenic and identified by the same author (Table 1). The Ph2, Ph4, Ph7 and Ph8 epitopes were not indicated as immunogenic in the assay by (23).

In the CBTope program, 143, 11 and 10 conformational epitope regions were predicted for the CP (Table 4), TDR1 (Table 2) and HSP70 proteins (Table 2). Of the 143

Table 1 Linear epitopes of the TDR1 and HSP70 proteins of *Leishmania infantum* predicted using IEDB, BepiPred and BcePred

Programs	Position	Sequence	Linear epitope
1;2;3[a]	39–54	REEMPQWYKQINPRET	Pt1
1;2;3	55–70	VPTLEVGNADKRFMFE	Pt2
1;2;3	195–214	AAQRASVRETSPTAAQCIEN	Pt3
1;2;3	310–324	ALVPRGDAEKEYEVG	Pt4
1;2;3	28–46	IIANDQGNRTTPSYVAFTD	Ph1
1;2;3	105–121	SVQYRGEEKTFTPEEIS	Ph2
1;2;3	149–165	AYFNDSQRQATKDAGTI	Ph3
1;2;3	183–197	YGLDKGDDGKERNVL	Ph4
1;2;3	241–261	**FFTEEFKRKNKGKNLASSHR**A[b]	Ph5
1;2;3	323–335	LQDAKMDKRSVHD	Ph6
1;2;3	380–393	FILTGGKSKQTEGL	Ph7
1;2;3	488–506	LNVSAEEKGTGKRNQITIT	Ph8
1;2;3	504–524	TITNDKG**RLSKDEIERMVNDA**[c]	Ph9
1;2;3	525–545	M**KYEEDDKAQRDRVEAKNGLE**[d]	Ph10
1;2;3	546–564	NYAYSMKNTLSDSNVSGKL	Ph11
1;2;3	558–576	SNVSGKLVSGKLEDSDKATLNKEI	Ph12
1;2;3	581–599	EWLSSNQEAAKEEYEHKQK	Ph13
1;2;3	587–605	QEAAKEEYEHKQKELESVC	Ph14

[a]IEDB (1), BepiPred (2), BcePred (3); Pt: TDR1 epitopes; Ph: HSP70 epitopes; [b]H17 immunodominant and species-specific epitope; bold amino acid residues are present in epitopes H17, H35[c] and H36[d] (32).

Table 2 B-cell conformational epitopes of the TDR1 (GI: 146097469) and HSP70 (GI: 758136) proteins of *Leishmania infantum* predicted using CBTope

No. of epitope residues[a]	Position	Sequence	Conformational epitope
36 (43)	16–57	FCHRVEIVAREKQVSYDRVAVGL**REEM PQWYKQINPRET**VPT[b]	Pect1
21 (24)	117–140	LRDPLSGEKRKAMDDNAAYVDGLL	Pect2
6 (09)	161–169	ALVPFLVRL	Pect3
10 (12)	208–219	**AAQCIEN**YRHLV	Pect4
28 (44)	238–281	LFCPFVDRARLASELRKFQMHIVEVPLH PQPEWYKYINPRDTVP	Pect5
5 (09)	293–301	ESQLIVQYI	Pect6
9 (11)	311–321	**LVPRGDAEKEY**	Pect7
4 (04)	374–377	FGGK	Pect8
9 (10)	389–399	LVRAKAFMPE	Pect9
11 (18)	413–430	LNGLAEAGMATPEAKSVF	Pect10
6 (06)	445–450	RRAQSG	Pect11
17 (20)	21–40	WQNERVD**IIANDQGNRTTPS**[b]	Pech1
22 (35)	48–82	ERLIGDAAKNQVAMNPHNTVFDAKRLI GRKFNDSV	Pech2
15 (29)	136–164	LGKQVKKAVVTVP**AYFNDSQRQATKDAGT**	Pech3
27 (38)	321–359	RV**LQDAKMDKRSVHD**VVLVGGSTRIPK VQSLVSDFFGGK	Pech4
6 (06)	381–386	**ILTGGK**	Pech5
21 (23)	427–449	SQIFSTYAD**NQPGVHIQVFEGER**[c]	Pech6
21 (32)	476–507	IEVTFDLDANGILNVSAEEKGTGKRNQI**TITN**	Pech7
4 (04)	539–542	**EAKN**	Pech8
11 (15)	561–575	**SGKL**EDSDKATLNKE	Pech9
5 (07)	603–609	**SVC**NPIM	Pech10

[a]Size of the conformational epitope region is in parenthesis; [b]amino acids in bold are part of the linear epitopes of the same region; Pect: TDR1 conformational epitope; Pech: HSP70 conformational epitope; [c]bold amino acids correspond to the immunodominant epitope H30 (32).

