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In vitro promastigote fitness of putative *Leishmania (Viannia) braziliensis*/*Leishmania (Viannia) peruviana* hybrids

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Abstract

In order to initiate studies on the phenotypic properties of hybrids vs. their putative parents, the in vitro growth behaviour of promastigotes was compared for 15 stocks characterised as *Leishmania (Viannia) braziliensis*, *Leishmania (Viannia) peruviana* and putative hybrids (isolated from the Eastern Andean valley of Huanuco, Peru). Five sets of three stocks, each set including a *L.(V.)braziliensis*, a *L.(V.)peruviana* and a putative hybrid, were constituted randomly and counted daily close to isolation from man (ten to 18 subcultures). Hybrids and *L.(V.)peruviana* presented similar growth characteristics, and they displayed a growth capacity (growth rate and cell density at stationary phase) significantly lower than the one of *L.(V.)braziliensis*. Following prolonged in vitro maintenance of one of the sets, the hybrid kept its lower growth capacity. The contrast between the difficulty to grow in vitro these putative hybrids, and their high isolation rate from natural populations is discussed.

Keywords: Leishmania; Hybrids; Peru; Growth characteristics

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1. Introduction

The reproductive mode of *Leishmania* and other parasitic Protozoa has been extensively studied during the latest years (Lanotte and Rioux, 1990; Tibayrenc et al., 1990; Gibson and Garside, 1991; Panton et al., 1991; Blaineau et al., 1992; Kreutzer et al., 1994; Mathieu-Daudé et al., 1995; Tait et al., 1996). With respect to *Leishmania*, an important role of sexual reproduction has been suggested, mainly through automixy (Blaineau et al., 1992). In contrast, according to Tibayrenc et al. (1990), reproduction of *Leishmania* is essentially clonal and patterns compatible with hybridisation between parasites of different genotypes (e.g. between *Leishmania(Leishmania)major* and *Leishmania(Leishmania)arabica* (Kelly et al., 1991), *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) panamensis* (Belli et al., 1994; Bañuls et al., 1997)) are observed rarely.

Phenotype characterisation properties of leishmanial hybrids might be useful for analysing to what extent basic biological features such as virulence, pathogenicity and dynamics of transmission are under the parasite's genetic control. Ideally, putative parents should display clear-cut phenotypic differences. This is the case for the putative *L.(V.)braziliensis*/*L.(V.)peruviana* hybrids we recently found in the Eastern Andean valley of Huanuco, Peru (Dujardin et al., 1995). Their putative parents differ eco-geographically and clinically: *L.(V.)braziliensis* circulates in the Amazonian lowlands and causes a severe form of cutaneous leishmaniasis which may evolve by metastasis to mucosal mutilations (Espundia) in up to 10% of the cases (Llanos-Cuentas, 1991), while *L.(V.)peruviana* is endemic in the Andean valleys only and is responsible for a benign form of cutaneous leishmaniasis (Uta) that never leads to Espundia (Herrer, 1962; Guerra, 1988).

The present work is a first attempt at addressing the hybrid phenotype. The in vitro growth behaviour of promastigotes characterised as *L.(V.)braziliensis*, *L.(V.)peruviana* and putative hybrids was measured. Putative hybrids and *L.(V.)peruviana* were found to grow slower and reached lower cell densities than *L.(V.)braziliensis*.

2. Materials and methods

2.1. Parasites

Five stocks from Huanuco, an Eastern Andean valley at the border between the biogeographical units (BGUs; Lamas, 1982) of Huanuco and Huallaga, Peru, have been defined as putative hybrids by their combination of genetic markers (two RAPD loci, one isoenzyme locus and the size of three chromosomes) characterising, respectively, *L.(V.)braziliensis* and *L.(V.)peruviana* from the focus of Sancos (Western Andean valley, BGU of Surco-South, Peru; Dujardin et al., 1995). These five stocks were compared with five stocks of each of their putative parents (Table 1).

