TRANSACTIONS OF THE ROYAL SOCIETY OF TROPICAL MEDICINE AND HYGIENE (2002) 96, SUPPLEMENT 1 S1/81–S1/86

Field epidemiology

Molecular epidemiology and diagnosis of *Leishmania*: what have we learnt from genome structure, dynamics and function?

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Abstract

This paper reviews our exploration of the dynamics of the *Leishmania* genome and its contribution to epidemiology and diagnosis. We used as a model Peruvian populations of *L. (Viannia) braziliensis* and *L. (V.) peruviana*, 2 species very close phylogenetically, but phenotypically very different in biotope and pathology. We initially focused on karyotype analysis. Our data showed that chromosomes were subject to a fast rate of evolution, and were sensitive indicators of genetic drift. Therefore, molecular karyotyping appeared an adequate tool for monitoring (i) emergence of close species, (ii) ecogeographical differentiation at the intraspecific level, and (iii) strain 'fingerprinting'. Chromosome size variation was mostly due to the number of tandemly repeated genes (*rDNA*, *mini-exon*, *gp63*, and *cysteine proteinase* genes), and could involve the deletion of unique genes (*L. (V.) braziliensis*-specific *gp63* families). Considering the importance of these genes in parasitism, their rearrangement might have functional implications: adaptation to different environments and pleomorphic pathogenicity. Our knowledge of genome structure and dynamics was used to develop new polymerase chain reaction (PCR) techniques. Amplification of *gp63* genes followed by cleavage with restriction enzymes and study of restriction fragment length polymorphism (*gp63* PCR–RFLP) allowed the discrimination of all species tested, even directly in biopsies with 95% sensitivity (compared with PCR amplification of kinetoplast deoxyribonucleic acid). At the intra-specific level, RFLP was also observed and corresponded to mutations in major immunogen domains of *gp63*. These seem to be under strong selection pressure, and the technique should facilitate addressing how the host's immune pressure may modulate parasite population structure. Altogether, *gp63* PCR–RFLP represents a significant operational improvement over the other techniques for molecular epidemiology and diagnosis: it combines sensitivity, discriminatory power and prognostic value.

Keywords: leishmaniasis, *Leishmania braziliensis*, *Leishmania peruviana*, chromosome size, molecular karyotyping, diagnosis, epidemiology, Peru

Introduction

Since the advent of molecular biology, technological advances have contributed to our insight into parasite population diversity: chiefly, isoenzyme analysis (CHANCE, 1979), study of restriction fragment length polymorphism (RFLP: BEVERLEY *et al.*, 1987), and random amplification of polymorphic deoxyribonucleic acid (RAPD: TIBAYRENC *et al.*, 1993). In trypanosomatids, correlation was observed between the genetic distances estimated by each of these methods (TIBAYRENC & AYALA, 1987). This correlation can be explained by the nature of the characters considered (nucleotide sequences), their variation (mostly point mutations), and their mode of inheritance (essentially clonal: TIBAYRENC *et al.*, 1990). An inherent constraint is the risk of redundancy among the information collected by the different methods. This risk can obviously be overcome by utilizing differences in the mutation rate: e.g., the sequence of ribosomal deoxyribonucleic acid (rDNA) intergenic spacers evolves more quickly than *rDNA* genes themselves, which allows discrimination at different taxonomic levels (VAN EYS *et al.*, 1989; CUPOLILLO *et al.*, 1995). However, instead of analysing the consequences of point mutations within DNA sequences, it is also possible to study how the DNA sequences themselves are (re-)arranged. The higher evolutionary importance of gene rearrangements (vs. point mutations) has already been emphasized in higher eukaryotes (WILSON *et al.*, 1974). Furthermore, in several prokaryotes, these mechanisms have been shown to play a role in differential pathogenesis (WOODS *et al.*, 1991; LIU & SANDERSON, 1995; DWORKIN & BLASER, 1997; BALL *et al.*, 1999; MURPHY & BELLAS, 1999). Accordingly, we assumed that studies of gene rearrangements might provide original information on parasite diversity. Since 1987, we have been exploring this hypothesis in the genus *Leishmania*, in order to understand better its extensive phenotypic diversity (ecology, hosts, pathology). The aim of the present paper is to show how genome dynamics contributes to the population diversity of *Leishmania*, and how its study has facilitated the development of original molecular epidemiological and diagnostic techniques: from molecular karyotyping to polymerase chain reaction (PCR) amplification followed by RFLP analysis (PCR–RFLP) at specific gene loci.

