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Comparative genomic analysis of three *Leishmania* species that cause diverse human disease

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Leishmania **parasites cause a broad spectrum of clinical disease. Here we report the sequencing of the genomes of two species of *Leishmania*: *Leishmania infantum* and *Leishmania braziliensis*. The comparison of these sequences with the published genome of *Leishmania major* reveals marked conservation of synteny and identifies only ~200 genes with a differential distribution between the three species. *L. braziliensis*, contrary to *Leishmania* species examined so far, possesses components of a putative RNA-mediated interference pathway, telomere-associated transposable elements and spliced leader–associated SLACS retrotransposons. We show that pseudogene formation and gene loss are the principal forces shaping the different genomes. Genes that are differentially distributed between the species encode proteins implicated in host-pathogen interactions and parasite survival in the macrophage.**

Leishmaniasis is an infectious disease that is prevalent in Europe, Africa, Asia and the Americas, killing thousands and debilitating millions of people each year. With 2 million new cases reported annually and 350 million people at risk, infection by the insect-transmitted *Leishmania* parasite represents an important global health problem for which there is no vaccine and few effective drugs (see TDR Leishmaniasis URL in Methods). At least 20 *Leishmania* species infect humans, and the spectrum of diseases that they cause can be categorized broadly into three types: (i) visceral leishmaniasis, the most serious form in which parasites leave the inoculation site and proliferate in liver, spleen and bone marrow, resulting in host immunosuppression and ultimately death in the absence of treatment; (ii) cutaneous leishmaniasis, in which parasites remain at the site of infection and cause localized long-term ulceration; and (iii) mucocutaneous leishmaniasis, a chronic destruction of mucosal tissue that develops from the cutaneous disease in less than 5% of affected individuals[1]. Infections, particularly those caused by visceralizing species, do not necessarily lead to clinical disease: despite the annual incidence of 0.5 million cases of life-threatening disease, most infections remain asymptomatic. Although host genetic variability and specific immune responses, together with the transmitting sandfly vector and environmental factors, are known to influence the outcome of infections[2], the main factor that determines clinical presentation is thought to be the species of infecting parasite. For example, the New World parasite *L. braziliensis* is the causative agent of mucocutaneous leishmaniasis, whereas the Old World species *L. major* and *L. infantum*, which are present in Africa, Europe and Asia, are parasites that cause cutaneous and visceral leishmaniasis, respectively.

Sequencing the genomes of three kinetoplastid parasitic protozoa, *L. major*[3], *Trypanosoma brucei*[4] (the causative agent of African trypanosomiasis) and *Trypanosoma cruzi*[5] (the causative agent of Chagas disease), previously revealed the preservation of large-scale gene synteny over 200–500 million years[6]. Despite a conserved core of ~6,200 trypanosomatid genes, more than 1,000 *Leishmania*-specific genes have been found, many of which remain uncharacterized. Architecturally, the chromosomes of *Leishmania* differ from those of

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the trypanosome species in not having extended subtelomeric regions containing species-specific genes.

Here we have extended these studies to the genomes of two other species, *L. infantum* (of the subgenus *Leishmania Leishmania*) and *L. braziliensis* (of the subgenus *Leishmania Viannia*), and we compare these genomes with that of *L. major*. Against a background of conserved gene content, synteny and architecture, we have identified roughly 200 differences at the gene or pseudogene content level, including 78 genes that are restricted to individual species. In particular, the genomes show significant differences to the only other *Leishmania* genome published (*L. major*), and there is evidence of the existence of RNA-mediated interference (RNAi) machinery and transposable elements in the genome of the most divergent species, *L. braziliensis*. These findings suggest that a few species-specific parasite genes are important in pathogenesis, that parasite gene expression levels differ considerably between species (perhaps as a consequence of variation in gene copy number) or that, contrary to expectation, the parasite genome plays only a small part in determining clinical presentation. This study therefore provides a framework for experimentally tractable investigations into the role of a few genes that might influence the tissue-specific expression of disease associated with different *Leishmania* species.

RESULTS

Genome content and architecture

The *L. infantum* and *L. braziliensis* genome sequences were produced by whole-genome shotgun sequencing to five- and sixfold coverage, respectively. Comparative-grade finished sequences were produced by aligning contigs against the reference *L. major* sequence[3] and by using PCR amplification between adjacent contig ends to confirm joins. The resulting assemblies of *L. infantum* and *L. braziliensis* contain 470 (N50 contig size of 150,519 bases) and 1,031 contigs (N50 contig size of 57,784 bases), respectively, corresponding to ~98% of the reference 33-Mb haploid genome size (**Table 1**). As compared with 8,395 annotated genes in the *L. major* genome[3], we found 8,195 and 8,314 genes in the genomes of *L. infantum* and *L. braziliensis*, respectively. Genes were manually annotated systematically, facilitated by the strong codon bias of *Leishmania* species[7], conservation of synteny, and the absence of a significant amount of *cis* splicing. Thus, despite the lack of functional information for more than 50% of the genes identified, these numbers are likely to reflect closely the true gene complement in these species.

About 3–4% of the predicted proteomes of *Leishmania* spp. comprise conserved amino acid repeats[8], which could potentially have a role in pathogenicity. For example, leucine-rich repeats comprise the largest class and can mediate interactions between the parasite surface and macrophage complement receptor[9]. DNA repeats comprise ~9–10% of the three *Leishmania* spp. genomes, and *L. braziliensis* has the largest number of these repeats (data not shown).

Despite an estimated 20–100 million years of separation between the *L. Viannia* spp. and the *L. Leishmania* spp. (depending on whether the *Leishmania* genus was separated by migration events or the breakup of the supercontinent Gondwanda[10,11]), synteny is conserved for more than 99% of genes between the three genomes. Conservation within coding sequences is also high: the average amino acid identity between *L. major* and *L. infantum* is 92%, and the average nucleotide identity is 94% (*L. major* versus *L. braziliensis*, 77% and 82%, respectively; *L. infantum* versus *L. braziliensis*, 77% and 81%, respectively). On the basis of sequence similarity and chromosome architecture, the New World *L. braziliensis* is clearly an outlier, consistent with its subgenus classification. *L. major* and *L. infantum* both have 36 chromosomes, whereas *L. braziliensis*, consistent with previous linkage analysis, has only 35 chromosomes owing to an apparent fusion of chromosomes 20 and 34 (ref. 12). Unlike many pathogenic protozoa in which subtelomeres play a central part in generating diversity, directional clusters of 'housekeeping' genes extend to within 5 kb of the telomeres.

Table 1 Summary of the *L. major*, *L. infantum* and *L. braziliensis* genomes

	L. major (V5.2)	*L. infantum* (V2)	*L. braziliensis* (V2)
Chromosome number	36	36	35
Contigs	36	562	1,041
Size (bp)	32,816,678	32,134,935	32,005,207
Overall G+C content (%)	59.7	59.3	57.76
Coding genes	8,298	8,154	8,153
Pseudogenes[a]	97	41	161
Coding G+C content (%)	62.5	62.45	60.38

[a]Pseudogenes include genes that have in-frame stop codons and/or frameshifts but have other characteristics of coding regions, as assessed by similarity to other genes or by codon bias.

Sexual reproduction is not an obligatory part of the *Leishmania* life cycle and may occur only rarely[13]. Nevertheless, strong selection clearly maintains both the organization and sequence of the *Leishmania* genomes. A plausible explanation is that there is a spatial constraint on the organization of genes into directional clusters, which are either polycistrons or groups of genes sharing uncharacterized regulatory elements.

Retrotransposons and RNAi

In addition to selection pressure acting against chromosomal rearrangements, *Leishmania* may lack some of the machinery that generates diversity in other eukaryotes. A lack of transposable elements would favor chromosome stability and is seen in the genomes of *L. major* and *L. infantum*. In other kinetoplastid parasites, namely *T. brucei* and *T. cruzi*, several classes of transposable elements are present (the non–long terminal repeat (LTR) retrotransposons, *ingi*/L1*Tc* and SLACS/CZAR and the LTR retrotransposon VIPER), but the *L. major* genome has only remnants of *ingi*/L1*Tc*-related elements (DIREs), suggesting their loss during evolution of the *Leishmania* lineage[14]. Similarly, *L. infantum* and *L. braziliensis* also contain the *ingi*/L1*Tc* DIREs.

Unexpectedly, we found evidence in *L. braziliensis* for the site-specific non-LTR retrotransposon SLACS/CZAR, which is associated with tandemly repeated spliced leader sequences in an arrangement similar to that of the SLACS or CZAR element in *T. brucei* or *T. cruzi*, respectively[15,16]. In addition, the telomeres of *L. braziliensis* contain a family of 20–30 previously unknown DNA transposable elements, each including putative reverse transcriptase, phage integrase (site-specific recombinase) and DNA and/or RNA polymerase domains, which we have called 'telomere-associated transposable elements (TATEs; **Supplementary Fig. 1** online). The TATEs and their bordering regions are highly conserved and are inserted only in the telomeric hexamer repeats at the same relative position (GGG↑TTA). As observed for most mobile elements, a duplicated motif (TT), present on either side of the transposable element, seems to correspond to a target site duplication. Unlike non-LTR retrotransposons, the TATEs do not contain an APE-like endonuclease domain but they do contain a putative integrase-like domain (site-specific recombinase), related to the transposase domains of other transposable elements, that may

contribute to the observed telomeric site specificity. The telomeres seem to contain clusters of tandemly arranged TATEs, including short elements probably derived from full-length elements by internal deletions. It has not been possible to determine the precise organization of the TATEs owing to their repetitive nature.

