

# Genetic analysis of multicase families of visceral leishmaniasis in northeastern Brazil: no major role for class II or class III regions of HLA

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*Familial aggregation, high relative risk to siblings, and segregation analysis, suggest genetic control of visceral leishmaniasis in Brazil. Class II gene effects in mice, and high circulating tumour necrosis factor  $\alpha$  in humans, provide reasons to target HLA. Fifteen polymorphic markers across 1.03 Mb (DQB1 to TNFa) were genotyped (87 multicase families; 638 individuals). Model-based parametric analyses using single-point combined segregation and linkage in COMDS, or multi-point linkage in ALLEGRO, failed to detect linkage. Model-free nonparametric affected sibling pair (SPLINK) or  $NPL_{all}$  score (ALLEGRO) analyses also failed to detect linkage. Information content mapping confirmed sufficient marker information to detect linkage. Analysis of simulated data sets demonstrated that these families had 100% power to detect  $NPL_{all}$  scores of 5 to 6 ( $>LOD4$ ;  $P < 0.00001$ ) over the range (7% to 61%) of age-related penetrances for a disease susceptibility gene. The extended transmission disequilibrium test (TDT) showed no consistent allelic associations between disease and the 15 loci. TDT also failed to detect significant associations between extended haplotypes and disease, consistent with failure to detect significant linkage disequilibrium across the region. Linkage disequilibrium between adjacent groups of markers (HLADQ/DR; 82–1/82–3/–238bpTNFA; LTA/62/TNFa) was not accompanied by significant global haplotype TDT associations with disease. The data suggest that class II/III regions of HLA do not contain major disease gene(s) for visceral leishmaniasis in Brazil.*

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## Introduction

Leishmaniasis occurs in most areas of the tropics and subtropics, including large areas of Africa, India and South America. Visceral leishmaniasis, caused by *Leishmania donovani*, *L. chagasi* and *L. infantum*, accounts for 500 000 of the 2 million new cases of leishmaniasis that occur annually (<http://www.who.int/emc/diseases/leish/leisburtre.html>). Although fatal in susceptible indi-

viduals, there is evidence to suggest that most people are resistant to clinical disease. Evidence from specific skin-test reactivity and lymphocyte proliferation assays<sup>1–3</sup> indicates that only a small subset of people infected with leishmanial parasites develop clinical disease. Familial aggregation is a feature of visceral leishmaniasis caused by *L. chagasi* in northeastern Brazil,<sup>4</sup> providing a high relative risk ( $\lambda_{25} = 34$ ) of disease in further siblings of affected sibling pairs.<sup>5</sup> Segregation analysis supports single dominant or additive gene control, with a frequency of  $\sim 0.002$  for the disease allele and penetrances ranging from 7% ( $<1$  year olds) to 61% (9–10 year olds) according to age liability classes. Although this does not discount minor contributions from multiple loci, a hypothesis worth testing is that susceptibility to visceral leishmaniasis in this region of Brazil is controlled by one (or a small number of) major gene(s).

In mice, genes encoding the IA (DQ in man) and IE (DR in man) class II molecules in the major histocompatibility complex (MHC: H-2 in mice, HLA in man) exert

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a profound influence on susceptibility to *L. donovani* infection.<sup>6</sup> Genetic mapping data<sup>6,7</sup> are supported by functional data showing that treatment *in vivo* with anti-IE monoclonal antibodies abrogates the non-cure response of IE-bearing H-2<sup>d</sup> mice.<sup>8</sup> Treatment of H-2<sup>b</sup> mice, which do not express IE, with monoclonal antibodies to IA reduces their ability to clear liver and spleen parasite burdens and self-cure. Transgenic introduction of the gene encoding IE  $\alpha$  chain to restore IE expression in non-IE expressing mice also abrogates the self-curing response.<sup>9</sup> Hence, there is strong evidence that polymorphism at class II molecules is itself functionally responsible for MHC-regulated control of infection.

In man, high levels of circulating tumour necrosis factor  $\alpha$  (TNF $\alpha$ ) associated with clinical visceral leishmaniasis<sup>10</sup> suggest that regulatory polymorphisms for genes encoded in other regions of the HLA complex might also be important in determining genetic susceptibility. This would be consistent with a range of other infectious diseases,<sup>11–17</sup> including mucocutaneous leishmaniasis,<sup>18</sup> where allelic associations between disease and polymorphisms in the gene (TNFA) encoding TNF $\alpha$  or the closely linked gene (TNFB) encoding lymphotoxin A (LTA or TNFB) have been observed. This may be functionally related to regulation of TNF $\alpha$  transcription by single nucleotide polymorphisms (SNPs) in the promoter region of TNFA.<sup>19–22</sup> Hence, there are multiple reasons to target the HLA complex as a candidate disease susceptibility locus for visceral leishmaniasis.

