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Genome-wide scan for visceral leishmaniasis susceptibility genes in Brazil

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Abstract

A genome-wide scan was conducted for visceral leishmaniasis in Brazil. Initially, 405 markers were typed in 22 multicase pedigrees (28 nuclear families; 174 individuals; 66 affected. Nonparametric multipoint analysis detected 9 chromosomal regions with provisional evidence (LOD scores 0.95 to 1.66; 0.003<P<0.018) for linkage. To confirm linkage 132 individuals (43 affected) from 19 independently ascertained families were genotyped across these regions. Three regions (6q27, 7q11.22, and 17q11.2-q21.3) retained evidence (LOD scores 1.08, 1.34, 1.14; P=0.013, 0.007, 0.011) for linkage. To determine which genes contribute to linkage at 17q11.2-q21.3, 80 single nucleotide polymorphisms were genotyped in 98 nuclear families with 183 affected individuals. FBAT analysis indicated associations at two chemokine genes, CCL1 and CCL16, that lie 1.6 Mb apart, show some extended linkage disequilibrium with each other, but each lie within different clusters of candidate CCL genes. Multiple genes may therefore contribute to the linkage peak for visceral leishmaniasis at 17q12.

Visceral leishmaniasis (VL) is caused by protozoan parasites of the *Leishmania donovani* complex: *L. donovani* and *L. infantum/chagasi*. Ninety percent of the estimated 500,000 new cases annually occur in Bangladesh, Brazil, India, Nepal and Sudan (Electronic Database Information 1). Clinical disease in susceptible individuals is fatal unless treated. A major problem is the epidemic nature of the disease, during which local death

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Electronic Database Information

[3]Primer3 software (http://frodo.wi.mit.edu/)

[1]WHO sites for leishmaniasis prevalence including Brazil http://www.who.int/leishmaniasis/en/ and http://www.who.int/leishmaniasis/burden/en/

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rates can influence a significant proportion of the population.1,2 Studies based on ethnic differences,3,4 familial aggregation,3,5 and a high relative risk (λ_{2S} = 34) of disease in further siblings of affected sibling pairs6 suggest that susceptibility to VL is genetically regulated in humans. Evidence from specific skin-test reactivity and lymphocyte proliferation assays7-9 also indicates that only a small subset (1 in 5-10) of people infected with leishmanial parasites develop clinical disease. One approach to identifying the genes involved in resistance and susceptibility to disease is to use family-based linkage analysis to undertake a genome scan. The first such study10 using multicase families of VL from the Aringa ethnic group in eastern Sudan reported genome-wide significance (LOD score 3.5; $p=3\times10^{-5}$) for a major gene on chromosome 22q12, with secondary loci at 2q23-q24 (LOD score 2.27; p=0.0006) and 2q35 (=*SLC11A1*; LOD score 1.00; p=0.015) for families negative for linkage at 22q12. A second study11 undertaken in two villages occupied by the related Masalit ethnic group in eastern Sudan provided evidence for major susceptibility loci at D1S1568 on 1p22 (LOD score 5.65; $p=1.72\times10^{-7}$) and D6S281 on 6q27 (LOD score 3.74; $p=1.68\times10^{-5}$) that were Y-chromosome-lineage and village-specific, with a second peak of linkage at D6S1719 (LOD score 2.13; $p=8.74\times10^{-4}$) ~4 Mb proximal to D6S281 that was common to both villages. Neither village supported a VL susceptibility gene on 22q12.10 The results suggested strong lineage-specific genes within villages due to founder effect and consanguinity in recently immigrant populations.

To determine whether the same or different regions of the genome carry susceptibility loci for VL in Brazil we carried out a genome scan using a two-staged linkage analysis in two separate cohorts of multicase pedigrees, followed by family-based allelic association testing. Details of the families used in this study are outlined in Table 1. These included families from São Luis that were used for the primary genome scan and refined mapping, an independently ascertained set of families from Natal used for linkage replication, and the full set of families from the Belem Family Study6,12 that were used for association mapping.