In many eukaryotes, the effects of retrotransposable elements can be regulated through a RNA silencing mechanism such as RNAi. Despite its demonstration and utility in *T. brucei*[17], RNAi has not been detected in other kinetoplastid species including *L. major* and *T. cruzi*[6,18]. Our comparison revealed genes in *L. braziliensis* that

Table 2 *Leishmania* genes of putative function that vary between species[a]

Product	*L. major*	*L. infantum*	*L. braziliensis*	*T. brucei*	*T. cruzi* (Tc00.1047053…)
Protein kinase	LmjF01.0750	LinJ01.0760	**LbrM01_V2.0720**	–	511585.160
Tagatose-6-phosphate kinase	LmjF02.0030	LinJ02.0030	**LbrM02_V2.0030**	–	–
Aminopeptidase P1	LmjF02.0040	LinJ02.0010	**LbrM02_V2.0060**	–	–
SLACS	–	–	LbrM02_V2.0550 LbrM02_V2.0720	Tb09.211.5015	–
31-0-demethyl-FK506 methyltransferase	**LmjF04.1165**	**LinJ04.1185**	LbrM04_V2.1180	–	–
Viscerotropic gene	LmjF05.0240	LinJ05.0340	**LbrM05_V2.0230**	–	503583.100
Flavoprotein subunit protein	LmjF07.0800	LinJ07.0870	**LbrM07_V2.0880**	–	–
CFAS, putative	–	LinJ08.0560	LbrM08_V2.0590	–	–
Argonaute	**LmjF11.0570**	**LinJ11.0500**	LbrM11_V2.0360	Tb10.406.0020	–
EF hand protein	LmjF13.1450	**LinJ13.1380**	–	–	–
PI3K	**LmjF14.0020**	LinJ14.0020	**LbrM14_V2.0020**	–	508859.90
Carboxypeptidase	LmjF14.0180	LinJ14.0180	**LbrM14_V2.0180**	–	–
Guanine nucleotide-binding protein	LmjF14.0760	LinJ14.0800	**LbrM14_V2.0740**	–	510989.30
Flagellar Ca^{2+}-binding protein	**LmjF16.0910**	LinJ16.0950	LbrM16_V2.0920	Tb08.5H5.30	507891.38
Flagellar Ca^{2+}-binding protein	**LmjF16.0920**	LinJ16.0960	LbrM16_V2.0930	Tb08.5H5.50	507891.47
Transporter (sugar)	LmjF18.0040	LinJ18.0040	**LbrM18_V2.0050**	Tb10.61.2747	507993.310
Glycerol uptake protein	LmjF19.1347	LinJ19.1260	**LbrM19_V2.1570**	Tb10.61.0380	511355.40
Phosphate-repressible phosphate permease	–	LinJ20.0040	LbrM10_V2.0990	–	–
Zn^{2+}-binding phosphatase	LmjF20.1480	LinJ20.1530	**LbrM20_V2.5730**	Tb927.1.3300	510636.50
Methylenetetrahydrofolate dehydrogenase	**LmjF22.0340**	LinJ22.0330	–	–	511809.20
Phosphoinositide-specific phosphatase C	**LmjF22.1680**	LinJ22.1500	LbrM22_V2.1590	Tb11.02.3780	504149.160
Argininosuccinate synthase	LmjF23.0260	LinJ23.0300	**LbrM23_V2.0290**	–	–
Oxoreductase	LmjF23.0670	LinJ23.0810	**LbrM23_V2.0770**	–	–
RNase III domain gene	–	–	LbrM23_V2.0390	Tb927.8.2370	–
HASPA	LmjF23.1040,1082, 1088	LinJ23.1160, 1200	–	–	–
SHERP	LmjF23.1050, 1080, 1086	LinJ23.1170, 1190	–	–	–
HASPB	LmjF23.1060, 1070	LinJ23.1180	–	–	–
Transcription elongation factor	LmjF24.0200	LinJ24.[a]	**LbrM24_V2.0190**	–	507669.104
Multi-pass transmembrane protein	LmjF24.0700	LinJ24.0350	**LbrM24_V2.0710**	Tb11.02.3050	503789.20
Lysophospholipase	LmjF24.1840	**LinJ10.0030**	LbrM24_V2.1910	–	–
RNase III gene	–	-	LbrM25_V2.1020	Tb927.3.1230	–
Glutathioneylspermidine synthase	**LmjF25.2380**	LinJ25.2500	**LbrM25_V2.1980**	–	508479.110
Adenine phosphoribosyltransferase[b]	–	–	LbrM26_V2.0120	Tb927.7.1790	507519.150
Eukaryotic translation release factor	**LmjF27.1710**	LinJ27.1220	LbrM27_V2.1850	Tb11.22.0012	506127.110
Lipase	LmjF29.1260	LinJ29.1500	**LbrM29_V2.1340**	Tb927.3.3860	504029.21
Multidrug resistance protein	–	LinJ30.1840	**LbrM24_V2.1400**	Tb927.8.2160	–
Triacyglycerol lipase	LmjF31.0830	LinJ31.0860	**LbrM31_V2.1010**	–	–
n-Acyl-l-amino acid amidohydrolase[b]	–	LinJ31.1490	–	–	–
p-Nitrophenylphosphatase[b]	–	LinJ31.3030	–	–	–
Helicase	**LmjF32.1590**	LinJ32.1990	LbrM32_V2.1760	Tb11.01.6420	503677.20
β-Galactofuranosyle transferase	–	–	LbrM20_V2.0480	–	504115.30
Aminophospholipid translocase	LmjF34.3220	LinJ34.2740	**LbrM20.2800**	Tb927.4.1510	511003.10
Galactokinase	–	–	LbrM35_V2.3650	–	–
Cysteine peptidase	LmjF35.3910	**LinJ35.4000**	**LbrM35_V2.3890**	–	–
l-Ribulokinase	LmjF36.0060	LinJ36.2610	**LbrM36_V2.0100**	–	–
Amino acid transporter	–	–	LbrM36_V2.1500	–	–
Phosphatidylinositol/phosphatidylcholine/ SEC14 cytosolic factor	–	LinJ36.2050	**LbrM36_V2.0690**	–	510293.20

[a]Gene found in the sequencing reads but not assembled into the genome. [b]Gene diversification after duplication.
Pseudogenes are indicated in boldface; coding genes, without boldface. **Table 1** identifies those genes with a putative function that are differently distributed between the three *Leishmania* species. The full list of genes, including those encoding hypothetical proteins, is given in **Supplementary Table 2**.

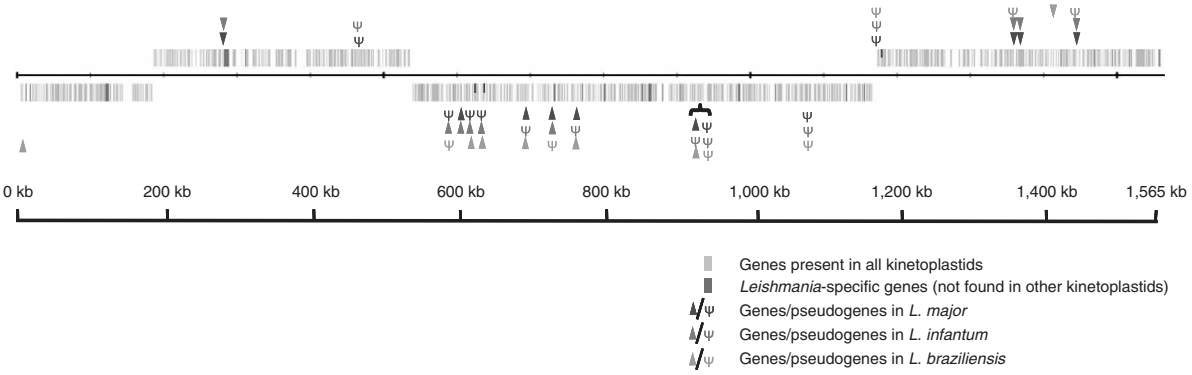

Figure 1 Chromosome 32 of *L. major* showing the positions of genes with a differential distribution between the three *Leishmania* species analyzed. The organization of chromosome 32 is shown schematically; both strands containing long, non-overlapping gene clusters[2]. Genes that are restricted to only one or two of the three *Leishmania* species are not concentrated in the subtelomeric regions or at the breakpoint between polycistronic transcription units, as seen in other kinetoplastid parasites[5], but are distributed more evenly along the chromosome. Most gene differences are a result of pseudogene formation rather than insertion or deletion of new sequences.

may be involved in the RNAi pathway (**Supplementary Fig. 2** online). A hallmark of this pathway in other eukaryotes is Dicer activity, which converts double-stranded RNA (dsRNA) into small interfering RNA (siRNA). A divergent gene (*Tb927.8.2370*) encoding a Dicer-like protein (TbDcl1) has been described in *T. brucei*[19]. The TbDcl1 protein bears the two RNAse III–like domains typical of Dicer and is required for generating siRNA-sized molecules, and its downregulation results in a less efficient RNAi response[19]. An ortholog of TbDcl1 has not been found in *T. cruzi* or *L. major*, trypanosomatids that lack a functional RNAi pathway. *L. braziliensis*, however, contains a similar gene (*LbrM23_V2.0390*) that is endowed with two conserved RNAse III domains. Dicer activity could also be carried out by a combination of independent proteins carrying the relevant dsRNA-binding domain, DEAD/H box RNA helicase and RNase III domains. The RNase genes implicated in this complex[19] are missing in *L. major* and *L. infantum*, but present in the *L. braziliensis* genome at regions of otherwise conserved synteny between the *Leishmania* species (**Supplementary Table 1** online).

