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# Isolation of intact *Leishmania amazonensis* large parasitophorous vacuoles from infected macrophages by density gradient fractionation

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1 **Isolation of intact *Leishmania amazonensis* large parasitophorous vacuoles from infected**  
2 **macrophages by density gradient fractionation**

3

4 Running title: Isolation of *L. amazonensis* PVs

5

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19

20 **Abstract (97 words)**

21

22 As the causative agent of hard-to-treat diffuse cutaneous leishmaniasis, *Leishmania*  
23 (*L.*) *amazonensis* persists in the host organism sheltered within large Parasitophorous  
24 Vacuoles (PVs) formed mainly in macrophages. In the present study, I present a simple and  
25 efficient method for *L. amazonensis* PV isolation. Isolated PVs are intact as demonstrated by

26 the conservation of lysosomal probes loaded into PVs before the procedure. The method is  
27 useful for studies aiming at a complete and accurate molecular profile of these structures, to  
28 better understand the biogenesis of this pathogen-containing vacuole and its implication in  
29 parasite persistence and in leishmaniasis pathogenesis.

30

31 **Keywords:** Leishmania amazonensis; Parasitophorous vacuoles; Density Gradient  
32 fractionation; PV isolation; Macrophages

33

34

35

## 36 **1. Introduction (299 words)**

37

38 *Leishmania* spp. are protozoan trypanosomatid parasites, which induce cutaneous,  
39 mucocutaneous or visceral leishmaniasis in man and other animals, being a matter of concern  
40 especially in poor and developing countries [1]. *Leishmania* spp. are dimorphic parasites  
41 which alternate between two developmental stages: the promastigote form (flagellated and  
42 elongated) is found extracellularly in the midgut of insect vectors and is transmitted to  
43 mammalian host upon insect blood meal. Once internalized by mammalian host cells such as  
44 macrophages mainly, but also neutrophils and dendritic cells, the parasite is sheltered within  
45 phagolysosome-like structures called Parasitophorous Vacuoles (PVs) where the promastigote  
46 differentiates into the round-shaped amastigote form that multiplies and propagates in the  
47 mammalian host organism [2].

48 During their intracellular lifecycle in mammals, these protozoan parasites remain  
49 enclosed in PVs [3]. The morphological and biochemical diversity of *Leishmania* PVs was  
50 not extensively studied. Most species – such as *L. major*, *L. donovani* and *L. braziliensis* – are

51 lodged in membrane-bound tight-fitting PVs, containing one or two amastigotes, that undergo  
52 fission as parasites divide. In contrast, species from the *L. mexicana* complex, such as *L.*  
53 *amazonensis*, *L. mexicana* and *L. pifanoi*, occupy large PVs which may contain many  
54 parasites [4], coinciding with their remarkable resistance to parasite killing mediated by  
55 interferon- $\gamma$  and lipopolysaccharide “classical” macrophage activation [5].

56 The molecular mechanisms involved in PV fission or PV volumetric expansion remain  
57 to be elucidated, and a complete profile of the molecular composition of these PVs is  
58 necessary to understand their participation in the different leishmaniasis outcomes [6]. This  
59 task will largely benefit from methods of PV isolation taking into account the different PV  
60 biogenesis displayed by *Leishmania* spp. In this study, such method is proposed based on  
61 previous studies on plant contractile vacuoles [7], exploiting the buoyant capacity of the large  
62 *L. amazonensis* PV for efficient isolation of intact pathogen-containing vacuoles.

63

## 64 **2. Material and Methods (828 words)**

65

### 66 2.1. Parasites

67

68 Amastigote forms of *L. amazonensis* (MHOM/BR/1973/M2269) are obtained from  
69 BALB/c mice footpad lesions induced by amastigote inoculation as described [8]. All  
70 animal procedures followed guidelines of the Escola Paulista de Medicina Ethics  
71 Committee (CEUA No 3398150715). Green fluorescent protein (GFP)-transfected *L.*  
72 *amazonensis* were a kind donation of Dr. Silvia R. Uliana, ICB-USP, Brazil.

73

### 74 2.2. Infection of primary macrophages

75

76 Primary mouse bone marrow-derived macrophages were obtained as described [9]  
77 and cultivated in 100 mm-diameter non-treated cell culture bacteriological Petri dishes  
78 (Corning Inc.) with RPMI 1640 medium supplemented with 10% fetal bovine serum  
79 (complete medium) in 34°C, 5% CO<sub>2</sub> environment. Lesion-derived amastigotes were  
80 added to macrophage cultures at a multiplicity of infection of 5 parasites per macrophage,  
81 for an overnight period of interaction. Then, macrophage cultures were washed  
82 with Hanks' Buffered Salt Solution (HBSS) for the removal of non-internalized parasites,  
83 fresh complete medium was added and cultures were proceeded to PV isolation after  
84 different time periods post-infection. The lysosomotropic probe for acidic compartments  
85 LysoTracker Red DND-99 (Thermo Fisher Scientific Inc.) was added to complete medium  
86 at 10 μM concentration for 15 minutes at 34°C, 5% CO<sub>2</sub> before PV isolation procedures.