Nonparametric linkage analysis performed on the initial 10cM genome wide scans undertaken on 22 multicase families from São Luis (Figure 1) provided evidence at $p<0.05$ for linkage of VL susceptibility to 9 regions of the genome on chromosomes 3, 4, 6, 8, 11, 16, 17, 19 and 22 (LOD scores 0.95 to 1.66; 1-sided p values $0.003<p<0.018$) for Brazil. Where broad regions of linkage were observed (chromosomes 11 and 17), and for chromosome 22 which had at that time independently been shown to control susceptibility to VL in Sudan,10 we saturated the regions by genotyping 19 additional markers in these families. This grid-tightened analysis removed evidence for linkage on chromosomes 11 and 22 (data not shown), but improved evidence for linkage on chromosome 17 (Figure 2A).

To determine whether the regions positive (i.e. chromosomes 3, 4, 6, 8, 16, 17 and 19) for linkage in São Luis could be replicated in an independently ascertained set of multicase VL families, 31 of the original genome scan markers, and 13 additional chromosome 17 markers,13 were genotyped in the Natal families (Table 1). This provided independent evidence for linkage of VL susceptibility to the regions on chromosomes 6, 7 (Table 2) and 17 (Figure 2B). Combined analysis of these regions for the São Luis and Natal families resulted in a single peak LOD score of 1.34 (p=0.007) at D7S502 on chromosome 7q11.22 (Table 2). There are no obvious candidate genes of interest under this peak of linkage. As we had observed before for VL in Sudan,11 the region of linkage on chromosome 6q27 (Table 2) that encompassed the markers D6S264 (LOD 0.71; p=0.035) and D6S281 (LOD 1.08; p=0.013) showed some population-specific heterogeneity, with Natal peaking at D6S264 (LOD 0.99; p=0.016) and Sao Luis peaking at D6S281 (LOD 0.97; p=0.017). Similarly, as we had observed for tuberculosis and leprosy in Brazil, we found evidence (Figure 2) for multiple peaks of linkage for VL susceptibility in Brazil at D17S1293 (LOD

0.76; p=0.031) on 17q12 (position 29.58 Mb), D17S1299 (LOD 1.14; p=0.011) at 17q21.2 (position 36.25 Mb), and at D17S1795 (LOD 0.91; p=0.02) on 17q21.33 (position 45.28 Mb).

The broad region of linkage on chromosome 17q11.1-q21.3 was of specific interest in the light of our previous demonstration13,14 of a cluster of genes across this region controlling susceptibility to leprosy and tuberculosis in multicase families from Belem in northeastern Brazil. We therefore undertook a more detailed analysis of 80 single nucleotide polymorphisms (SNPs) in 30 candidate immune response genes (see Supplementary Information - Table 1) across the region 17q11.2-q21.33. The 80 SNPs were genotyped in 98 nuclear families containing 183 affected offspring from the Belem Family Study (Table 1). Allelic association tests performed using FBAT15 showed significant associations (Table 3) at *CCL1* and *CCL16*. None were robust to application of a strict Bonferroni correction (i.e. multiplying by the number of informative SNPs typed across the region). The two SNPs at *CCL1* were in strong LD with each other (D'=1; r^2=0.84), within a strong haplotype block (CCL13-CCL1) in the first chemokine gene cluster at 29.61 to 29.71 Mb on 17q12 (Figure 3), i.e. at the first peak of linkage (Figure 2B). D' also provided some evidence for haplotypes that extended from *CCL1* in this cluster to *CCL16* (white rectangle, Figure 3), the first gene in the second strongest haplotype block (CCL16-CCL18) in the cluster of chemokine genes at 31.22 to 31.47 Mb on 17q12. Both *CCL1* and *CCL16* are valid candidate susceptibility genes for VL, as are other members of these clusters of chemokine genes. In particular, CCL1 interacts with CC chemokine receptor 8, which is preferentially expressed in polarized T helper 2 and in T regulatory cells. Recent work16 shows that CCL1 is produced by monocytes activated by ligation of FcγRII and exposure to IL-1β or bacterial lipopolysaccharide, so-called M2 or alternatively activated macropaphages. *L. donovani* also causes alternative activation of macrophages17, and is associated with T cells that produce IL-10.18,19 CCL16 is also upregulated in macrophages following stimulation with IL-10, and itself differentially induces other CCL family members in the 17q12 cluster in the presence of IL-10.20 We cannot conclude, either statistically or functionally, that CCL1 and CCL16 have separate effects. It is possible that SNPs at both of these genes are haplotype tagging a long range response element(s) that co-ordinately regulates multiple chemokines across the region. Despite the large number of SNPs (see Supplementary Information Table 1) that we examined in 30 candidate genes across the broader region of linkage across 17q11.1-q21.3, we did not find evidence for other significant associations using the complete set of Belem Family Study families. A larger sample of families across northeastern Brazil, with sufficient power to examine heterogeneity across study sites, may detect further associations within the 17q11.1-q21.3 region.