Argonaute, an endonuclease involved in the dsRNA-triggered cleavage of mRNA, is another crucial component of the RNAi machinery and, unlike *L. major*, *L. braziliensis* contains an ortholog of the functional argonaute gene (*TbAGO1*) present in *T. brucei*. A second gene containing an argonaute PIWI domain (*TbPWI1*), which was originally identified in *T. brucei* and has orthologs in both *Leishmania* and *T. cruzi*, has been shown not to be involved in the RNAi pathway[20]. *TbAGO1* can be functionally replaced by the human gene encoding Argonaute2, suggesting that *TbAGO1* encodes the endonuclease activity required for mRNA target degradation in the trypanosome RNAi pathway[21]. The *L. braziliensis* gene contains the typical argonaute domains PAZ and PIWI, the latter of which contains key amino acids essential for TbAGO1 activity[22]. In addition, the *L. braziliensis AGO1* gene encodes an amino-terminal RGG domain, which is present in TbAGO1 and shown to be essential for association with polyribosomes[22].

Examination of the syntenic regions on chromosome 11 in *L. major* and *L. infantum* revealed remnants of *AGO1*, suggesting that the RNAi machinery has been lost from the *Leishmania* subgenus to which they both belong (**Supplementary Table 1**). In the alternative subgenus *L. viannia* (which includes *L. braziliensis*), RNA viruses have been characterized[23], however, suggesting that this lineage could have retained RNAi as an antiviral defense mechanism. The RNAi machinery may also have a role in regulating the functions of transposable elements.

Genes differentially distributed between species

So far, only one gene locus has been directly implicated in *Leishmania* disease tropism. In *Leishmania donovani*, the causative agent of visceral leishmaniasis, A2 gene products are required for parasite survival in visceral organs; by contrast, *L. major* contains only A2 pseudogenes[24]. Given this precedent, we systematically searched the three genomes in parallel (using ACT software[25]) for species-specific genes that might contribute to differences in disease presentation, immune response and pathogenicity. Despite the broad differences in disease phenotype, we found that few genes are specific to individual *Leishmania* species. **Table 2** lists those genes that have been ascribed a putative function (the full list is given in **Supplementary Table 2** online). We found 5 *L. major*–specific genes, 26 *L. infantum*–specific genes and ~47 *L. braziliensis*–specific genes, which were distributed throughout the genome (**Fig. 1**) rather than concentrated in subtelomeric regions or breakpoints of directional gene clusters, as previously observed across kinetoplastid species[6]. In addition to the 47 genes specific to *L. braziliensis*, an almost equivalent number of genes are present in *L. major* and *L. infantum* but absent or degenerate in *L. braziliensis*.

Given 20–100 million years of divergence within the *Leishmania* genus, the small number of species-specific differences in gene content is unexpected. For example, more than 1,000 genes differ between the human infective *Plasmodium falciparum* and the rodent malarial species[26], which may have diverged over a similar timescale because the mouse and human lineages diverged from their common ancestor 75 million years ago[27].

We found no obvious breaks in synteny or evidence that translocations or segmental duplications have served to generate lineage-specific diversity in *Leishmania*. We did, however, find clear instances where tandem duplication, followed by diversification, accounts for species-specific differences; for example, copies of a hydrolase gene in *L. infantum* (*LinJ31.3030*) and an adenine phosphoribosyltransferase gene in *L. braziliensis* (*LbrM26_V2.0120*) seem to have arisen and diverged from an adjacent gene. Larger tandem gene arrays are a characteristic feature of all kinetoplastid parasite genomes[6], facilitating increased protein expression in the absence of gene regulation by transcription initiation. Although correctly assembling highly

repetitive regions is technically difficult from randomly sequenced DNA, the depth of assembled reads provides an indication of the number of repeat units present in specific regions. The largest family of surface-expressed protein genes in *Leishmania*, the amastins, are specifically expressed by intracellular parasites in the host[28]. In *L. major*, the largest amastin array (comprising 21 out of 54 amastin genes) is interspersed with repeat units of the unrelated tuzin genes that encode proteins of unknown function. Although similar in organization, the amastin-tuzin array seems to be reduced in size by at least half in *L. braziliensis* (on the basis of the depth of coverage of reads across this repeat region). By contrast, the surface-expressed GP63 zinc metalloproteinases, which function in host cell binding and parasite protection from complement-mediated lysis[29], are encoded by a repeated gene cluster that seems to be enlarged fourfold in *L. braziliensis* as compared with *L. major* or *L. infantum*.

A major determinant of lineage-specific differences in gene content seems to be pseudogene formation. The species specificity of ~80% of the genes listed in **Table 2** and **Supplementary Table 2** can be attributed to the deterioration of an existing coding sequence in the two other species: in each case, there is a degenerate sequence in the corresponding region of synteny in the species that lacks the 'functional' gene. This observation contrasts with an analysis of other kinetoplastid species, where gene insertions or substitutions were found more commonly to generate genus-specific sequences[6].

We identified 23 pseudogenes, present in all three species, that show little degeneracy, suggesting that they have become pseudogenes recently or are under positive selection (**Supplementary Table 2**). In addition, they are interrupted by both frameshifts and in-frame stop codons in different positions across the three species (**Fig. 2**), indicating that they have arisen independently three times in the *Leishmania* lineage. Strong codon bias, a feature of *Leishmania* coding sequences, and sequence similarity are maintained in each pseudogene, and in-frame UAG or UAA stop codons are present in almost all, thereby ruling out translation through selenocysteine incorporation, a process that has been described in *Leishmania*[30]. For several pseudogenes, non-degenerate orthologs were identified in *T. brucei* and *T. cruzi*. Functions could be conceptually ascribed, on the basis of sequence similarity, to 12 pseudogenes, and in many cases relate to housekeeping (for example, carboxypeptidase, phosphoglycerate kinase, oxidoreductase, glutamamyl carboxypeptidase, aminoacyclase, epsilon-adaptin and beta-adaptin).

Of ~200 genes with a differential distribution between *Leishmania* species, the functions of only 34% could be annotated on the basis of sequence similarity or protein domain searches (**Table 2** and **Supplementary Table 2**). Some gene products have similarity to proteins of unknown function in different organisms, whereas others are unique to the *Leishmania* species analyzed. Not surprisingly, a single candidate that might explain the different disease tropisms of the individual species did not emerge; however, many significant gene differences were identified.

One gene in *L. infantum*, which has become a pseudogene in *L. braziliensis* but seems to be absent from *L.* major, encodes a putative phosphatidylinositol or phosphatidylcholine transfer protein (PITP), SEC14 cytosolic factor. An apparently intact ortholog is present in *T. cruzi* but not in *T. brucei*. Although the precise role of this protein is unknown, it has been implicated in the budding of secretory vesicles from the *trans*-Golgi network[31] and could therefore influence cell-surface molecule expression in *L. infantum*, affecting host-parasite interactions as a result.

Another *L. infantum* gene, which is a pseudogene in the other *Leishmania* species and *T. brucei* (but not in *T. cruzi*), encodes a putative phosphatidylinositol 3-kinase (PI3K). This PI3K has the remnants of a Ras-binding domain, a C2 lipid-binding domain, and accessory and catalytic domains reminiscent of class I PI3Ks present in other eukaryotes, including *Dictyostelium discoideum*, yeast and mammals. The only true PI3K identified in trypanosomatids so far is VPS34, a class III PI3K present in *T. brucei*[32]. Orthologs of VPS34 are present in all *Leishmania* species, but the *L. infantum*–specific class I PI3K is novel. Evidence suggests that PI3Ks and PITPs can work synergistically at the *trans*-Golgi to facilitate vesicle budding[33] but, given the properties of class I PI3Ks in other systems and the large number of downstream effectors, the *L. infantum* PI3K might influence as yet unidentified processes that may have an impact on parasite tropism.

Another *L. infantum*–specific gene encodes glutathionylspermidine synthase (GspS), which is required for synthesis of the unusual thiol trypanothione that functions in protecting the parasite against oxidative stress. Although both GspS and trypanothione synthetase (TryS) are required to generate trypanothione in the related organism *Crithidia fasciculata*, a broad specificity trypanothione synthetase substitutes for both GspS and TryS in *T. brucei* and *T. cruzi*[34]. The gene encoding TryS in *L. major* is also sufficient to generate trypanothione, although a GspS pseudogene is also present in the genome[35] and, with only four mutations, could be the result of a recent acquisition. Despite a much greater predicted period of separation, the *L. braziliensis* genome also has a clearly identifiable GspS pseudogene (with approximately ten mutations) with highly conserved domains.

Cyclopropane fatty acids (CFAs), although rare in eukaryotes, are common plasma membrane components in some bacteria and have been previously detected in lipid extracts from some but not all *Leishmania* species[36]. Consistent with that analysis, a single gene encoding cyclopropane fatty acyl phospholipid synthase (CFAS) is present in both *L. infantum* and *L. braziliensis* but not in *L. major*. In bacteria, cyclopropanation by CFAS requires *S*-adenosyl methionine (as a methylene donor) in a modification predicted to maintain the integrity of the plasma membrane—an important factor in the innate immune response to *Mycobacterium tuberculosis* infection[37]. The *Leishmania* CFAS gene is most similar to its bacterial homologs, and strong phylogenetic evidence (**Supplementary Fig. 3** online) suggests that the *Leishmania* lineage acquired this gene by horizontal transfer (and secondary loss from *L. major*). Given that neither the enzyme nor its fatty acid modification are present in humans, CFAS is a putative chemotherapeutic target for the most severe form of leishmaniasis. In addition, the presence of this gene in some species but not others may explain published experimental data[38] on the effects of the *S*-adenosyl methionine analog sinefungin, a compound with known antiparasitic properties. This drug inhibits the growth of *L. donovani* parasites (which are closely related to *L. infantum* and also have a CFAS gene) but has little effect on *L. major*[38].

A notable absence from the *L. braziliensis* genome is the multigene HASP/SHERP locus, which encodes the HASP family of hydrophilic acylated surface proteins (expressed exclusively in infective stages of *L. major* and *L. donovani*) and the vector-stage–specific SHERP protein[39]. Although deletion of this region in *L. major* does not influence virulence, its overexpression causes increased sensitivity to complement-mediated parasite lysis and reduced viability in host macrophages[40].