To date, only a few studies have examined polymorphisms across the HLA complex in relation to susceptibility to visceral leishmaniasis. These have included population-based case-control studies,<sup>23–25</sup> as well as family-based genetic analysis.<sup>26</sup> Linkage or allelic associations have been negative<sup>23,26</sup> or weak,<sup>24,25</sup> but sample sizes have been small. Here we examine 15 polymorphic loci spanning 1.03 Mb from HLADQB1 to the TNFA microsatellite in 87 multicase families of visceral leishmaniasis from northeastern Brazil.<sup>5,27</sup> We use parametric and nonparametric linkage analyses, information content mapping, power calculations, single marker and haplotype transmission disequilibrium testing, and analysis of linkage disequilibrium, to evaluate the role of HLA genes in determining disease susceptibility.

## Results

### Linkage analyses

Allele frequencies for markers, and cumulative physical distances between markers, are provided in Table 1. A breakdown of the family structures is provided in Table 2. Combined segregation and linkage analysis performed within COMDS under an additive model provided no evidence for positive linkage between a putative disease susceptibility locus (DSL) controlling visceral leishmaniasis and HLA markers (Table 3). Multipoint parametric linkage analysis using this model and performed within ALLEGRO (Table 3) met criteria (LOD scores  $\ll -2$ ) for excluding loci in both class II and class III regions of HLA in controlling susceptibility to visceral leishmaniasis. However, since HLA does not appear to be the major gene regulating visceral leishmaniasis in this population, the model of inheritance predicted by segregation analysis may be inappropriate. Hence, nonparametric model-

free linkage analyses were performed. Singlepoint (data not shown) and multipoint nonparametric analysis in ALLEGRO (Figure 1a) again failed to provide evidence (all NPL<sub>all</sub> scores  $< 1$ ; lowest *P* value 0.23) for HLA genes in controlling visceral leishmaniasis, despite the fact that the information content across the region (Figure 1b) was high (0.59 to 0.85). Affected sib-pair analyses performed within SPLINK also failed to demonstrate significant linkage (Table 4). The highest LOD score observed in SPLINK was 0.701 (*P* = 0.055) for HLADQA1 with 0:1:2 identity-by-descent (IBD) allele sharing of 20:41:39. This equates to a locus-specific risk for siblings of patients ( $\lambda_s$ ; calculated from the ratio of the expected proportion of affected sibling pairs sharing zero alleles identical by descent (0.25) and the observed proportion<sup>28</sup>) of 1.25.

### Power to detect linkage

In order to be sure that our families had sufficient power to detect linkage, simulations were performed within ALLEGRO. One hundred replicates were generated using parameters (single gene additive model; allele frequency for disease gene *q* = 0.0016; extremes of age-related penetrances of 7% and 61%) defined initially by segregation analysis performed for these families,<sup>4</sup> and actual HLA marker information for all individuals in the pedigrees. The simulated data sets were then used to carry out nonparametric linkage analysis within ALLEGRO. NPL<sub>all</sub> scores for over the range of critical values (1.65, 2.33, 3.09, 3.27, 4.27) equivalent to *P* values of 0.05, 0.01, 0.001, 0.0001 and 0.00001 are shown in Table 5. These critical values are equivalent to allele-sharing LOD scores of 0.59, 1.17, 2.07, 3.00 and 3.95. This analysis demonstrates that these families have 100% power to detect NPL<sub>all</sub> scores of 5 to 6 (=LOD $>4$ ; *P*  $< 0.00001$ ) over the range of age-related penetrances for a putative DSL in this population.

### Family-based allelic association testing

Although linkage of visceral leishmaniasis susceptibility to HLA markers was not observed, it was possible that family-based allelic association testing performed would be more powerful in detecting weaker associations. Using the extended transmission disequilibrium test (ETDT) and transmission to all affected offspring (Table 6), significant genotype-wise association was observed for microsatellite marker 82–1 ( $\chi^2_{29} = 46$ , *P* = 0.024), and for the adjacent (30 kb distal) marker 82–3 ( $\chi^2_{42} = 61.4$ , *P* = 0.027). The allele-wise ETDT was not significant for these markers. The significant  $\chi^2$  for goodness of fit comparing allele-wise and genotype-wise tests (Table 6) indicates poor fit to the data that most likely results from multiple rare alleles at these markers. A single test statistic showing significant biases in transmission of individual alleles (82–1<sub>94</sub> +ve *P* = 0.03) did not survive correction for multiple testing. No other markers showed significant allele-wise or genotype-wise ETDT statistics. Again, tests for significant biases (+ve indicates higher transmission of this allele to affected offspring than expected) in transmission of individual alleles (DQB1<sub>0603</sub> +ve *P* = 0.01; DQA1<sub>0103</sub>; DR<sub>15</sub> +ve *P* = 0.04; LH1<sub>99</sub> +ve *P* = 0.03; 62<sub>168</sub> +ve *P* = 0.04) did not survive correction for multiple testing. Another reason for a significant goodness of fit test may be the use of ETDT to test multiple transmissions within a family. The more conservative use of haplotype relative risk (HRR) that examines transmission to a single affected individual in each family failed to provide evidence for

