Evaluation of an *in vitro* and *in vivo* model for experimental infection with *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana*

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SUMMARY

Leishmania (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana* are two parasite species characterized by a very different pathogenicity in humans despite a high genetic similarity. We hypothesized previously that *L.* (*V.*) *peruviana* would descend from *L.* (*V.*) *braziliensis* and would have acquired its '*peruviana*' character during the southward colonization and adaptation of the transmission cycle in the Peruvian Andes. In order to have a first appreciation of the differences in virulence between both species, we evaluated an *in vitro* and *in vivo* model for experimental infection. A procedure was adapted to enrich culture forms in infective stages and the purified metacyclics were used to infect macrophage cell lines and golden hamsters. The models were tested with 2 representative strains of *L.* (*V.*) *braziliensis* from cutaneous and mucosal origin respectively and 2 representative strains of *L.* (*V.*) *peruviana* from Northern and Southern Peru respectively. Our models were reproducible and sensitive enough to detect phenotypic differences among strains. We showed *in vitro* as well as *in vivo* that the *L.* (*V.*) *braziliensis* was more infective than *L.* (*V.*) *peruviana*. Furthermore, we found that *in vitro* infectivity patterns of the 4 strains analysed, were in agreement with the geographical structuring of parasite populations demonstrated in our previous studies. Further work is needed to confirm our results with more strains of different geographical origin and their specific clinical outcome. However, our data open new perspectives for understanding the process of speciation in *Leishmania* and its implications in terms of pathogenicity.

Key words: *Leishmania* (*V.*) *braziliensis*, *Leishmania* (*V.*) *peruviana*, infectivity, virulence, macrophage, hamster.

INTRODUCTION

The genus *Leishmania* contains parasite species causing a spectrum of clinical phenotypes in humans. Understanding the factors that underlie this pleomorphism is important for the design of rational diagnosis and surveillance strategies, as well as for the development of new drugs and vaccines. Obviously, parasite and host factors interact in producing a clinical phenotype (de Almeida *et al.* 2003) and their elucidation requires, among others, good natural and experimental models.

The 2 neotropical species *Leishmania* (*Viannia*) *braziliensis* and *L.* (*V.*) *peruviana* fit perfectly in this context. The former is the most aggressive species of subgenus *Viannia*, causing severe cutaneous and mucosal lesions, in patients throughout the Amazonian basin (Guerra, 1988). In contrast, *L.* (*V.*) *peruviana* causes benign cutaneous lesions and it was never found to be associated with the mucosal phenotype (Lucas *et al.* 1998). To date, these species have only been encountered in Peru, being (essentially) endemic in the Andean highlands (between 800 and 3000 metres above sea level). Despite these major clinical differences, the 2 parasites are genetically very similar: a few markers only allow their discrimination (Dujardin *et al.* 1995*a*; Victoir *et al.* 1995; Bañuls *et al.* 2000; Garcia *et al.* 2005; Zhang *et al.* 2006). On the basis of karyotype data, it has been hypothesized that *L.* (*V.*) *peruviana* would descend from *L.* (*V.*) *braziliensis* and would have acquired its 'peruviana' character during the southward colonization of the transmission cycle in the Peruvian Andes (Dujardin *et al.* 1993, 1995*a*, *b*, 1998, 2000, 2002).

Molecular studies showed that some of the genetic characters distinguishing both species concerned genes encoding putative virulence factors (virulence

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being defined as the degree of pathogenicity of a microorganism genetically endowed with that capacity, as manifested against a host with an intact immune system under normal conditions; Chang *et al*. 2003): the metalloprotease gp63 (Victoir *et al*. 1995) and the cysteine proteinase b (Garcia *et al*. 2005). This might support the contribution of parasite factors to the difference in pathogenicity observed in humans. However, these molecular markers might be confounding factors and further documentation of the role of the parasite and host should come from experimental studies *in vitro* and *in vivo* but these were mainly done for *Leishmania* strains from subgenus *Leishmania* (mostly *L*. (*L*.) *major*) (Garin *et al*. 2001; Achour *et al*. 2002; Baldwin *et al*. 2003; Vladimirov *et al*. 2003; Sádlová *et al*. 2006).