Table 3 Linear epitopes of the putative calpain-like cysteine peptidase (GI:146090707) of *Leishmania infantum* predicted using IEDB, BepiPred and BcePred

Programs	Position	Sequence	Linear epitope
1;2;3[a]	81–93	CFVTKKVRQDGRY	P1
1;2;3	243–261	LHNPFEDEEYVYKGPLNSK	P2
1;2;3	264–182	TWDVKQRAKHDVDDERSIF	P3
1;2;3	348–369	VVIKQEDQRRFTSPDEMTKYLQ	P4
1;2;3	470–488	ELCQKEKDRVDFYVDEGTD	P5
1;2;3	493–512	MHQTKPYVSKSGGDAMTEDY	P6
1;2;3	516–527	YLYDDTDRKIAG	P7
1;2;3	617–634	AHHDEQAESDSPFEDKRF	P8
1;2;3	699–717	CFISKNPRKDGRYTFQFHR	P9
1;2;3	859–877	VKMYNPYEDSPYTGPMHRD	P10
1;2;3	1055–1073	YWFLRKGDKDKLDIERLNT	P11
1;2;3	1060–1078	KGDKDKLDIERLNTDVARQ	P12
1;2;3	1132–1154	YLYDANDKRISPSTQATNNREIG	P13
1;2;3	1241–1259	RLHHKPHRNEDEILALERK	P14
1;2;3	1292–1318	LDSDPEYMNAERERHNLKKDPRNAGKV	P15
1;2;3	1442–1459	NRKPKRDAKAIKDLQRTL	P16
1;2;3	1510–1528	RKEDPLGNRDDIKTLEDEL	P17
1;2;3	1526–1544	DELNDRARELAKDQQANQR	P18
1;2;3	1537–1555	KDQQANQRAFLDQDPYGVP	P19
1;2;3	1627–1645	PLLENPEFKELDTKRRRVL	P20
1;2;3	1646–1664	NRGGDTSKVPDMEDRMNDV	P21
1;2;3	1671–1689	DMNVAERPDYMDTTYKGIP	P22
1,2,3	1705–1733	EVKRQQQKQDPRRNARGIA DTEQELNDRA	P23
1;2;3	1769–1781	AEMEAQRAKLKKD	P24
1;2;3	1772–1791	EAQRAKLKKDMRRNTKPIAE	P25
1;2;3	1793–1811	ENLLNDRAHELAKSLKEKE	P26
1;2;3	1801–1819	HELAKSLKEKERPKFLDAR	P27
1;2;3	1973–1991	LEAQYRDLKKSPKANPQDV	P28
1;2;3	1981–1999	KKSPKANPQDVADCEELMN	P29
1;2;3	2028–2046	EELPLDTDDEFSNLEAQRA	P30
1;2;3	2044–2062	QRARLMRQPAKNKKAIDEI	P31
1;2;3	2248–2268	VEMRANPKKNAQSIKSAEEDL	P32
1;2;3	2273–2291	MEMAKEKAAEEREDYIDPE	P33
1;2;3	2291–2309	EPEGRKLNDLGLDDDPTFV	P34
1;2;3	2310–2328	GIEEQYRRSRKDPYADQDR	P35
1;2;3	2329–2347	LRDLEQMMNDRAHDLAKMK	P36
1;2;3	2346–2364	MKNARDRDMYLDRAPRNVP	P37
1;2;3	2495–2513	LGIPSEDLNPYLDEDPHFH	P38
1;2;3;	2516–2534	EDMYRDAKNNPTKAKKASQ	P39
1;2;3	2530–2548	KKASQLLDQMNERAREIAQ	P40
1;2;3	2712–2734	FHELETRRAKLKSEDPRAHQKAI	P41
1;2;3	2785–2803	KHLRDLKSDSKRNAAAIRE	P42
1;2;3	2853–2871	ERAKLKARDSVKNAKKIQA	P43
1;2;3	2872–2893	LEDQLNERANQLAEAQKQEDL RGLDPKPEGIP	P44
1;2;3	2885–2903	EAQKQEDLRGLDPKPEGIP	P45
1;2;3;	2920–2939	PQLRDMKADPRTRPEDLQQV	P46
1;2;3	2993–3014	LKAQDPRRNAAKIRDSEDRLRE	P47
1;2;3	3015–3037	RSYELAEQQRTKDLENLDQVPEG	P48
1;2;3	3057–3075	QHRQLAKDSVKDSAKNSEL	P49
1;2;3	3076–3092	LTKLEEKMNDRAHELAK	P50
1;2;3	3262–3280	RARLKLRDPKRNARAIKDL	P51
1;2;3	3328–3346	PQLRALKKDPKKNAEAIRR	P52
1;2;3	3346–3364	RVENEMNNRANELARQLLE	P53