**Table 1** Candidate gene marker information for visceral leishmaniasis families, northeastern Brazil

Marker	Kb	<i>n</i>	<i>H</i>	Size range (bp)	Allele frequencies
HLADQB1	0	12	0.87	–	0201=0.127; 0301=0.255; 0302=0.088; 0303=0.147; 0401=0.000; 0501=0.105; 0502=0.014; 0503=0.057; 0601=0.005; 0602=0.061; 0603=0.008; 0604=0.064
HLADQA1	20	8	0.81	–	0101=0.160; 0102=0.147; 0103=0.094; 0201=0.231; 0301=0.118; 0401=0.000; 0501=0.250; 0601=0.000
HLADRB1	80	13	0.88	–	DR1=0.064; DR2/15=0.072; DR2/16=0.008; DR3=0.068; DR4=0.125; DR6/13=0.155; DR6/14=0.087; DR7=0.163; DR8=0.023; DR9=0.045; DR10=0.011; DR11=0.129; DR12=0.049
LH1	280	14	0.86	77–103	0.197, 0.1241, 0.070, 0.032, 0.028, 0.028, 0.178, 0.189, 0.048, 0.041, 0.032, 0.035, 0.043, 0.018
D3A	400	12	0.82	109, 118–138	0.086, 0.001, 0.001, 0.021, 0.162, 0.297, 0.201, 0.075, 0.071, 0.035, 0.043, 0.007
9N1	800	10	0.83	88–106	0.028, 0.001, 0.029, 0.059, 0.115, 0.257, 0.216, 0.133, 0.152, 0.010
82–2	830	10	0.82	114–132	0.024, 0.042, 0.099, 0.217, 0.254, 0.154, 0.178, 0.018, 0.004, 0.010
T2	860	13	0.85	151–175	0.027, 0.016, 0.104, 0.071, 0.135, 0.275, 0.092, 0.122, 0.073, 0.043, 0.023, 0.015, 0.004
82–1	900	12	0.78	90–112	0.004, 0.049, 0.347, 0.225, 0.076, 0.057, 0.017, 0.176, 0.024, 0.021, 0.003, 0.001
82–3	930	13	0.86	148, 151–159, 162–174	0.021, 0.009, 0.025, 0.024, 0.169, 0.181, 0.094, 0.127, 0.176, 0.119, 0.017, 0.021, 0.017
TNFA-308	970	2	0.04	–	0.885, 0.115
TNFA-238	975	2	0.21	–	0.977, 0.023
LTA/TNFB	990	2	0.48	–	0.599, 0.401
62	1010	15	0.86	149–165, 168–178	0.125, 0.074, 0.022, 0.028, 0.059, 0.104, 0.265, 0.059, 0.018, 0.075, 0.091, 0.044, 0.011, 0.015, 0.010
TNFA	1030	13	0.84	101–125	0.021, 0.129, 0.013, 0.126, 0.051, 0.268, 0.144, 0.009, 0.019, 0.130, 0.063, 0.090, 0.018

Kb = cumulative physical distance between markers in kilobase pairs; *n* = number of alleles; *H* = heterozygosity.

**Table 2** Breakdown of number of affected sibs per nuclear family for 117 nuclear families contained within the 87 multicase families. Nuclear families with only one affected offspring were always part of more complex pedigree with multiple affected relative pairs that contribute to the NPL<sub>all</sub> linkage analysis

No. affected sibs	No. nuclear families	No. nuclear families with an affected parent
1	38	12
2	61	4
3	14	1
4	1	1
5	2	0
6	1	0
Total	117	18

allelic associations for any of the markers across HLA (data not shown). There was no evidence for significant linkage disequilibrium that extended across the class II and class III regions in this population (data not shown). No significant global ETDT statistics were obtained using TDT Phase for any haplotype combinations across or within the class II and class III regions. Individual haplotype associations were observed for DQA1-DRB1 (3–13;

passed 17, not passed 7;  $\chi^2 = 4.2$ ,  $P = 0.041$ ) and DQB1-DQA1-DRB1 (11–3–13; passed 14, not passed 4;  $\chi^2 = 5.6$ ,  $P = 0.018$ ) when transmissions to all affected sibs in a family were analysed. This is consistent with data showing strong linkage disequilibrium between DQB1 and DQA1 ( $D' = 0.849$ ;  $P = 2.6e-132$ ), DQB1 and DR ( $D' = 0.818$ ;  $P = 3.2e-138$ ) and DQA1 and DR ( $D' = 0.801$ ;  $P = 9.4e-94$ ). However, these individual haplotype associations were not significant using the more conservative association test examining transmissions to a single affected sib per family, and did not survive correction for multiple testing. No significant individual haplotype associations were observed involving haplotypes with 82–1/82–3/–238bpTNFA or LTA/62/TNFA (data not shown), even though some evidence ( $D' > 0.7$ ;  $P < 1.6e-6$ ) for linkage disequilibrium was observed for markers within these two clusters.