In the present study, we developed *in vitro* and *in vivo* models for experimental infections and compared the phenotypic differences between *L*. (*V*.) *braziliensis* and *L*. (*V*.) *peruviana* strains. To that end, we selected 4 clinical isolates from Peru: (i) 2 representative strains of *L*. (*V*.) *braziliensis* isolated from patients with cutaneous and mucosal lesions, respectively and (ii) 2 representative strains of *L*. (*V*.) *peruviana* originating from the North and the South of the Andean endemic area. A key issue for this type of experimental study is to work with populations that are as homogenous as possible. Therefore, a procedure was adapted to enrich infective-stage parasites (metacyclics) in culture. These purified parasites (homogeneous population) were used to infect macrophage cell lines as well as golden hamsters. We showed *in vitro* as well as *in vivo* that under our experimental conditions the *L*. (*V*.) *braziliensis* isolates used here were more infective than the *L*. (*V*.) *peruviana* ones.

MATERIALS AND METHODS

Leishmania *strains*

The 4 representative strains used in the present study were isolated from Peruvian patients with cutaneous or mucosal lesions. The *L*. (*V*.) *braziliensis* were sympatric isolates originating from the Amazonian basin: MHOM/PE/91/LC2043 (mucosal origin), and MHOM/PE/91/LC2177 (cutaneous origin); the *L*. (*V*.) *peruviana* isolates were both of cutaneous origin: MHOM/PE/90/HB86 (Andes from the North) and MHOM/PE/90/LCA08 (Andes from the South). In order to ensure the homogeneity of the parasite population, the strains were cloned by the micro-drop method (Van Meirvenne *et al*. 1975) and characterized by multi-locus enzyme electrophoresis (13 enzymes, Bañuls, 1998). Identity of the strains was confirmed by Pulsed Field Gradient Electrophoresis before the beginning of the experiments, as described elsewhere (Dujardin *et al*. 1987). For each strain, cryostabilates made from parasites with a minimum number of sub-inoculations were thawed and first cultivated at 26 °C in a biphasic agar medium supplemented with 15% defibrinated rabbit blood and 0·85% saline solution. They were then adapted to grow at 26 °C in an enriched medium, M199 (Sigma), supplemented with 20% (v/v) heat-inactivated fetal calf serum (FCS) and adjusted to pH 7·4. In order to have enough parasites for the experiments, promastigotes at early stationary phase (1×10^6 parasites/ml) were subcultivated into 2 bottles (50 ml each) with medium M199, one at pH7·4 and the other adjusted to pH 5·5 (modified method by Bates and Tetley, 1993; Zakai *et al*. 1998; Almeida *et al*. 1993), and both were incubated at 26 °C. The growth curve was monitored by daily counting of parasites using a Neubauer brightline haemocytometer.

Ficoll centrifugation gradient

In order to obtain purified metacyclic parasites from the pH 5·5 culture, we adapted the method described by Späth and Beverley (2001). Ficoll Type 400 (Sigma) was used to prepare a 20% stock solution. The gradient was formed using Ficoll 20% at the bottom and Ficoll 10% on the top of a 15 ml or 50 ml tube, according to the volume of parasite culture to be purified. The parasite culture at early stationary phase (4th day of culture, according to the growth curve) was resuspended in M199 (2–6 ml according to the parasite concentration) and poured slowly on the top of the Ficoll 10% and centrifugated at 2500 rpm for 15 min. After centrifugation, the fraction above or inside the Ficoll 10% was recovered, and an aliquot was observed under the microscope to count and verify the morphology and viability of the parasites. The remaining fraction of the purified parasites was used to evaluate their resistance to complement, and their infectivity *in vitro* and *in vivo*. From now on, these purified parasites will be called 'Met-pH5·5'. The metacyclic population has become relatively more homogeneous in comparison with the stationary promastigotes where mixed populations coexist. The homogenization process achieved by the Ficoll gradient centrifugation experiment facilitates separation of the parasites based on the physical properties: body and flagellum size, body shape (characteristics of the metacyclic forms) (Bates and Tetley, 1993).

Complement lysis test

Resistance to complement lysis of the Met-pH5·5 parasites and promastigotes obtained from stationary phase culture at pH 7·4 (4th day according to the growth curve, and from now on called 'Pro-pH7·4') was tested. The parasites (3×10^6) were exposed to serial dilutions of human serum from a healthy

individual (from 1 : 1 to 1 : 128 in phosphate buffer solution). In order to estimate the percentage of surviving parasites, we counted in duplicate the parasites showing flagellar movement using a Neubauer brightline haemocytometer. With the program Probit SPSS 3.2, the concentration of serum that produces 50% parasite lysis (LD_{50}) was estimated. This experiment was carried out in duplicate to ensure the reproducibility of the counting.