The stocks qualifying for the present study were selected according to the following criteria:

1. uncloned stocks as close as possible to the initial isolate, for optimal representativity of the natural population sample;
2. within each set of parasites, all five stocks did display the same genotype as defined by the three kinds of markers mentioned above, and particularly by the size of chromosomes recognised, respectively, by DNA probes pLb-22, pLb-134Sp and pLb-168 (the most discriminant of these markers; Dujardin et al., 1993a,b): *L.(V.)braziliensis* = 1300-, 700- and 640-kb, *L.(V.)peruviana* = 1100-, 600- and 700-kb, and *L.(V.)braziliensis*/*L.(V.)peruviana* putative hybrids = 1300/1100-, 700/600- and 640/700-kb;
3. when estimating growth parameters, all the stocks, putative parents and hybrids, did show a similar number of subinoculations ($n = 10–18$, Table 1) since their primary isolation from man; and
4. the stocks were isolated either from the same focus or from the closest related one (Table 1): eight were originated from Huanuco (five hybrids and three sympatric *L.(V.)braziliensis*), five from Sancos (five *L.(V.)peruviana*) and two from the Peruvian and Bolivian Amazonian forest, respectively (two stocks of *L.(V.)braziliensis*).

Table 1
Stocks used in this study

Designation	Geographical origin	Exp.	*N*
L.(V.)braziliensis			
MHOM/PE/91/LC1417	Huanuco, Huancapallac, Limapampa	1	8
MHOM/BO/92/CUM005	Alto Beni	2	7
MHOM/PE/93/LC2141	Cuzco, Paucartambo, Pilcopata	3	13
MHOM/PE/91/LC1412	Huanuco, Huancapallac, Limapampa	4	8
MHOM/PE/91/LC1409	Huanuco, Huancapallac, Limapampa	5	7
L.(V.)peruviana			
MHOM/PE/90/LCA06	Ayacucho, Lucanas, Sancos	1	5
MHOM/PE/90/LCA08	Ayacucho, Lucanas, Sancos	2	11
MHOM/PE/90/LCA01	Ayacucho, Lucanas, Sancos	3	9
MHOM/PE/90/LCA05	Ayacucho, Lucanas, Sancos	4	11
MHOM/PE/90/LCA04	Ayacucho, Lucanas, Sancos	5	10
L.(V.)braziliensis/*L.(V.)peruviana* hybrids			
MHOM/PE/91/LC1408	Huanuco, Huancapallac, Limapampa	1	9
MHOM/PE/91/LC1419	Huanuco, Huancapallac, Puyac	2	11
MHOM/PE/91/LH1099	Huanuco, Ambo	3	10
MHOM/PE/91/LC1407	Huanuco, Huancapallac, Limapampa	4	7
MHOM/PE/91/LC1418	Huanuco, Huancapallac, Limapampa	5	9

Exp. = code number of the experimental set in which three stocks, one for each genotype, were counted in parallel; *N* = Number of passages (since primary isolation from man) of the first promastigote stabilate used for initiating counting experiments.

2.2. In vitro methodology

Five sets of three stocks, each set including one *L.(V.)braziliensis*, one *L.(V.)peruviana* and one hybrid, were constituted randomly. Each set was grown consecutively as follows. For each of the three stocks of a given set, the first promastigote stabilate since primary isolation (called S1) was retrieved from liquid nitrogen (where it was stored in 5% DMSO) and cultivated in blood agar medium (Tobie et al., 1950) at 26°C, until the end of the experiment. After five subcultures following thawing (S1 + 5) in order to eliminate the possible influence of DMSO, cultures of the three stocks were counted in a Bürker chamber and inoculated on the same day at a density of 1×10^6 parasites/ml into a flask containing 7 ml of blood agar (same batch of rabbit blood for the three stocks) and 15 ml of Locke solution. They were counted daily until day 8, when lysis usually became noticeable, with the same protocol: (i) the dilution used for counting was vortexed in order to reduce rosettes as much as possible; (ii) the sometimes remaining rosettes were not counted; and (iii) viable parasites only were considered (well-shaped promastigotes, refringent under phase contrast). For one of the five sets, a second promastigote stabilate (called S2, and prepared a few subcultures later than S1) was thawn, further maintained in vitro, and counted at five, 14 and 23 passages after thawing (respectively called S2 + 5, S2 + 14 and S2 + 23). Identity of the stocks was not known by the responsible of cultivation and counting.

2.3. Growth parameters and data processing

Two parameters are classically considered for characterising growth behaviour of a parasite in a given culture medium: growth rate during logarithmic phase; and cell density at stationary phase. For individual description of each growth curve, these two parameters were estimated, respectively, by: (i) computing the slope of the regression line between day 0 and day 3; and (ii) fitting the cell densities from day 0 to day 8 to a pK determination equation, and calculating the limit (Program Enzfit, Elsevier).