Table 3 (Continued)

Programs	Position	Sequence	Linear epitope
1;2;3	3396–3314	ERAKLKAQDPRRNQRRIAD	P54
1;2;3	3406–3424	RRNQRRIADLEDRLNDRAV	P55
1;2;3	3525–3544	FQQLRQECANLKAKDPRRN	P56
1;2;3	3542–3561	RNADKVKSLEDQMRSRVHEL	P57
1;2;3	3597–3615	LPELRRAKKSLRDTQRAQG	P58
1;2;3	3609–3627	DTQRAQGLLNELNERIHEL	P59
1;2;3	3719–3736	EAVRPHNNPDFHNLATRAR	P60
1;2;3	3732–3750	ATRARELRKDSRRNADKLA	P61
1;2;3	3829–3849	HKLAEAQKREDLRGLNSAPLG	P62
1;2;3	3855–3873	LNPHDDPRFAAKLPELRAQ	P63
1;2;3	3870–3894	LRAQKKEGFPRAQSRLNDTQAKLDE	P64
1;2;3	3928–3946	ADPEFHQLEAERLDLISKN	P65
1;2;3	3944–3962	SKNPKANKDAIKDLEAALN	P66
1;2;3	3967–3983	ELAREHRKGDRGYLNAE	P67
1;2;3	4076–4094	RLLKADPSADPKKVSDLEQ	P68
1;2;3	4087–4105	KKVSDLEQDMNDRAHELAE	P69
1;2;3	4148–4166	LQEPKKNQAKAAELEGRLN	P70
1;2;3	4167–4191	RLNDRAHELAKEQRARDLQDLDEAPRGI	P71
1;2;3	4204–4223	FASLAFQRRDPNNTLRKALD	P72
1;2;3	4334–4352	DLPLDKDPQFHQMEVERAK	P73
1;2;3	4339–4367	ERAKLKAQDLTKNANKIKD	P74
1;2;3	4360–4378	KNANKIKDLEDKLNDRAEN	P75
1;2;3	4379–4397	LAEVQKKEDLRNLDGKPRG	P76
1;2;3	4393–4411	GKPRGIPLESLNPHDDAEF	P77
1;2;3	4414–4432	HLPELRRLKNEQPNHPKIK	P78
1;2;3	4433–4451	DLQAKLDNRADELAKAQID	P79
1;2;3	4469–4487	LPLDSDKLFTSLEKQLRQA	P80
1;2;3	4478–4496	TSLEKQLRQAKQDLKRNAD	P81
1;2;3	4488–4506	KQDLKRNADKITDLQDCMN	P82
1;2;3	4495–4513	ADKITDLQDCMNKRVHELA	P83
1;2;3	4542–4560	AMFRELEAQRAKLKEDPKR	P84
1;2;3	4560–4578	RNADKIKDLEGKLNDRAHE	P85
1;2;3	4571–4589	KLNDRAHELAKAQKEAARG	P86
1;2;3	4611–4631	FVKMEQQLRRLNKDPKRSASA	P87
1;2;3	4640–4658	QDRADELGENLLKGARDKY	P88
1;2;3	4650–4668	LLKGARDKYLDPNPEGVPV	P89
1;2;3	4708–4726	LNDRAAELAKEQRQKDRAF	P90
1;2;3	4721–4739	QKDRAFLDPEPEGIPIADV	P91
1;2;3	4752–4770	DYLRKLKKDPRRNADAIAD	P92
1;2;3	4766–4784	DAIADTQESMNDRAHELAK	P93
1;2;3	4813–4831	LKFRDAANRRRDAKRRRLP	P94
1;2;3	4817–4835	DAANRRRDAKRRRLPTTDI	P95
1;2;3;	4874–4892	PLDADKEFAALEAERRRRS	P96
1;2;3	4885–4904	EAERRRRSKDPRAAKRNKDV	P97
1;2;3	4905–4923	IRDLENQMSDRAHQLAKEE	P98
1;2;3	4921–4939	KEEFAKQRDFMDQEPEGVP	P99
1;2;3	4940–4958	LERLPLDTDPEFKDAEIAR	P100
1;2;3	4959–4977	YKAKTDPKADPKKVAALEK	P101
1;2;3	5010–5028	LPLSDDPEFNVLAKQRQAL	P102
1;2;3	5023–5041	KQRQALKNTRRGRDPEMKD	P103
1;2;3	5033–5051	RGRDPEMKDLEERMNDRVH	P104
1;2;3	5088–5106	MENELLKAMKDPRSNAGKI	P105
1;2;3	5152–5170	LNPLERKRRDIKKNPKRNA	P106
1;2;3	5165–5183	NPKRNADVLRNLEREIAAR	P107
1;2;3	5189–5207	RDFLAKERAFLDQEPEGVQ	P108

