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

Marine Ginouvès, Stéphane Simon, Eliane Bourreau, Vincent Lacoste, Catherine Ronet, Pierre Couppié, Mathieu Nacher, Magalie Pierre Demar, Ghislaine Prévot

Title: Prevalence and distribution of Leishmania RNA Virus 1 in Leishmania parasites from French Guiana

Running title: Leishmania RNA Virus 1 in French Guiana

Authors: Ginouvès Marine[1,2*], Simon Stéphane[1,2], Bourreau Eliane[3], Lacoste Vincent[4], Ronet Catherine[5], Couppié Pierre[1,2,6], Nacher Mathieu[1,7], Demar Magalie[1,2,8], and Prévot Ghislaine[1]

[1]Ecosystemes Amazoniens et Pathologie Tropicale – EA 3593 – Labex CEBA – Medicine department, University of French Guiana, Cayenne, French Guiana

[2]Laboratoire Associé – Centre National de Référence Leishmania, Laboratory of Parasitology and Mycology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana

[3]Immunology Laboratory of Leishmaniasis, Pasteur Institute of French Guiana, Cayenne, French Guiana

[4]Laboratory of Virus-Host Interactions, Pasteur Institute of French Guiana, Cayenne, French Guiana

[5]Department of Biochemistry, University of Lausanne, Epalinges, Switzerland

[6]Guianan Institute of Tropical Dermatology; Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana

[7]Centre d'Investigation Clinique Epidémiologie Clinique Antilles Guyane CIC EC 1424, Cayenne General Hospital, Cayenne, French Guiana

[8]Laboratory of Parasitology and Mycology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana

* Corresponding author: Ecosystemes Amazoniens et Pathologie Tropicale – EA 3593 – Labex CEBA – Medicine department, University of French Guiana, Cayenne, French Guiana, E-mail : marine.ginouves@univ-guyane.fr, Phone: + 594 594 29 62 31

Abstract

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

Keywords

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

Word counts

Abstract: 82 words

Text: 1601 words

Number of figures and tables: 2 figures and 1 table

Financial support

This work was supported by the University of the French West Indies and French Guiana and the Ministère Français de l'Enseignement Supérieur et de la Recherche Scientifique. It has benefited from an Investissement d'Avenir grant managed by Agence Nationale de la Recherche (CEBA, reference no. ANR-10-LABX-25-01).

Conflicts of Interest

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

Introduction

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

The LRV cDNA was first completely sequenced by Stuart et al. for the prototype virus termed LRV1-1.[12] Then, complete cDNA sequences were reported by Scheffter et al. for the LRV1-4 and LRV2-1 isolates.[13, 14] The LRV1-1 and LRV1-4 sequences share 77% nucleotide identity between themselves.[14]

In French Guiana, Leishmania species predominantly diagnosed are L. guyanensis, L. braziliensis and occasionally L. amazonensis (Simon et al., submitted data).[15] Leishmaniasis represents a significant risk for those in contact with the forest. Our study aimed to determine the prevalence of LRV1 in the circulating strains of Leishmania spp. in French Guiana and to establish its repartition on the territory.

Material and methods

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

Leishmania isolate identification was performed by PCR-RFLP as previously described.[16]

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

Total RNA extraction. Total RNA was extracted using Trizol® according to the manufacturer's recommendations, with minor modifications. Pellets were thawed at room temperature (RT) and homogenized with 1 ml of Trizol®. After a 15 to 30 minutes incubation at RT, 0.2 ml of chloroform per ml of Trizol® was added, vortexed 15 seconds and incubated 2 to 15 minutes at RT. Phase separation by centrifugation 10 minutes at 12 000 g and 4°C allowed recovering the upper phase, to which 0.5 ml of isopropanol was added. The mixture was then incubated 10 minutes at RT and centrifuged for 10 minutes at 12 000 g at 4 ° C. The RNA pellet was then washed with 75% ethanol, dried and dissolved in 10 µl of DEPC water. The RNA was stored at -80°C.

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

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

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

Results

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

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

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

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

Discussion

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

Concerning the type of biological material used for the search of LRV, in all previous studies this search was performed on human biopsies containing low amounts of the amastigote form of the parasite.[18-20, 22] In our study, we used cultured parasites corresponding to the promastigote form (10^7 cells) in which the quantity of LRV has been reported to be higher due to virus multiplication in culture [25] (personal communication). This could thus explain the large difference of prevalence observed between our results and those previously published.[18-20, 22]