For global analysis and statistical comparison of the data from the five sets of three stocks, a covariance analysis in GLIM (Crawley, 1993) was performed, using F statistics: the outcome variable was parasite density (expressed as 10^6 promastigotes/ml) and the explanatory variables were day (treated as a continuous variable, i.e. a covariate), experiment (each one of the five sets) and genotype (three in each experiment). The covariance analysis was used for a global data interpretation: logarithmic and stationary phases were not anymore considered separately, and a linear relationship was assumed between parasite density and day, together with normal errors. These assumptions were validated by a check on the fit of the final model (Minimal Adequate Model (MAM)), i.e. by checking the plots of: (i) residuals versus fitted values, which showed a random scatter; (ii) the sorted residuals against the ordered standard normal residuals which showed a straight line; and (iii) the fitted values versus the observed values which showed a random scatter around the line $x = y$.

3. Results

3.1. Evolution of growth behaviour during in vitro maintenance

In order to estimate the influence of in vitro maintenance on growth, a first set of three stocks each characterised as *L.(V.)braziliensis* (LC1417), *L.(V.)peruviana* (LCA06) and hybrid (LC1408) between these two genotypes was constituted: (i) thawed a first time at passages 8, 5 and 9 (since primary isolation from man), respectively, and counted daily at passages +5 after thawing (S1 + 5); and (ii) thawed a second time at passages 15, 13 and 17 (since primary isolation from man), respectively, and counted daily at passages +5, +14 and +23 after thawing (respectively, called S2 + 5, S2 + 14 and S2 + 23, Fig. 1 and Table 2).

Even if absolute variations in growth parameters were observed for a given stock, among these four maintenance steps, two constants were observed independently of the passage number. Firstly, the logarithmic growth rate of *L.(V.)braziliensis* was higher than the one of hybrids. Second, average cell density at stationary phase ranked (from high to low values) from *L.(V.)braziliensis* to *L.(V.)peruviana* and then to hybrids. In contrast, the logarithmic growth rate of *L.(V.)peruviana*, which was similar to that of hybrids at the beginning of maintenance, progressively reached values characteristic of *L.(V.)braziliensis*.

3.2. Growth differences among genotypes

In order to evaluate whether the relative growth differences observed here above could be expressed reproducibly by other stocks with the same genotypes, five sets (including the one previously studied) of three stocks (each stock characterised, respectively, as *L.(V.)braziliensis*, *L.(V.)peruviana* and putative hybrid) were selected randomly and analysed successively at subculture S1 + 5 (Fig. 2, Table 2) by daily counting.

Comparison between *L.(V.)braziliensis* and hybrids showed that four out of the five hybrids growed slower and reached lower densities at stationary phase than *L.(V.)braziliensis* (Fig. 2(A–D), Table 2). For one set (set nr5), the hybrid LC1418 grew faster and reached a higher density than *L.(V.)braziliensis* (Fig. 2(E), Table 2) at the difference of the other experiments. In order to verify whether a noticeable change of genotype did take place in this uncloned stock during maintenance, its karyotype was checked before and after the experiment, and no difference was observed (not shown).

Comparison of *L.(V.)braziliensis* to *L.(V.)peruviana* showed that three out of the five *L.(V.)peruviana* isolates presented a much lower growth rate than *L.(V.)braziliensis*; one was equivalent and one higher. Four out of the five *L.(V.)peruviana* stocks reached a lower cell density at stationary phase than *L.(V.)braziliensis* (Fig. 2(A–C and E), Table 2). In one set (Fig. 2(D), Table 2), similar density was reached by *L.(V.)peruviana* LCA05 and *L.(V.)braziliensis* LC1412.