Table 3 (Continued)

Programs	Position	Sequence	Linear epitope
1;2;3	5226–5244	LRALKKQPAKNRDAIEDLE	P109
1;2;3	5235–5253	KNRDAIEDLEERMNDRAHH	P110
1;2;3	5254–5272	LAKDYLAKDRDYLEKEPLG	P111
1;2;3	5269–5287	EPLGVPVEELPLNEDVTFR	P112
1;2;3	5284–5306	VTFRDAEEKRRALKRDPRGNAKA	P113
1;2;3	5307–5325	IKDLEDQLNDRAEQLAQQK	P114
1;2;3	5322–5340	AQQKLDKERAFLDPRPEGI	P115
1;2;3	5343–5361	KDMQLDRDKAFKDMERQLR	P116
1;2;3	5362–5380	QLRCDPRKNANAIRDMEED	P117
1;2;3	5371–5389	ANAIRDMEEDMNSRAHVLA	P118
1;2;3	5390–5408	KRQLADDRNFLNPEPRGVP	P119
1;2;3	5413–5431	ALEDDPEFRKTELARREAK	P120
1;2;3	5427–5445	RREAKRNPKNADRVRELES	P121
1;2;3	5476–5494	EELPLDTDPDFHGMEVDRR	P122
1;2;3	5490–5508	EVDRRKLNKDPAKNSRTIK	P123
1;2;3	5509–5527	DLEEQLNNRARELARDKKG	P124
1;2;3	5528–5546	YQDPVFHEANEDIAEQWPR	P125
1;2;3	5594–5612	SRLFQDKAHPQNQPYRVTL	P126
1;2;3	5613–5631	FNPDSSPVTVEVDDRVPCD	P127
1;2;3	5630–5648	CDDKREPKFTQVPSRMWYP	P128
1;2;3	5823–5841	RLSSPGEWNNYTAGGTSKY	P129
1;2;3	5980–5993	SLHPGDEEGERLDF	P130

[a]IEDB (1), BepiPred (2), BcePred (3); P: CP epitopes.

conformational epitopes of CP, 67 contained, entirely or partially, amino acid residues of this proteins linear epitopes. Regarding the TDR1 protein, three linear epitopes (Pt1, Pt3 and Pt4) were partially or entirely in conformational epitope region. For the HSP70 protein, two epitopes were exclusively conformational, and only eight contain either partial or entire linear epitopes (Table 2).

The structures predicted by the PHD program were the α helix, extended strand and random coil. For the three proteins, the β sheet was not predicted. The H17 immunodominant epitope residues of HSP70 (23), which correspond to the Ph5 linear epitope, are located in the α helix (241–249) and random coil (250–260) of this proteins secondary structure regions. TDR1 exhibits a similar frequency in terms of the amounts of α helices (45·1%) and random coils (43·8%). In evaluating the location of the linear and conformational epitopes, it is apparent that they are primarily located in the α helices and random coils. The linear epitopes were mainly composed of amino acids E, A, K, R, D, L and T.