Gene evolution

In addition to the small number of species-specific and differentially distributed genes, other genetic factors are likely to define the

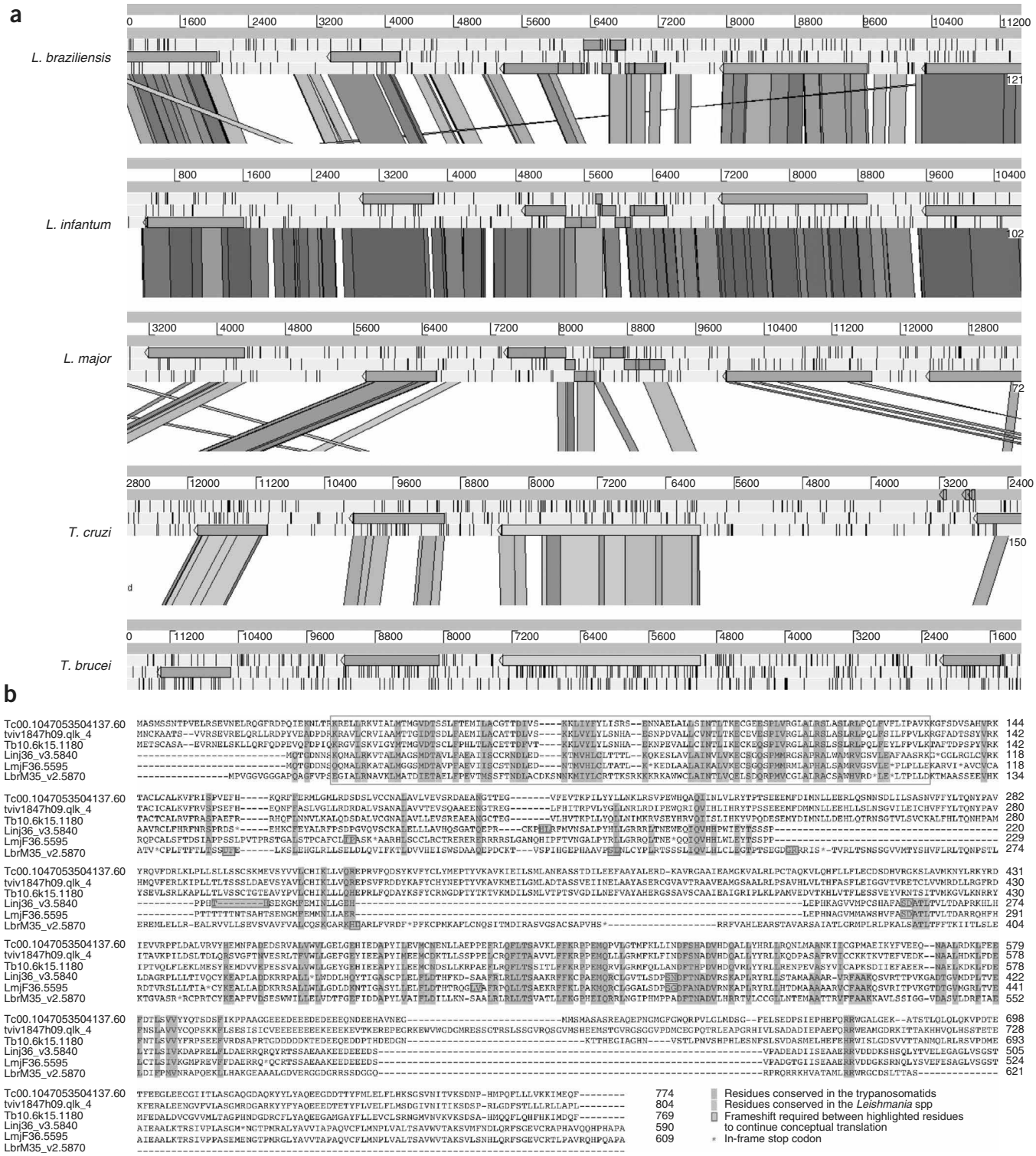

Figure 2 Conserved pseudogenes in *Leishmania* species. Many *Leishmania* genes present in all three species retain sequence conservation but have frameshifts and/or in-frame stop codons. Some of these pseudogenes have intact syntenic orthologs in other kinetoplastids. (**a**) Comparison, using the sequence tool ACT, of a region of conserved synteny containing orthologs of the beta-adaptin 4 gene (gray/yellow) and the adjacent syntenic genes (brown) from *L. major*, *L. infantum*, *L. braziliensis*, *T. cruzi* and *T. brucei*. Gray bars represent the forward and reverse strands of DNA. The red-pink lines between sequences represent sequence similarity (tBLASTx). Each of the *Leishmania* orthologs of the beta-adaptin 4 gene (gray) contains several frameshifts and stop codons, whereas the two trypanosome species have uninterrupted intact copies (yellow). Gene prediction of the *Leishmania* pseudogenes was done by using similarity and codon bias. (**b**) Alignment of amino acid sequences from the three *Leishmania* species with their orthologs in *T. cruzi*, *T. brucei* and *Trypanosoma vivax*, showing that there are conserved domains across all species. The N-terminal β-adaptin domain (boxed region) shows conservation between all six species, and the most conserved residues correspond to residues that are restricted in higher eukaryotes.

differences between the species. We therefore searched for genes with signatures of positive selection as an indicator that they may be involved in host-pathogen interactions (Methods). Those genes with the highest ratios of non-synonymous to synonymous mutations (dN/dS) were, for the most part, involved in undefined biological processes (**Supplementary Table 3** online). We found, however, that ~8% of genes seem to be evolving at different rates between the three *Leishmania* species (**Supplementary Table 4** online) and are involved in a spectrum of core processes (including transport, biopolymer metabolism, cellular metabolism, lipid metabolism and RNA metabolism), which might influence parasite survival in the host and disease outcome (**Supplementary Table 5** online).

DISCUSSION

Comparisons of the complete genomes of three species of *Leishmania* have revealed a greater extent of synteny and similarity than would be expected, given their predicted period of separation. Contrary to previous comparisons of distantly related kinetoplastid genomes, gene loss and pseudogene formation are the principal factors shaping the *Leishmania* genomes. We have found little evidence of lineage-specific genetic acquisition accounting for differences between these parasite species.

Given our poor understanding of the way in which different human-infective species of the *Leishmania* genus cause diverse clinical disease, the identification of only a few differentially distributed parasite genes should facilitate timely experimental verification of their role in disease development. In addition, the unexpected identification of a putative RNAi pathway increases the likelihood that the findings from the three genome projects can be translated into insights into gene function. The potential to manipulate gene expression by RNAi, perhaps by using a tetracycline-inducible promoter system (as demonstrated in *L. donovani* [41]), may be especially useful to complement the classical 'two-step gene knockout' strategy for disruption of *Leishmania* gene function[42]. Identification of a few genes that are either species-specific or under positive selective pressure provides a comprehensive and manageable resource to target efforts in identifying parasite factors that influence infection. Conversely, factors that are unique to the *Leishmania* genus but common to all species may be used as potential drug targets or vaccine candidates.

METHODS

DNA preparation. Details of the sequenced *L. major* strain have been published[3]. *L. infantum* JPCM5 (MCAN/ES/98/LLM-877)[43] and *L. (Viannia) braziliensis* M2904 (MHOM/BR/75M2904)[44] were the strains selected for analysis here. The *L. infantum* JPC (MCAN/ES/98/LLM-724) strain, from which the JPCM5 clone used in the sequencing project was derived, was isolated in the WHO Collaborating Centre for Leishmaniasis, ISCIII, Madrid, Spain, from the spleen of a naturally infected dog residing in the area in 1998 (ref. 43). The parasites were tested for virulence by inoculation into hamsters: parasites were recovered from the spleen 15 weeks after infection. The parasites also infected the human U937 macrophage cell line and the dog DH82 macrophage cell line[43].

L. (Viannia) braziliensis clone LB2904 (MHOM/BR/75M2904) is a reference strain from Evandro Chagas Institute, Belém, Brazil. This strain was isolated by direct culture from a lesion on the right side of the thorax of a man who had been performing survey work in Serra dos Carajás, Brazilian Amazonia. The LB2904 clone is infective in hamsters and BALB/c mice and can be genetically transfected and cloned on plates. The *L. infantum* and *L. braziliensis* strains used are available on request from D.F.S. or J.C.M., and A.K.C., respectively.

Sequencing. The following methodology for sequencing, assembly, finishing and annotation applies to both *L. infantum* and *L. braziliensis*. A whole-genome shotgun strategy was used and produced roughly sixfold coverage of the whole genome from plasmid clones containing small fragments of up to 4 kb inserted into the pUC19 vector (Sanger Institute). Problems associated with high G+C sequence were addressed by optimizing the sequencing mixture (a 4:1 ratio of standard Big Dye terminator mix and dGTP Big Dye mix with the addition of dimethylsulfoxide). Sequence reads were assembled with PHRED/PHRAP on the basis of overlapping sequence and were edited in a GAP4 database[45]. The quality of the reads for both projects was similar: 91.5% of *L. infantum* and 92.7% of *L. braziliensis* bases had a quality score (derived from the PHRED score generated by GAP4; ref. 45) >70 ($P = 1.0^{-7}$). In comparison, in the finished genome of *L. major* 96.8% of bases exceeded this value.

Regions containing repeat sequences or with an unexpected read depth were manually inspected. We used positional information from sequenced read-pairs to help to resolve the orientation and position of contigs. Pre-finishing used an automated in-house software program (Auto-Prefinish) to identify primers and clones for additional sequencing to close physical and sequence gaps by oligo-walking. In addition, end sequences from a *L. braziliensis* fosmid library (4–5-fold clone coverage) were produced to provide paired-read information from 40-kb inserts. The assembled contigs were iteratively ordered and orientated by alignment to the *L. major* genome sequence and by manual checking. In particular, we re-examined regions with apparent breaks in synteny for potential mis-assembly errors or genuine breaks. Information from orientated read-pairs, together with additional sequencing from selected large insert clones, was used to resolve potential mis-assemblies. Version 2 of the *L. infantum* and *L. braziliensis* genomes were used for the subsequent analyses reported here.

