

Prevalence and Distribution of Leishmania RNA Virus 1 in Leishmania Parasites from French Guiana

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27

28 Abstract

29 In South America, the presence of the Leishmania RNA Virus type 1 (LRV1) was described
30 in Leishmania guyanensis and Leishmania braziliensis strains. The aim of this study was to
31 determine the prevalence and distribution of LRV1 in Leishmania strains in French Guiana
32 given that, in this French overseas department, most Leishmania infections are due to these
33 parasite species. The presence of the virus was observed in 74% of Leishmania sp. strains,
34 with a highest presence in the internal areas of the country.

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36 Leishmania RNA virus, Leishmania guyanensis, Leishmania braziliensis, French Guiana.

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46 Conflicts of Interest

47 Conflicts that the editors consider relevant to the content of the manuscript have been
48 disclosed.

49

50 Introduction

51 Virus Like-Particles (VLPs) discovery began in the sixties with the first report in the
52 parasitic protozoan *Entamoeba histolytica*.¹ Since then, similar structures have been identified
53 in an ever-expanding list of unicellular eukaryotes: *Leishmania*,² *Plasmodium*,³ *Naegleria*,³
54 *Giardia*,⁴ *Trichomonas*⁵ as well as in other parasite species and in fungi.⁶ The first discovery
55 of VLPs in *Leishmania* was reported by Molyneux in *Leishmania hertigi*, a non-human
56 parasite species.² The presence of VLPs in another *Leishmania* species was then highlighted
57 by Tarr et. al, namely the *Leishmania (braziliensis) guyanensis* species.⁷ *Leishmania* RNA
58 Virus (LRV) is an icosahedral encapsidated double-stranded 5.3Kb RNA virus, belonging to
59 the Totiviridae family. This virus has been observed in the New World *Leishmania* species,
60 *Leishmania (Viannia) guyanensis* and *Leishmania (Viannia) braziliensis*, in one strain of the
61 Old World parasite *L. major* and in *L. aethiopica*.^{8, 9} New World strains are grouped into the
62 LRV1 type, which is divided in 14 subtypes (LRV1-1 to LRV1-14) that spread over South
63 America.^{7, 10, 11} Old World strains belong to the LRV2 type.⁹

64 The LRV cDNA was first completely sequenced by Stuart et al. for the prototype virus
65 termed LRV1-1.¹² Then, complete cDNA sequences were reported by Scheffter et al. for the
66 LRV1-4 and LRV2-1 isolates.^{13, 14} The LRV1-1 and LRV1-4 sequences share 77% nucleotide
67 identity between themselves.¹⁴

68

69

In French Guiana, *Leishmania* species predominantly diagnosed are *L. guyanensis*, *L.*
braziliensis and occasionally *L. amazonensis* (Simon et al., submitted data).¹⁵ Leishmaniasis

70 represents a significant risk for those in contact with the forest. Our study aimed to determine
71 the prevalence of LRV1 in the circulating strains of *Leishmania* spp. in French Guiana and to
72 establish its repartition on the territory.

73

74 Material and methods

75 Parasites. A total of 129 strains of *Leishmania* spp. isolated from 424 patients
76 (including 164 available cultures) infected in French Guiana between 2012 and 2014 were
77 kindly supplied by the parasitology and mycology laboratory of the Cayenne General
78 Hospital, with strict respect for patient anonymity. Promastigote parasites were cultured in
79 RPMI 1640 (Gibco) containing L-glutamine, 20 mM HEPES, phenol red and supplemented
80 with 20% heat-inactivated fetal calf serum (Gibco), 50 IU/ml penicillin (Invitrogen), 0.05
81 mg/ml streptomycin (Invitrogen) and nonessential amino acids (Gibco).

82 *Leishmania* isolate identification was performed by PCR-RFLP as previously
83 described.¹⁶

84 When parasites reached a stationary phase, counting was performed in a Malassez cell.
85 Pellets containing 1.10^7 parasites were constituted after culture by centrifugation 5 minutes at
86 514 g and removal of the supernatant. The pellets were stored at -80°C until RNA extraction.

87 Total RNA extraction. Total RNA was extracted using Trizol® according to the
88 manufacturer's recommendations, with minor modifications. Pellets were thawed at room
89 temperature (RT) and homogenized with 1 ml of Trizol®. After a 15 to 30 minutes incubation
90 at RT, 0.2 ml of chloroform per ml of Trizol® was added, vortexed 15 seconds and incubated
91 2 to 15 minutes at RT. Phase separation by centrifugation 10 minutes at 12 000 g and 4°C
92 allowed recovering the upper phase, to which 0.5 ml of isopropanol was added. The mixture

93 was then incubated 10 minutes at RT and centrifuged for 10 minutes at 12 000 g at 4 ° C. The
94 RNA pellet was then washed with 75% ethanol, dried and dissolved in 10 µl of DEPC water.
95 The RNA was stored at -80°C.

