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In Vitro Sensitivity of Cutaneous *Leishmania* Promastigote Isolates Circulating in French Guiana to a Set of Drugs

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Abstract. Anti-leishmaniasis drug resistance is a common problem worldwide. The aim of this study was to inventory the general in vitro level of sensitivity of *Leishmania* isolates circulating in French Guiana and to highlight potential in vitro pentamidine-resistant isolates. This sensitivity study was conducted on 36 patient-promastigote isolates for seven drugs (amphotericin B, azithromycin, fluconazole, meglumine antimoniate, miltefosine, paromomycin, and pentamidine) using the Cell Counting Kit-8 viability test. The IC_{50} values obtained were heterogeneous. One isolate exhibited high IC_{50} values for almost all drugs tested. Pentamidine, which is the first-line treatment in French Guiana, showed efficacy at very low doses (mean of 0.0038 μg/mL). The concordance of the in vitro pentamidine results with the patients' clinical outcomes was 94% (K = 0.82).

INTRODUCTION

Cutaneous leishmaniasis (CL) is the most common form of leishmaniasis worldwide with 0.7–1.3 million new cases each year.[1] In the New World, it is mainly caused by *Leishmania* (*Viannia*) *braziliensis*, *Leishmania* (*Leishmania*) *amazonensis*, and *Leishmania* (*Viannia*) *guyanensis*. CL and disseminated cutaneous leishmaniasis (DCL) potentially cause disfiguring scars, and muco-cutaneous leishmaniasis (MCL) can potentially cause obstruction or destruction of the nose, pharynx, and larynx. Treatments are available to cure this infection, but therapeutic options are threatened by the emergence of resistant strains.

Many drugs used as first- or second-line treatment are approved by the World Health Organization (WHO) and are used in different world regions, depending on the species and the clinical presentation. The conventional treatments based on meglumine antimoniate, sodium stibogluconate, and pentamidine cause severe side effects and require parenteral administration. Visceral leishmaniasis (VL) has become increasingly drug resistant with a non-response rate to antimonials of over 60% in Bihar, India.[2] In CL, the efficacy of antimonials varies: 94.2% in Bolivia,[3] 84% in Brazil,[4] 75.6–78% in Peru,[5,6] and 61–67% in Colombia.[7] The efficacy of pentamidine is 35% in Peru.[6] Other treatments have been used, such as amphotericin B as a first- or second-line treatment to cure VL, MCL, and CL. Trials have shown the effectiveness of a less toxic form, liposomal amphotericin B (Ambisome®), in the treatment of CL.[8–11]

The efficacy of azithromycin against CL ranges from an 85% cure rate in *L. braziliensis* infections in Araçuaí and Varzelândia, Brazil[12] to 45.5% for the same species in Salta, Argentina.[13] Fluconazole has good efficacy, which increases with the given dose, for the treatment of *L. major* infections.[14,15] High doses of fluconazole are required for *L. braziliensis* infections.[16] The miltefosine cure rate varies from 82% in Guatemala to 33% in Colombia.[17] The cure rate is dose dependent and can reach 94%[18] for New World strains. The use of injectable paromomycin is not effective against CL.[19,20] However, its topical use as an ointment cured CL caused by species of the New and Old World, especially when supplemented with gentamicin.[21–25] However, the efficacy may vary.[26]

Until 1980, meglumine antimoniate (Glucantime®) was the first intention treatment of leishmaniasis in French Guiana. It was replaced in 1980 by pentamidine (Lomidine®) and since 1992, by pentamidine isethionate (Pentacarinat®), for *L. guyanensis* infections. Recurrences were reported in French Guiana in two studies. One reported a relapse rate of 6.8% for *L. guyanensis* in 219 patients followed from 1981 to 1987.[27] The other reported a 33% recurrence rate in 21 military patients monitored between 2004 and 2005.[28] The mechanism of late recurring leishmaniasis is poorly understood. Several mechanisms may be involved, such as late onset reactivation of persistent living parasites or the presence of *Leishmania* clones with lower drug sensitivity within isolates.