In our genome-wide search we have identified three chromosomal regions, 6q27, 7q11.22 and 17q11.1-q22, as carrying genes contributing to VL in northeastern Brazil. In this study we failed to replicate the major linkage peaks previously reported on chromosomes 22q1210 and 1p2211 reported for different villages in the Sudan. Although differences in disease phenotype and parasite strain could account for this, we did observe one region of overlap with Sudanese VL at 6q27.11 In Sudan, two peaks of linkage were observed, one at D6S1719 that was contributed to by two villages studied, and a second ~4 Mb distal at D6S281 that was village-specific. Although the evidence for linkage in this admixed Brazilian population does not achieve the level of significance observed in ethnically uniform Sudanese villages, we did also observe population heterogeneity with the Natal peak of linkage at D6S264 and the São Luis peak ~3 Mb distal at D6S281. D6S264 lies 0.7 Mb proximal to the gene (*CCR6*) encoding CC chemokine receptor 6, providing an interesting parallel with the involvement of the CC chemokine clusters on chromosome 17q12. The CC chemokine CCL20, also known as liver and activation-regulated chemokine or macrophage inflammatory protein-3α, is encoded on chromosome 2q36.3 and is the only

chemokine ligand for CCR6 (reviewed[21]). Ligation of CCL20 to CCR6 is responsible for chemoattraction of immature dendritic cells, effector and memory T cells, and B cells to inflammatory sites. Expression of CCR6 on immature dendritic cells is dependent on transforming growth factor-β, IL-10 and IL-15, all of which are elevated in the bone marrow or serum of patients with acute VL.19,22,23 At the other 6q27 peak of linkage, D6S281 lies 0.7-1 Mb proximal to *DLL1* encoding the Notch ligand delta-1 and *PSMB1*encoding the proteasome subunit beta-type 1. Proteasome function is important in degradation of proteins for presentation to T cells by antigen processing cells, which use the Notch pathway to instruct T cell differentiation.24 Specifically, DLL1 induces T helper 1 cells which release interferon-γ that is crucial for immune control of *L. donovani* infection.25

One of the major aims of studies of genetic susceptibility to disease is to identify genes/mechanisms/pathways that contribute to the pathogenesis of disease. As we11 and others10 have demonstrated, the genes that are polymorphic, and therefore act as risk factors for disease, may differ between populations and geographical regions. In this study we have seen some commonality for chromosomal regions involved in VL across Brazil and Sudan, and across different diseases caused by intracellular pathogens of macrophages. Further functional and genetic analysis of the regions identified in this study will contribute to our global understanding of the mechanisms of disease for these important intramacrophage pathogens.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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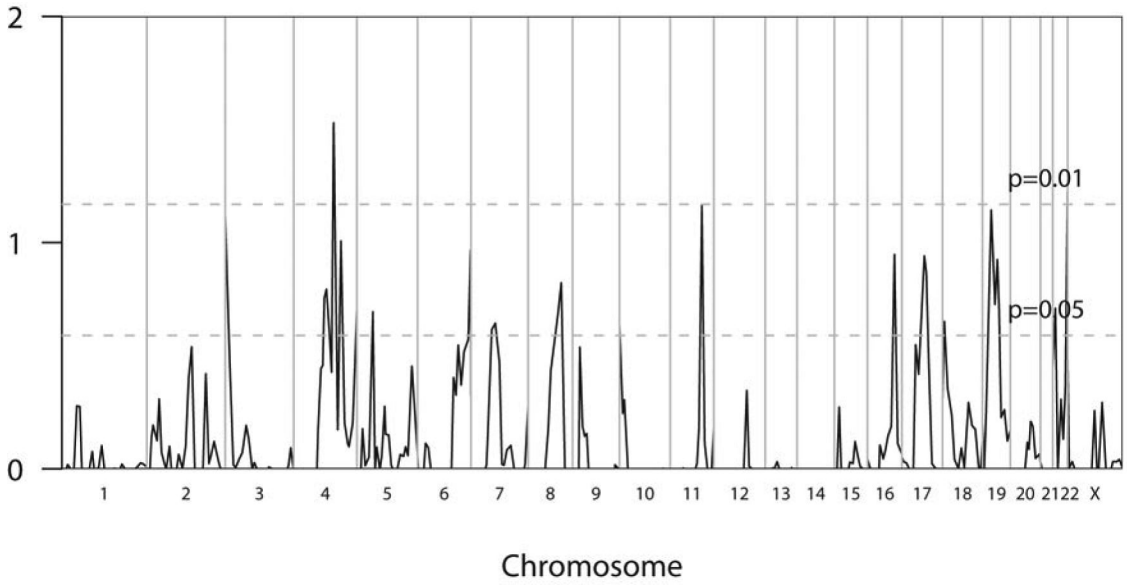