96 Reverse transcription PCR. cDNA was synthesized using the SuperScript™ II
97 Reverse Transcriptase (Invitrogen), according to the manufacturer's recommendations with
98 random hexamers (Invitrogen). Then, PCR amplification of a 124-bp LRV fragment located
99 in the most conserved LRV1 region was done with the LRV1 forward primer, LRV1-F1: 5'-
100 CTGACTGGACGGGGGTAAT-3' and LRV1 reverse primer, LRV1-R1: 5'-
101 CAAAACACTCCCTTACGC-3' enabling to amplify all LRV1 subtypes that have been
102 slightly modified from Ives et al.¹⁷ These primers were based on LRV1-1 and LRV1-4
103 genome sequences (Genbank accession number : LRV1-1 : NC002063 and LRV1-4 :
104 NC003601) A denaturation step at 94°C for 2 minutes was followed by 40 cycles at 94°C for
105 30 seconds, 54°C for 30 seconds and 72°C for 1 minute. The PCR was completed by a final
106 elongation at 72°C for 5 minutes. PCR products were analyzed on a 2% gel agarose to verify
107 the presence of amplification products at the expected size. The reference strain of
108 *Leishmania guyanensis* (MHOM/GF/97/LBC6) and water were used as positive and negative
109 controls, respectively, in each RT-PCR experiment.

110 Ethical aspects. The study was retrospective. All patients were informed (during
111 consultation, with posters) that data and analysis of results may be used in research and that
112 they have a right to refuse.

113 Statistical analysis. Statistical significance between proportion of LRV from coastal
114 communes and inland communes and proportion of LRV from coastal area (seaside) and the
115 rest of the territory were determined by chi 2 test; $P < 0.05$ was considered significant.

116

117 Results

118 Search for LRV1 was performed on 129 Leishmania isolates including 112 isolates of
119 *L. guyanensis*, 11 isolates of *L. braziliensis* and 6 isolates of *L. amazonensis*. Its presence was
120 detected in 96 of the 129 isolates (74%).

121 Figure 1 shows RT-PCR products obtained from a panel of different LRV1-positive or
122 negative Leishmania species. Table 1 groups the results per site and Leishmania species.
123 Eighty percent (90/112) of *L. guyanensis* isolates and 55% (6/11) of *L. braziliensis* isolates
124 were LRV positive. Sequencing of the PCR fragment obtained from a *L. braziliensis* isolate
125 confirmed the detection of LRV1-4. No LRV1 was detected from *L. amazonensis* isolates.

126 Figure 2 shows the distribution of Leishmania isolates carrying or not LRV across
127 French Guiana. There were no significant differences between the proportion of LRV from
128 coastal communes and inland communes ($p=0.22$), however, there was a significant
129 difference between the seaside and the rest of the territory ($p < 0.001$). Indeed, apart from
130 Kourou, where the only available isolate was positive to LRV, isolates from Mana, Iracoubo,
131 and Macouria were negative. Half isolates from Rémire were positive to LRV. In all inland
132 communes, more than half of the isolates were LRV positive. In communes with the highest
133 sampling effort, LRV presence was predominant: Regina with 82% (14/17) of positive
134 isolates, Maripasoula 82% (14/17), Saül 100% (10/10), Cacao 75% (6/8) and Papaïchton 86%
135 (6/7).

136 The presumed site of infection was unknown, or outside of French Guiana ($n=2$), for
137 37 out of 129 isolates. Among them, 73% proved to be LRV positive. The two isolates, whose
138 suspected infection sites were outside of French Guiana, were contracted in Manaus and
139 Suriname and were LRV positive *L. guyanensis* isolates.

140

141 Discussion

142 Leishmania RNA viruses can infect different Leishmania species. Surveys of New
143 World parasites have identified LRV1 only in isolates that originated from the Amazon basin,
144 such as *L. guyanensis* and *L. braziliensis*.^{7, 10, 18-20} The average molecular prevalence of LRV
145 that we report here in *Leishmania* sp. isolates circulating in French Guiana is equivalent to the
146 one described by Bourreau et al. (in press)²¹ but substantially higher (74%) than those reported
147 in other South American countries, which ranged from 5.8% in Colombia, 18% in Peru, and
148 25.5% in the Brazilian city of Caratinga, Minas Gerais.¹⁸⁻²⁰ This difference can first be
149 explained by the geographical origin of the *Leishmania* isolates and, secondly, by the type of
150 biological material used for LRV detection. Indeed, in Colombia, the virus was detected from
151 parasites originating from the Amazon area of the country while no virus was detected in
152 other regions.¹⁸ In Brazil, Pereira et al. ²² reported two LRV1 positive isolates out of 48 tested
153 (4.1%). These two *L. guyanensis* positive isolates were from the Amazon region. In the
154 present study, we report the presence of LRV throughout the whole territory. This could be
155 correlated to tropical climate conditions encountered in French Guiana. In contrast, the
156 weaker presence of LRV along the coastal area was presumably due to the low density of
157 vectors and reservoirs implying a low parasite transmission in this more urbanized area.^{23, 24}
158 Ecologic and epidemiologic factors related to Amazon region might be involved in the
159 repartition of *Leishmania* parasites susceptible to harbor the LRV.¹⁸