The annual incidence of CL in French Guiana is 0.5 ‰, with 86.2% of cases due to *L. guyanensis*, 9.7% due to *L. braziliensis*, 2.8% due to *L. amazonensis*, and 1.3% due to *L.(Viannia) lainsoni* (Simon and others, submitted). The first-line treatment against the predominant species is pentamidine[29] and the second-line treatment is meglumine antimoniate for *L. braziliensis* infections. Some cases of clinical resistance to these treatments have been reported in French Guiana.[27,28]

This study aimed to determine the levels of in vitro sensitivity of *Leishmania* spp. isolates circulating in French Guiana to available treatments, and the pentamidine threshold resistance value. We performed in vitro *Leishmania* spp. sensitivity tests, using promastigote forms, for seven drugs: amphotericin B, azithromycin, fluconazole, meglumine antimoniate, miltefosine, paromomycin, and pentamidine.

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TABLE 1

IC_{50} of *Leishmania* isolates and *Leishmania guyanensis* reference strain according to the medium used and the drug tested

			Promastigote IC_{50}							
			Amphotericin B		Azithromycin		Fluconazole		Meglumine antimoniate	
Species	Medium	Isolate number	µg/mL	95% CI	µg/mL	95% CI	µg/mL	95% CI	µg/mL	95% CI
L. guyanensis	R	1	4.16	3.50–4.94	> 3,000	–	3,759	3,252–4,345	9,510	8,768–10,316
		2	11.51	11.17–11.87	> 3,000	–	5,000–10,000	–	7,500–15,000	–
		3	4.19	3.52–4.98	192.4	175.9–210.4	3,607	495.2–26,267	5,222	3,666–7,438
		4	1.87	1.63–2.15	–	–	–	–	3,057	2,711–3,447
		5	1.56–3.12	–	–	–	–	–	2,781	2,103–3,676
		6	1.58	1.43–1.74	–	–	–	–	3,353	3,065–3,669
		7	5.38	4.70–6.16	–	–	–	–	3,034	2,860–3,218
		8	1.89	1.61–2.21	–	–	–	–	2,562	1,009–6,504
		9	2.57	2.39–2.77	–	–	–	–	2,802	2,458–3,193
		10	0.78–1.56	–	–	–	–	–	< 937.5	–
		11	9.81	8.86–10.85	–	–	–	–	15,000–30,000	–
		12	6.25–12.5	–	–	–	–	–	15,000–30,000	–
		13	1.56–3.12	–	–	–	–	–	1,597	1,494–1,708
		14	1.03	0.85–1.23	–	–	–	–	< 937.5	–
		15	3.76	3.12–4.53	–	–	–	–	1,875–3,750	–
		Ref*	**1.4**	**1.31–1.492**	**< 93.75**	**–**	**3,117**	**3,016–3,222**	**10,705**	**9,830–11,657**
Isolates interval values in R medium†			1.03–12.5		192.4–> 3,000		3,607–10,000		< 937.5–30,000	
L. guyanensis	S	16	6.25–12.5	–	–	–	–	–	15,000–30,000	–
		17	23.89	0.3079–1,854	–	–	–	–	> 30,000	–
		18	6.25–12.5	–	–	–	–	–	13339	10,841–16,412
		19	> 25	–	–	–	–	–	> 30,000	–
		20	–	–	–	–	–	–	–	–
		21	3.12–6.25	–	–	–	–	–	–	–
		22	3.1	–	–	–	–	–	< 937.5	–
		23	3.12–6.25	–	–	–	–	–	> 30,000	–
		24	3.12–6.25	–	–	–	–	–	18,699	4,757–73,501
		25	6.25–12.5	–	–	–	–	–	–	–
		26	3.92	–	–	–	–	–	< 937.5	–
		27	4.93	4.82–5.04	102.3	88.52–118.2	3,145	2,995–3,303	18,642	8,707–39,913
		Ref	**2.08**	**0.002–2,044**	**< 93.75**	**–**	**< 312.5**	**–**	**< 937.5**	**–**
Isolates interval values in S medium†			3.1–> 25		102.3		3,145		< 937.5–> 30,000	
L. guyanensis	R-BH	28	6.25–12.5	–	187.5–375	–	2,500–5,000	–	13,130	12,902–13,362
		29	4.41	4.11–4.73	35.15	17.68–69.89	2,993	2,951–3,035	7,349	4,966–10,875
		30	13.31	12.71–13.94	83.72	74.91–93.58	3,327	3,244–3,411	–	–
		31	> 25	–	177.7	139.3–226.5	4,638	3,505–6,138	> 30,000	–
		32	3.29	3.21–3.37	< 93.75	–	2,011	1,893–2,137	4,744	3,343–6,730
		33	7.83	7.38–8.31	< 93.75	–	2,500–5,000	–	–	–
		Ref	**2.39**	**2.27–2.54**	**< 93.75**	**–**	**2,992**	**2,773–3,228**	**10,635**	**9,424–12,001**
Isolates interval values in R–BH medium†			3.29–> 25		< 93.75–375		2,011–5,000		4,744–> 30,000	
Leishmania braziliensis	R–BH	34	1.27	1.08–1.48	83.31	59.73–116.2	830.7	687.6–1,004	–	–
L. braziliensis	R	35	< 0.78	–	–	–	–	–	–	–
Leishmania amazonensis	R–BH	36	8.35	7.77–8.98	< 93.75	–	3,105	2,983–3,232	9,822	9,384–10,282
Total isolates interval values‡			< 0.78–> 25		< 93.75–> 3,000		830.7–10,000		4,744–> 30,000	