87

### 88 2.3. Isolation of *L. amazonensis* Parasitophorous Vacuoles

89

90 *L. amazonensis* PVs were isolated from *in vitro*-infected macrophages using a  
91 protocol adapted from plant cell vacuole isolation [7]. The procedure is schematized in  
92 Fig. 1. Macrophages infected for 24-72 hours (time post-infection, p.i.) were detached  
93 from >5 culture dishes after HBSS washes and treatment with a pre-warmed detachment  
94 solution composed of 1% w/v Ethylenediaminetetraacetic Acid Tetrasodium Tetrahydrate  
95 Salt (EDTA 4Na, Sigma-Aldrich, catalogue 03699) in Phosphate Buffer Saline without  
96 Calcium and Magnesium (PBS -/-), for 10 min at 34°C 5% CO<sub>2</sub>. Detachment solution is  
97 discarded and macrophages were fully detached by flushing cultures with complete  
98 medium, collecting detached cells in 50 ml centrifuge tubes. After centrifugation at 300 g,  
99 10 minutes, 4°C, macrophages were resuspended in Lysis buffer composed of 200 mM  
100 Sucrose (Sigma-Aldrich, catalogue S7903), 10% v/v Ficoll Type 400 (Sigma-Aldrich,

101 catalogue F4375), 10 mM EDTA (Sigma-Aldrich, catalogue EDS, adjusted to pH 8.0),  
102 and 5 mM Sodium Phosphate pH 8.0 (mixture of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  Sigma-Aldrich  
103 catalogue S9390 and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  Sigma-Aldrich catalogue S9638, adjusted to pH 8 as  
104 described [7]) in water.

105         Next, macrophages in Lysis buffer were passed multiple times through 30-gauge  
106 12mm (1/2 inch) needles coupled to 50 ml syringe, conserving the collection 50 ml tube  
107 on ice during the passages. This will mechanically disrupt macrophages, releasing intact  
108 PVs and intracellular amastigotes. Disruption process was inspected by observing the  
109 presence of intact PVs containing parasites under a regular microscope using Neubauer  
110 chambers.

111         Disrupted macrophage samples with intact PVs were then transferred to Ultra-  
112 clear centrifugation tubes, 14 ml (Beckman Coulter, catalogue 344060), carefully layering  
113 first 3 ml of Lysis buffer containing disrupted macrophages, followed by 1.5 ml of 4% v/v  
114 Ficoll Type 400 solution (mixture of Lysis buffer and Vacuole buffer, as described [7])  
115 and finally by 0.5 ml of Vacuole buffer composed of 450 mM sucrose, 2 mM EDTA  
116 (Sigma-Aldrich, catalogue EDS, adjusted to pH 8.0), and 5 mM Sodium Phosphate pH 7.5  
117 (mixture of  $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$  Sigma-Aldrich catalogue S9390 and  $\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$  Sigma-  
118 Aldrich catalogue S9638, adjusted to pH 7.5 as described [7]), in water. Layering were  
119 performed on ice, using ice-cold solutions.

120         Samples are centrifuged using ultra-centrifuges for 50 minutes at 71000g 10°C  
121 using slow acceleration and slow deceleration settings. Isolated PVs were retrieved in the  
122 Vacuole buffer (upper) fraction of the gradient and isolated amastigotes with PV remnants  
123 were retrieved in the intermediary Ficoll 4% fraction. Intact PVs were kept in Vacuole  
124 buffer and used immediately after isolation for downstream experiments. Free parasites  
125 and parasites sheltered in intact isolated PVs found in Vacuole buffer of Ficoll 4%

126 fractions were differentially counted in Neubauer chambers. Purity of isolated PV  
127 preparation is expressed as the percentage of parasites sheltered in intact PVs among total  
128 parasites (free or within PVs) counted in the Vacuole buffer fraction.

129         Quantitation of total proteins from free parasite preparations (Ficoll 4% fraction)  
130 or isolated PV preparations (Vacuole buffer fraction) was performed by Pierce BCA  
131 Protein Assay kit (Thermo Fisher Scientific Inc.) following manufacturer instructions.  
132 Data was normalized by the number of parasites counted in these fractions.

133

#### 134 2.4. Confocal Microscopy

135

136         For live imaging acquisition, the isolated PVs were placed in round microscopy  
137 dishes ( $\mu$ -Dish 35 mm, ibidi GmbH) and imaging of isolated PVs was performed in live,  
138 non-fixed samples, using 100 $\times$  objective lenses from a SP5 II Tandem Scanner Confocal  
139 system (Leica Microsystems). Samples were maintained at 34°C and 5% CO<sub>2</sub> within the  
140 incubators coupled to the microscope system.