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

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

Authors' addresses

Ginouvès Marine and Simon Stéphane, Ecosystemes Amazoniens et Pathologie Tropicale – EA 3593 – Labex CEBA – Medicine department, University of French Guiana, Cayenne, French Guiana, Laboratoire Associé – Centre National de Référence Leishmania, Laboratory of Parasitology and Mycology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, E-mails : marine.ginouves@univ-guyane.fr, stephane.simon@guyane.univ-ag.fr, Bourreau Eliane, Immunology Laboratory of Leishmaniasis, Pasteur Institute of French Guiana, Cayenne, French Guiana, E-mail : ebourreau@pasteur-cayenne.fr, Lacoste Vincent, Laboratory of Virus Hosts Interaction, Pasteur Institute of French Guiana, Cayenne, French Guiana, E-mail : vlacoste@pasteur-cayenne.fr, Ronet Catherine, Department of Biochemistry, University of Lausanne, Epalinges, Switzerland, E-mail : catherine.ronet@unil.ch, Couppié Pierre, Ecosystemes Amazoniens et Pathologie Tropicale – EA 3593 – Labex CEBA – Medicine department, University of French Guiana, Cayenne, French Guiana and Guianan Institute of Tropical Dermatology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, E-mail : pierre.couppie@ch-cayenne.fr, Nacher Mathieu, Ecosystemes Amazoniens et Pathologie Tropicale – EA 3593 – Labex CEBA – Medicine department, University of French Guiana, Cayenne, French Guiana, Centre d'Investigation Clinique Epidémiologie Clinique Antilles Guyane CIC EC 1424, Cayenne General Hospital, Cayenne, French Guiana, E-mail : mathieu.nacher@ch-cayenne.fr, Demar Magalie, Ecosystemes Amazoniens et Pathologie Tropicale – EA 3593 – Labex CEBA – Medicine department, University of French Guiana, Cayenne, French Guiana, Laboratoire Associé – Centre National de Référence Leishmania, Laboratory of Parasitology and Mycology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana and Laboratory of Parasitology and Mycology, Centre Hospitalier Andrée Rosemon, Cayenne, French Guiana, E-mail : magalie.demar@ch-cayenne.fr and Prévot Ghislaine, Ecosystemes Amazoniens et Pathologie Tropicale – EA 3593 – Labex CEBA – Medicine department, University of French Guiana, Cayenne, French Guiana, E-mail : fac.prevot@gmail.com

References

1. Miller JH, Schwartzwelter JC, 1960. Virus-like particles in an Entamoeba histolytica trophozoite. J Parasitol 46: 523-524.

2. Molyneux DH, 1974. Virus-like particles in Leishmania parasites. Nature 249: 588-589.

3. Diamond LS, Mattern CF, 1976. Protozoal viruses. Adv Virus Res 20: 87-112.

4. Wang AL, Wang CC, 1986. Discovery of a specific double-stranded RNA virus in Giardia lamblia. Mol Biochem Parasitol 21: 269-276.

5. Wang AL, Wang CC, 1986. The double-stranded RNA in Trichomonas vaginalis may originate from virus-like particles. Proc Natl Acad Sci U S A 83: 7956-7960.

6. Tipper,D.J., Bostian,K.A., 1984. Double-stranded ribonucleic acid killer systems in yeasts. Microbiol Rev 48: 125–156

7. Tarr PI, Aline RF Jr, Smiley BL, Scholler J, Keithly J, Stuart K., 1988. LR1: A candidate RNA virus of Leishmania. Proc Natl Acad Sci U S A 85: 9575-9575.

8. Cadd TL, Keenan MC, Patterson JL, 1993. Detection of Leishmania RNA virus 1 proteins. J Virol 67: 5647-5650.

9. Zangger H, Hailu A, Desponds C, Lye LF, Akopyants NS, Dobson DE, Ronet C, Ghalib H, Beverley SM, Fasel N, 2014. Leishmania aethiopica field isolates bearing an endosymbiontic dsRNA virus induce pro-inflammatory cytokine response. PLoS Negl Trop Dis 8: e2836.

10. Guilbride L, Myler PJ, Stuart K, 1992. Distribution and sequence divergence of LRV1 viruses among different Leishmania species. Mol Biochem Parasitol 54: 101-104.

11. Widmer G, Dooley S, 1995. Phylogenetic analysis of Leishmania RNA virus and Leishmania suggests ancient virus-parasite association. Nucleic Acids Res 23: 2300-2304.

12. Stuart KD, Weeks R, Guilbride L, Myler PJ, 1992. Molecular organization of Leishmania RNA virus 1. Proc of the Natl Acad Sci USA 89: 8596-8600.

13. Scheffter SM, Ro YT, Chung IK, Patterson JL, 1995. The complete sequence of Leishmania RNA virus LRV2-1, a virus of an old world parasite strain. Virology 212: 84-90.

14. Scheffter S, Widmer G, Patterson JL, 1994. Complete sequence of Leishmania RNA virus 1-4 and identification of conserved sequences. Virology 199: 479-483.