In vitro *infections*

For the *in vitro* infections 3 independent experiments were performed using the macrophage cell line RAW 264 (murine origin), which were infected with the Met-pH5·5 or Pro-pH7·4 parasites in a ratio of 30 : 1 (parasites : macrophage). After 2 h incubation at 34 °C and 5% CO_2, free parasites were removed by repeated washings and the cultures were incubated for an additional 3 days at 34 °C and 5% CO_2. The infection was monitored (24 h, 48 h and 72 h post-infection) using parallel culture plates which were fixed with 0·25% glutaraldehyde and stained with Giemsa and May-Grünwald (dilution 1 : 4) in order to visualize the intracellular amastigotes. Up to 200 cells were counted in order to determinate the percentage of infected macrophages and the average number of amastigotes by infected macrophages. The product of the 2 previous parameters was used to define an infection index.

In vivo *infections*

In this case, for each species, we used the strain with the higher rate of *in vitro* infectivity. For each strain, 15 male golden hamsters (*Mesocricetus auratus*) of 4 weeks age, obtained from the Peruvian National Institute of Health, were randomly distributed in 3 groups, 5 animals in each, (i) one group inoculated at the right footpad with 1×10^6 promastigotes at stationary phase (Pro-pH7·4) resuspended in 100 μl of saline solution, (ii) the second group inoculated with 1×10^6 purified metacyclic promastigotes (Met-pH5·5) resuspended in 100 μl of saline solution and (iii) the third group inoculated at the left footpad with 100 μl of saline solution as a control. The diameter of the lesions was measured with a micrometer (0–25 mm), with a precision of 0·01 mm, every 2 weeks at the beginning of the experiment, and every week later on.

Statistical analysis

The data were reported as the average $\pm$ S.D. and using the PROBIT program to calculate the LD_{50} for the complement test. The *in vitro* and *in vivo* data did not have a normal distribution, so a non-parametric test i.e. the Two-sample Wilcoxon Rank-sum (Mann-Whitney) was used for comparing both *in vitro* and *in vivo* data. The differences were considered as statistically significant at $P < 0{\cdot}05$.

RESULTS

Enrichment and purification of metacyclics

For the 4 representative strains used here, enrichment and purification of metacyclics parasite forms (by parasite culture at pH 5·5 and using the Ficoll centrifugation gradient, respectively) were verified by comparing 3 parameters between Pro-pH7·4 and Met-pH5·5 parasites (Table 1). (1) The complement lysis test showed that twice as many Met-pH5·5 parasites survived serum concentrations compared to Pro-pH7·4 parasites. (2) Macrophage *in vitro* infection showed infection indices that were mostly higher for Met-pH5·5 parasites, and that the effect was more pronounced for the 48 h and 72 h infection groups, with differences that were statistically significant for all strains except for LCA08 at 48 h post-infection (95% confidence interval). (3) *In vivo* infection of hamsters revealed that lesions induced by Met-pH5·5 parasites were larger than those induced by Pro-pH7·4 parasites. For LC2043, differences were statistically significant (ANOVA, $P < 0{\cdot}05$) from the 2nd week post-infection until the end of the experiment (6 weeks follow up). Metacyclics of HB86 also showed significant differences at the 14th week post-infection (ANOVA, $P < 0{\cdot}05$). The maximum difference between Met-pH5·5 and Pro-pH7·4 size lesion was 3·16 mm (5th week post-infection) and 0·70 mm (14th week post-infection) for LC2043 and HB86 respectively. The difference between lesion pattern produced by Pro-pH7·4 and Met-pH5·5 parasites was larger in *L.* (*V.*) *braziliensis* than in *L.* (*V.*) *peruviana*, which could be explained by a higher proportion of metacyclics in Pro-pH7·4 parasites of HB86 used for the infection than the corresponding parasites of LC2043.