(continued)

TABLE 1
Continued

Species	Medium	Isolate number	Promastigote IC_{50} Miltefosine µg/mL	Miltefosine 95% CI	Paromomycin µg/mL	Paromomycin 95% CI	Pentamidine µg/mL	Pentamidine 95% CI	Clinical outcome§
L. guyanensis	R	1	3.91	3.25–4.34	< 156.25	–	0.0013	0.012–0.014	Sensitive presumed
		2	3.9–7.8	–	< 156.25	–	0.00078–0.0015	–	Sensitive
		3	< 3.9	–	< 156.25	–	0.0011	0.0009–0.001	–
		4	< 3.9	–	1,211	982.7–1,492	–	–	Sensitive
		5	< 3.9	–	> 5,000	–	–	–	Sensitive presumed
		6	< 3.9	–	701.8	542.7–907.4	–	–	–
		7	< 3.9	–	1,154	928.8–1,434	0.0065	–	Sensitive
		8	< 3.9	–	2,314	826.1–6,484	> 0.01	–	Resistant
		9	< 3.9	–	953.4	365.1–2,489	0.004	0.004–0.005	Sensitive
		10	5.27	4.69–5.92	< 156.25	–	0.0025	0.002–0.003	Sensitive
		11	10.53	7.97–13.90	96.27	53.84–172.2	0.0056	0.005–0.006	Sensitive presumed
		12	10.63	9.10–12.42	359.7	338.5–382.2	0.006	0.003–0.01	–
		13	–	–	269.7	237.1–306.7	0.0094	0.003–0.02	Resistant
		14	< 3.9	–	56.47	39.93–79.86	0.0059	0.005–0.006	Sensitive
		15	6.64	5.33–8.26	98.12	89.52–107.5	0.0057	0.005–0.006	Sensitive
		Ref*	**3.9–7.8**	**–**	**110**	**75.5–160.3**	**0.002119**	**0.0017–0.0025**	
Isolates interval values in R medium†			< 3.9–10.63		< 156.25–> 5,000		0.001–> 0.01		
L. guyanensis	S	16	> 125	–	48.12	14.82–156.3	0.0041	0.003–0.004	–
		17	10.6	9.17–12.26	< 156.25	–	0.00312–0.00625	–	Sensitive presumed
		18	7.6	6.89–8.39	< 156.25	–	0.003	0.002–0.003	Sensitive
		19	> 125	–	267.3	241.9–295.2	> 0.01	–	Resistant
		20	< 3.9	–	< 156.25	–	0.00312–0.00625	–	Sensitive presumed
		21	9.95	8.17–12.13	< 156.25	–	0.0025	0.0022–0.0027	Resistant
		22	6.66	5.92–7.50	< 156.25	–	0.0033	0.002–0.005	–
		23	7.8–15.6	–	< 156.25	–	0.0026	0.001–0.004	Sensitive
		24	11.7	10.81–12.66	< 156.25	–	0.0036	0.0033–0.0039	Intermediate
		25	3.09	2.57–3.72	98.52	34.45–281.7	0.0015	0.0013–0.0019	Intermediate
		26	1.55	0.17–13.96	< 156.25	–	0.0033	0.002–0.004	–
		27	< 3.9	–	738.9	425.7–1,283	0.00312–0.00625	–	–
		Ref	**8.85**	**4.9–15.9**	**186.8**	**134.6–259.2**	**0.002329**	**0.00084–0.0064**	
Isolates interval values in S medium†			< 3.9–> 125		48.12–738.9		0.0015–> 0.01		
L. guyanensis	R-BH	28	3.52	3.24–3.82	159.8	134.5–189.9	0.00312–0.00625	–	Sensitive presumed
		29	< 3.9	–	358.1	275.5–465.4	0.0015–0.00312	–	–
		30	1.61	0.52–5.01	714.3	611.7–834.2	0.0035	0.003–0.004	Sensitive presumed
		31	< 3.9	–	4,461	303.6–65,551	0.0028	0.002–0.003	–
		32	< 3.9	–	–	–	0.0015–0.00312	–	–
		33	–	–	–	–	–	Intermediate	
		Ref	**6.2**	**5.9–6.52**	**77.89**	**46.15–131.5**	**0.002537**	**0.0023–0.0027**	
Isolates interval values in R–BH medium†			1.61–3.52		159.8–4,461		0.001–0.006		
Leishmania braziliensis	R–BH	34	3	1.31–6.85	–	–	0.0016	0.001–0.002	–
L. braziliensis	R	35	< 3.9	–	< 156.25	–	0.00625–0.0125	–	–
Leishmania amazonensis	R–BH	36	1.95	0.45–8.29	129.6	122.1–137.5	0.00312–0.00625	–	–
Total isolates interval values‡			< 3.9–> 125		< 156.25–> 5,000		0.001–> 0.01		