15. Rotureau B, Ravel C, Nacher M, Couppié P, Curtet I, Dedet JP, Carme B, 2006. Molecular epidemiology of Leishmania (viannia) guyanensis in French Guiana. J Clin Microbiol 44: 468-473.

16. Simon S, Veron V, Carme B, 2010. Leishmania spp. identification by polymerase chain reaction–restriction fragment length polymorphism analysis and its applications in French Guiana. Diagn Microbiol Infect Dis 66: 175-180.

17. Ives A, Ronet C, Prevel F, Ruzzante G, Fuertes-Marraco S, Schutz F, Zangger H, Revaz-Breton M, Lye L, Hickerson SM, Beverley SM, Acha-Orbea H, Launois P, Fasel N, Masina S, 2011. Leishmania RNA virus controls the severity of mucocutaneous leishmaniasis. Science 331: 775-778.

18. Salinas G, Zamora M, Stuart K, Saravia N, 1996. Leishmania RNA viruses in Leishmania of the Viannia subgenus. Am J Trop Med Hyg 54: 425-429.

19. Saiz M, Llanos-Cuentas A, Echevarria J, Roncal N, Cruz M, Muniz MT, Lucas C, Wirth DF, Scheffter S, Magill AJ, Patterson JL, 1998. Short report: Detection of Leishmaniavirus in human biopsy samples of leishmaniasis from Peru. Am J Trop Med and Hyg 58: 192-194.

20. Ogg MM, Carrion R,Jr, Botelho AC, Mayrink W, Correa-Oliveira R, Patterson JL, 2003. Short report: Quantification of Leishmaniavirus RNA in clinical samples and its possible role in pathogenesis. Am J Trop Med Hyg 69: 309-313.

21. Bourreau E, Ginouves M, Prévot G, Hartley M, Gangneux J, Robert-Gangneux F, Dufour J, Marie DS, Bertolotti A, Pratlong F, Martin R, Schütz F, Couppié P, Fasel N, Ronet C, 2015. Leishmania-RNA virus presence in L. guyanensis parasites increases the risk of first-line treatment failure and symptomatic relapse. J Infect Dis.

22. Pereira Lde O, Maretti-Mira AC, Rodrigues KM, Lima RB, Oliveira-Neto MP, Cupolillo E, Pirmez C, de Oliveira MP, 2013. Severity of tegumentary Leishmaniasis is not exclusively associated with Leishmania RNA virus 1 infection in Brazil. Mem Inst Oswaldo Cruz 108: 665-667.

23. Rotureau B, Gaborit P, Issaly J, Carinci R, Fouque F, Carme B, 2006. Diversity and ecology of sand flies (diptera: Psychodidae: Phlebotominae) in coastal French Guiana. Am J Trop Med Hyg 75: 62-69.

24. Fouque F, Gaborit P, Issaly J, Carinci R, Gantier JC, Ravel C, Dedet JP, 2007. Phlebotomine sand flies (diptera: Psychodidae) associated with changing patterns in the transmission of the human cutaneous leishmaniasis in French Guiana. Mem Inst Oswaldo Cruz 102: 35-40.

25. Croft SL, Molyneux DH, 1979. Studies on the ultrastructure, virus-like particles and infectivity of Leishmania hertigi. Ann Trop Med Parasitol 73: 213-226.

26. Ronet C, Beverley SM, Fasel N, 2011. Muco-cutaneous Leishmaniasis in the new world: The ultimate subversion. Virulence 2: 547-552.

27. Ives A, Masina S, Castiglioni P, Prevel F, Revaz-Breton M, Hartley MA, Launois P, Fasel N, Ronet C, 2014. MyD88 and TLR9 dependent immune responses mediate resistance to Leishmania guyanensis infections, irrespective of Leishmania RNA virus burden. PLoS One 9: e96766.

Table 1. Leishmania LRV positive or negative summary strains

Species	L. guyanensis		L. braziliensis		L. amazonensis		Leishmania spp.		%
LRV	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/outisde	25	4	2	3	0	3	27	10	73%
Total	90	22	6	5	0	6	96	33	
%	80%		55%		0%		74%		

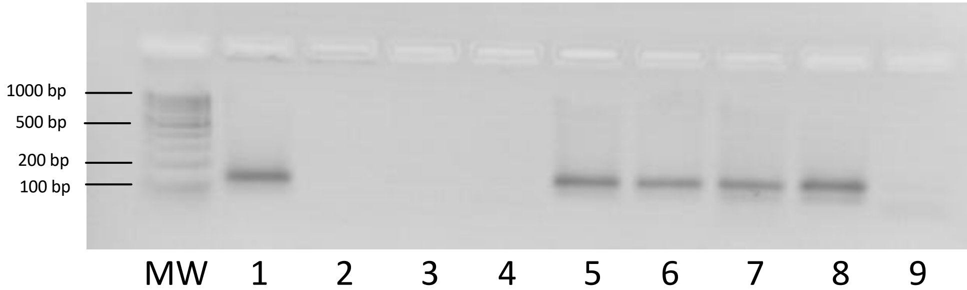

Figure 1 . LRV1 detection in different *Leishmania* isolates.
MW (Molecular Weight Marker 100bp, Sigma ®); 1,5,6 : LRV1 positive *Leishmania guyanensis* isolates; 2 : LRV1 negative *Leishmania guyanensis* isolate; 3, 4 : LRV1 negative *Leishmania amazonensis* isolates, 7 : LRV1 positive *Leishmania braziliensis*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