## Discussion

In a previous study of visceral leishmaniasis in northeastern Brazil, Amendoeira and colleagues<sup>26</sup> typed polymorphisms at HLA A, B, C, DR, DQ, Bf, C2, C4a or C4b in 10 families with multiple cases of visceral leishmaniasis caused by *L. chagasi* and found no evidence for linkage. This result might be put down to small sample size. However, we have also failed to find evidence for linkage

**Table 3** Combined segregation and linkage analysis using COMDS, and multipoint parametric linkage analysis using ALLEGRO. For COMDS, the best-fit additive single locus model was employed<sup>5</sup> with parameters D = Dominance = 0.50, displacement on liability scale between homozygotes = T = 6.04, frequency of disease allele = Q = 0.0016. For each locus examined for linkage, the model was tested iterating the recombination fraction theta ( $\theta$ ), and with a fixed value of  $\theta = (0.5)$ . Results are expressed as  $\chi^2_1$  one-sided tests and Lod (Z). For ALLEGRO (multipoint LOD), the best-fit additive single locus model was employed<sup>5</sup> with parameters D = 0.50, Q = 0.0016, and the extremes of age-related penetrances (7% and 61%) as computed in COMDS

Marker	$\theta$	-2lnL	$\chi^2_1$	Z	P (1-tailed)	Multipoint LOD	
						7%	61%
HLADQB1	(0.5) 0.294	1245.7 1244.3	1.4	0.304	0.240	-27.428	-33.379
HLADQA1	(0.5) 0.274	1109.6 1107.8	1.8	0.391	0.175	-30.365	-36.585
HLADRB1	(0.5) 0.274	1230.4 1227.9	2.5	0.543	0.114	-34.164	-42.149
LH1	(0.5) 0.5	1179.1 1179.1	0	0		-36.896	-43.753
D3A	(0.5) 0.5	1200.0 1200.0	0	0		-38.867	-46.309
9N1	(0.5) 0.5	1200.9 1200.9	0	0		-45.591	-53.729
82-2	(0.5) 0.5	1159.1 1159.1	0	0		-48.430	-56.959
T2	(0.5) 0.5	1248.9 1248.9	0	0		-51.152	-60.680
82-1	(0.5) 0.5	1086.3 1086.3	0	0		-49.702	-58.468
82-3	(0.5) 0.5	1237.1 1237.1	0	0		-49.016	-58.306
TNFA-308	(0.5) 0.5	319.0 319.0	0	0		-38.091	-44.714
TNFA-238	(0.5) 0.273	634.1 633.6	0.5	0.109	0.465	-38.090	-44.716
LTA/TNF $\beta$	(0.5) 0.267	724.0 723.2	0.8	0.174	0.362	-39.255	-46.593
62	(0.5) 0.5	1237.7 1237.7	0	0		-42.268	-50.253
TNF $\alpha$	(0.5) 0.5	1206.7 1206.7	0	0		-42.251	-50.475

following extensive analysis using 15 polymorphic markers spanning a 1.03 Mb region of HLA from DQB1 to the TNFA microsatellite in 638 individuals from 87 two and three-generation pedigrees with multiple cases of visceral leishmaniasis. We approached this first as a test of the hypothesis that the HLA region might contain the major single gene predicted by segregation analysis carried out using extended information on disease status from these families.<sup>5</sup> Hence, we applied parametric linkage analyses, performed as combined segregation and linkage analyses within COMDS, and as multipoint analysis in ALLEGRO to maximise marker information across the region. All of these tests failed to demonstrate linkage. This is in striking contrast to similar analysis<sup>17</sup> of 73 multicase families (376 individuals) of leprosy from the same region of Brazil. In this case, combined segregation and linkage analysis performed in COMDS provided highly significant linkage to DQB1 (LOD = 4.978;  $P = 0.00000171$ ), DQA1 (4.870; 0.00000221), DRB1 (5.783; 0.00000025), TNF-308