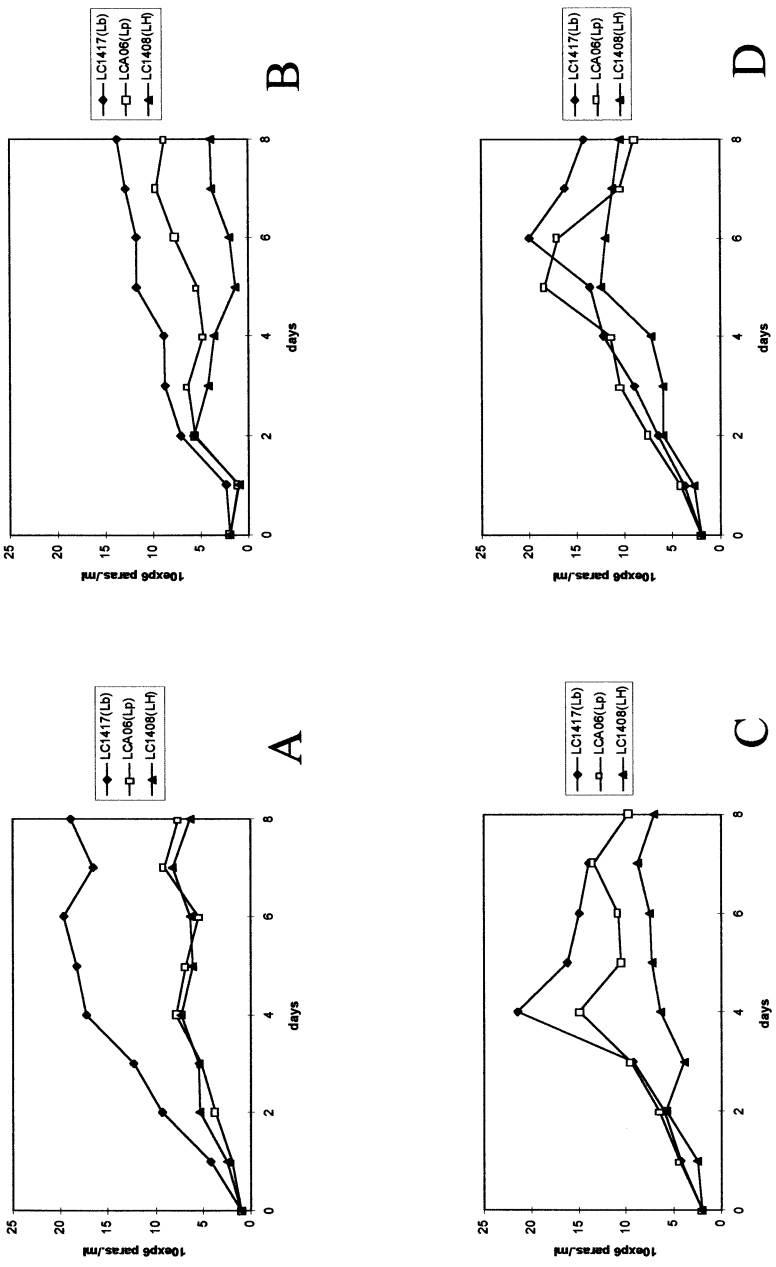

Fig. 1. Growth curves of *L.(V.)braziliensis* (LC1417), *L.(V.)peruviana* (LCA06) and putative hybrid (LC1408), at five passages after thawing of first stabilate since primary isolation (S1 + 5, A), and at five (B), 14 (C) and 23 (D) passages after thawing of second stabilate (respectively, S2 + 5, + 14 and + 23).