Using the IEDB, BepiPred and BcePred programs, 14, 4 and 130 linear epitopes were predicted for the HSP70, TDR1 and CP proteins, respectively. TDR1 and CP were nor previously reported as antigens and were discovered by our regular screening procedure. On the other hand, the HSP70 protein was used as a prediction control because its epitopes have been experimentally tested by Wallace *et al.* (25) and Quijada *et al.* (23). The three programs predicted the entire region of the H17 epitope (241–260) of *L. infantum* HSP70. According to Quijada *et al.* (23), the H17 peptide is an immunodominant and species-specific B-cell epitope, as it is different from the equivalent region in the HSP70 of *Trypanosoma cruzi*. Additionally, the programs predicted one epitope located in the carboxy-terminal region (from Met-525 to Glu-545) identified herein as Ph10, which includes some amino acids residues (EADDRA) previously described as immunodominant by Wallace *et al.* (25). Among the 14 linear epitopes predicted, five are above the cut-off point established by Quijada *et al.* (23). Additionally, seven are within the limit or slightly below the cut-off point. Only two epitopes predicted were not equivalent to the immunogenic peptides identified by the authors cited above. The exclusively conformational Pech2 and Pech6 epitopes coincide with five more peptides described by Quijada *et al.* (23). Pech6 contains 14 amino acids (NQPGVHIQVFEGER) of the 20 that are part of the immunodominant H30 peptide, as described by the above authors.

Combining the results of the prediction of linear and conformational HSP70 epitopes, seven epitopes were clearly immunogenic, seven were within the cut-off limit determined by Quijada *et al.* (23), and only two epitopes

Table 4 B-cell conformational epitopes of the calpain-like cysteine peptidase (GI: 146090707) of *Leishmania infantum* predicted using CBTope

No. of epitope residues[a]	Position	Sequence	Conformational epitope
19 (62)	19–62	DRENAHIAREWQRITEVYPAGVNQPLLPEVFSREQFG QGNHYEC	Pecc1
6 (6)	82–87	**FVTKKV**	Pecc2
38 (20)	95–132	FQFFRGQEWVKVEIDDIIAMEEGEVLYARSPTEHWWPL	Pecc3
36 (28)	164–199	PVLNIPMDAKLAKAAGAEVTEGFYWLVLAQRIQSGQ	Pecc4
24 (15)	208–231	DIELETMGLQREQQYGVLEIFSLT	Pecc5
14 (12)	252–265	**YVYKGPLNSK**DTTW	Pecc6
70 (58)	304–373	EADATYFHDEWKGESAGGNPTSVSWRKNPLYFVRNSGSTA FEIV**VVIKQEDQRRFTSPDEMTKYLQ**CGMV	Pecc7
4 (4)	396–399	IHKS	Pecc8
9 (8)	420–428	YLVPSCMHK	Pecc9
21 (17)	461–481	WANSATKNV**ELCQKEKDRVDF**	Pecc10
7 (6)	487–493	**TD**IHIL**M**	Pecc11
4 (4)	501–504	SKSG	Pecc12
15 (10)	527–541	**G**VHAATNFREISIIH	Pecc13
5 (5)	552–556	SITCP	Pecc14
16 (7)	575–590	NVRIVDPPEDATMFDD	Pecc15
103 (74)	624–726	**ESDSPFEDKRF**YVDNRGATSEPWVHIGDLYPEGKTRPLL PNELSRDQFGQ GDHYDCSTLTAFAALMEHHPDVIRN**CFISKNPRKDGRY TFQFHR**YGQWIKVEI	Pecc16
11 (10)	742–752	SPTHHWWPLLL	Pecc17
4 (4)	787–790	PMEA	Pecc18
5 (5)	804–808	QFWRD	Pecc19
24 (21)	858–881	L**VKMYNPYEDSPYTGPMHRD**DSSW	Pecc20
25 (16)	921–945	HPSYNFNSEWGDTTSGGNVSLVTWR	Pecc21
15 (14)	959–973	PVQIIGMIRQPDQRH	Pecc22
5 (4)	1003–1007	TYLVT	Pecc23
9 (4)	1018–1026	LYLHNREVA	Pecc24
10 (10)	1038–1047	YIIPTGMRRD	Pecc25
12 (7)	1125–1136	AQDFLSM**YLYDA**	Pecc26
27 (22)	1140–1166	**RISPSTQATNNREIG**LVQHVSKPGRYA	Pecc27
6 (4)	1241–1246	**RLHHKP**	Pecc28
10 (9)	1265–1274	HELARALLGK	Pecc29
19 (18)	1285–1303	IEKLAPL**LDSDPEYMNAER**	Pecc30
5 (4)	1353–1357	RDDIE	Pecc31
9 (7)	1381–1389	NARKINDME	Pecc32
14 (8)	1402–1415	DMHKKERTYLDPEP	Pecc33
7 (6)	1474–1480	ERELFLD	Pecc34
8 (6)	1502–1509	KEIERLQL	Pecc35
22 (17)	1546–1567	**FLDQDPYGVP**LEKLCLDYNDDF	Pecc36
4 (4)	1574–1577	LRAL	Pecc37
4 (4)	1586–1589	TAIA	Pecc38
6 (5)	1606–1611	EAARDR	Pecc39
5 (4)	1649–1653	**GDTSK**	Pecc40
29 (27)	1666–1694	LEIAH**DMNVAERPDYMDTTYKGIP**VEDLP	Pecc41
11 (8)	1703–1713	SL**EVKRQQQKQ**	Pecc42
5 (5)	1731–1735	**DRA**ME	Pecc43
5 (5)	1738–1742	AEKLK	Pecc44
25 (18)	1746–1770	NYLDPEPEGVPLRLVPLDSDAAF**AE**	Pecc45
6 (5)	1805–1810	**KSLKEK**	Pecc46
12 (9)	1822–1833	GIPYTELPLDAD	Pecc47
29 (26)	1849–1877	QPHKNATAIQDLEEALNDRAGELAKEKLA	Pecc48
19 (13)	1927–1945	LQGEMDDMVNALAAEELAR	Pecc49
45 (39)	1957–2001		Pecc50