Infection differences between L. (V.) braziliensis *and* L. (V.) peruviana *representative strains*

To compare virulence between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana*, we compared *in vitro* and *in vivo* results obtained with Met-pH5·5 parasites from the representative strains assayed here. These representative strains were chosen because of their genomic and genetic characteristics very well described elsewhere (Dujardin *et al.* 1995*b*; Bañuls *et al.* 2000). (i) The *in vitro* infectivity was expressed as the infection index rate at 24 h, 48 h, and 72 h post-infection of each representative strain. In all 3 independent experiments for each strain, the 4 strains ranked as following (from high to low): LC2043, LC2177, HB86 and LCA08 (Table 1 and

Table 1. Results for *in vitro* (panel A) and *in vivo* (panel B) infections using stationary non-purified promastigotes (Pro-pH7·4) and purified metacyclics (Met-pH5·5) from *Leishmania* (*Viannia*) *braziliensis* (Lb) and *L.* (*Viannia*) *peruviana* (Lp)

(*Program Probit SPSS 3.2 used to calculate the LD_{50} for the complement test. ** Differences in the *in vitro* infections using 95% confidence interval. *** Differences statistically significant in the *in vivo* infections (ANOVA; $P<0{\cdot}05$, $n=5$).)

Panel A.

		Enrichment metacyclics			*In vitro* infections (Infection index)					
		Serum % producing 50% lysis (95% confidence*)			24 h		48 h		72 h	
Strain		Pro-pH7·4	Met-pH5·5	Fold-time increased	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5
Lb	LC2043	0·059	0·16	2·7	170·43±6·74**	232·06±8·8**	235·78±8·36**	457·99±14·08**	368·37±12·77**	563·04±15·90**
Lb	LC2177	0·069	0·153	2·2	165·00±6·37	160·59±5·83	231·12±8·19**	301·96±9·3**	366·03±13·26**	491·6±15·04**
Lp	HB86	0·056	0·107	1·91	106·64±4·15**	148·11±5·9**	190·92±6·53**	236·74±8·22**	281·41±9·55**	379·5±13·63**
Lp	LCA08	0·069	0·136	1·97	86·66±3·38	97·62±3·87	150·54±5·28	148·25±6·07	136·78±5·06**	188·46±6·5**

Panel B.

	Time post-infection (*in vivo*)									
Strain	2 weeks***		3 weeks***		4 weeks***		5 weeks***		6 weeks***	
Lb	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5
LC2043	0±0	2·21±0·56	0·13±0·12	3·29±0·48	0·25±0·21	3·24±0·54	0·16±0·19	3·3±0·31	0·21±0·36	2·77±0·24
	11 weeks		12 weeks		13 weeks		14 weeks***			
Lp	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5	Pro-pH7·4	Met-pH5·5		
HB86	0·44±0·48	0·92±0·12	0·75±0·40	1·1±0·09	0·99±0·57	1·6±0·66	1·06±0·24	1·76±0·47		

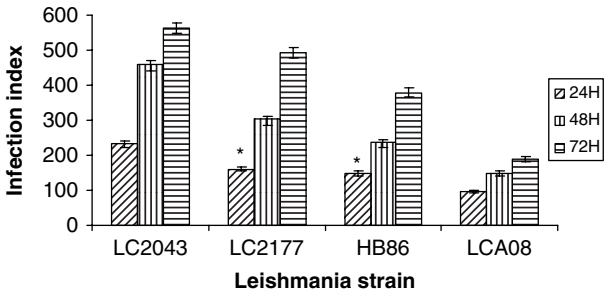

Fig. 1. *In vitro* infection of macrophages with purified metacyclic promastigotes: infection indices at different times post-infection for the *L.* (*V.*) *braziliensis* (LC2043 and LC2177) and *L.* (*V.*) *peruviana* (HB86 and LCA08) strains. For a given time-point, differences between strains were statistically different ($P=0{\cdot}000$, Mann-Whitney test) except for LC2177 and HB86 at 24 h (*).

Fig. 1). Noteworthy was that, despite the fact that the experiments were carried out with different culture batches of parasites and macrophages, the *in vitro* infection parameters were quite reproducible. Thus, for example, the average number of amastigotes per macrophage followed the same trend, at 72 h the values were $6{\cdot}83\pm0{\cdot}73$ (LC2043), $6{\cdot}3\pm0{\cdot}26$ (LC2177), $5{\cdot}3\pm0{\cdot}26$ (HB86) and $3{\cdot}2\pm0{\cdot}26$ (LCA08). A similar trend was observed with the percentage of infected macrophages (data not shown). Statistically, infection indices differed significantly between the 4 strains. This was observed at different times except for LC2177 and HB86 at 24 h post-infection (95% confidence interval). (ii) The *in vivo* infection experimental model was carried out with the more infective representative strain from each species observed after the *in vitro* experiments. In the case of the LC2043-infected animals, the development of the lesion was faster (2 weeks post-infection) than in the animals infected with HB86 (14 weeks post-infection). In addition, LC2043 lesions were larger in size than those produced by HB86 (maximum size lesion: 3·32 mm and 1·76 mm respectively) (Fig. 2A and B respectively). To assure that the lesion was due to the presence of the parasite, an aspirate from the lesion was taken after 1 month of infection and placed on blood agar medium, cultures were positives after 1 week of incubation (data not shown).