141         Immunostaining of isolated PVs were performed using antibodies against  
142 Lysosomal-Associated Membrane Proteins 1 and 2 (LAMP1 and LAMP2) and *L.*  
143 *amazonensis* membrane markers (2A3-26 antibody; kindly donated by Dr. Eric Prina,  
144 Institut Pasteur, France) after formaldehyde fixation as described [4].

145         Confocal image acquisition was performed by acquiring focal stacks of 20 to 30  
146 optical sections from live samples with an optical section separation (*z*-interval) of 0.2 to  
147 1  $\mu$ m. Acquired images were processed by Leica Application Suite X (LAS X) and Imaris  
148 v.7.4.2 software (Bitplane AG, Andor Technology).

149

#### 150 2.5 Statistical analysis

151

152 The results are presented as the means with standard errors (SEM). Statistical tests  
153 were analyzed using SPSS software (IBM), considering normal (parametric tests) or non-  
154 normal distributions (nonparametric tests), and significant differences were indicated by *p*  
155 values below 0.05.

156

### 157 **3. Results (307 words)**

158

#### 159 3.1. Isolated PVs are intact, preserving structure, acidification and membrane content

160

161 After the density gradient fractionation, *L. amazonensis* PVs were observed devoid of  
162 host macrophage remnants; isolated PVs and clusters of multiple aggregated PVs could be  
163 detected, containing 1 to 3 intracellular amastigotes (Fig. 2A). When PVs were isolated from  
164 macrophage cultures infected with GFP-expressing *L. amazonensis*, amastigotes were found  
165 inside isolated PVs conserving the GFP fluorescence, indicating the viability of these  
166 parasites (Fig. 2B). Furthermore, by loading the phagolysosome-like PVs with the  
167 lysosomotropic probe LysoTracker, administrated to infected macrophage cultures before PV  
168 isolation, LysoTracker signal is retained by the isolated PVs, indicating that these vacuoles are  
169 intact, still sheltering amastigotes (Fig. 2C, Movie 1). Importantly, isolated PVs containing  
170 parasites conserve PV membrane markers such as LAMP proteins (Fig. 2D).

171

#### 172 3.2. Isolated PVs fraction presents good degree of purity up to 72 hours post-infection

173

174 The parasite counting comparing the free parasites and the intact PVs with parasites  
175 fractions after 24, 48 and 48 hours post-infection (p.i.) indicates that, although parasite



176 numbers in the free parasites fraction increase with time p.i., the parasite numbers found in  
177 the intact PVs with parasites fraction remains unaltered (Fig. 3A). The differential counting of  
178 free parasites and parasites found within intact PVs in the isolated PV fraction allowed for  
179 retrieving a purity index in these three time points p.i. Accordingly, purity does not differ  
180 among time points, presenting an aggregated mean of 78.2% ( $\pm 4.8$  SEM)(Fig. 3B). The  
181 isolation method yields preparations of free parasites and isolated parasite-containing PVs  
182 from which protein amounts are in the range of  $\mu\text{g}$ . The normalization of the total protein  
183 amount per parasite counted in each fraction (free parasites *versus* intact PVs with parasites)  
184 indicates more proteins retrieved per parasite in the latter fraction, highlighting an important  
185 contribution of PV protein content in the preparation of isolated PVs (Fig. 3C).

186

#### 187 **4. Discussion**

188

189 Most non-viral intracellular pathogens gain entrance into human and animal host cells  
190 by “classic” or modified phagocytosis and are thus lodged in phagosomes which they may or  
191 not continue to occupy in the course of infection [10]. Their survival, multiplication and  
192 persistence within host cells depend on modulation of the compositional and functional  
193 phenotypes of the phagosomes they occupy, including vacuolar pH (regulated by vacuolar  
194 ATPases), substrate acquisition through membrane transporters and channels, and fusion with  
195 lysosomes, phagosomes and other vesicles. Each phagosome thus exhibits particular features,  
196 whose biogenesis is conditioned to different signals triggered by both the host cell and the  
197 internalized particle/microorganism. Once internalized by host cells, some pathogens interfere  
198 with phagosomal maturation, leading to the development of customized phagosomes excluded  
199 from host cell endocytic and secretory pathways, displaying thus selective  
200 fusogenic/functional properties [10, 11].

201           Considering that the differential, species-specific biogenesis of *Leishmania* PVs might  
202 account for their long-term relationship with host macrophages, a complete and precise  
203 profiling of these structures could contribute not only to the understanding of how  
204 intracellular pathogens persist hidden inside host cells vacuoles, but also to the development  
205 of novel therapeutical strategies, targeting parasite- or host-derived components of the PV  
206 [12-15].