IC_{50} = 50% inhibitory concentration; R = RPMI 1640 medium; R-BH = RPMI 1640 medium + L-Biopterin and Hemin Chloride; S = Schneider medium (+L-Biopterin and Hemin Chloride).
*Ref = *Leishmania guyanensis* reference strain.
†Isolates interval values for each medium according to the drug tested.
‡Isolates interval values for all medium included, according to the drug tested.
§Sensitive, cured after one course of pentacarinat; sensitive presumed, presumed cured after one course of pentacarinat (did not consult again); intermediate, cured after two courses of pentacarinat; resistant, cured after three or more courses of pentacarinat.

MATERIALS AND METHODS

Parasites and cultures. There were 221 patients consulting the dermatology department of Cayenne hospital or one of the health centers across French Guiana between April 2013 and May 2014, who were diagnosed as *Leishmania* positive using the polymerase chain reaction restriction fragment length polymorphism identification technique.[30] Biopsies collected from patients for diagnosis were cultured at 26°C in Roswell Park Memorial Institute medium 1640 medium (Gibco®, Paisley, Scotland) containing L-glutamine, 20 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, and phenol red, supplemented with 20% heat-inactivated fetal calf serum (Gibco, Paisley, Scotland), 50 IU/mL penicillin (Invitrogen®, Carlsbad, CA), 0.05 mg/mL streptomycin (Invitrogen, Carlsbad, CA), and nonessential amino acids (Gibco, Paisley, Scotland). Thirty-six culture isolates, representing approximately 28.7% of all annual cases, were suitable for drug-sensitivity tests.

The MHOM/GF/97/LBC6 reference *L. guyanensis* strain was originally from French Guiana.