DISCUSSION

In the presented work, an experimental model was adapted and evaluated for analysing the *in vitro* and *in vivo* infectivity of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* representative strains. These two parasite species cause dramatically different pathologies in humans, despite their great genetic similarity.

The use of purified metacyclic promastigotes is very important for infectivity studies, not only because they indeed represent the infective forms inoculated by the vector, but also because they represent a well-defined and relatively homogeneous population. Such a population is essential to compare different *Leishmania* strains at a corresponding developmental stage. So far, most studies on metacyclogenesis have focused on parasites of subgenus *Leishmania*: *L.* (*L.*) *major*, *L.* (*L.*) *donovani*, *L.* (*L.*) *tropica*, *L.* (*L.*) *infantum* and *L.* (*L.*) *mexicana* (Bates and Tetley, 1993; Zakai *et al.* 1998). In comparison there are fewer reports on parasites of the subgenus *Viannia* (Almeida *et al.* 1993; Pinto-da-Silva *et al.* 2002), despite the fact that the latter comprise some species highly pathogenic for humans. Our experimental procedure was adapted from that evaluated in subgenus *Leishmania* species and combined the acidic induction of metacyclogenesis (Bates and Tetley, 1993; Zakai *et al.* 1998) with the purification of metacyclics by a Ficoll gradient centrifugation method (Späth and Beverley, 2001). Production of *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* metacyclics was verified by measuring 3 parameters (and comparison with non-purified stationary promastigotes): (i) increased resistance to complement lysis, (ii) higher infection indices in macrophages and (iii) larger lesions in hamsters. All parameters indicated an homogeneous metacyclic population in the strains tested here. Previous work done by our group identified a molecular marker (the infective-insect stage protein also known as the Meta 1 protein) using metacyclic-enriched preparations (without purification) of *L.* (*V.*) *braziliensis* LC2043 (also produced by acid induction, Gamboa *et al.* 2007), one of the strains here studied. This marker, and maybe others identified in the future, might be studied by measuring their expression profile using purified preparations of metacyclic parasites from this and other *Leishmania* species from subgenus *Viannia* using the methodology described here (manuscript in preparation).

Using purified metacyclics as sources of infection, comparison of *in vitro* and *in vivo* patterns could be made between *L.* (*V.*) *braziliensis* and *L.* (*V.*) *peruviana* representative strains, giving us the real image of the infection capacity of these strains, that normally hide when mixed parasite populations are used. *In vitro* (tested for 4 representative strains) as well as *in vivo* (2 representative strains), it was clear that for the representative strains tested here, *L.* (*V.*) *braziliensis* was more infective than *L.* (*V.*) *peruviana*, showing higher infection indices and faster appearance and larger size of lesions respectively. This correlates with the clinical outcome observed for *L.* (*V.*) *braziliensis*, associated with severe cutaneous and mucosal lesions in the Amazonian basin (Guerra, 1988). Meanwhile, *L.* (*V.*) *peruviana* produces benign cutaneous lesions, never associated with the mucosal phenotype (Lucas *et al.* 1998). Thus our model was sensitive enough to detect phenotypic differences among the representative