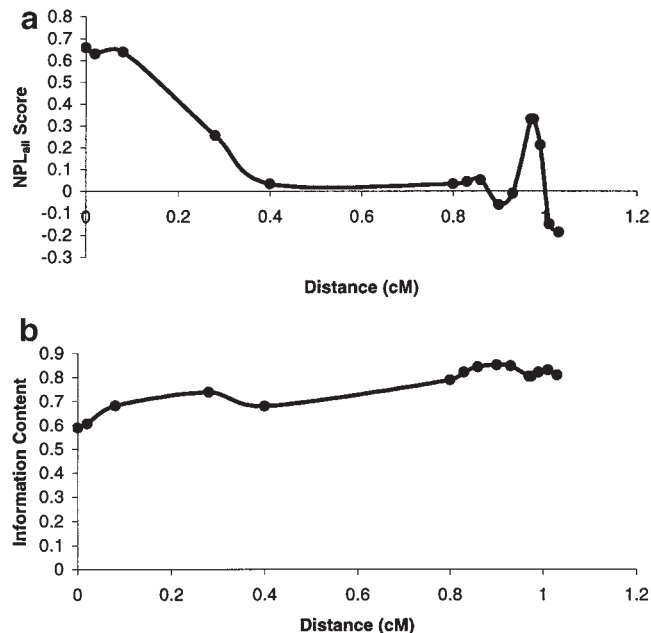
(4.000; 0.00001791), and LTA (1.935; 0.00285171). This was supported by nonparametric multipoint analysis in GENEHUNTER with  $P$  values ranging from  $P = 0.0009$  to  $P = 0.003$ , and locus-specific  $\lambda_s$  values of 1.66 for DQA1 and 1.79 for DQB1 (M-A Shaw and JM Blackwell, unpublished analysis) compared to the 1.25 obtained for DQA1 in the present study. The information content mapping carried out within ALLEGRO shows that our result with visceral leishmaniasis is unlikely to be due to insufficient marker information to detect linkage. Our conclusion is that HLA does not contain the major gene controlling visceral leishmaniasis as predicted by segregation analysis.

To determine whether HLA contained a disease susceptibility locus with lesser effect, or not detectable under the model of inheritance predicted by COMDS, we extended our analyses to nonparametric linkage analyses and transmission disequilibrium testing. Because of the small effective sample sizes (Table 4), our set of families may not have been ideal for the affected sibling pair

**Table 4** Affected sib-pair (SPLINK) analyses for HLA for visceral leishmaniasis families from northeastern Brazil

Marker	No. families	No. sib-pairs	IBD			ESS	MLS	P
			0	1	2			
HLADQB1	47	62	23	45	32	40.8	0.266	0.180
HLADQA1	47	62	20	41	39	32.6	0.701	<b>0.055</b>
HLADRB1	60	76	23	46	31	57.2	0.249	0.193
LH1	67	83	24	48	28	57.4	0.075	0.355
D3A	68	84	23	50	27	58.3	0.028	0.445
9N1	65	81	24	48	28	56.2	0.059	0.379
82-2	68	84	24	47	29	56.2	0.132	0.285
T2	67	83	25	50	25	60.1	0.002	0.540
82-1	68	84	25	50	25	55.1	0	0.583
82-3	68	84	23	47	30	64.8	0.196	0.279
TNFA-238	57	69	23	50	27	9.6	0.009	0.490
TNFA-308	68	83	24	49	27	18.1	0.013	0.492
LTA/TNFB	68	84	23	46	31	27.6	0.212	0.219
62	64	80	24	49	27	61	0.004	0.533
TNFa	67	83	24	48	28	61.3	0.054	0.391

No. families = number of families informative for this marker. ESS = effective sample size (equivalent to number of fully informative affected sib-pairs).



**Figure 1** Multipoint nonparametric analysis for linkage between a putative disease susceptibility allele for visceral leishmaniasis and 15 markers tested across the HLA class II/III region. (a)  $NPL_{all}$  scores, none achieved a  $NPL_{all}$  score of 1.5 required for statistical significance at the  $P = 0.05$  level. (b) Information content mapping across the region. CentiMorgan (cM) distances on the X axes based on physical distances (see Table 1) where 1 cM = 1 Mb.

analysis performed using SPLINK. Others have shown<sup>29</sup> that in excess of 4000 affected sibs may be required to demonstrate linkage if the disease allele is at low ( $q = 0.01$ ) frequency. In an attempt to maximise information over our larger two or three-generation pedigrees, we performed multipoint analysis of all affected relative pair combinations in ALLEGRO. This should have improved our power to detect linkage. Again, we failed to find evidence for linkage of a putative DSL to HLA, even though analysis of simulated data sets within ALLEGRO demon-

**Table 5** Percent power to detect linkage at critical values of the  $NPL_{all}$  score determined by ALLEGRO linkage simulations at extremes (7% and 61%) of age-related penetrance for a putative disease susceptibility gene in 87 multicaser families from northeastern Brazil