Drugs. The stock concentrations of drugs were 250 µg/mL for amphotericin B (liquid solution, Sigma-Aldrich, St. Louis, MO), 30 mg/mL for azithromycin (Sigma-Aldrich, St. Louis, MO) diluted in ethanol, 100 mg/mL for fluconazole (Sigma-Aldrich, St. Louis, MO) diluted in DMSO, 300 mg/mL for meglumine antimoniate (liquid solution supplied by the CHC, Glucantime, Aventis, France), 1.25 mg/mL for miltefosine (Sigma-Aldrich, St. Louis, MO) diluted in ethanol, 50 mg/mL for paromomycin (Sigma-Aldrich, St. Louis, MO) diluted in sterile water, and 100 mg/mL for pentamidine (Sigma-Aldrich, St. Louis, MO) diluted in sterile water. Solutions were stored at –20°C.

The optimal concentration ranges were first determined for each drug. Based on these results, serial 2-fold dilutions were performed to obtain the final testing concentrations, which were 0.78–25 µg/mL for amphotericin B, 93.75–3,000 µg/mL for azithromycin, 312.5–10,000 µg/mL for fluconazole, 937.5–30,000 µg/mL for meglumine antimoniate, 3.9–125 µg/mL for miltefosine, 156.25–5,000 µg/mL for paromomycin, and 0.00039–0.0125 µg/mL for pentamidine.

In vitro promastigote sensitivity tests. *Leishmania* promastigotes were cultured in different media: either in RPMI 1640 medium (Gibco, Paisley, Scotland) containing L-glutamine, 20 mM HEPES, without phenol red, supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, Scotland), 50 IU/mL penicillin (Invitrogen, Carlsbad, CA), 0.05 mg/mL streptomycin (Invitrogen, Carlsbad, CA), nonessential amino acids (Gibco, Paisley, Scotland), which was further supplemented with 0.6 mg/mL L-Biopterin (Santa Cruz Biotechnology®, Heidelberg, Germany) and 5 mg/mL Hemin Chloride (Santa Cruz Biotechnology, Heidelberg, Germany) (called R-BH medium) or not (called R medium); or in Schneider's drosophila medium (Sigma®, St. Louis, MO) containing L-glutamine and supplemented with 10% heat-inactivated fetal calf serum (Gibco, Paisley, Scotland), 0.6 mg/mL L-Biopterin (Santa Cruz Biotechnology, Heidelberg, Germany), and 5 mg/mL Hemin Chloride (Santa Cruz Biotechnology, Heidelberg, Germany) (called S medium). Viability tests were performed in triplicate using the Cell Counting Kit-8 assay (Sigma, St. Louis, MO), according to the procedure of Ginouves and others.[31] Briefly, 10^6 parasites/well, in the exponential growth phase, were placed in contact with different drug concentrations in a 96-well plate for 48 hours at 26°C. Then, 10% of WST-8 was added and the parasites were incubated for a further 24 hours at 26°C. Absorbance was measured at 450 nm using a Tristar LB941 spectrophotometer (Berthold Technologies®, Wildbad, Germany) or Multiskan (Thermo Scientific® Waltham, MA). The percentage of inhibition was obtained as follows: % inhibition = $[(A_{control} - A_{test})/A_{control}] \times 100$. The 50% inhibitory concentration (IC_{50}) was calculated using GraphPad Prism6® software (La Jolla, CA).

Ethical aspects. The study of patient outcomes was retrospective and monocentric. All patients were informed using written documents and posters during consultation that case records and biological data might be further used in research and that they had the right to refuse. The monocentric audit of retrospective anonymized case record data is permitted by the CNIL (National Commission for Informatics and Liberties) (number 1805118v0), and the project did not raise any specific concerns by the Ethical Committee at Cayenne General Hospital.

Statistical analysis. The 95% confidence interval (95% CI) was determined using GraphPad Prism6 software for each test. Kappa test values were determined using STATA® (College Station, TX), to assess the concordance between the in vitro phenotype "susceptible or resistant" to pentamidine and patient outcomes.

RESULTS

***Leishmania* sensitivity levels.** The in vitro sensitivity tests performed on the 36 promastigote isolates included 33 isolates of *L. guyanensis*, two of *L. braziliensis*, and one of *L. amazonensis* (Table 1). IC_{50} values varied widely: they ranged from 1.03 (or even < 0.78) to 23.89 (> 25) µg/mL for amphotericin B, 35.15 to 192 (or even > 3,000) µg/mL for azithromycin, 830.7 to 4,638 (> 5,000) µg/mL for fluconazole, 1,597 (< 937.5) to 18,699 (> 30,000) µg/mL for meglumine antimoniate, 1.55 to 11.7 (> 125) µg/mL for miltefosine, 48.12 to 4,461 (> 5000) µg/mL for paromomycin, and 0.001 to 0.0094 (> 0.01) µg/mL for pentamidine (Table 1).