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Peruvian leishmaniasis, a unique ecoepidemiological model

For testing our hypothesis, the best model would correspond to parasites that differed phenotypically but were very similar at the level of their DNA sequence and so would allow a better estimate of the weight of genome rearrangements. These conditions are fulfilled in Peruvian leishmaniases. The 2 main forms of tegumentary leishmaniasis in Peru are very different from the clinical and ecological points of view. The form causing the major health problem is endemic in the Amazonian lowlands: it is caused by *L. (Viannia) braziliensis* and is characterized by severe cutaneous ulcers which, in up to 10% of the cases, may evolve by metastasis to a disfiguring mucosal condition called espundia (LLANOS-CUENTAS, 1991). A second form, uta, is caused by *L. (V.) peruviana* and is endemic on the Pacific slopes of the Andes and in certain inter-Andean valleys, mostly in xerophytic environments (GUERRA, 1988). It is a leishmaniasis of altitude (1300–2800 m), characterized by

benign cutaneous lesions which, in contrast to sylvatic leishmaniasis, have never been reported to involve mucosal metastasis; in a few cases, mucosal lesions resulting from contiguity have been reported (E. A. Llanos-Cuentas, unpublished observations). At the beginning of our work, these 2 etiological agents were indistinguishable by molecular techniques, and it was only in 1990 that isoenzyme analysis permitted their discrimination (ARANA *et al.*, 1990). Actually, the 2 species were shown to differ by only one of 16 enzymatic loci (mannose phosphate isomerase: BAÑULS *et al.*, 2000) and they are the 2 phylogenetically closest species of the subgenus *Viannia* (see CHOUICHA *et al.*, 1997). Why thus are such similar parasites associated with broad ecological and clinical differences? Without excluding the possible role of host factors, we considered these 2 species to be ideal for investigating the potential involvement of gene rearrangements.

Analysis and interpretation of chromosome size polymorphism

A first, macroscopic approach to gene rearrangement was molecular karyotyping by pulsed field electrophoresis. This method of chromosome separation was initially applied to trypanosomatids (VAN DER PLOEG *et al.*, 1984). Later, a series of papers asserted—with a limited sample of organisms—the potential of molecular karyotyping for taxonomic and epidemiological studies of several protozoa. Subsequent publications revealed problems in the application of the technique in the field, and concluded that molecular karyotyping was not applicable to molecular epidemiology. The major reason was the extensive chromosome size polymorphism observed among strains. This property could be of interest from the point of view of strain 'fingerprinting': the more polymorphic a marker, the higher its power to test the null hypothesis of similarity between 2 isolates. We applied this principle to comparing, in the same Andean valley, human and sand fly isolates of *Leishmania*: the identity of the whole karyotype, together with entomological arguments, allowed us to incriminate *Lutzomyia ayacuchensis* as a vector in that region (A. Caceres, unpublished data).