207           This study provides a useful methodology for the isolation of large *Leishmania* PVs,  
208 with good degree of purity and protein amount for further molecular and/or biochemical  
209 analyses. Previous works attempting to isolate *Leishmania* PVs were performed notably on  
210 tight-fitting PVs formed by *L. donovani*, exploiting the expression of Endoplasmic reticulum  
211 (ER)-derived proteins such as calnexin to target and isolate PVs in antibody-based affinity  
212 columns [16]. However, when applying the same method to large PVs formed by other  
213 species, *L. pifanoi*, only early formed, small PVs (2 to 8 hours of intracellular infection) could  
214 be isolated, a time window before multiplication of the parasites and possibly before  
215 extensive parasite-mediated customization of these vacuoles. Although *L. amazonensis* PVs  
216 could reach several  $\mu\text{m}$  in diameter and may contain dozens of multiplying parasites in  
217 primary macrophages along 72 hours of intracellular infection, the methods here presented  
218 allowed for isolation of PVs with a moderate size, containing few parasites – what represents  
219 one important limitation of the protocol and corroborate these previous studies where only  
220 small PVs could be retrieved.

221           Another important limitation of this study is that the presence of macrophage  
222 remnants, e.g. other organelles, was evaluated only morphologically under the microscope.  
223 Highly sensitive molecular techniques to investigate mitochondria, ER and Golgi remnants on  
224 isolated PV preparations must be performed before accomplishing a complete profile of  
225 proteins displayed specifically by these structures.

226 In conclusion, this methodological advance adds to the investigation on the importance  
227 of a species-specific PV to *Leishmania* intracellular parasitism and whether there is a  
228 connection between PV biogenesis, parasite persistence and leishmaniasis outcome.

229

### 230 **Conflicts of interest**

231

232 The author has no potential conflict of interest.

233

### 234 **Acknowledgments**

235

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241

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282

### 283 **Figure Legends**

284

#### 285 **Figure 1: Scheme of PV isolation by density gradient fractionation.**

286 From left to right: Primary macrophages infected for 24, 48 and 72 hours were detached  
287 from culture plates and resuspended in Lysis buffer (see Methods) after centrifugation.

288 Infected macrophages suspension was passaged several times through 30-gauge needles  
289 for mechanical disruption and release of PVs and intracellular parasites. The disrupted  
290 suspension was then layered by Ficoll 4% followed by Vacuole buffer solutions (see  
291 Methods) to form the density gradient. After ultracentrifugation, Isolated PVs containing  
292 parasites are found in the upper fraction of Vacuole Buffer, while free parasites are found  
293 in the middle fraction.

294

#### 295 **Figure 2: Microscopy images of isolated, intact PVs containing live parasites**

296 A) Phase contrast images of isolated PVs. Arrows indicate PVs and numbers indicate how  
297 many amastigotes were detected in each isolated PVs. Bar=5 $\mu$ m. B) Phase contrast  
298 images merged with GFP green-fluorescent signal from live amastigotes within isolated

299 PVs. Bar=4 $\mu$ m. C) Confocal live imaging of isolated PV loaded with LysoTracker probe  
300 (red) containing live GFP-expressing amastigotes (green). Upper image shows the three-  
301 dimensional projection of the isolated PV; lower row shows the same isolated PV  
302 observed in different focal planes (z-stack 1-4). Bar=4 $\mu$ m. Confocal image of  
303 formaldehyde-fixed isolated PV immunostained for host LAMP1 and LAMP-2 PV  
304 membrane proteins and for *L. amazonensis* amastigote-specific surface antigen (2A3-26  
305 antibody), visualized in two different angles (upper and lower images). Arrows indicate  
306 LAMP-1/LAMP-2 surrounding amastigotes (indicated by asterisk). Bar=1 $\mu$ m.

307

308 **Figure 3: Quantification of parasites and total proteins in the density gradient**  
309 **fractions.**

310 A) Parasite counts in the free parasite fraction and in the fraction containing isolated PVs  
311 with parasites after fractionation of PVs formed at 24, 48 and 72 hours post-infection  
312 (p.i.). Results are expressed as means with SEM after n=3 independent experiments for  
313 the 24h p.i. group, n=6 independent experiments for the 48h p.i. group, and n=2  
314 independent experiments for the 72h p.i. group. B) Purity of isolated PV preparations after  
315 24, 48 and 72 hours p.i., calculated in isolated PV fractions as percentage of parasites  
316 contained within intact PVs among all parasites counted in the fraction. C) Total protein  
317 quantification per parasite ( $\mu$ g per  $10^5$  parasites) in the free parasites fraction and in the  
318 fraction containing isolated PVs with parasites. Mann-Whitney test, asterisk indicate  
319 statistical significance at  $p < 0.05$ .