The reference strain, a strain isolated in 1997 in French Guiana, allowed assessment of the evolution of the sensitivity of patient isolates. Moreover, this strain had the lowest IC_{50} and was, therefore, considered to be the sensitive reference strain for interpretation of the IC_{50} results.

IC_{50} values for patient isolates were variable relative to the reference strain values, except for fluconazole, for which the values were close to those of the reference strain. One isolate, number 19, was of interest because it presented high IC_{50} values for four of the five drugs tested (amphotericin B, meglumine antimoniate, miltefosine, and pentamidine) and the corresponding patient was also very difficult to treat.

Various media were used in this study. RPMI medium was first used, because of its use in parasite cultures from diagnostic biopsies. The parasites are generally difficult to maintain in vitro. The culture medium was thus improved by adding essential factors, such as biopterin[32,33] and hemin chloride,[34] to the RPMI medium and another *Leishmania* medium, Schneider's Drosophila medium. The addition of these essential factors improved parasite growth, but may have influenced the drug sensitivity of the isolates.[35] Indeed, we tested two patient isolates with the three

different media and observed large differences depending on the drug tested for one of the two isolates, from 10 to more than 100 times (data not shown). These differences may result from the influence of the medium composition on drug activity.[35] However, the IC_{50} values obtained in this study were globally in the same range, suggesting that the media were generally equivalent.

The lowest concentrations required for the in vitro tests were for pentamidine (average of 0.0038 μg/mL), miltefosine, and amphotericin B (average of 3.00 and 5.81 μg/mL, respectively).

Phenotypic variation and culture conditions. We performed sensitivity tests at two random time intervals with four *L. guyanensis* isolates in R-BH medium (Table 2) to estimate the temporal phenotypic variability of *Leishmania* isolates. The random variability of the IC_{50s} did not appear to result from the time in culture (each with low passages of 1–4), but was associated with the drug tested. This variability was particularly pronounced for azithromycin (up to a 1.107-fold difference).

Comparison of in vitro sensitivity to pentamidine and clinical outcome. The in vitro sensitivity of *Leishmania* to pentamidine was related to clinical features (Table 1). Isolates from patients cured after a single course of pentamidine were considered to be sensitive. Patients who received a single course of pentamidine isethionate and did not consult again were considered to be cured (in previous studies conducted at the reference center for leishmaniasis treatment in French Guiana, patients indicated that they were better and did not see the point in returning when asked why they failed to come to their control consultation[36]) and the isolate to be sensitive. Isolates from patients who were cured after two courses of pentamidine were considered to be intermediate. Isolates from patients who were cured after three or more courses of pentamidine were considered to be resistant.

There was a strong correlation (r = 0.94 [17/18 without considering the intermediate status; K = 0.82]) between the in vitro results and patient outcomes.

DISCUSSION

This first study on the anti-leishmanial drug sensitivity of cutaneous *Leishmania* isolates from French Guiana showed great heterogeneity between isolates, and revealed one in vitro-resistant isolate to four of the five drugs tested.

We performed the tests using the promastigote form, because it was the easiest form to handle on a large scale and allowed us to make a first assessment of the drug sensitivity of the circulating isolates. The promastigote form model is not recommended in the literature for in vitro sensitivity tests because several parameters can influence the sensitivity results (such as cell density, growth rates, the drug tested, medium composition)[35]; it is not the mammalian form, and is generally less sensitive to some drugs or plant compounds, unlike the intracellular amastigote or axenic amastigote forms. Indeed, it appears that intracellular amastigote forms better reflect the observed sensitivity in patients, especially to pentavalent antimonials,[37] which require conversion by the host cell to a trivalent form.[38] Moreover, promastigote sensitivity has been shown to be variable for the drugs tested, with low sensitivity to paromomycin and higher sensitivity to pentamidine than