Figure 1.

Results of primary genome scan undertaken using 23 multicase VL pedigrees (29 nuclear families; 180 individuals; 68 affected individuals) from São Luis, Brazil6,26. Multipoint allele-sharing34 LOD scores (plotted as sign(dhat)•LOD35) are shown on the vertical axis and the distance in cM (Genethon Map) from the p terminus for all chromosomes on the horizontal axis. One-sided p = 0.05 and 0.01 cut-offs are indicated by the horizontal dashed lines at LOD = 0.59 and 1.17. DNA was genotyped for the 400 markers that make up the Applied Biosystems ABI Prism Linkage Mapping Set version 2 with markers at ~10cM intervals (see Electronic Database Information 2) across the genome. Microsatellite allele frequencies were determined from families using SPLINK.31 Nonparametric multipoint linkage analyses were performed in ALLEGRO.35 The S_{pairs} scoring function with 0.5 weighting was used to take account of differences in pedigree size.35 The S_{pairs} scoring function compares allele-sharing identical-by-descent across all relative pairs within the extended pedigrees, including the singleton families within larger pedigrees. Unaffected members of pedigrees were included to assist ALLEGRO to infer missing parents' genotypes. Information content, which indicates the fraction of the total inheritance information extracted by the available marker data, was Mean±SD 0.61±0.11 across all chromosomes. Simulations (100) performed in ALLEGRO using a standard set of 6 microsatellite markers (7-10 alleles; 0.73 heterozygosity) showed that the primary genomes scan family set from São Luis families had 88% power to detect linkage to a major gene at an allele sharing LOD score of 3 (p=0.0001) for VL.