160 Concerning the type of biological material used for the search of LRV, in all previous
161 studies this search was performed on human biopsies containing low amounts of the
162 amastigote form of the parasite.^{18-20, 22} In our study, we used cultured parasites corresponding
163 to the promastigote form (10^7 cells) in which the quantity of LRV has been reported to be
164 higher due to virus multiplication in culture ²⁵ (personal communication). This could thus

165 explain the large difference of prevalence observed between our results and those previously
166 published.^{18-20, 22}

167 We here report the detection of LRV1 from different *L. guyanensis* and *L. braziliensis*
168 isolates.^{7, 10, 18-20} None of the six *L. amazonensis* isolates tested were positive for LRV1. With
169 the exception of the *L. panamensis* species for which no LRV1 has been identified,¹⁸
170 no information is currently available for the other New World *Leishmania* species. Therefore,
171 LRV1 has so far only been detected in parasite species of the *L. Viannia* subgenus.

172 LRV infection of parasites seems to affect their virulence in a murine model of
173 mucosal cutaneous leishmaniasis.^{17, 26, 27} To determine if the clinical evolution of infected
174 patients can be affected by the presence of the virus or can be correlated, at least in part, to the
175 genetic variability of the virus for which at least 14 subtypes have been identified, we
176 envision, based on the present results, to characterize the genetic diversity of different LRV1
177 isolates at the genomic level. These results should enable us to gain insights into the role
178 LRV1 plays in the evolution of leishmaniasis lesions and deepen the achievements of
179 Bourreau et al. (in press)²¹describing the impact of LRV on first-line treatment failure and
180 symptomatic relapse.

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279 9: e96766.
- 280
- 281
- 282
- 283

Table 1. Leishmania LRV positive or negative summary strains

Species LRV	L. guyanensis		L. braziliensis		L. amazonensis		Leishmania spp.		%
	POS	NEG	POS	NEG	POS	NEG	POS	NEG	POS
Presumed site of infection									
Cacao	6	2	0	0	0	0	6	2	75%
Camopi	3	1	0	0	0	0	3	1	75%
Grand santi	3	0	1	0	0	3	4	3	57%
Iracoubo	0	1	0	0	0	0	0	1	0%
Kourou	1	0	0	0	0	0	1	0	100%
Macouria	0	4	0	0	0	0	0	4	0%
Mana	0	1	0	0	0	0	0	1	0%
Maripasoula	12	2	2	1	0	0	14	3	82%
Montsinery	1	0	0	0	0	0	1	0	100%
Nouragues	1	0	0	0	0	0	1	0	100%
Papaïchton	6	1	0	0	0	0	6	1	86%
Régina	14	2	0	1	0	0	14	3	82%
Rémire	1	1	0	0	0	0	1	1	50%
Roura	4	0	0	0	0	0	4	0	100%
Saint Elie	1	1	0	0	0	0	1	1	50%
Saint Georges de l'Oyapock	2	1	0	0	0	0	2	1	67%
Saint Laurent du Maroni	1	1	0	0	0	0	1	1	50%
Saül	9	0	1	0	0	0	10	0	100%
Unknown/outside	25	4	2	3	0	3	27	10	73%
Total	90	22	6	5	0	6	96	33	
%	80%		55%		0%		74%		

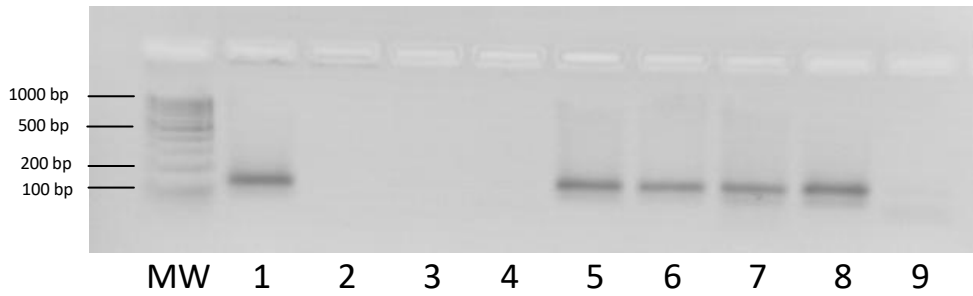


Figure 1 . LRV1 detection in different *Leishmania* isolates.