However, in order to search for any correlation between the size of homologous chromosomes (as defined by hybridization with specific probes) and taxonomic or biological features, numerical processing is required. The core of the problem was to discriminate significant chromosome size variation from the 'background' caused by inhomogeneity of electrical fields, DNA concentration, and telomere expansion and contraction. In a first numerical attempt, size classes of 25 kb were created and chromosomes within the same size class were considered as being similarly sized (GIANNINI *et al.*, 1990). By doing so, the chromosomes were considered as a discrete variable with weighting of size variation: 2 chromosomes differing, for instance, by 25 or 200 kb were considered as genomically different. We developed a second method, the absolute chromosome size difference index (aCSDI), which considers chromosomes as a continuous variable and introduces a weighting of size variation: we assumed that the more the size of 2 chromosomes differed, the more were they dissimilar in a strict genomic sense (DUJARDIN *et al.*, 1995a). This method gave more weight to significant changes in chromosomal size resulting from any kind of rearrangement, and proved to fit with stepwise DNA rearrangements, a mechanism that was demonstrated later to apply to several chromosomes (see below). Data generated by aCSDI were easily analysed by agglomerative algorithms like UPGMA (SNEATH & SOKAL, 1973) or the Fitch–Margoliash method (FELSENSTEIN, 1984), and led to significantly structured dendrograms which were more meaningful biologically than dendrograms built up from discrete characters, in which hierarchization was almost abolished and relationships between isolates could not be interpreted (DUJARDIN *et al.*, 1995a); aCSDI was therefore selected for the analysis of natural populations of Peruvian *Leishmania*.

Molecular epidemiology: karyotyping

In order to understand the influence of ecogeography on karyotype diversity, an allopatric sample of parasites was collected from different biogeographical units (BGUs) of the Amazonian forest and the Andes (Fig. 1). BGUs were defined on the basis of the endemicity of butterfly species (LAMAS, 1982). Our working hypothesis was that ecogeographical factors responsible for structuring butterfly populations were also responsible for structuring populations of parasites and their vectors (DUJARDIN *et al.*, 1993a). Five different chromosomes (among 35 in the subgenus *Viannia*; BRITTO *et al.*, 1998) identified by specific probes (DUJARDIN *et al.*, 1993b) were selected according to their extensive polymorphism and analysed by aCSDI. The resulting dendrogram revealed clear differences between *L. (V.) braziliensis* and *L. (V.) peruviana*, and identified within the latter species a population structure corresponding to the BGUs of origin, along a north–south cline (DUJARDIN *et al.*, 1995a; Fig. 1).

Analysis of the same populations by isoenzymes (the 'gold standard') revealed a significant correlation between chromosome and isoenzyme genetic distances, which (i) implied that rearrangements responsible for chromosome size polymorphism and point mutations associated with isoenzyme polymorphism were evolutionarily correlated, and (ii) therefore validated the aCSDI methodology (DUJARDIN *et al.*, 1998). However, in the case of isoenzymes, both the extent of polymorphism and its relationship with geographical distance were much lower. A close relationship between molecular and geographical distances may be due to genetic drift (TABACHNICK & BLACK, 1995), optimal conditions for which certainly exist in the present case: small parasite populations in isolated Andean valleys.

Chromosomes appear to be subject to a faster evolution rate and to be more sensitive indicators of genetic drift than isoenzymes (DUJARDIN *et al.*, 1998). However, the adaptive significance of chromosome plasticity cannot be excluded as the type of relationship with geography has been shown to vary from one chromosome to another (DUJARDIN *et al.*, 1998). A similar observation was reported in natural populations of *Drosophila melanogaster* and was accounted for by the action of selective factors, as genetic drift alone would affect all loci similarly (SINGH & RHOMBERG, 1987). In the case of *L. (V.) peruviana*, at least 2 selective factors can be identified: the different ecogeographical origin of the populations studied (LAMAS, 1982) and their transmission by different sand fly vectors all along the cline (A. Caceres, unpublished data; DAVIES *et al.*, 1993; VILLASECA *et al.*, 1993).

Another interesting result was the high similarity of the karyotypes of northern *L. (V.) peruviana* isolates and *L. (V.) braziliensis* to each other, in contrast to those of southern *L. (V.) peruviana*. This genomic gradient corresponds to an ecogeographical one: northern *L. (V.) peruviana* isolates originated from the BGU close to Porculla, the only natural pass in the Peruvian Andes between the Amazonian forest and the Pacific slopes (Fig. 1). We proposed the hypothesis that the Andean *L. (V.) peruviana* diverged from *L. (V.) braziliensis*, by colonization of the Andean region near Porculla, and underwent karyotype differentiation while migrating from one Pacific BGU to the other, further south (DUJARDIN *et al.*, 1995a; DUJARDIN, 1997).