TABLE 2
Temporal phenotypic variability of *Leishmania* isolates

Isolate	Interval time between 2 tests	Test number	Amphotericin B		Azithromycin		Fluconazole		Meglumine antimoniate		Miltefosine		Paromomycin		Pentamidine	
			CI50 (μg/mL)	Ratio (test2/ test1)	CI50 (μg/mL)	Ratio (test2/ test1)	CI50 (μg/mL)	Ratio (test2/ test1)	CI50 (μg/mL)	Ratio (test2/ test1)	CI50 (μg/mL)	Ratio (test2/ test1)	CI50 (μg/mL)	Ratio (test2/ test1)	CI50 (μg/mL)	Ratio (test2/ test1)
A	1 month	Test 1	4.935	1.9	102.3	1.3	3,145	1.3	18,642	2.2	0.963	3.2	738.9	1.2	0.00319	2.2
		Test 2	9.147		81.5		4,076		8,357		3.12		611.7		0.001458	
B	1 month	Test 1	4.412	0.9	35.15	**0.18.10^7**	2,993	1.2	7,349	1.7	3.103	17	358.1	2.8	0.00278	0.5
		Test 2	3.79		0.000019		3,494		4,269		0.18		1,006		0.001379	
C	1 month	Test 1	3.294	3.6	9.684	**1.6.10^7**	2,011	2.0	4,744	2.5	0.1324	1.2	–	–	0.002657	0.5
		Test 2	11.968		6.1E–07		4,039		11,951		0.16		< 156.25		0.00133	
D	3 month	Test 1	2.059	–	–	–	–	–	6,559	2.5	5.457	0.8	105.6	5.3	0.00704	0.7
		Test 2	–		217.6		3,334		16,574		4.357		568		0.00504	

In bold: highest values among each pair of test (ratio > 1.5).

amastigote[39,40] or axenic amastigote forms.[41] However, the promastigote and amastigote forms display similar sensitivity to miltefosine and amphotericin.[42] Though, there is no correlation between in vitro results using promastigotes and patient clinical outcomes for visceral leishmaniasis antimonial assays, unlike for the amastigote form[43,44] for which in vitro tests correlate well with clinical outcome. In contrast, Grogl and others showed an 86–89% correlation coefficient for the patient response to sodium stibogluconate and meglumine antimoniate treatment and in vitro susceptibility of promastigotes from CL and MCL *Leishmania* isolates.[45] Here, we observed a 94% correlation between the in vitro pentamidine results and patient outcomes. Moreover, there is concordance between in vitro promastigotes and intracellular amastigotes[46] for antimonials, when they are in the identical environment.[47]

The axenic amastigote form has been suggested to be a possible alternative, because of its morphological and metabolic similarity to the intracellular macrophage amastigote form,[48] but it shares the same drawback with the promastigote form because of its inability to accumulate drugs as macrophages do.[41] The mammalian intracellular amastigote model has been recommended as the gold standard. However, several factors may bias the response to drugs in this form also, including the type of macrophage used,[49] the variable macrophage infection rate,[49] macrophage infectivity depending on the *Leishmania* species,[50] incomplete intracellular transformation into the amastigote[37] and, as with axenic amastigote forms, the long process of adaptation to the environment and transformation, which leads to the selection of subpopulations.[51,52] In vitro amastigote intracellular results also do not always correlated with the clinical outcome of the patients,[53] particularly due to host factors. Finally, this model is inappropriate for large-scale in vitro monitoring of drug efficacy. Overall, each model has its benefits and drawbacks.

As mentioned above, there are some potential limitations in this study. Comparison tests using the intracellular amastigote form may be informative. A larger number of isolates would refine and confirm the promastigote sensitivity threshold for pentamidine (determined to be $\geq$ 0.009 µg/mL in this study), as well as the in vitro and in vivo consistency. Another important limitation was the large variation in the results of the same isolate when tested in different media, depending on the drugs used, making it challenging to compare the results from one study to another.

Despite these limitations, this study may provide the first baseline to monitor the evolution of the drug sensitivity of *Leishmania* isolates in French Guiana.

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