[2]ABI products https://products.appliedbiosystems.com/ab/en/US/adirect/ab

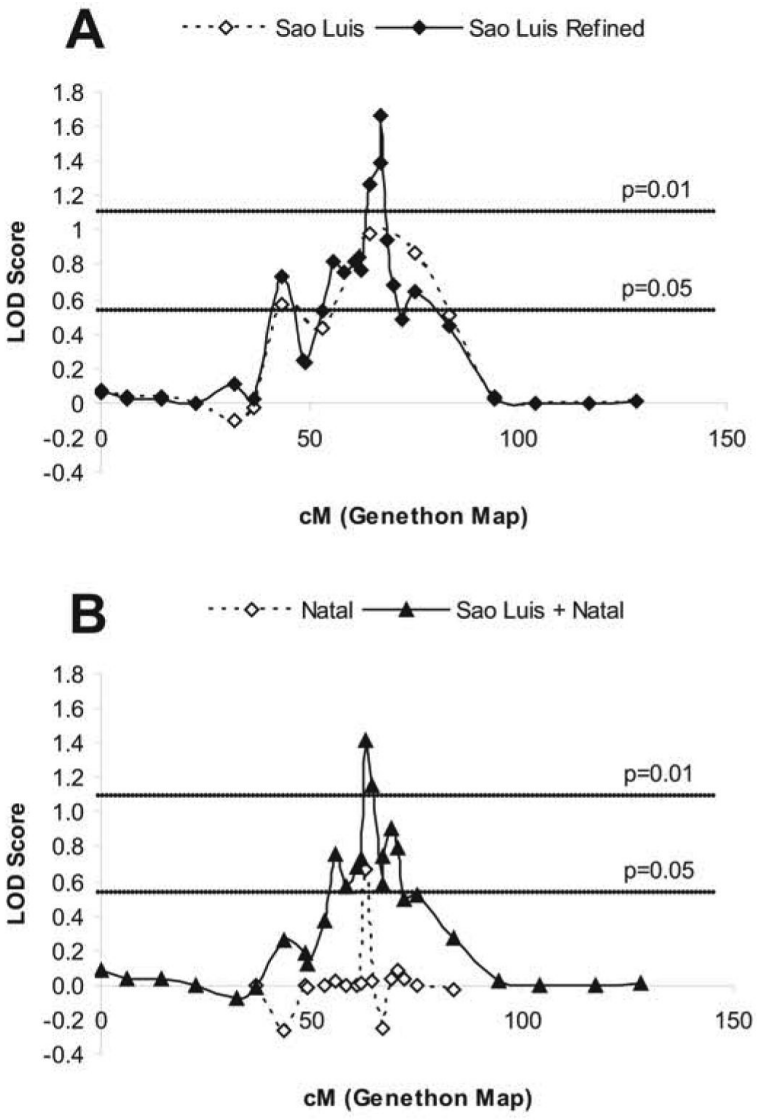

Figure 2.

Comparison of (A) primary and refined mapping of São Luis families, and (B) replication using the independently ascertained Natal families, for the chromosome 17q11.2-q23.3 region. Multipoint allele-sharing34 LOD scores (plotted as sign(dhat)•LOD35) are shown on the vertical axis and the distance in cM (Genethon Map) from the p terminus on the horizontal axis. One-sided p = 0.05 and 0.01 cut-offs are indicated by the horizontal dashed lines at LOD = 0.59 and 1.17. Refined mapping for Sao Luis families was carried out by genotyping 13 additional chromosome 17 microsatellites.13 These plus 6 of the original primary scan markers were genotyped in the Natal families. Analysis was performed as before (see legend to Figure 1). The Natal families had 70% power to detect a major gene at LOD = 2.07, p=0.001. The combined families had 98% power to detect a major gene at LOD = 3, p=0.0001.

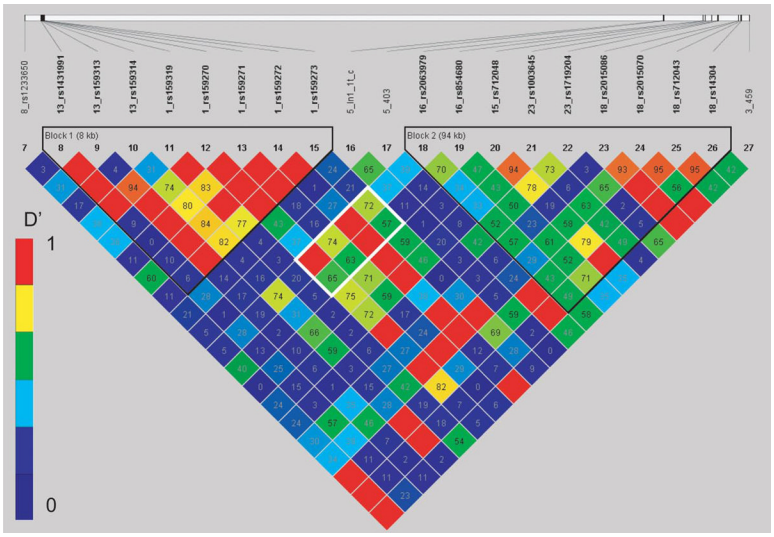

Figure 3.

LD plot generated using Haploview available from the HapMap Project site (Electronic Database Information 7). LD between pairs of markers across chromosome 17q11.1-q22 was determined within Haploview using Hedrick's definition of Lewontin's D' statistic. 36 LD values between markers are indicated at the intercept of the two markers on the matrix. Two main haplotype blocks (CCL13-CCL1 and CCL16-CCL18; the CCL number and rs number for each SNP are shown) within each of the chemokine gene clusters on chromosome 17q12 are outlined within the black triangles. Some evidence for longer haplotypes that extend between these two haplotype blocks is indicated by higher LD values within the white rectangle. Only markers with a minor allele frequency >0.1 were included in the analysis.