Altogether, our data indicate that molecular karyotyping is an adequate tool for monitoring (i) the emergence of close species, (ii) ecogeographical differentiation at the intraspecific level, and (iii) strain 'fingerprinting'. Further work is required in order to evaluate its capacity to discriminate at higher taxonomic levels.

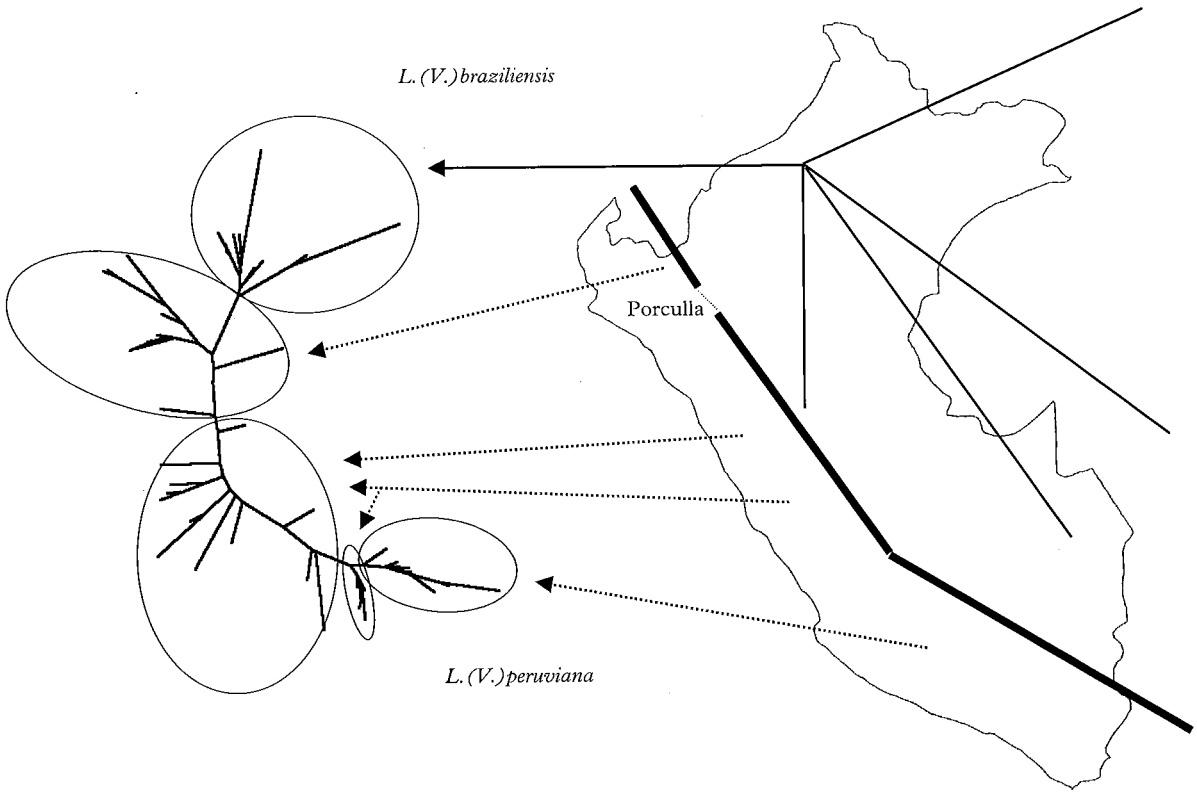

Fig. 1. Size polymorphism of chromosomes 2, 10, 11, 27 and 134Sg in Peruvian isolates of *Leishmania (Viannia) braziliensis* and *L. (V.) peruviana*. Agglomeration by the Fitch–Margoliash method after aCSDI calculation. Porculla: natural pass between forest and Pacific slopes of the Andes (thick line). (From DUJARDIN *et al.*, 2000; reproduced with the permission of Instituto Oswaldo Cruz, Rio de Janeiro, Brazil.)