Critical value	P value	$NPL_{all}$ 7% penetrance	$NPL_{all}$ 61% penetrance
1.65	0.005	100	100
2.33	0.01	100	100
3.09	0.001	100	100
3.27	0.0001	100	100
4.27	0.00001	100	100
5	<0.00001	100	100
6	<0.00001	98	100
7	<0.00001	84	89
8	<0.00001	26	29

strated that these families had 100% power to detect  $NPL_{all}$  scores of 5 to 6 (=LOD4;  $P < 0.00001$ ) over the range (7% to 61%) of age-related penetrances for disease. Nor did transmission disequilibrium testing provide any powerful evidence of strong allelic associations between alleles at markers across the HLA complex and susceptibility to visceral leishmaniasis. In this case, our maximum number of transmissions tested (244, Table 6) may also have fallen short of that required<sup>29</sup> to detect associations at low frequencies ( $q = 0.01$ ) of the putative disease gene. Hence, an extended sample from northeastern Brazil may find evidence for allelic associations between visceral leishmaniasis and HLA. All of our primary data are available on request for meta-analysis by others. It is also possible that HLA associations will be observed in other geographical regions. For example, Faghiri and coworkers<sup>24</sup> found that the relative risk of disease was 13.27-higher in individuals carrying HLA A26 when 52 cases (allele frequency 15.38%) of antibody diagnosed visceral leishmaniasis caused by *L. infantum* in Iran were compared with 226 matched controls (allele frequency 1.35%) (Fisher's exact test  $P = 0.0001$ ;  $P = 0.004$  after correction according to the number of antigens studied). No other

**Table 6** ETDT analyses for HLA markers for visceral leishmaniasis in northeastern Brazil

Marker	No. alleles	NT ETDT	NT HRR	Allele-wise			Genotype-wise		
				$\chi^2$	df	P	$\chi^2$	df	P
HLADQB	11	161	62	14.52	10	0.151	42.98	35	0.167
HLADQA	6	141	62	8.05	5	0.153	21.49	15	0.122
HLADRB	13	225	80	15.12	12	0.235	45.91	41	0.277
LH1	14	228	100	13.46	13	0.413	68.54	51	0.052
D3A	10	209	102	3.51	9	0.941	28.23	28	0.452
9N1	9	209	96	2.97	8	0.936	30.76	27	0.282
82-2	9	220	104	8.25	8	0.409	31.37	25	0.178
T2	13	238	100	9.52	12	0.658	40.70	43	0.571
82-1*	12	212	106	10.95	11	0.448	46.0	29	0.024
82-3**	13	243	104	12.17	12	0.432	61.39	42	0.027
TNFA-238	2	11	90	2.35	1	0.124	2.35	1	0.124
TNFA-308	2	44	100	0.82	1	0.365	0.82	1	0.365
LTA/TNFB	2	116	92	0.31	1	0.577	0.31	1	0.577
62	14	230	96	17.56	13	0.175	51.72	43	0.171
TNFA	13	244	106	12.58	12	0.4	43.56	39	0.285

No. alleles = number of alleles contributing to ETDT analysis. NT ETDT = No. of transmissions to all affected offspring used in ETDT. NT HRR = No. of transmissions to a single affected offspring in each family used for Haplotype Relative Risk analysis.

\* $\chi^2 = 35.06$ ; df = 18;  $P = 0.009$  for goodness of fit between allele-wise and genotype-wise statistics. \*\* $\chi^2 = 49.217$ ; df = 30;  $P = 0.015$  for goodness of fit between allele-wise and genotype-wise statistics.

significant HLA A or Cw allele  $P$  values survived correction for multiple testing. No significant associations were observed for HLA B alleles. Overall, the evidence for HLA associations in Iran was weak. In India, no significant bias in transmission of HLA A, B, or DR alleles from heterozygous parents to 51 unrelated parasitologically proven *L. donovani* kala-azar patients was observed.<sup>23</sup> More recently Meddeb-Garnaoui and coworkers<sup>25</sup> examined HLA-DRB1, -DQB1, TNFalpha, TNFbeta, HSP70-2 and HSP70-hom genetic polymorphisms in 156 unrelated patients with mediterranean visceral leishmaniasis caused by *L. infantum* compared with 154 unrelated leishmanin skin test positive healthy controls. They found reduced allele (DR2) and genotype (DR2/DR13; DQB1\*0201/-) frequencies in patients compared to controls but none of these remained significant after application of a Bonferroni correction factor. A higher frequency of homozygotes for the HSP70-2/PstI negative allele in cases compared to controls also failed to achieve significance after Bonferroni correction. No associations were found for the -308 base pair TNFA gene polymorphism or the NcoI polymorphism in the first intron of the TNFB/LTA gene. Like us, these authors<sup>25</sup> conclude that their results do not support association between susceptibility to visceral leishmaniasis and the MHC class II and class III loci.

In conclusion, evidence available to date does not support a major role for polymorphism in genes of the HLA complex in determining susceptibility to human clinical visceral leishmaniasis. This is of interest considering the previous genetic and functional studies in mice.<sup>6,8,9</sup> Also in view of the major role which class I and II genes of the MHC have in presenting antigen to protective or disease exacerbatory CD4<sup>30,31</sup> or CD8<sup>32</sup> T cells, and the high levels of circulating TNF $\alpha$  associated with clinical disease in man.<sup>10</sup> There are, of course, many other genes/mechanisms that regulate these important immunological pathways. The negative result obtained here simply fuels the search for the non-MHC genes and mechanisms

influencing the severe clinical manifestations of human visceral leishmaniasis.