[7]HapMap Project Site http://www.hapmap.org/

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Table 1

Details of family structure for families used in 2-stage genome scan (São Luis and Natal) and allelic association mapping (Belém Family Study6,12,26) of VL in Brazil.

Family Structure	***Numbers Genotyped***		
	São Luis - Primary Scan and Refined Linkage	***Natal - Linkage Replication***	***Belém Family Study – Association Mapping***
No. families	22	19	72
No. nuclear families	28	28	98
Nuclear families with 1 affected sib	5	16	35
Nuclear families with 2 affected sibs	14	10	50
Nuclear families with 3 affected sibs	7	1	8
Nuclear families with 4 affected sibs	1	0	3
Nuclear families with 5 affected sibs	1	0	1
No. affected offspring	63	39	176
No. of affected parents	4	4	10
Total No. of affected individuals	66	43	183
Total No. individuals	174	132	448

Study sites and diagnosis: Multicase families with VL were collected during 1991-1994 for the Belém Family Study6,12,26, and during 2000-2004 for independently ascertained families from Natal, Rio Grande do Norte.27-29 Families were ascertained from medical records of the Fundacão National de Saude in the States of Para, Maranhão, Piaui and Rio Grande do Norte. Families were collected on the basis of data from the 1983-85, 1993-94 epidemics for the Belém Family Study, and incident cases during 2000-2004 for Natal. Families were visited at home when medical records indicated that additional family members had been, or were currently, affected with VL. For the Belém Family Study, the total sample comprised 11 pedigrees (79 individuals) from Santarem, Marajo and Igaripe Miri in Para; 18 pedigrees (125 individuals) from Teresina in Piaui; and 43 pedigrees (244 individuals) from the island of São Luis in Maranhão. The Natal families were from peri-urban sites around Natal. Epidemiological and demographic details relating to the study sites are described in detail elsewhere.6,27,29 The populations studied are long-term (>200 years) admixtures of Caucasian, Negroid and Native Indian ethnic backgrounds. All families were of equivalent socio-economic status. Blood was collected by venepuncture from all available family members. DNA was prepared directly from blood (Natal), or from Epstein-Barr virus-transformed B cells cultured from fresh or cryopreserved peripheral blood mononuclear cells (Belém Family Study). Whole genome amplification using multiple displacement amplification (MDA, Qiagen) was used to replenish DNA stocks. Ethical approvals were obtained from the Institutional Review Boards of (i) the Instituto Evandro Chagas, Belém, Para, Brazil; (ii) the Universidade Federal do Rio Grande do Norte (Project No. 19-01 and 10-04), Natal, RN, Brazil; and federally from Comissão Nacional de Ética em Pesquisa (CONEP; Project No. 4572 and 11019). Informed consent for sample collection was obtained from adults, and from parents of children <18 years old. All individuals classified as affected were diagnosed with clinical VL requiring treatment. Data on sub-clinical disease or asymptomatic infections were not included. Diagnosis of clinical VL was made on the basis of clinical, parasitological and serological criteria as described.6,26,27 At initial presentation, symptoms suggestive of VL included fever, often prolonged and not cyclical (differential diagnosis for malaria), pale continence 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. Examinations were carried out by experienced local clinicians. Bone marrow aspirates were taken from all suspected cases. For the Belém Family Study, 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, depending on location.6 Cultured parasites from a subset (~10% over 4 States) of VL patients were confirmed as *L. chagasi* based on monoclonal antibody or isoenzyme identification. *L. amazonenesis* and *L. mexicana* were never observed. For the Natal study, all patients were either parasitologically confirmed by direct observation of parasites in bone marrow biopsies, and/or by a combination of clinical symptoms and positive antibody responses using a *L. chagasi* recombinant K39 (rK39)-based enzyme-linked immunosorbent assay (ELISA) and ELISA using total *L. chagasi* promastigote antigen.28,30

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Table 2

Replication of linkage at chromosomes 6 and 7.