Deciphering the mechanisms of chromosome size variation

A deeper analysis of chromosome size variation among Peruvian leishmanial populations revealed another interesting feature. Large variants of 4 chromosomes (of the 5 mentioned above) were observed in *L. (V.) braziliensis*, in contrast to smaller variants in southern *L. (V.) peruviana*. These chromosomes contained long arrays of tandemly repeated genes: *gp63* (chromosome 10: DUJARDIN *et al.*, 1994; VICTOIR *et al.*, 1995), *rDNA* (chromosome 27: BRITTO *et al.*, 1998; INGA *et al.*, 1998), *mini-exon* (chromosome 2; KEBEDE *et al.*, 1999), and *cysteine proteinase b* (chromosome 8; POLET, 1999). This type of organization is common in trypanosomatids, and has a functional meaning: the production of large amounts of protein through large polycistronic transcripts (STILES *et al.*, 1999). Theoretically, such structures are highly sensitive to amplification/deletion through unequal crossing-over (SZOSTAK & WU, 1980). The occurrence of this mechanism was tested by densitometric analysis of chromosome hybridization and/or physical mapping of the corresponding loci. In the 4 chromosomes, a significant correlation was encountered between chromosome size and copy number of the genes (DUJARDIN *et al.*, 1994; VICTOIR *et al.*, 1995; INGA *et al.*, 1998; KEBEDE *et al.*, 1999; POLET, 1999). Gene content ('dosage') thus appears to be one of the main mechanisms responsible for genomic plasticity. It accounts for the stepwise chromosome size variation observed among natural populations, therefore further validating the use of aCSDI for numerical analysis. Most of all, considering the importance of the genes involved, it allows us to address the question of the functional implications of chromosome size polymorphism in terms of (i) the adaptation potential of the parasites to different environments and, more fundamentally, (ii) the pleomorphic pathogenicity of *Leishmania*. Further sampling in sympatric conditions—in order to exclude ecogeographical effects—is necessary to address the link with pathogenicity.

Functional effects of gene rearrangements: the *gp63* model

Gene 'dosage' itself (ASHBURNER, 1989), modifications in regulatory intergenic regions (RAMAMOORTHY *et al.*, 1995), or deletions of single genes (BOURKE *et al.*, 1996) may have important effects on phenotype. In addition, synergistic effects on the fitness of the parasites might be expected from the accumulation of deletions affecting different kind of genes, coding for general (mRNA splicing for the *mini-exon* gene, protein translation for *rDNA*) and specific (internalization and survival within the macrophage for *gp63* and *cysteine proteinase b*) functions.

We concentrated on chromosome 10 and particularly the *gp63* gene locus, as a prime model with its major genomic rearrangements: variation of 100 kb and halved copy number of *gp63* genes that allowed a clear-cut differentiation between all *L. (V.) braziliensis* and *L. (V.) peruviana* isolates tested. Cosmid clones covering most of the locus were isolated from an *L. (V.) braziliensis* library (a generous gift from D. C. Barker). This species was chosen because it would give access to the largest *gp63* gene locus. Physical mapping revealed heterogeneity in the *gp63* repeats, suggesting the existence of several gene families (or isogenes) as in *L. (V.) guyanensis* (see STEINKRAUS *et al.*, 1993). Isolation of the different restriction variants and their sequencing confirmed this. The strong genic reduction observed within the global pool of *gp63* genes in *L. (V.) peruviana* suggested that one *gp63* isogene could have been totally deleted. This was tested by intragenic amplification of all *gp63* iso-