## Methods

### Ascertainment of families

The study was based in three sites (Santerem, Marajo and Igaripe Miri) in the State of Para, one site (São Luis Island) in the State of Maranhão, and one site (Terasina) in the State of Piauí in northeastern Brazil. Ethical approval for the study was obtained from the Ethics Committee of the Instituto Evandro Chagas, Belem, Para, Brazil. Epidemiological and demographic details relating to the study sites are described in detail elsewhere.<sup>5</sup> Multicase families were ascertained from the medical records of the Fundação Nacional de Saude in the States of Para, Maranhão and Piauí. Families were collected on the basis of data from the 1983–85 and the 1993–94 epidemics. Families were pursued when there was indication from their medical records that additional family members had been, or were currently, affected with visceral leishmaniasis. All of the specific sites studied were areas of low incidence of cutaneous leishmaniasis, although the northeastern region of Brazil as a whole contains areas of high incidence of cutaneous disease.<sup>33</sup> The separation of cutaneous and visceral disease areas relates to microgeographical/ecological differences.<sup>34,35</sup> The populations studied in northeastern Brazil represent admixtures of Caucasian, Negroid and Native Indian ethnic backgrounds that have interbred extensively for over 150 years. Family-based analysis was therefore considered preferable to a population-based genetic study. The total data for segregation analysis<sup>5</sup> consisted of 87 two or three-generation pedigrees with 824 individuals yielding 138 nuclear families (117 with at least one affected individual). Families were collected from the five sites. For genetic studies, 15 families (99 individuals) were from Santerem, Marajo and Igaripe Miri in the State

of Para; 23 families (175 individuals) from Terasina in the State of Piauí; and 49 (364 individuals) from the island of São Luís in the State of Maranhão. All families were of equivalent socio-economic status. Blood was collected by venepuncture from all available members of the families and peripheral blood mononuclear cells cryopreserved for later preparation of Epstein-Barr virus (EBV)-transformed B cells. Informed consent for venepuncture was obtained from adults, and from the parents of children <18 years old. EBV cells were expanded and DNA prepared for genetic analysis. A total of 87 multicase families (117 nuclear families; 638 individuals) were available for analysis after checking for genetic integrity within all families.

### Diagnosis

Diagnosis was made on the basis of clinical, parasitological and serological criteria as described.<sup>5</sup> All of the individuals classified as affected in these families were diagnosed with clinical visceral leishmaniasis requiring treatment with pentavalent antimony. Data on sub-clinical disease or asymptomatic infections were not available. At initial presentation, symptoms suggestive of visceral leishmaniasis included fever, often prolonged and not cyclical (differential diagnosis for malaria), pale countenance of skin due to anaemia, weight loss and hepatosplenomegaly (predominantly splenomegaly in this region of Brazil). A hard and palpable spleen was a significant clinical indicator. These examinations were carried out by experienced local clinicians. Bone marrow or splenic aspirates were taken from all suspected cases. Diagnosis of 98–100% of patients was supported by immunofluorescent antibody tests to detect leishmania-specific antibodies using antigen prepared from *L. chagasi* promastigotes, and 84–100% by direct observation of Giemsa stained parasites in bone marrow or splenic smears, depending on location.<sup>5</sup> Cultured parasites from a subset (~10% over the three sites) of visceral leishmaniasis patients were confirmed as *L. chagasi* based on monoclonal antibody or isoenzyme identification performed at the Instituto Evandro Chagas in Belem. *L. amazonensis* and *L. mexicana* were never observed.

### Genetic typing

Fifteen polymorphic loci were typed across a 1.03 Mb region of HLA from DQB1 to the TNF $\alpha$  microsatellite distal to the genes encoding TNF $\alpha$  (TNFA) and LTA. The HLA class II DQA1, DQB1 and DRB1 loci were typed by PCR amplification and sequence-specific oligonucleotide (PCR-SSO) hybridization as described.<sup>36,37</sup> HLA-DRB1 probe sequences were taken from the 1991 HLA Workshop.<sup>38</sup> HLA-DQB1 and DQA1 sequences were from the same workshop and from other published sources.<sup>39,40</sup> SSO probes were labelled with P<sub>32</sub> and hybridization observed by exposure to X-ray film (Kodak). Typings were read in duplicate for each individual DNA. Nine novel microsatellite markers (LH1, D3A, 9N1, 82-2, T2, 82-1, 82-3, and 62; Hsieh *et al*<sup>41</sup>) and the TNF $\alpha$  microsatellite<sup>42</sup> were typed by PCR amplification using marker-specific FAM-, TAMRA-, TET- or HEX-labelled forward and unlabelled reverse primers. PCR products were co-loaded with ROX -350 fluorescent labelled size standard (Applied Biosystems, Foster City, CA, USA) onto 6% polyacrylamide gels and run on an ABI 373 or ABI377 sequencer analyser (Applied Biosystems). Gels were ana-