Annotation. Manual annotation of the *L. major* genome[3] was transferred to the assembled genomes of both *L. infantum* and *L. braziliensis* on the basis of BLASTp matches and positional information by using an in-house Perl script. Gene models were manually inspected and further edited, where appropriate, with Artemis software[46]. New gene models were identified by using a combination of CodonUsage[47] and Hexamer[48], and by visualizing tBLASTx comparisons of regions with conserved synteny using ACT software[25]. We compared protein sequences against the non-redundant protein database UniProt and an in-house kinetoplastid-only database. Repetitive regions can largely account for small discrepancies in apparent sequence coverage and gene number.

Evolutionary analysis. For the dN/dS analysis, three-way positional orthologs were identified by a combination of reciprocal BLAST and manual curation of conserved synteny regions. Codon-based alignments were produced by using codeml from the PAML package[49] and the settings: model = 0 (one dN/dS estimate over whole tree) for the dN/dS_{tree} estimates, and model = 1 (one dN/dS estimate for each branch of tree) for the dN/dS_{branch} estimates, with the assumption that orthologous rates were equivalent. dN/dS estimates were considered significantly different between species if $2(\ln L_{model1} - \ln L_{model0}) > 5.911$ (5% χ^2 critical value with 2 d.f.). Genes with dN/dS > 5, or $2(\ln L_{model1} - \ln L_{model0}) \leq 0$ were excluded from further analysis. Mann-Whitney tests were used to determine whether groups of genes had significantly higher or lower dN/dS values as compared with all other genes. A Kruskall-Wallis test was used to determine whether differences in dN/dS_{branch} values were significant between species for genes grouped by gene ontology category.

For repeat sequences, genome-wide searches were undertaken with RepSeq[8] to identify amino acid repeats. We used RepeatScout[50] and RepeatMasker to identify nucleic acid repeats.

CFAS phylogeny. The CFAS gene was identified as a potential lateral transfer by similarity searching (BLASTp) against the GenBank non-redundant protein database using the *L. infantum* CFAS sequence as query. To assemble the data set for phylogenetic analysis, all sequences with an *e*-value of $<10^{-30}$ were downloaded. Note that, although eukaryotes were not specifically excluded from this process, none of the eukaryotic sequences in GenBank, which includes the completely sequenced genomes of *Trypanosoma cruzi* and *Trypanosoma brucei*, met the *e*-value cut-off criterion.

Sequences were aligned with MUSCLE using default parameters. Regions of poor alignment where homology could not be ascertained with confidence were identified by eye and excluded. We conducted preliminary analyses of all

sequences by unweighted parsimony using PAUP. The data set was narrowed down through successive rounds of analysis and sequence removal to obtain a final subset of sequences that were broadly representative of the full data set.

The final tree was derived by bayesian inference using a mixture of amino acid models. Alignment positions were weighting according to evolutionary rate by using a four-category γ-distribution with the shape parameter α calculated by the program on the basis of a neighbor-joining tree. Analyses consisted of two sets of four chains run for 600,000 generations with results saved every 1,000 generations. Analyses were run until both sets of chains converged (split frequency = 0.007), and tree topology and posterior probabilities were calculated after discarding a 25% burn-in (150 trees). The tree topology was further tested with 100 replicates of maximum likelihood bootstrapping by the program PhyML using a JTT substitution model with a four-category γ-distribution and with the shape parameter α calculated by the program.

Accession codes. European Molecular Biology Laboratory (EMBL): *L. infantum* chromosomes 1–36, AM502219 to AM502254; *L. braziliensis* chromosomes 1–35, AM494938 to AM494972.

URLs. The *L. infantum* and *L. braziliensis* genome sequencing reads, quality files and annotated consensus sequences can be accessed from the following FTP sites: ftp://ftp.sanger.ac.uk/pub/pathogens/L_infantum/, ftp://ftp.sanger.ac.uk/pub/pathogens/L_braziliensis/. The fully annotated genomes for all three species of *Leishmania* are also available for searching, viewing and downloading at the GeneDB database (http://www.genedb.org). Other URLs: MUSCLE, http://phylogenomics.berkeley.edu/cgi-bin/muscle/input_muscle.py; PAUP, http://www.molecularevolution.org/software/paup*/; PhyML, http://atgc.lirmm.fr/phyml/; pUC19 vector information, http://www.sanger.ac.uk/Teams/Team53/psub/sequences/pUC19.shtml; RepeatMasker, http://www.repeatmasker.org/; TDR Leishmaniasis URL, http://www.who.int/tdr/diseases/leish.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

C.S.P., M.B., D.F.S., A.K.C., J.C.M. and B.B. worked on all aspects of work, contributed to the design of the project and wrote the article. C.S.P. and J.C.R. annotated the genomes; K.S., D.H. and L.M. carried out the assembly and finishing of the genomes; A.F., T.C., Z.H., K.J., S.M., D.O., S.R., R.S., S.W., C.A. and B.W. sequenced the genomes and M.A.Q., H.N., E.R. and S.T. made the clone libraries. N.P., E.A., A.T., M.A., A.K., A.I., M.-A.R. and T.C. wrote and developed software for annotation and comparative analysis of the three genome sequences. F.B. worked on identifying the transposable elements, and S.L.B. and A.F. worked on the phylogenetic analysis of CFA synthase. A.K.C., L.O.B. and L.R.O.T. elucidated the RNAi pathway. D.J. performed the evolutionary analysis, and D.P.D. analyzed the amino acid repeats. D.F.S., J.C.M., S.O.O. and J.D.H. worked on some of the species-specific genes.

COMPETING INTERESTS STATEMENT

The authors declare no competing financial interests.

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RESEARCH ARTICLE

Proteomic analysis of antigens from *Leishmania infantum* promastigotes

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Leishmaniasis is a zoonotic disease caused by the species of the genus *Leishmania*, flagellated protozoa that multiply inside mammalian macrophages and are transmitted by the bite of the sandfly. The disease is widespread and due to the lack of fully effective treatment and vaccination the search for new drugs and immune targets is needed. Proteomics seems to be a suitable strategy because the annotated sequenced genome of *L. major* is available. Here, we present a high-resolution proteome for *L. infantum* promastigotes comprising of around 700 spots. Western blot with rabbit hyperimmune serum raised against *L. infantum* promastiogote extracts and further analysis by MALDI-TOF and MALDI-TOF/TOF MS allowed the identification of various relevant functional antigenic proteins. Major antigenic proteins were identified as propionil carboxilasa, ATPase beta subunit, transketolase, proteasome subunit, succinyl-diaminopimelate desuccinylase, a probable tubulin alpha chain, the full-size heat shock protein 70, and several proteins of unknown function. In addition, one enzyme from the ergosterol biosynthesis pathway (adrenodoxin reductase) and the structural paraflagellar rod protein 3 (PAR3) were found among non-antigenic proteins. This study corroborates the usefulness of proteomics in identifying new proteins with crucial biological functions in *Leishmania* parasites.

Keywords:
Antigens / *Leishmania infantum* / MALDI-TOF / Promastigotes

1 Introduction

Leishmaniasis is a zoonotic disease caused by different species of the genus *Leishmania*, heteroxenous flagellated protozoa that infect macrophages and dendritic cells of mammalian tissues and are transmitted by the bite of the sandfly vector (*Phlebotomus and Lutzomya* spp.). In these two hosts, the parasite alternates several developmental forms: the multiplicative flagellated promastigote resides in the gut of the vector whereas the metacyclic promastigote is a non-dividing form that accumulates in the mouthparts of the female sandfly for its transmission within saliva into the dermis of the mammalian host during a blood meal [1]. The amastigote forms replicate in the phagolysosomal compartment of cutaneous or visceral mammalian macrophages [2]. Consequently, in the vertebrate host the infection can progress in different manifestations that range from self-limiting cutaneous or diffuse mucocutaneous forms to visceral infections that course with fever, substantial loss of weight, swelling of the spleen and liver and anemia [3]. Death can eventually occur if successful treatment is not applied.

The disease is widespread in many countries of the Old and New World, with an estimated prevalence of 12–14 million and an annual incidence of 2 million people (WHO, www.who.int/tdr/diseases/leish/files/leish-poster.pdf). The population at risk increases up to 368 million of each the majority are concentrated in South East Asia and North Africa [3]. In the Mediterranean countries the disease has emerged as an opportunistic infection, due to its close association with human immunodeficiency virus infection [4, 5], where dogs are the principal reservoir [6].

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Abbreviations: ABC, ammonium bicarbonate; **HSP**, heat shock proteins

From the old time, leishmaniasis continues to be an important public health problem due to, not only environmental risk factors such as massive migrations, urbanization, deforestation, new irrigation schemes, but also to individual risk factors: HIV, malnutrition, genetics, etc. Therefore, leishmaniasis is part of those diseases, which still requires improved control measures [7].

Effective control measures rely on effective treatment and vaccination programmes, but despite the relatively large amount of drugs commercially available and on clinical trials [8, 9], fully effective treatment of visceral forms is still lacking and a vaccine has not yet been successfully developed. Consequently, among the WHO/TDR recommendations in leishmaniasis research, those focused on the development of new tools such as diagnostic tests, drugs and vaccines are specially stressed [4]. Since the ~33.6-Mb genome (~8300 protein coding genes, http://www.genedb.org/leish/index.jsp) of *Leishmania major* has been sequenced proteomics became a very useful technology for the precise identification of *Leishmania* proteins produced by different developmental stages [10–12] that may be involved in parasite virulence and pathogenicity [13]. Some of these proteins are seen as potential targets for drugs and resistant mechanisms [14]. Despite the great deal of studies on the immunogenic properties of some leishmanial proteins and their suitability as candidates for diagnosis and vaccination purposes against canine and human leishmaniasis [9, 15–19], information on the functional role of relevant immunodiagnostic antigens is rather scarce [20, 21]. Therefore, in the present report we used a proteomic approach to track some antigens that are mainly recognized by antibodies generated against immunizations of rodents with leishmanial proteins in an attempt to identify new immunogenic components or to provide additional functional information on already know antigens.