Table 4 (Continued)

No. of epitope residues[a]	Position	Sequence	Conformational epitope
		RLVESLPLNDDPQFHK**LEAQYRDLKKSPKANPQDVA DCEELMN**DR	
13 (10)	2086–2098	DEAPQNIPLKYIP	Pecc51
12 (7)	2146–2157	ALQQQVRASVLP	Pecc52
17 (13)	2215–2231	WEERANLGNPLGFSPED	Pecc53
10 (10)	2290–2299	**PEPEGRKLND**	Pecc54
12 (12)	2310–2321	**GIEEQYRRSRKD**	Pecc55
5 (4)	2368–2372	LPLDT	Pecc56
21 (16)	2377–2397	GRLEAQRAKLCQNPVRNAQSI	Pecc57
4 (4)	2407–2410	RADV	Pecc58
4 (4)	2415–2418	ALKN	Pecc59
4 (4)	2452–2455	ALKS	Pecc60
9 (7)	2465–2473	RAVEEQMND	Pecc61
19 (19)	2488–2506	RDMEPNS**LGIPSEDLNPYL**	Pecc62
9 (9)	2516–2524	**EDMYRDAKN**	Pecc63
4 (4)	2568–2571	ETLP	Pecc64
17 (17)	2589–2605	RKGPSGGKKSAERLADV	Pecc65
11 (5)	2618–2628	NAARKQYVDAM	Pecc66
9 (6)	2638–2646	KLGDDPPFV	Pecc67
22 (12)	2711–2732	V**FHELETRRAKLKSEDPRAHQK**	Pecc68
23 (19)	2738–2760	EDQLNDRAHELAKEVKEGELRAL	Pecc69
16 (11)	2771–2786	VIIPHNDVEFNNCA**KH**	Pecc70
33 (26)	2812–2844	GAELAEAMLKQDRSYLKPQAAAVPLKYLPLDTD	Pecc71
19 (10)	2899–2917	**PEGIP**LYVIDPHSDAKFAS	Pecc72
21 (14)	2933–2953	**PEDLQQV**VDAMNDRAHELASE	Pecc73
10 (7)	2962–2971	YLEEEPKGVP	Pecc74
32 (19)	3026–3057	**KDLENLDQVPEG**LPITLVNPHDDPAFAKMVNQ	Pecc75
10 (10)	3065–3074	**SVKDSAKNSE**	Pecc76
6 (5)	3091–3096	**AK**LMLE	Pecc77
10 (4)	3183–3192	NPHADSQFAE	Pecc78
13 (12)	3231–3243	DRDYLDPEPEGVA	Pecc79
13 (13)	3253–3265	PEFHDMEVG**RARL**	Pecc80
9 (8)	3278–3286	**KDL**EQRLND	Pecc81
4 (4)	3293–3296	RRQL	Pecc82
7 (5)	3330–3336	**LRALKKD**	Pecc83
36 (21)	3368–3403	GYLEPNPENVALEYLSLDKDPEIAEMEV**ERAKLKAQ**	Pecc84
67 (43)	3422–3488	**RAVE**LAVAKKAEELAHFAPQYNGIETAAMRPYDDPE FAALVDQLRKLEKASAGASPEAEKVLTDMDA	Pecc85
19 (10)	3496–3514	EKVEGDLWFLDKEPEGIPL	Pecc86
52 (25)	3524–3575	I**FQQLRQECANLKAKDPRRNADKVKSLEDQMSRRVH EL**AKHLKESDFDGVDT	Pecc87
8 (6)	3613–3620	**AQGLLNEL**	Pecc88
8 (7)	3631–3638	ALSGDRSA	Pecc89
17 (11)	3650–3666	SDLPLDTDGIYSGLEVE	Pecc90
34 (26)	3703–3736	DDLKNVDPKPHGIPI**EAVRPHNNPDFHNLATRAR**	Pecc91
18 (17)	3766–3783	EMLGNDRGYLDPEPEGVP	Pecc92
51 (42)	3795–3845	FHEMEVQRAVLVAQDQVKNRQAIADLEGRLNDCA**H KLAEAQKREDLRGLNS**	Pecc93
13 (5)	3865–3877	**AKLPELRAQKKEG**	Pecc94
5 (5)	3892–3896	**LDE**IL	Pecc95
17 (14)	3907–3923	DRARYLYPTPEGIPVAA	Pecc96
16 (7)	3929–3944	**DPEFHQLEAERLDLIS**	Pecc97
7 (7)	3955–3961	**KDLEAAL**	Pecc98
15 (12)	3969–3983	**AREHRKGDRGYLNAE**	Pecc99
4 (4)	4018–4021	NAAA	Pecc100