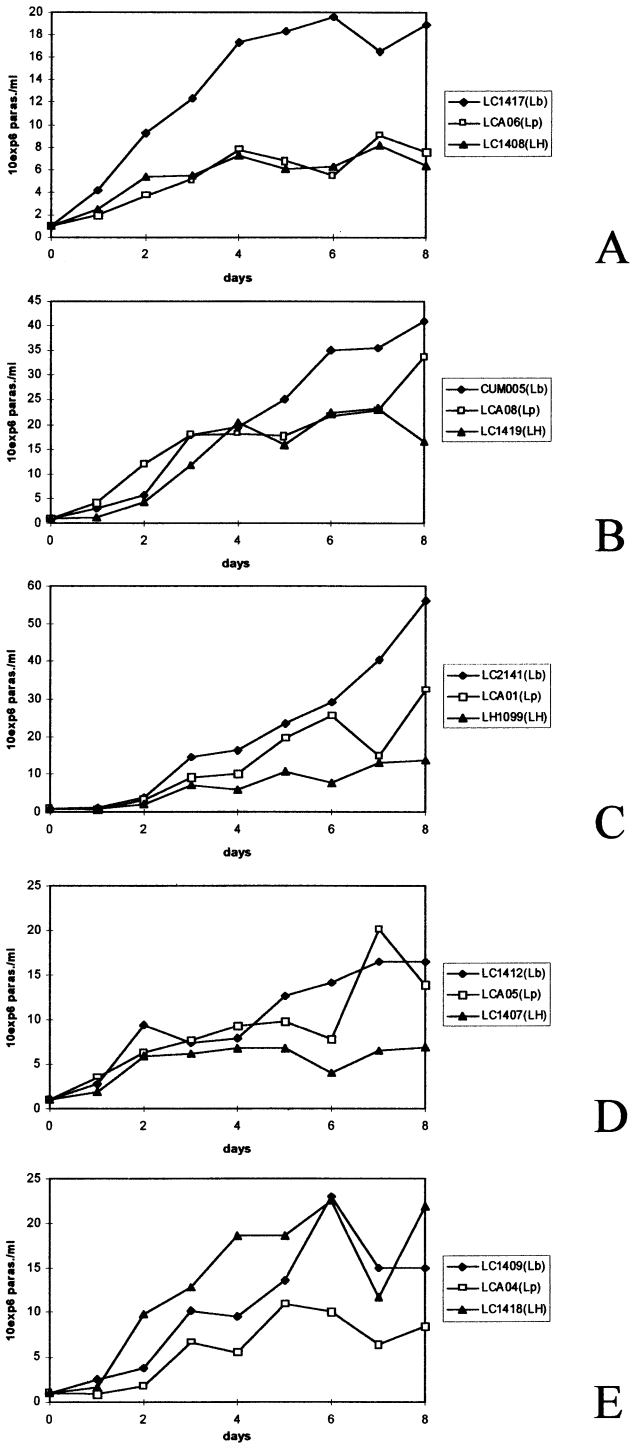

Fig. 2. Growth curves of *L.(V.)braziliensis*, *L.(V.)peruviana* and hybrids between both species in five experimental sets (A–E) each with one different isolate of the three genotypes: all measures performed at S1 + 5.

Table 2
Growth behaviour of *L.(V.)braziliensis* (*Lb*), *L.(V.)peruviana* (*Lp*) and putative hybrids (*Lb*/*Lp*)

	Slope	Limit	*N*
a. In vitro maintenance			
S1+5			
Lb LC1417	3.90	17.5	13
Lp LCA06	1.43	7.1	10
Lb/*Lp* LC1408	1.64	6.6	14
S2+5			
Lb LC1417	2.51	11.5	20
Lp LCA06	1.76	7.2	18
Lb/*Lp* LC1408	1.16	3.3	22
S2+14			
Lb LC1417	2.37	16.1	29
Lp LCA06	2.46	11.4	27
Lb/*Lp* LC1408	0.9	6.8	31
S2+23			
Lb LC1417	2.38	14.8	38
Lp LCA06	2.89	12.8	40
Lb/*Lp* LC1408	1.52	10.1	36
b. Comparison of stocks at S1+5			
Lb CUM005	5.30	32.5	12
Lp LCA08	5.85	22.4	16
Lb/*Lp* LC1419	3.52	19.8	16
Lb LC2141	4.34	40.1	18
Lp LCA01	2.74	23.3	14
Lb/*Lp* LH1099	1.97	10.4	15
Lb LC1412	2.58	12.7	13
Lp LCA05	2.25	11.6	16
Lb/*Lp* LC1407	1.93	6.2	12
Lb LC1409	2.89	15.5	12
Lp LCA04	1.79	8.4	15
Lb/*Lp* LC1418	4.36	18.1	14

Slope of the growth curves from day 0 to day 3 (millions parasites/ml) and limit (estimation of stationary phase, calculated from fit with pK-like equation, see Materials and methods; millions parasites/ml) from growth curve plots shown in Figs. 1 and 2; *N*, number of passages since primary isolation at the moment of growth parameter measuring; S1 and S2, respectively first and second promastigote stabilate since primary isolation.

Comparison of *L.(V.)peruviana* to hybrids showed less pronounced differences (Table 2). Logarithmic growth rate was higher for *L.(V.)peruviana* in 3/5 cases and lower in the two other ones. At stationary phase cell density was higher for *L.(V.)peruviana* in 4/5 cases; one hybrid, LC1418 presented a higher density.

Covariance analysis of data from the five experiments did not show any significant difference in growth rate between *L.(V.)peruviana* and hybrids. Accordingly,

both genotypes were considered together and compared to *L.(V.)braziliensis*. A MAM was generated, where the three explanatory variables (day, experiment and genotype) were found to be significant. After accounting for variation in parasite density with time and experiment, genotypic difference (*L.(V.)braziliensis* vs. *L.(V.)peruviana*/hybrids) was shown to explain 28% (1548/5456) of the remaining variance, a result significant at a *P*-value less than 0.01 (*F* statistics, Table 3). Altogether, these results indicate thus that at S1 + 5, in our experimental conditions, the average growth behaviour of *L.(V.)peruviana* and hybrids was similar, and that both sets of stocks showed a lower capacity for in vitro growth than *L.(V.)braziliensis*.