2 Materials and methods

2.1 Cell culture of parasites

Promastigotes of the *Leishmania* strain MHOM/FR/78/LEM75 were grown in Schneider medium supplemented to a final concentration of 0.4 g/L $NaHCO_3$, 4 g/L HEPES, 100 U penicillin/mL (Gibco), 100 μg streptomycin/mL (Gibco) and 10% fetal bovine serum (Gibco), pH 6.8 at 26°C.

2.2 Sample preparation

Mid-log promastigotes were recovered on day 7 post-inoculum (p.i.) and the parasites were centrifuged at 3000 rpm for 10 min at 4°C. The resulting pellet was washed five times with Tris-HCl pH 7.8, and resuspended in 0.1 mL of this same buffer. The sample was sonicated for 10 s with a Virsonic 5 (Virtis, NY) set at 70% output power on ice bath. The homogenate was extracted in 5 mM Tris-HCl buffer pH 7.8 containing 1 mM PMSF as a protease inhibitor, at 4°C overnight and, subsequently centrifuged at 10 000 × g for 1 hour at 4°C (Biofuge 17RS: Heraeus Sepatech,Osterode, Denmark). The supernatant was dialyzed overnight at 4°C in 0.5 mM Tris-HCl buffer. To eliminate contaminants such as salts, nucleic acids, etc., proteins were precipitated in 20% TCA in acetone with 20 mM DTT for 1 hour at −20°C, added 1:1 to the homogenate. Then, the sample was centrifuged at 10 000 rpm for 15 min and the pellet was washed with cold acetone containing 20 mM DTT. Residual acetone was removed by air-drying. To achieve a well-focused first-dimension separation, sample proteins must be completely disaggregated and fully solubilized. Therefore, we proved five different sample buffers: Ts1 (containing 7 M Urea, 2 M Thiourea, 4% CHAPS, 60 mM DTT, 5 mM CO_3K_2, 2% IPG buffer-Amersham Bioscience); Ts2 (8M Urea, 2% CHAPS, 60 mM DTT, 5 mM CO_3K_2, 2% IPG buffer); Ts3 (8M Urea, 2% CHAPS, 60 mM DTT, 5 mM, 2% IPG buffer); Ts4 (7M Urea, 2M Thiourea, 4% CHAPS, 60 mM DTT, 5mM NaOH, 2% IPG buffer); and Ts5, with the same components as Ts1 but replacing DTT by Destreak buffer (Amersham Biosciences). We added 50 μL of these buffers and incubated at room temperature for 30 min. Following clarification by centrifugation at room temperature (12 000 rpm, 10 min) the supernatant was stored frozen (−80°C).

2.3 2-DE

Re-hydration buffer (340 μL) was added to promastigote solubilized extracts (7 M urea, 2 M thiourea, 2% CHAPS, 0.75% IPG buffer 4–7, bromophenol blue), which were immediately adsorbed onto 18-cm pH 4–7 IPG strips (Amersham Biosciences). Optimal IEF was carried out at 20°C, with an active re-hydration step of 12 h (50 V), and then focused on an IPGphor IEF unit (Amersham Biosciences) by using the following program: 2 h at 150 V, 1 h at 500 V, 1 h at 1000 V, 1 h at 1000–2000 V and 6 h at 8000 V. After focusing, IPG strips were equilibrated for 15 min in 10 mL of 50 mM Tris-HCl, pH 8.8, 6 M urea, 30% v/v glycerol, 2% w/v SDS, traces of bromophenol blue containing 100 mg of DTT. Further incubation was carried out for 25 min in the same buffer but replacing DTT by 300 mg of iodoacetamide. After equilibration, the IPG strips were placed onto 12.5% SDS-polyacrylamide gels and sealed with 0.5% w/v agarose. SDS-PAGE was run at 15 mA/gel for 20 min as initial migration and increased to 30 mA/gel for separation until front dye reached the bottom of the gel.

The 2-D gels were stained with silver staining MS compatible. Briefly, the gels were fixed in 40% ethanol v/v, 10% v/v acetic acid overnight, then they were sensitized with sodium acetate 0.68% w/v and 0.05% sodium thiosulfate for 30 min and washed with deionized water three times for 5 min. The gels were incubated in 0.25% w/v silver nitrate for 30 min. After incubation, they were rinsed twice with deionized water for 50 s followed by adding the developing solution, containing 2.5% w/v sodium carbonate with 0.04% v/v formaldehyde until desired intensity. Development was

terminated by adding 1.5% w/v EDTA. The protein images were scanned and analyzed with PD-Quest 6.1 software (Bio-Rad).

2.4 Production of rabbit anti-sera specific to *Leishmania infantum* promastigotes

Anti-*L. infantum* polyclonal antibodies were raised by rabbit immunization. A rabbit was immunized by intramuscular injection of 1 mg of crude extract of promastigotes emulsified in equal volume of Freund's complete adjuvant, followed by two additional administrations of the same amount of antigenic proteins in Freund's incomplete adjuvant at 2-week interval.

Animal handling was carried out according to the Council Directive 86/609/EEC of 24 November 1986. Accreditation rules for laboratories and people carrying out animal research are being approved by the Spanish Government.

2.5 Immunoblot analysis

Proteins from 2-DE gels were transferred to an NC membrane (Amersham Biosciences) on a Trans-blot semidry Transfer Unit (Amersham Biosciences). The membranes were rinsed with TBS–Tween buffer (20 mM Tris, 500 mM NaCl, pH 7.4) and incubated with blocking buffer (5% w/v skimmed milk buffer) overnight at 4°C. The blotted membranes were incubated with rabbit anti-*Leishmania* serum 1:100 diluted in 1% w/v milk blocking buffer for 2 h at room temperature. After washing three times in 0.05%TBS–Tween for 15 min, the membranes were incubated with horseradish peroxidase-conjugated goat anti-rabbit IgG antibody (Caltag) diluted 1:3500 in blocking buffer, for 2 h at room temperature. Membranes were washed three times with TBS–Tween buffer for 15 min and once with TBS for 20 min, and then the membranes were treated with ECL solution (Amersham Biosciences) and exposed to X-ray film for 1 min.

2.6 MALDI-TOF MS and MALDI-TOF/TOF MS

Spots of interest were manually excised from silver stained 2-DE gels after being distained as described by Gharahdaghi *et al.* [22]. Briefly, gel spots were incubated in 100 mM sodium thiosulfate and 30 mM potassium ferricyanide, rinsed twice in 25 mM ammonium bicarbonate (ABC) and once in water, shrunk with 100% ACN for 15 min, and dried in a Savant SpeedVac for 20–30 min. Then, the spots were reduced with 10 mM dithioerythritol in 25 mM ABC for 30 min at 56°C and subsequently alkylated with 55 mM iodoacetamide in 25 mM ABC for 20 min in the dark. Gel pieces were alternately washed with 25 mM ABC and ACN, and dried under vacuum. Gel pieces were incubated with 12.5 ng/μL sequencing grade trypsin (Roche Molecular Biochemicals) in 25 mM ABC overnight at 37°C. After digestion, the supernatants (crude extracts) were separated. Peptides were extracted from the gel pieces first into 50% ACN, 1% TFA and then into 100% ACN. Then, 1 μL of each sample and 0.4 μL of 3 mg/mL CHCA matrix (Sigma) in 50% ACN, 0.01% TFA were spotted onto a MALDI target. MALDI-TOF MS analysis was performed on a Voyager-DE STR mass spectrometer (PerSeptive Biosystems, Framingham, MA). Spectra were acquired over the m/z range of 700–4500 Da. Spectra were internally calibrated using trypsin autodigestion products. Tryptic, monoisotopic peptide mass lists were generated and used for database searching. MS/MS sequencing analysis was carried out using the MALDI-TOF/TOF mass spectrometer 4700 Proteomics Analyzer (Applied Biosystems, Framingham, MA). MS was performed at the Complutense University of Madrid Proteomics Facility.

2.7 Database search

The PMF and peptide fragment-ion data obtained from MALDI-TOF and MS/MS analyses, respectively, were used to search for protein candidates in two sequence databases: Swiss-Prot/TrEMBL non-redundant protein database (www.expasy.ch/sprot) and a complete genomic database from the related species *L. major*, namely ftp://ftp.sanger.ac.uk/pub/databases/L.majorsequences/LEISHPEP/, using the MASCOT (www.matrixscience.com) software program. Initial search parameters were as follows: Cys as *S*-carbamidomethyl derivative and Met in oxidized form, one missed cleavage site, peptide mass tolerance of 50 ppm, and MS/MS tolerance of ±0.5 Da. When this approach failed, amino acid sequences were deduced manually from the charge-state deencrypted spectra and used as queries for searches using basic local alignment search tool (BLAST).

3 Results

3.1 Optimization of sample preparation for 2-DE separation

We verified that the presence of salts in initial protein homogenate (by employing PBS or Tris-HCl buffer) interfered with the sample focusing. Thus, we employed a precipitation step with 20%TCA, 20 mM DTT in acetone, (added 1:1 to homogenate) and just one wash step with 20 mM DDT: acetone provided better resolution than higher TCA concentrations and two acetone wash steps. Moreover, we compared the efficacy of different conditions to achieve an *L. infantum* protein extract full solubilization.