genes and subsequent restriction cleavage (*gp63* PCR–RFLP; VICTOIR *et al.*, 1998). An *L. (V.) braziliensis gp63* isogene (now called *gp63spe*) was not found among *L. (V.) peruviana*, suggesting full deletion (Fig. 2). In contrast, in the *L. (V.) braziliensis* genome, *gp63spe* was present as a small cluster of 3 copies, besides large clusters of other *gp63* isogenes. Because of the possibility of a lower copy number and competition with other more abundant isogenes during the amplification process, we could not exclude the possibility that *gp63spe* was present but not detected in *L. (V.) peruviana*. However, a further PCR assay, amplifying *gp63spe* only, confirmed the presence of the specific isogene *gp63spe* in *L. (V.) braziliensis* and its absence from *L. (V.) peruviana* isolates (K. Victoir, unpublished data).

In other *Leishmania* species, the presence of different *gp63* isogenes has been associated with different isoforms of the proteinase gp63 expressed differentially throughout the life cycle, and possibly differing in function (MEDINA-ACOSTA *et al.*, 1993). Considering the marked difference in pathogenicity between *L. (V.) braziliensis* and *L. (V.) peruviana*, and the potential role of gp63 in parasite virulence during its life cycle (resistance to complement-mediated lysis, adhesion to the macrophage, and survival within the phagolysosome), it appeared justified to check whether the genomic differences mentioned above were of consequence at transcription level. Therefore, *gp63*-specific reverse transcription PCR assays were developed to compare promastigotes and amastigotes (from macrophages *in vitro*) of both species. Preliminary results indicated that *gp63spe* is transcribed in both life stages of *L. (V.) braziliensis*, and obviously not in *L. (V.) peruviana*.

These data illustrate how gene 'dosage' may lead to deletion of whole sets of specific genes. Transcription activity of the latter provides direct support for our hypothesis of the functional impact from these genomic rearrangements. Furthermore, indirect consequences of these events may not be excluded if one considers the presence of regulatory genes among the rearranged genes (like the *mag* genes for gp63; MCCOY *et al.*, 1998). Additional work is in progress to document further these observations, and their direct association with the differences in pathogenicity observed between *L. (V.) braziliensis* and *L. (V.) peruviana*.

Molecular epidemiology: PCR–RFLP

Besides better understanding of leishmanial genome structure, the *gp63* locus offered prospects for the development of new PCR tools for molecular epidemiology and diagnosis. Repeated *gp63* genes guarantee the sensitivity of detection of *Leishmania*, and genomic heterogeneity (existence of different isogenes) might allow discrimination at specific and intraspecific levels. Therefore, *gp63* PCR–RFLP was evaluated on purified DNA: the resulting patterns discriminated the first four *Viannia* species tested, *L. braziliensis*, *L. peruviana*, *L. guyanensis* and *L. lainsoni* (see VICTOIR *et al.*, 1998), and provided evidence for intraspecific polymorphism within *L. peruviana*.

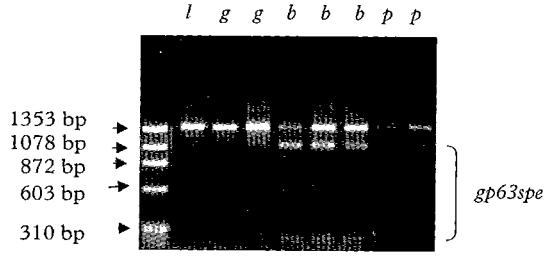

Fig. 2. *Sal*I restriction fragment length polymorphism patterns after polymerase chain reaction amplification of *gp63* genes of isolates of *Leishmania (Viannia) lainsoni (l)*, *L. (V.) guyanensis (g)*, *L. (V.) braziliensis (b)* and *L. (V.) peruviana (p)*. Fragments corresponding to a specific *L. (V.) braziliensis gp63* isogene (*gp63spe*) are indicated.