4. Discussion

Difference of growth behaviour among *Leishmania* species is a well-documented fact. The representatives of subgenus *Viannia*, the former *braziliensis* complex, are less prolific than those of the *mexicana* complex and this criterion has been used for their biological discrimination (Lainson and Shaw, 1987). To our knowledge, however, no study has compared the behaviour of subgeneric genetic populations nor hybrids (Kelly et al., 1991; Belli et al., 1994; Bañuls et al., 1997) with their putative parents.

In the present work, this question was addressed by comparing three categories of closely related parasites: *L.(V.)braziliensis*, *L.(V.)peruviana* and their putative hybrids, all characterised previously with a large set of genetic markers (Dujardin et al., 1995). With a collection of 15 stocks (five for each category of parasites) under strict and comparable experimental conditions (five passages after thawing, ten to 18 passages since primary isolation from man), *L.(V.)braziliensis*/*L.(V.)peruviana* hybrids and *L.(V.)peruviana* showed an average lower growth capacity than *L.(V.)braziliensis*. Furthermore, in vitro maintenance of one set of three stocks through 23 passages after thawing did not affect the lower growth capacity of the hybrids while a progressive decrease in the relative growth differences between *L.(V.)braziliensis* and *L.(V.)peruviana* did take place.

Table 3
Growth behaviour of *L.(V.)braziliensis*, *L.(V.)peruviana* and putative hybrids

Explanatory variables	Deviance explained	*F*(df)	*P*
Day	5661	133.8(1,129)	<0.01
Experiment	1579	12.9(4,128)	<0.01
Genotype-day interaction	1548	50.7(1,128)	<0.01
Residual	3908		
Total	12 695		

Covariance analysis of data from five experiments involving different stocks of the three genotypes (Table 2); due to a similar growth rate between *L.(V.)peruviana* and hybrids, both genotypes were considered together and compared to *L.(V.)braziliensis*; *F* statistics (df = respective degrees of freedom).

Exceptions (1/5 hybrids and 1/5 *L.(V.)peruviana*) to this general trend can be accounted for by various reasons: (i) differences in adaptation to in vitro cultivation as suggested by *L.(V.)peruviana* during prolonged in vitro maintenance; (ii) uncontrolled differences in experimental procedures; and chiefly, (iii) unidentified genetic differences. All the stocks of *L.(V.)braziliensis*, *L.(V.)peruviana* and hybrids were selected within each group of organisms according to a same or very similar genotype (molecular karyotyping, MLEE and RAPD; Bañuls, 1993; Dujardin et al., 1993b, 1995). However, small genetic differences not apprehended by the characterisation tools we used cannot be excluded and might have played a role in the generation of these exceptions. This possibility is sustained by the fact that some of the stocks here studied were not sympatric. Genetic differences within a given stock should also be considered. Indeed, all the stocks here analysed were willingly not cloned in order to be as close as possible from the population present at isolation time, and a new genetic subpopulation (e.g. in the case of the 'abnormally' fast growing hybrid LC1418) could have arisen during maintenance, by outgrowing of an initially minor subpopulation or by genomic rearrangement. However, within the limits of the discriminatory power of our characterisation methods, this hypothesis was not validated.

Despite of these few exceptions, the general trend indicated by our results appears to be significative and deserves several comments. A low in vitro growth of putative hybrids is not unique to *Leishmania*. In a recent study of *Trypanosoma cruzi* (Laurent et al., 1997), parasites of clonet 39 showed the highest doubling time and the lowest concentration at the end of the log phase. This genotype is a putative *T. cruzi* hybrid (Souto et al., 1996) and it was also characterised by several different-sized homologous chromosomes (Henriksson and Dujardin, unpublished results). The lowered in vitro growth of hybrids could be accounted for by two mechanisms: (i) dominance of growth characters from the slowly growing parent (*L.(V.)peruviana* in this paper); or (ii) hybrid breakdown as observed in other Eukaryotes (Li et al., 1997). The latter phenomenon can be promoted by differences in gene organisation in the two homologues of a chromosome. This possibility should be further explored, as for three chromosomes of the *Leishmania* here studied, size difference between variants of putative parents was accompanied by changes in the organisation of important genes (respectively: rDNA, Inga et al., 1998; gp63, Victoir et al., 1995; mini-exon, Dujardin, unpublished results).