These experiments showed that buffers Ts1 and Ts2 containing 2 M thiourea and 7 M urea improved spot resolution when compared to the use of 8 M urea as the only chaotropic agent. To avoid protein lose, degradation or modification by residual acidity of precipitation solution, the best results were obtained with the addition of 5 mM CO_3K_2 (Ts1 and Ts2) in sample buffer instead of 5 mM NaOH (Ts3 and Ts4). However, the streaking in the acidic part of gel was the main

problem and to diminish it we replaced DTT by Destreak buffer (Amersham Bioscience). Therefore, buffer Ts5 (7 M urea, 2 M thiourea, 4% CHAPS, 5 mM CO_3K_2, 2% IPG buffer and Destreak buffer) was the most effective in generating large numbers of discrete spots.

Besides, for the IEF step, we added a pre-step of 150 V for 2 hours, which allowed the ions in the sample to move to the ends of the IPG strips, and to reduce horizontal streaking.

Under these conditions, we obtained a spot resolution good enough to provide reliable image analysis from at least 8–10 runs, being able to detect around 700 spots (Fig. 1).

3.2 Identification of *Leishmania* promastigote proteins by MS and the use of protein databases

At present, the number of *L. infantum* protein sequences in public databases is very small and a version of nucleotide database available in GeneDB is still in revision. In consequence, it was necessary to attempt to identify *L. infantum* proteins by reference all known *Leishmania spp.* sequences in the Swiss-Prot/TrEMBLE database (http://us.expasy.org/sprot/) and the more comprehensive *L. major* GeneDB protein database (http://www.genedb.org/genedb/leish/index.jsp). This strategy has been applied with success for other *Leishmania* species that lack a fully sequenced genome [12, 14].

To establish a reference map for mid-log promastigotes, we tried to identify a number of spots to serve as landmark proteins. Well-resolved protein spots (72 in total) covering a wide range of molecular weights, p*I* and relative intensities were randomly selected and excised from the gels for MS and MS/MS analyses. Of spots analyzed by MALDI-TOF, 35% were clearly identified by their homology with those of *L. major*. This percentage increased to almost 50% when MS/MS was used. Reliable identification was only referred to *Lesihmania* databases. We did not consider reliable protein identification from the homology between *Leishmania* peptides and peptides of known proteins for other organisms due to the existence of peptide compositions and masses, which are not well conserved across species [23]. Parameters used to specify a reliable protein identification for MALDI-TOF were the number of matched peptides, the percentage of coverage, the MOWSE score and observed p*I*/Mr. The results are summarized in Table 1.

Among the identified proteins the most remarkable was the relative high frequency of appearance of the heat shock proteins (HSP)70, both the full sized protein (spots 17, 60), fragments (spots 11, 20) or precursors and/or related proteins (spots 18, 19, 44). We also found one enzyme from the ergosterol biosynthesis pathway (adrenodoxin reductase), a small number of enzymes from diverse metabolic routes (transketolase, mannose-1-phosphate guanyltransferase, succinyl-diaminopimelate desuccinylase, etc.) and some

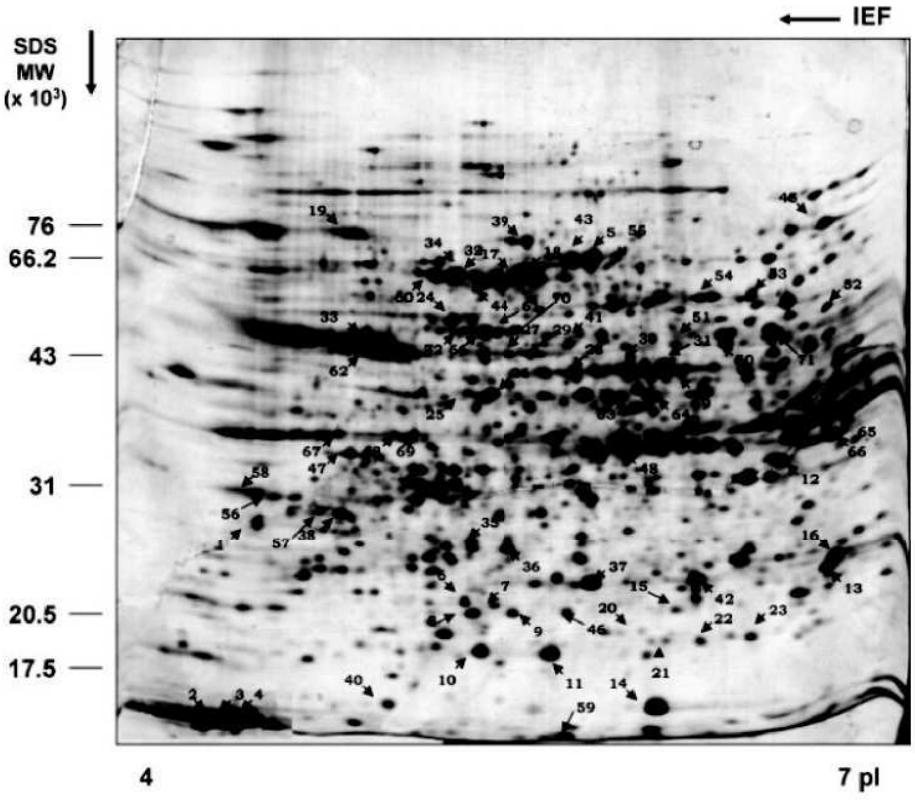

Figure 1. Two-DE analysis of proteins from *L. infantum* promastigotes. IEF was performed with 800 μg of proteins using a 4–7 pH range strips. SDS-PAGE was performed on 12.5% polyacrylamide gels and stained with silver. The numbers refer to the spot identity used in the Tables and text. This figure is a reliable representation of 8–10 gel runs.

Table 1. Identification of *L. infantum* promastigotes proteins in non-redundant sequence databases using data from MALDI-TOF and MALDI-TOF/TOF MS

Leishmania protein	Spot	Database accession[a)]	Analysis way	MOWSE score	Protein coverage	Matched peptides (total)	Anti-geni-city	EXP p*I*[b)]	EXP Mr Da[b)]
LmjF36.6010, hypothetical	3	LmF36.6010	MS	56	20	7	No	4.31	8 600
Propionil carboxilase	5	L549.5	MS	64	16	14	Yes	5.78	66 000
Heat shock 70-related protein 1, mitochondrial precursor, probable	11	CHR30_tmp. 227c	MS	56	19	9	No	5.63	18 000
PGKB phosphoglycerate kinase	14	CHR16–22_tmp. 959c	MS	57	25	11	No	6.01	8 750
HSP 70, putative	17	CHR28_tmp. 09	MS	245	36	8	Yes	5.50	66 000
Heat shock 70 kDa protein, mitochondrial precursor, probable	18	CHR30_tmp. 17	MS	248	25	12	Yes	5.59	66 000
Heat shock 70 kDa protein, mitochondrial precursor, probable	19	CHR30_tmp. 17	MS	221	25	12	Yes	4.89	71 000
Heat shock 70 kDa, fragment	20	CHR28_tmp. 09	MS	171	39	12	Yes	5.87	19 000
Hypothetical 7.9 KDa protein	25	CHR26_tmp. 151.c	MS	53	10	13	Yes	5.31	39 000
ATPase beta subunit, probable	33	LmjF25.1180c	MS	89	34	14	Yes	4.93	53 000
Unknown function	34	CHR16–22_tmp. 96	MS	56	25	8	Yes	5.26	66 000
Hypothetical protein L6066.01	35	L6066.01	MS	57	45	10	No	5.31	26 700
Unknown function	36	LmjF15.0680	MS	53	25	8	No	5.45	26 200
Phosphomannomutase	38	LmjF36.1960	MS-MS/MS	113		1	Yes	4.89	30 000
PAR, probable	39	CHR29_tmp. 86	MS	169	17	11	No	5.54	73 500
Propionil carboxilase	43	PCACA	MS	66	16	14	Yes	5.70	66 000
Heat shock 70 kDa protein, Mitochondrial precursor, probable	44	CHR30_tmp. 17	MS	72	28	14	Yes	5.33	65 800
Transketolase, probable	45	LM24.206	MS-MS/MS	25		1	Yes	6.67	73 500
Proteasome subunit	47	P1295.09	MS-MS/MS	26		1	Yes	4.88	34 600
at5g50850/k16e14_l	49	LmjF25.1710c	MS-MS/MS	141		3	Yes	6.00	42 000
Disulfide isomerase PDI	50	Q818E1_	MS	76	25	9	Yes	6.25	43 200
20S proteasome alpha 5 subunit	57	L3640.16	MS	130	60	14	Yes	4.8	30 000
P28 protein, probable	59	LmjF25.2010c	MS-MS/MS	51		2	No	5.65	5 000
HSp70, heat shock protein, putative	60	CHR28_tmp. 09	MS-MS/MS	79		1	Yes	5.21	66 000
Succinyl-diaminopimelate desuccinylase	61	LmjF31.1890c	MS-MS/MS	41		1	Yes	5.46	53 000
Probable tubulin alpha chain	62	P1046.22	MS-MS/MS	24		1	Yes	4.93	52.000
Tcc2i18.6	63	CHR16–22_tmp. 58	MS-MS/MS	26		1	Yes	5.92	39 400
L3836.5	64	L3836	MS-MS/MS	27		1	No	5.96	39 400
Mannose-1-phospathe guanyltransferase	65	L2581.06	MS-MS/MS	47		1	No	6.72	37 000

a) Protein accession numbers are available in databases at the Sanger Institute.
b) Experimental molecular weight (Mr) and experimental p*I* were derived from the migration of related markers during 2-DE.

structural proteins such as paraflagellar rod protein 3 (PAR-3) and α-tubulin. The functional classification of the identified proteins is shown in Table 2.