lysed using the computer software programmes genescan and genotyper (Perkin-Elmer, Foster City, CA, USA). The -238 bp<sup>43</sup> SNP for TNFA was typed by PCR-restriction fragment length polymorphism PCR-RFLP) by designing a primer that would incorporate a *Bsa*WI restriction site into one of the variants. The -308 bp<sup>44</sup> SNP for TNFA, and the LTA *Nco*I biallelic polymorphism<sup>45</sup> were typed as described. The gene order across the HLA class II and class III regions is DQB1, DQA1, DRB1, LH1, D3A, 9N1, 82-2, T2, 82-1, 82-3, -238 bp TNFA, -308 bp TNFA, LTA*Nco*I, 62 and TNFA.<sup>41</sup>

### Parametric linkage analysis

Combined segregation and linkage analysis was performed within the computer program COMDS.<sup>46</sup> This is achieved by estimating segregation model parameters with the recombination fraction (TH) fixed at 0.5, and against the model with segregation parameters and recombination fraction estimated. The model employed was generated using COMDS to carry out segregation analysis of the complete pedigree sets for visceral leishmaniasis.<sup>5</sup> The difference between -2 log likelihood (-2lnL) in the two models is a  $\chi^2$  with 1 degree of freedom. This can be expressed as a lod (Z) through division by 2 log(10). COMDS is only capable of using nine allele frequencies so for microsatellite markers with >9 alleles, the alleles of lowest frequency were pooled and used as a single 'allele'. Multipoint parametric linkage analysis was performed within the computer program ALLEGRO,<sup>47</sup> using the COMDS<sup>5</sup> best-fit model (additive single gene; gene frequency  $q = 0.0016$  for the putative disease susceptibility allele; penetrances 7% and 61%, the extremes predicted for different age liability classes).

### Nonparametric linkage analyses

Single- and multi-point nonparametric linkage analyses were performed within ALLEGRO.<sup>47</sup> This analysis compares IBD allele sharing for all affected relatives in a pedigree to generate an NPL<sub>all</sub> score and associated *P* value. Since complex pedigrees were used, this score has more power to detect linkage than scores generated only by comparison of IBD allele sharing between affected sibling pairs.<sup>48</sup> For comparison, affected sib-pair linkage analysis was performed within SPLINK version 1.08 that uses the maximum likelihood IBD method,<sup>49,50</sup> modified to allow for uncertainty of IBD assignment due to missing or incomplete parental data.<sup>51,52</sup> In nuclear families with more than one affected sib-pair, all comparisons are weighted at 2/A where A equals the number of affected siblings. This is considered to be a conservative strategy.<sup>52</sup> Gene frequencies for marker loci were derived from unrelated individuals using SPLINK (Table 1).

### Linkage simulations

To determine whether the 87 multicase families had sufficient power to detect linkage, simulations were performed within ALLEGRO.<sup>47</sup> One hundred replicates were generated using parameters (single gene additive model; allele frequency for disease gene  $q = 0.0016$ ; extremes of age-related penetrances of 7% and 61%) defined initially by segregation analysis performed for these families,<sup>4</sup> and actual HLA marker information for all individuals in the pedigrees. The simulated data sets were then used to carry out nonparametric linkage analysis within ALLEGRO to determine power to detect linkage at critical values of NPL<sub>all</sub> scores.

### Allelic association using transmission disequilibrium testing

The extended transmission disequilibrium test<sup>53,54</sup> was used to determine whether there was any bias in transmission of particular marker alleles from heterozygous parents to affected offspring. Analysis was performed using the program ETDT.<sup>54</sup> Nuclear families with marker information for only one parent were discarded by ETDT from the analysis. ETDT considers a genotype-wise (saturated model) where transmission probabilities for each allele of each genotype are individually examined to determine deviation from 50% transmission to affected offspring. An alternative allele-wise (parsimonious model) considers transmission probabilities of each allele across genotypes, to test the extent to which each allele is associated with disease. The test also provides information on transmission of individual alleles of the locus to affected offspring. In this case, a Bonferroni correction for multiple testing was applied by multiplying the *P* value by the number of alleles informative for transmission testing. When testing families where there are multiple transmissions, significant *P* values observed may be due to linkage rather than association.<sup>54</sup> Hence the haplotype relative risk (HRR; Falk and Rubinstein<sup>55</sup>) was also used to restrict the analysis to one informative transmission per family, usually to the eldest affected child in each family. ETDT for haplotypes across multiple loci was performed within the computer program TDT Phase<sup>56</sup> that provides a global ETDT statistic for haplotype associations, as well as individual haplotype associations.

### Linkage disequilibrium testing

Parents of all pedigrees were used as a sample of genetically independent population sample to measure linkage disequilibrium between markers. Conventional pairwise disequilibrium statistics (*D'*)<sup>57</sup> and associated *P* values, were determined for all pairwise combinations of markers across the class II and class III regions.

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