There is an apparent paradox between data obtained in vitro and data from natural populations. The lower growth capacity of hybrids we have observed in vitro should correspond to a low isolation rate of these hybrids from natural infection, and so the more in mixed infections where the quickly growing parent (here, *L.(V.)braziliensis*) could be present. At the contrary, in our initial sampling in Huanuco (1991), out of seven isolates, four were putative hybrids and three were *L.(V.)braziliensis* (Dujardin et al., 1995). A few years later the putative hybrids still represented a relatively high proportion of the characterised isolates made from patients in Huanuco (Nolder, D. and Davies, C.R., personal communication). Hybrids could have been better fit to the conditions of in vitro isolation: isolation medium was very similar to the Tobie here used, but the influence of extrinsic

factors was not considered, such as the average temperature prevailing in the field at the time of isolation, as well as frequency of subinoculations. Alternatively, mixed infections with *L.(V.)braziliensis* and hybrids might well be very rare, and this fact could be in favour of distinct eco-epidemiological cycles.

At this stage however, it has to be kept in mind that in vitro growth behaviour of promastigotes does not necessarily correspond to the behaviour of promastigotes in natural conditions: different sandfly species with different promastigote-interacting lectins (Svobodova et al., 1996) or different feeding behaviours (Cameron et al., 1995). Further studies in other experimental conditions closer to natural conditions—development in macrophages and in the vector—will help explore further hybrid fitness and their dynamics of transmission relevant to natural populations.

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References

Bañuls, A.L., 1993. Analyse génétique d'isolats naturels sud-américains de Leishmania montrant de possibles phénomènes de recombinaison. DEA thesis, Université de Montpellier II.

Bañuls, A.L., Guerrini, F., Le Pont, F., Barrera, C., Espinel, I., Guderian, R., Echeverria, R., Tibayrenc, M., 1997. Evidence for hybridisation by multilocus enzyme electrophoresis and random amplified polymorphic DNA between *Leishmania braziliensis* and *Leishmania panamensis/guyanensis* in Ecuador. J. Euk. Microbiol. 44, 408–411.

Belli, A.A., Miles, M.A., Kelly, J.M., 1994. A putative *Leishmania panamensis/Leishmania braziliensis* hybrid is a causative agent of human cutaneous leishmaniasis in Nicaragua. Parasitology 109, 435–442.

Blaineau, C., Bastien, P., Pagès, M., 1992. Multiple forms of chromosomes I, II and V in a restricted population of *Leishmania infantum* contrasting with monomorphism in individual strains suggest haploidy or automixy. Mol. Biochem. Parasitol. 50, 197–204.

Cameron, M.M., Milligan, P.J.M., Llanos-Cuentas, A., Davies, C.R., 1995. An association between phlebotomine sandflies and aphids in the Peruvian Andes. Med. Vet. Entomol. 9, 127–132.

Crawley, M.J., 1993. GLIM for Ecologists. Blackwell Science, Oxford, 379 pp.

Dujardin, J.C., Gajendran, N., Arevalo, J., Llanos-Cuentas, A., Guerra, H., Gomez, J., Arroyo, J., De Doncker, S., Jacquet, D., Hamers, R., Le Ray, D., 1993a. Karyotype polymorphism and conserved characters in the *Leishmania (Viannia) braziliensis* complex explored with chromosome-derived probes. Ann. Soc. Belge Méd. Trop. 73, 101–118.

Dujardin, J.C., Llanos-Cuentas, A., Caceres, A., Arana, M., Dujardin, J.P., Guerrini, F., Gomez, J., Arroyo, J., De Doncker, S., Jacquet, D., Hamers, R., Guerra, H., Le Ray, D., Arevalo, J., 1993b. Molecular karyotype variation of *Leishmania (Viannia) peruviana* evidences geographical populations in Peru along a North–South cline. Ann. Trop. Med. Parasitol. 87, 335–347.

Dujardin, J.C., Bañuls, A.L., Llanos-Cuentas, A., Alvarez, E., DeDoncker, S., Jacquet, D., Le Ray, D., Arevalo, J., Tibayrenc, M., 1995. Putative *Leishmania* hybrids in the Eastern Andean valley of Huanuco. Peru. Acta Tropica 59, 293–307.

Gibson, W.C., Garside, L.H., 1991. Genetic exchange in *Trypanosoma brucei brucei*: variable chromosomal location of housekeeping genes in different trypanosome stocks. Mol. Biochem. Parasitol. 45, 77–90.

Guerra, H., 1988. Distribution of *Leishmania* in Peru. In: Walton, B.C., Wijeyaratne, P.M., Modabber, F. (Eds.), Research on Control Strategies for the Leishmaniases, IDRC-MR 184e. Ottawa, pp. 135–145.

Herrer, A., 1962. The incidence of uta (cutaneous leishmaniasis) among the child population of Peru. Sci. Repts Ist. Super. Sanità 2, 131–137.