MW (Molecular Weight Marker 100bp, Sigma ®); 1,5,6 : LRV1 positive *Leishmania guyanensis* isolates; 2 : LRV1 negative *Leishmania guyanensis* isolate; 3, 4 : LRV1 negative *Leishmania amazonensis* isolates, 7 : LRV1 positive *Leishmania braziliensis* isolate; 8 : LRV1 positive control (reference strain of *Leishmania guyanensis* MHOM/GF/97/LBC6); 9 : LRV1 negative control (water).

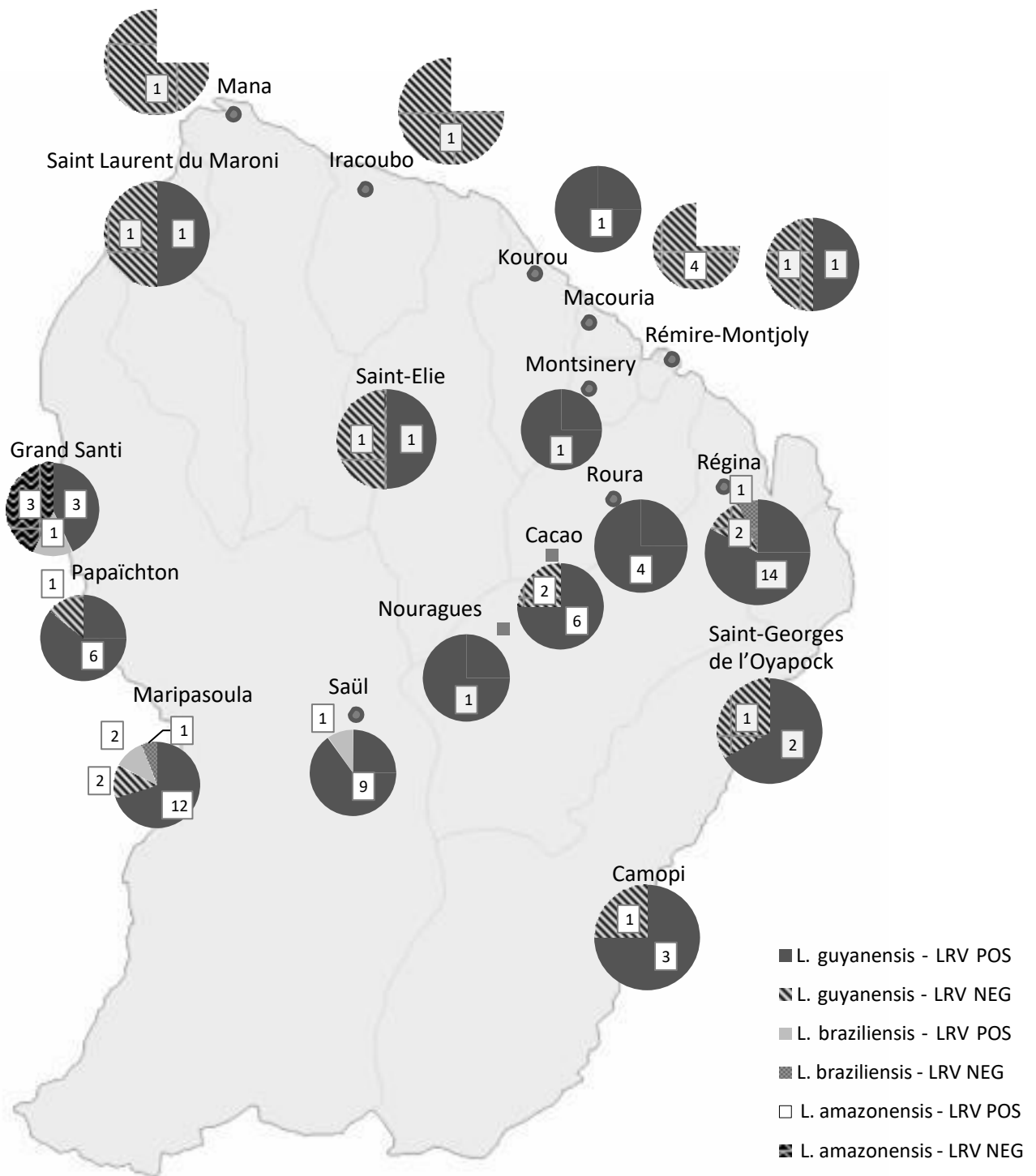


Figure 2. Distribution of *Leishmania* isolates carrying or not the LRV, in French Guiana. Pie charts full correspond to positive *Leishmania* LRV and pie charts with patterns correspond to negative *Leishmania* LRV.