Table 4 (Continued)

No. of epitope residues[a]	Position	Sequence	Conformational epitope
8 (6)	4056–4063	PVRMLKPH	Pecc101
32 (30)	4081–4112	**DPSADPKKVSDLEQDMNDRAHELAE**EALAGDR	Pecc102
11 (5)	4119–4129	KPEGIAIESLP	Pecc103
15 (12)	4152–4166	**KKNQAKAAELEGRLN**	Pecc104
18 (15)	4192–4209	PVDLLNPHEDET**FASLAF**	Pecc105
35 (30)	4250–4284	GDRDYLDPNPEGVPLRVLPLNEDPEFHEMEVQRAV	Pecc106
5 (4)	4317–4321	NGDRG	Pecc107
48 (43)	4394–4441	**KPRGIPLESLNPHDDAEF**AS**HLPELRRLKNEQPN** **HPKIKDLQAKLDNR**	Pecc108
10 (9)	4474–4483	**DKLFTSLEKQ**	Pecc109
9 (7)	4495–4503	**ADKITDLQD**	Pecc110
11 (10)	4522–4532	RYLDPEPENVP	Pecc111
11 (5)	4547–4557	**LEAQRAKLKED**	Pecc112
37 (28)	4571–4607	**KLNDRAHELAKAQKEAARG**FLNPTSHRVPK ALLPLDE	Pecc113
45 (30)	4646–4690	**LGENLLKGARDKYLDPNPEGVPV**GYLPLDSDP QYSHAELQRAVLK	Pecc114
13 (12)	4703–4715	DLEKVL**NDRAAEL**	Pecc115
10 (6)	4735–4744	**PIADV**PLDDD	Pecc116
49 (41)	4766–4814	**DAIADTQESMNDRAHELAK**GVVAEDLACLPRAAY RGIPKEDLNLHTY**LK**	Pecc117
12 (9)	4873–4884	**VPLDADKEFAAL**	Pecc118
11 (8)	4903–4913	**DVIRDLENQMS**	Pecc119
47 (41)	4952–4998	**KDAEIARYKAKTDPKADPKKVAALEK**RMNDRA HELAKVELAKDRAFL	Pecc120
45 (40)	5042–5086	**LEERMNDRVH**DIAREFLSKHRGYLNPEPQNV PIADIPLNRDPIFR	Pecc121
11 (6)	5100–5110	**RSNAGKI**AELQ	Pecc122
11 (9)	5244–5254	**EERMNDRAHHL**	Pecc123
23 (21)	5265–5287	YLEK**EPLGVPVEELPLNEDVTFR**	Pecc124
24 (20)	5378–5401	**EEDMNSRAHVLAKRQLADDRNFLN**	Pecc125
9 (9)	5464–5472	AYLDPEPEG	Pecc126
12 (6)	5485–5496	**DFHGMEVDRRKL**	Pecc127
8 (5)	5508–5515	**KDLEEQLN**	Pecc128
6 (6)	5523–5528	**RDKKGY**	Pecc129
32 (23)	5536–5567	**ANEDIAEQWPR**IRELYPEGVYDPVTPDTTLPS	Pecc130
16 (13)	5599–5614	**DKAHPQNQPYRVTLFN**	Pecc131
12 (11)	5629–5640	**PCDDKREPKFTQ**	Pecc132
23 (13)	5648–5670	**P**LLLEKAYAKFVGGYENFNNCNA	Pecc133
14 (13)	5684–5697	HISLEDPKHAAATN	Pecc134
14 (12)	5729–5742	PDGLHPQCSYALMD	Pecc135
46 (34)	5753–5798	PLDIVIKVHNCYTDAPHYNGPLRKGDSNWT ADVKRACSFSPDESDM	Pecc136
27 (23)	5813–5839	MQRCHINCGD**RLSSPGEWNNYTAGGTS**	Pecc137
40 (27)	5856–5895	TSRPATILAEVRHTNPLYVDETNCKQYPY TGLTLMQPSNA	Pecc138
15 (13)	5929–5943	PPSSTCYLIPYTKDR	Pecc139
12 (5)	6004–6015	LLHQTKISDPNS	Pecc140
6 (6)	6038–6043	KIGTTG	Pecc141
4 (4)	6056–6059	KAPQ	Pecc142
8 (8)	6136–6143	RRTDSLPP	Pecc143