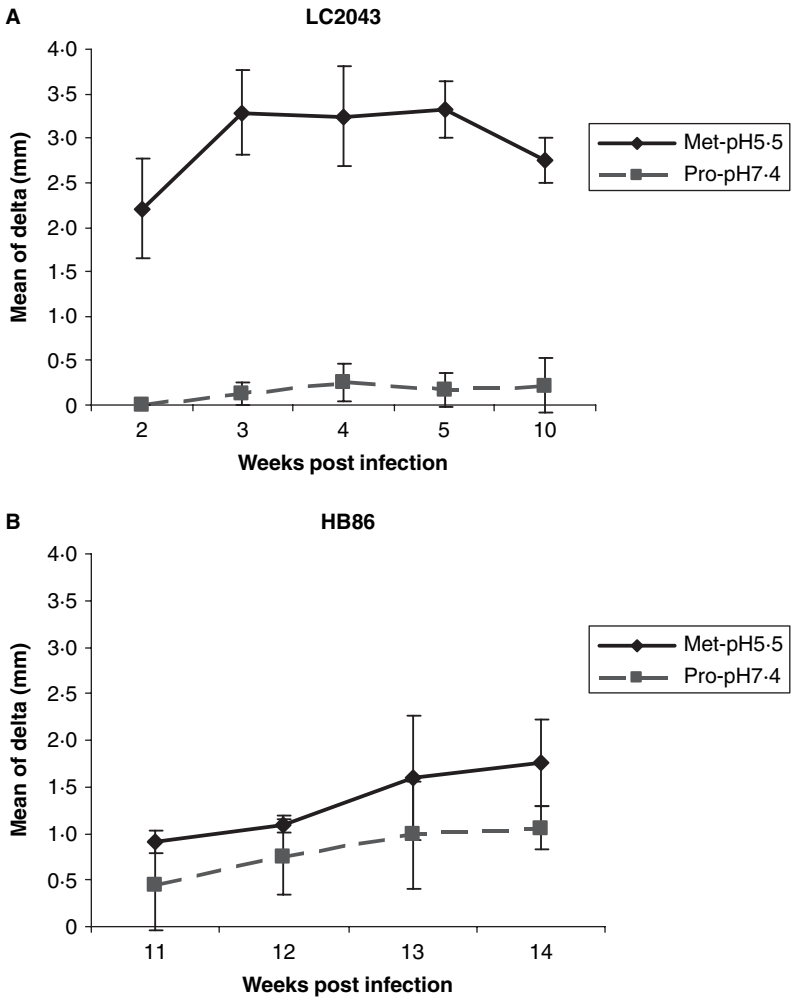

Fig. 2. *In vivo* infections of hamsters using purified metacyclic promastigotes from LC2043 (A) and HB86 (B). Differences were statistically significant from 2 weeks post-infection until the end of the experiment for LC2043 and only at week 14 post-infection for HB86 (ANOVA; $P<0{\cdot}05$, $n=5$).

strains. Our results should be confirmed with a larger sample of the 2 species (from different geographical origin and clinical outcome); but at this preliminary stage, they already converged with different pathogenicities in humans: *L.* (*V.*) *braziliensis*, the most pathogenic in humans, being also more infective in our experimental models. Considering the higher average number of amastigotes observed by *in vitro*-infected macrophages in *L.* (*V.*) *braziliensis*, it would be interesting to measure if this is related to an increased resistance to nitric oxide. This feature could indeed confer a survival benefit for the parasites inside the macrophage and was shown to correlate with disease severity in American tegumentary leishmaniasis (Giudice *et al.* 2007).

A more extensive analysis of the *in vitro* data revealed another interesting result. Indeed, the 4 strains ranked from high to low infectivity as follows: LC2043, LC2177, HB86 and LCA08. It is noteworthy that this corresponded to the gradient of karyotype dissimilarity previously observed among the same strains, which correlated itself with the distance between their geographical origins (Dujardin *et al.* 1993, 1995*b*, 1998). We hypothesized that this dynamic picture would reflect a recent speciation of *L.* (*V.*) *peruviana* from *L.* (*V.*) *braziliensis* (Dujardin *et al.* 1993, 1995*a*, *b*, 1998, 2000, 2002). According to our hypothesis, a *L.* (*V.*) *braziliensis*-like parasite would have colonized the Pacific slopes of the Andes, in the North of Peru (close to the lowest pass across the Andes along the whole Peruvian territory). Then, through a North-South migration and isolation, it would have increased its genomic differentiation. Further work on a larger set of strains is needed to verify this possible correlation between infectivity and geographical location of parasite populations. This may open new perspectives to understand the process of speciation in *Leishmania* and its implications in terms of pathogenicity.

In conclusion, we thus have a model able to demonstrate, in a reproducible way, differences in infectivity among representative strains from *Leishmania* of subgenus *Viannia*. Our results support the role of the parasite in the differences in human pathogenicity observed between *L.* (*V.*) *braziliensis* and

L. (*V.*) *peruviana*. However, further extrapolation should be made with great caution. There is still a huge gap between experimental models and the real situation in humans. *In vitro* models lack the immunological context. On the other hand immunological response may be different between animals and humans and, last but not least, there is a major difference between an infection caused by a needle or by a sandfly bite (immuno-modulatory effect of saliva) (Almeida *et al*. 2003). Further work is thus needed to upgrade *in vitro* as well as *in vivo* models and gather information to extrapolate the data to human infection.

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