Table 2. Functional classification of *Leishmania infantum* promastigote proteins identity by MS

1. Carbohydrate metabolism

-Transketolase
-Mannose-1-phosphate guanyltransferase
-Phosphoglycerate kinase
-Phosphomannomutase

2. Protein synthesis and catabolism

- Proteasome subunit
-20S proteasome alpha 5 subunit

3. Amino acid metabolism and synthesis

-Succinyl-diaminopimelate desuccinylase (Lysine biosynthesis)

4. Structural

-Paraflagellar rod protein 3 (PAR 3)
-α-tubulin (probable)

5. Stress response/chaperone

-Heat shock 70-related protein 1, mitochondrial
-Heat shock 70

6. Ergosterol biosynthesis

-Adrenodoxin reductase

7. Fatty acid metabolism

-Propionyl-CoA carboxylase

8. Hypothetical proteins

-L3836.5
-LmjF36.6010
-Hypothetical 7.9 kDa protein
-Hypothetical protein L6066.01
-LmjF15.0680

9. Others

-Unknown function LmjF15.0680
-ATPase beta subunit probable
-Unknown function (CHR16–22_tmp. 96)
-at5g50850/k16e14_1
-Disulfide isomerase
-p28 protein, probable
-Tcc2i18.6

3.3 Immunoblot analysis of 2-DE maps with rabbit hyperimmune sera

To investigate the immunogenicity of proteins identified in the promastigotes stage, immunoblots with anti-*L. infantum* rabbit hyperimmune sera were performed. Following autoradiography, antiserum was able to recognize more than 50 antigenic spots, located between 90 and 30 kDa (Fig. 2). When the same immunoblot was probed with sera from naive rabbit, no proteins were detected (data not shown).

A number of large antigenic spots were detected in the acidic range (4–5.5), thus we tried to standardize IEF in the pH range 3.5–5, but the spot resolution was not good enough to provide reliable image (data not shown). By comparison with silver-stained gels, proteins previously identified as propionil carboxilasa, ATPase beta subunit, transketolase, proteasome subunit, succinyl-diaminopimelate desuccinylase, a probable tubulin alpha chain, and several proteins of unknown function appeared antigenic on immunoblots. In the case of HSP70, both full-size and related proteins were highly immunogenic whereas the fragment of 20 kDa corresponding to an HSP70-related protein 1 and a similar size fragment of the native protein did not show immunogenicity.

For the identification of a group of antigenic spots with a high quality mass spectra (53, 54, 49, 50 and 71, Fig. 1) we had to turn to deduce manually their amino acid sequences plus homology searching in BLAST. In spite of analyzing at least six peaks per spot, no consistent identifications were obtained in databases. It is likely that differences in primary amino acid sequences of these proteins in *L. infantum* account for this unsuccessful identification when using the *L. major* database.

4 Discussion

To obtain reproducible results, it was important to standardize both sample preparation and the source of material. Therefore, we selected promastigotes from mid-log phase because this is the parasite form commonly used in serodiagnosis and because it contains increased amount of infective forms [24]. Several reagents are available for improving protein solubilization, such as NP-40 [25], or the non-detergent sulfobetaines [26]. The relatively simple extraction (sonication), precipitation (in TCA-acetone) and solubilization (Ts5 buffer) methods used in our study gave good solubility for a relatively important number of proteins.

Herein we present a high-resolution 2-DE gel map of *L. infantum*, resolving more than 700 spots. Our analysis allowed sampling a lower proportion of the genome than that reached in previous works on developmentally regulated stages with *L. mexicana* [12], *L. infantum* [11] or *L. donovani* [27], where about 25% of the predicted ORF (about 2000 spots) of *L. major* was tracked. However, whereas they cover wider overlapping pH gradients (*e.g.* from 4 to 11) we restricted to 4 to 7 pH since most of antigenic proteins that constitute our main objective co-localized within this range.

Numerous studies had shown complex antigenic patterns in human and canine visceral leishmaniasis by Western blot. Among the antigens frequently recognized were those with molecular weights of 14, 16, 26, 28, 30, 33, 46, 63, 68, 70, 73, 83 and 117 kDa [28–31]. Some of them are well characterized like HSP70, gp63, HSP83, several ribosomal

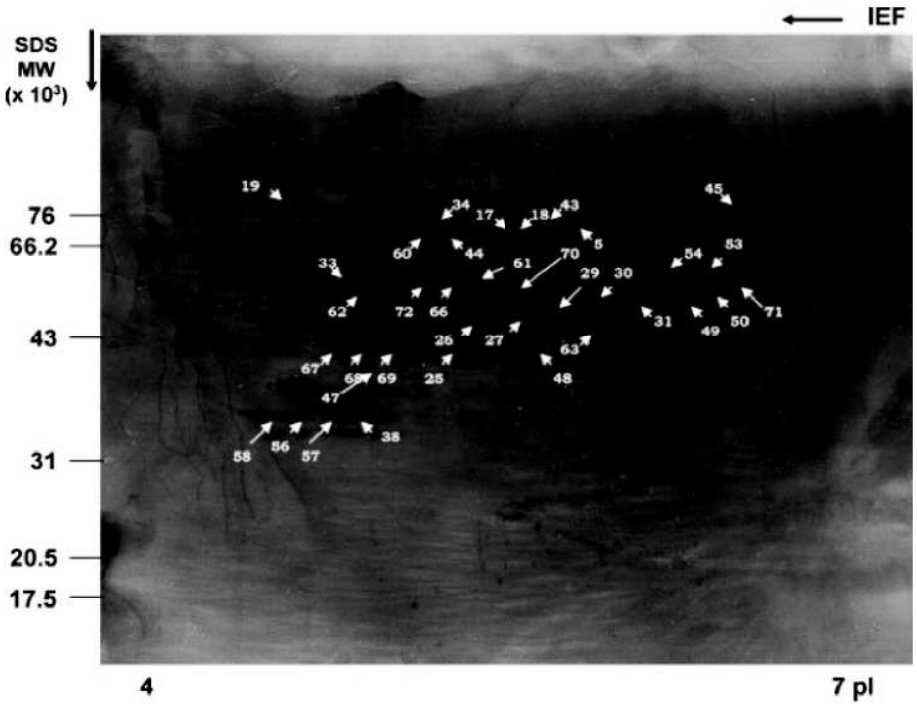

Figure 2. Two-DE immunoblot analysis of proteins from *L. infantum* promastigotes. Two-DE separated proteins were transferred to NC membrane and probed with rabbit hyper-immune sera raised against *L. infantum*. Bound antibodies were detected with goat anti-rabbit IgG antibodies at 1:3500 dilution.

proteins, histones, KMP11 or LACK and they are used in diagnostic or in vaccination assays. Others still await identification. In the present study, the most abundant antigens belong to HSP70 family. Among stress or heat shock proteins, HSP70 constitutes the most conserved and ubiquitous protein known to date [32], likely to play important role in humoral response against various infectious diseases and autoimmune syndromes. For their special immunogenic properties they are considered as "panantigens" [33]. Surprisingly, despite the high sequence identity between host and *Leishmania* HSP70, the immune response elicited during this parasitic infection is particularly directed against some specific epitopes of *Leishmania* HSP70. Besides, high levels of anti-HSP70 antibodies are observed in early phases of the disease, thus making this protein a useful tool in the praecox diagnosis of leishmaniasis [34].

Several enzymes belonging to crucial metabolic routes also exhibited antigenic properties.

These are the propionyl-CoA carboxylase, a highly antigenic protein that is present in all organisms but with unreported antigenicity; the vacuolar ATPase and the succinyl-diaminopimelate desuccinylase. Electrogenic vacuolar ATPases ($V\text{-}H^+$-ATPases) are important for maintenance of the acidic environment inside protozoan cell vesicles [35] and therefore they may be involved in regulating *Leishmania* adaptation mechanisms to both vertebrate and invertebrate hosts, which, among others, include marked changes in ionic composition and pH [36]. Although, to our knowledge, no information has been provided as to whether $V\text{-}H^+$-ATPases are antigenic in other organisms, various studies award their autoantigenicity and consequent relationship with autoimmune diseases [37, 38]. The succinyl-diaminopimelate belongs to the diaminopimelate pathway for the synthesis of the D,L-diaminopimelic acid, which is also a component of the peptidoglycan layer of Gram-negative and mycobacterial cell walls [39].To our knowledge diaminopimelate is not synthesized by mammals therefore *Leishmania* enzymes involved in its production could be promising targets for new drugs as well as vaccine development.

For replication of *L. chagasi* promastigotes and survival of the amastigotes inside the vertebrate host cell intact proteasome function is required [40]. Herein we identified a subunit of the *L. infantum* proteasome with antigenic properties. The antigenicity of the *Leishmania* proteasome has also been confirmed by Christensen *et al.* [41] and their role as panantigen was suggested [42, 43]. Together, the immunological and physiological features of the proteasome prompt this structure as another ideal target for parasite control.

Among non-antigenic proteins identified, the following could be of interest as targets for chemotherapeutic control, the paraflagellar rod-3 protein and the mannose phosphate guanyltransferase (MPT) enzyme. Paraflagellar rod-2 protein is a components of the paraflagellar rod units, a singular structure of kinetoplastids, euglenoids, and dinoflagellates [44]. MPT is an enzyme of the carbohydrate metabolism not previously identified in *Leishmania* by proteomics analysis. This enzyme plays a key role in the synthesis of LPG (Lipophosphoglycan), an essential virulent factor of *Leishmania* promastigotes [45].

In summary, by applying a proteomic approach we were able to identify a substantial number of proteins present in crude extracts of *L. infantum* mid-log promastigotes. Most of them are antigenic and have been proved associated to crucial physiological and virulence functions of this parasite. Therefore, they may be suitable targets for both vaccination and chemotherapeutic strategies. Unraveling new target candidates in this parasite awaits further approaches to the sub-cellular proteome in combination with new powerful innovative technologies together with the imminent availability of the annotated genome of *L. infantum*.

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