[a]Size of the conformational epitope region is in parenthesis; amino acids in bold are part of the linear epitopes of the same region; Pecc: calpain-like cysteine peptidase conformational epitope.

were falsely predicted. Considering the linear and conformational epitopes, it is clear that they are concentrated in the carboxy-terminal region of HSP70, which is recognized as the proteins most divergent region (26). These results indicate that the predictions may be more reliable when the tools are used in combination for identifying epitopes.

Using the same methodology, among the conformational epitopes predicted, 46·8% and 27·3% are part of the linear epitopes of the CP and TDR1 proteins, respectively.

The arginine (R), glutamic acid (E) and lysine (K) amino acids are among the most frequent in the CP, TDR1 and HSP70 proteins, respectively. These amino acids contain side chains with basic (arginine, lysine) and acidic (glutamic acid) functional groups that allow the formation of ionic bonds (electrostatic bonds) and hydrogen bonds (27), which are interactions that also occur when the antibody recognizes the epitope. These amino acids are also residues with greater scalar values of accessibility to the solvent (8), an important factor for immunogenicity of an antigen or synthetic peptide (28). These findings indicate that the linear B-cell epitopes simultaneously predicted in this study using the three programs are good candidates to be tested in diagnostic assays and vaccines for visceral leishmaniasis.

Using the PHD program, it appears that the proteins analysed are composed of α helices, extended strands and random coils, with a predominance of α helices for the CP and TDR1 proteins. There is a strong tendency for certain amino acids to form a specific secondary structure, in which case the residues glutamate (E), alanine (A), leucine (L), methionine (M), glutamine (Q), lysine (K), arginine (R) and histidine (H) are preferably found in α helices (29). HSP70 and TDR1 are, respectively, composed of 39·4% and 45·1% α helix, which corroborates the amino acid composition.

For the TDR1 protein, most of the amino acids residues that compose the linear epitopes are located in the secondary structure of α helices and random coils. In HSP70, the immunodominant H17 epitope is located in an α helix and random coils region. None of the proteins were predicted to have loop or turn secondary structures. Secondary structures, loops and turns that are present on the surface of proteins may participate in interactions with other molecules.

The identification of epitopes using bioinformatics still has limitations, and studies are therefore necessary to improve the accuracy of B-cell epitope prediction methods. According to Greenbaum *et al.* (30), higher accuracy can be obtained by improving the quality of existing databases, which contain incorrectly delineated epitopes. This is important because the prediction methods use epitope databases to evaluate the methods efficiency. As the prediction results produced by several methods may be different (28), it may be more appropriate to use multiple tools to obtain more consistent and accurate results, which was the approach used in this study. Subsequently, bioinformatics data must be confirmed by laboratory assays so that the information is fed into the databases more accurately.

Most B-cell epitopes in proteins are conformational or discontinuous (31); however, most of the prediction methods available only identify linear or continuous epitopes. The main obstacle is the necessity of knowing the antigen structure to predict conformational epitopes, and protein-model databases are still limited in the amount of tertiary and quaternary structures available. Nevertheless, the identification of linear epitopes has shown promising research results for identifying vaccine antigens and allergens, as well as for the immunodiagnosis of leishmaniasis (23,32).

This work describes for the first time the use of a combination of different *in silico* epitope prediction methods and an assessment of secondary structures for the identification of *Leishmania* epitopes.

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CONFLICT OF INTERESTS

The authors declare that they have no conflict of interests.

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