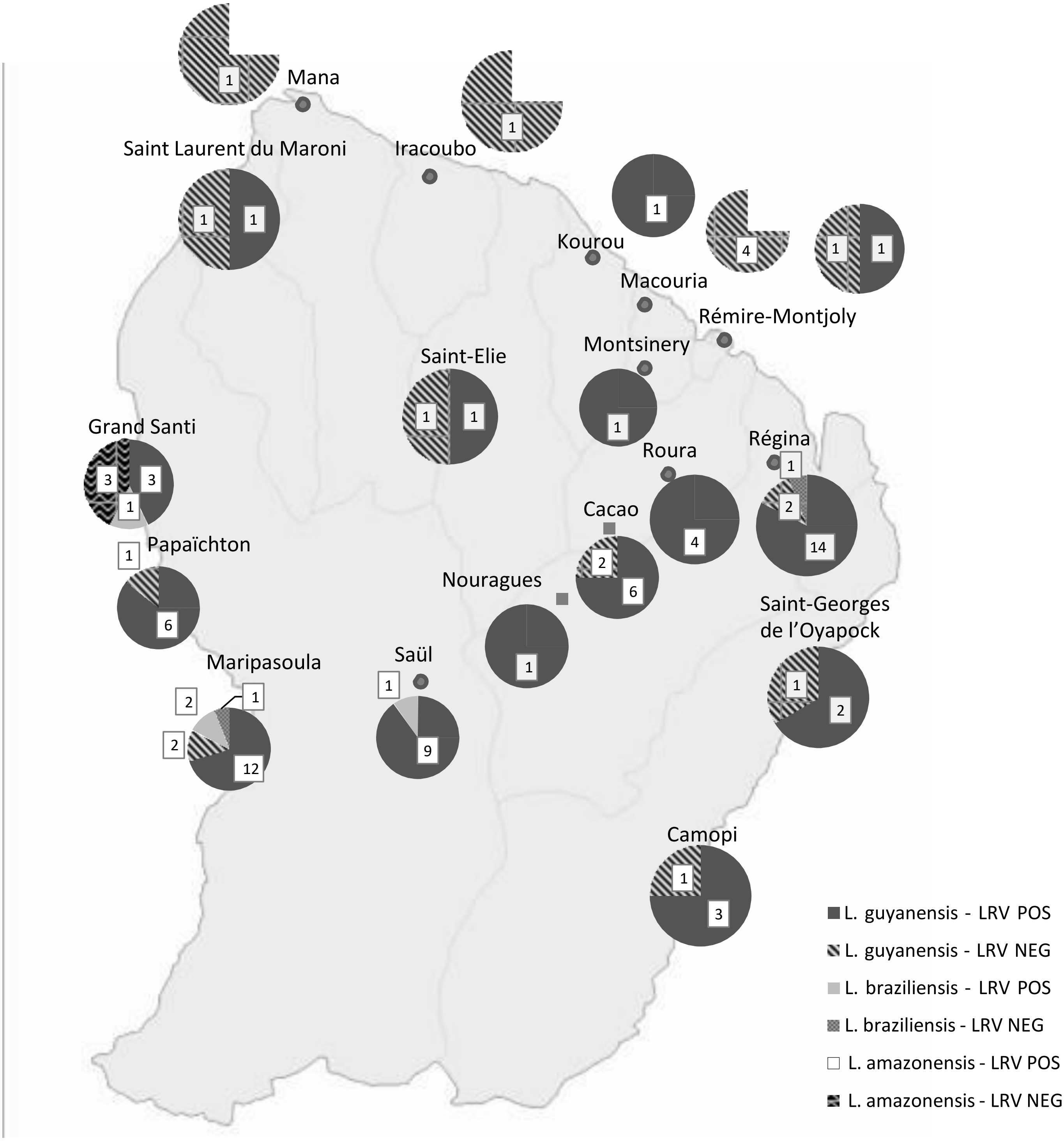

Figure 2. Distribution of *Leishmania* isolates carrying or not the LRV, in French Guiana. Pie charts full correspond to positive *Leishmania* LRV and pie charts with patterns correspond to negative *Leishmania* LRV.