The next step was to evaluate *in situ* the detection sensitivity of our system, by comparison to a reference technique with high sensitivity, PCR amplification of kinetoplast DNA (kDNA PCR: SCHUBACH *et al.*, 1998). Preliminary results on 38 biopsies from Peruvian cutaneous and mucosal leishmaniasis patients indicated a sensitivity of 95% (K. Victoir, unpublished results). This level of sensitivity is higher than expected, considering the number of target copies in both assays (up to 10 000 in kDNA minicircles vs. 30–70 *gp63* genes), and it could be increased by further optimization of the assay and the DNA extraction procedure.

The *gp63* PCR–RFLP technique thus represents a significant operational improvement combining sensitivity and discrimination of species associated with different clinical patterns i.e., a potential prognostic value. Other PCR assays with similar advantages have been developed, such as that targeting intergenic DNA sequences (CUPOLILLO *et al.*, 1995). These sequences, however, could be under different selective pressure (intergenic non-coding sequences for rDNA spacers versus intragenic sequences for *gp63*), and their respective discriminatory power ought to be compared in one and the same large sample of isolates. For diagnostic purposes, *gp63* PCR–RFLP and similar tests should not be used on their own on biopsies, but certainly as second line assays for (i) confirmation of kDNA PCR and, most of all, (ii) identification of the parasites at specific and subspecific levels.

Another potential application of *gp63* PCR–RFLP is to address how immune pressure on important immunogens like gp63 modulates parasite population structure. The relevance of this question was documented recently by our study of another *Leishmania* model, the Mediterranean *L. (L.) infantum*. Using 26 isolates from 4 countries (France, Spain, Algeria and Tunisia), *gp63* PCR–RFLP patterns were compared to isoenzyme profiles as neutral markers. Significant geographical structuring was observed with *gp63* data, together with unexpectedly low overlap with the isoenzyme-based population structure (GUERBOUJ *et al.*, 2000b). For instance, representatives of the 2 zymodemes considered in the study (MON-1 and MON-24) did not cluster together, but were scattered across the different branches of the *gp63* dendrogram. Interestingly, *gp63* genetic polymorphism was restricted to gene regions coding for the surface domains of the protein, known to be variable (SCHLAGENHAUF *et al.*, 1998). These results support the concept of antigenic polymorphism among *gp63* genes (MORALES *et al.*, 1997). The strong geographical structuring at DNA level could result from the strong pressure exerted on gp63 by the mammalian hosts, humans and dogs. How the host's immune response can shape the population structure of pathogens is a question crucial for the design of vaccines, where protective determinants of candidate immunogens can be either conserved or pleomorphic (GUPTA & ANDERSON, 1999; GUERBOUJ *et al.*, 2000a). In this context, future molecular epidemiology of leishmaniases should focus on antigen genes, as has already been done with malaria (GUPTA *et al.*, 1994).

Concluding remarks

In their constant struggle for life, *Leishmania* organisms need to innovate and adapt themselves to the different challenges and stresses inherent in their parasitic nature. These parasites cannot (TIBAYRENC *et al.*, 1990), or can only rarely (DUJARDIN *et al.*, 1995b), rely on sexuality for genetic innovation. Our results suggest that they rearrange and adequately adjust their genome structure, rich in tandemly repeated genes, with consequences at expression level and, most probably, in terms of fitness and pathogenicity. In addition to the extraordinary lesson of life we are taught by the leishma-

nial genome, we can use the genomic mechanisms to develop new molecular tools and gain with them information complementary to what is already known. We hope and expect that these innovative tools can be simplified and adapted to conditions in endemic countries, where the health systems urgently need rapid, sensitive, specific and low-cost diagnostic tests.

Acknowledgements

The various studies discussed here received financial support from the European Commission (contracts TS2-CT90-0315, TS3-CT92-0129, IC18-CT96-0123 and IC18-CT98-0256), Fonds voor Wetenschappelijk Onderzoek (Nationale Loterij, grants 346/1990 and 9.0024.90), and the Belgian Agency for Co-operation in Development.

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