Inga, R., De Doncker, S., Gomez, J., Lopez, M., Garcia, R., Le Ray, D., Arevalo, J., Dujardin, J.C., 1998. Relation between variation in copy number of ribosomal RNA encoding genes and size of harbouring chromosomes in *Leishmania* of subgenus *Viannia*. Mol. Biochem. Parasitol. 92, 219–228.

Kelly, J.M., Law, J.M., Chapman, C.J., Van Eys, G.J.J.M., Evans, D.A., 1991. Evidence of genetic recombination in *Leishmania*. Mol. Biochem. Parasitol. 46, 253–264.

Kreutzer, R.D., Yemma, J.J., Grogl, M., Tesh, R.B., Martin, T.I., 1994. Evidence of sexual reproduction in the Protozoan parasite *Leishmania* (Kinetoplastida: Trypanosomatidae). Am. J. Trop. Med. Hyg. 51, 301–307.

Lamas, G., 1982. A preliminary zoogeographical division of Peru, based on butterfly distributions (Lepidoptera, Papilionoidea). In: Prance, T.P. (Ed.), Biological Diversification in the Tropics. Columbia University Press, New York, pp. 336–357.

Lainson, R., Shaw, J.J., 1987. Evolution, classification and geographical distribution. In: Peters, W., Killick-Kendrick, R. (Eds.), The Leishmaniases in Biology and Medicine. Academic Press, London, pp. 1–120.

Lanotte, G., Rioux, J., 1990. Fusion cellulaire chez les *Leishmania* (Kinetoplastida, Trypanosomatidae). C. R. Acad. Sci. Paris 310, 285–288.

Laurent, J.P., Barnabe, C., Quesney, V., Noel, S., Tibayrenc, M., 1997. Impact of clonal evolution on the biological diversity of *Trypanosoma cruzi*. Parasitology 114, 213–218.

Li, Z., Pinson, S.R., Paterson, A.H., Park, W.D., Stansel, J.W., 1997. Genetics of hybrid sterility and hybrid breakdown in an intersubspecific rice (*Oriza sativa* L.) population. Genetics 145, 1139–1148.

Llanos-Cuentas, E.A., 1991. Tratamiento de Leishmaniasis Mucosa: Analisis de los Factores Asociados con la Repuesta Terapeutica a los Antimoniales Pentavalentes. Tesis de Doctorado, Universidad Peruana Cayetano Heredia, Lima.

Mathieu-Daudé, F., Stevens, J., Welsh, J., Tibayrenc, M., McClelland, M., 1995. Genetic diversity and population structure of *Trypanosoma brucei*: clonality versus sexuality. Mol. Biochem. Parasitol. 72, 89–101.

Panton, L.J., Tesh, R.B., Nadeau, K.C., Beverley, S.M., 1991. A test for genetic exchange in mixed infections of *Leishmania major* in the sand fly *Phlebotomus papatasi*. J. Protozool. 38, 224–228.

Souto, R.P., Fernandes, O., Macedo, A.M., Campbell, D.A., Zingales, B., 1996. DNA markers define two major phylogenetic lineages of *Trypanosoma cruzi*. Mol. Biochem. Parasitol. 83, 141–152.

Svobodova, M., Volf, P., Killick-Kendrick, R., 1996. Agglutination of *Leishmania* promastigotes by midgut lectins from various species of phlebotomine sandflies. Ann. Trop. Med. Parasitol. 90, 329–336.

Tait, A., Buchanan, N., Hide, G., Turner, C.M.R., 1996. Self-fertilisation in *Trypanosoma brucei*. Mol. Biochem. Parasitol. 76, 31–42.

Tibayrenc, M., Kjelleberg, F., Ayala, F.J., 1990. A clonal theory of parasitic protozoa: The population structures of *Entamoeba*, *Giardia*, *Leishmania*, *Naegleria*, *Plasmodium*, *Trichomonas*, and *Trypanosoma* and their medical and taxonomical consequences. Proc. Natl. Acad. Sci. USA 87, 2414–2418.

Tobie, E.J., Von brand, T., Mehlman, B., 1950. Cultural and physiological observations on *Trypanosoma rhodesiense* and *Trypanosoma gambiense*. J. Parasitol. 36, 48–54.

Victoir, K., Dujardin, J.C., De Doncker, S., Barker, D.C., Arevalo, J., Hamers, R., Le Ray, D., 1995. Plasticity of gp63 gene organisation in *Leishmania (Viannia) braziliensis* and *Leishmania (Viannia) peruviana*. Parasitology 111, 265–273.