Marker	*Chr. Location*	*Physical Map Location (Mb)*	*Genethon Location (cM)*	*São Luis Primary Scan*			*Natal*			*São Luis + Natal*		
				LOD	Z_{lr}	*P*	*LOD*	Z_{lr}	*P*	*LOD*	Z_{lr}	*P*
D6S1581	6q25.3	160.2	165	0.52	1.54	NS	0.03	0.38	NS	0.38	1.33	NS
D6S264	6q27	166.6	179.1	**0.57**	**1.62**	**0.053**	**0.99**	**2.14**	**0.016**	**0.71**	**1.81**	**0.035**
D6S281	6q27	169.7	_*	**0.97**	**2.11**	**0.017**	**0.65**	**1.73**	**0.042**	**1.08**	**2.22**	**0.013**
D6S446	6q27	170.4	188.4	0.05	0.49	NS	0.32	1.22	NS	0.14	0.79	
D7S519	7p13	46.0	70.5	**0.75**	**1.86**	**0.032**	0.13	0.78	NS	**0.86**	**2.00**	**0.023**
D7S502	7q11.22	66.7	79.6	**0.60**	**1.66**	**0.048**	**1.04**	**2.18**	**0.014**	**1.34**	**2.49**	**0.007**

Replication for linkage was undertaken for chromosomes 3, 4, 6, 8, 16, and 19 in the Natal families (see Table 1) by genotyping 25 markers from the Applied Biosystems ABI Prism Linkage Mapping Set version 2 (Electronic Database Information 2). Only chromosomes 6 and 7 were independently positive for linkage in Natal. Linkage analysis was performed as before (see legend to Figure 1).

*location in Genethon map inconsistent with physical or decode maps; position used based on physical map

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Table 3

FBAT analysis for chromosome 17q11.2-q23.3 SNPs that showed positive allelic associations with VL at *CCL1* and *CCL16* under an additive model.

			Additive Model		
Gene/SNP	**Allele**	**Frequency**	**Z score**	***P value***	***Pc***
***CCL1*/rs159271**	C	0.87	+2.18	**0.02**	**0.03**
	T	0.13	−2.18	**0.02**	**0.03**
***CCL1*/rs159273**	G	0.88	+1.95	**0.02**	**0.05**
	T	0.12	−1.95	**0.02**	**0.05**
***CCL16*/rs2063979**	A	0.58	+2.21	**0.03**	**0.03**
	G	0.42	−2.21	**0.03**	**0.03**
***CCL16*/rs854680**	G	0.62	+2.06	**0.02**	**0.04**
	T	0.38	−2.06	**0.02**	**0.04**

A total of 80 SNPs (see Supplementary Information Table 1) within or adjacent to candidate genes across the chromosome 17q11.2-q23.3 region were genotyped for all DNAs from the Belém Family Study (Table 1). SNP allele frequencies were determined from unrelated individuals in the families using SPLINK.31 Tests for deviation from Hardy-Weinberg equilibrium (HWE) were performed within STATA v8.0 (Electronic Database Information 4) using the GenAssoc package (Electronic Database Information 5). One marker that showed significant deviation from HWE was eliminated from the analysis. Nine markers with minor allele frequencies <0.1 (see Supplementary Information Table 1) were not informative for allelic association testing. Association tests were performed using the family-based transmission disequilibrium test (TDT)32 generalized to allow additive, dominant and genotype analyses within FBAT.15 An empirical estimator of the variance (fbat –e) was used to detect allelic association in the presence of linkage (P corrected = *Pc*). FBAT is available online (Electronic Database Information 6). TDT power approximations for trios were carried out using the method of Knapp.33 A sample of 200 child-parent trios, equivalent to the Belém Family Study families, has 99% power to detect an effect (OR = 2; p=0.05) for SNPs with variant allele frequency of 0.4; 72% power for SNP with variant allele frequency of 0.1.

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[4]Stata v8.2 http://www.stata.com/

[5]Cambridge Institute for Medical Research – David Clayton software http://www-gene.cimr.cam.ac.uk/clayton/software/

[6]FBAT http://www.biostat.harvard.edu/~fbat/default.html