International Scholars Journals

African Journal of Parasitology Research ISSN 2756-3391 Vol. 9 (1), pp. 001-005, November, 2024. Available online at www.internationalscholarsjournals.com © International Scholars Journals



*Research*

# Molecular diagnosis of the first cases of *Leishmania tropica* cutaneous leishmaniasis in elementary school pupils in the Tchiamba-Nzassi health district in Pointe Noire, Republic of Congo

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Received: 01-Nov-2024, Manuscript No. AJPROAJ-24-151554; Editor assigned: 06-Nov-2024, PreQC No. AJPROAJ-24-151554 (PQ); Reviewed: 20-Nov-2024, QC No. AJPROAJ-24-151554; Revised: 22-Nov-2024, Manuscript No. AJPROAJ-24-151554 (R); Published: 20-Dec-2024

**Introduction: Leishmaniasis is a neglected tropical disease caused by a flagellate parasite of the genus Leishmania transmitted to humans by the bite of a sandfly.**

**General objective: To contribute to the molecular diagnosis of cutaneous leishmaniasis in a population of elementary school pupils in the Tchiamba-Nzassi health district with suspicious chronic lesions in the Pointe-Noire department.**

**Methodology: This was a descriptive cross-sectional study including 23 suspected cases of cutaneous leishmaniasis among elementary school pupils in the DS of Tchiamba-Nzassi in the department of Pointe-Noire, which took place from June to November 2022, a period of 06 months. Sociodemographic characteristics (age, sex) were collected using a survey form.**

**Two diagnostic methods were used to detect Leishmania: Parasitological examination of MGG-stained smears and molecular diagnosis, which involved amplifying Leishmania DNA in lesion secretions by real-time PCR using the TECHNE "Pro qPCR Reagents Leishmania" kit.**

**Results: A total of 23 patients were studied. The mean age of our subjects was 11.7 ± 2 years, with extremes ranging from 8 to 15 years. Males were predominantly represented (67%), with a sex ratio (2H/1F) of 2.**

**Parasitological diagnosis did not reveal the amastigote form of leishmaniasis on the slides. Molecular analysis revealed Leishmania DNA in three (3) samples, *i.e.,* 13% of the study population. The lesions in which *L. tropica* was detected were the legs (2 cases) and the toe (1 case).**

**Conclusion: The incidence of cutaneous leishmaniasis was low in our study. This pathology affected children of all ages with chronic skin lesions located preferentially on the lower limbs. Molecular biology was used to diagnose this neglected tropical disease and the species identified was *Leishmania tropica*.**

**Keywords:** Cutaneous leishmaniasis, Molecular diagnosis, *Leishmania tropica*, Students, Pointe-Noire, Congo.

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## INTRODUCTION

Leishmaniasis is an infectious disease affecting mammals, including humans. They are caused by the multiplication in cells of the mononuclear phagocyte system of flagellate protozoa belonging to the genus Leishmania. Over 20 different species of Leishmania are known to cause disease in humans, with varying degrees of pathology. The species is the main determinant of the clinical course of the disease [1]. Leishmaniasis is transmitted to humans by the bite of the hematophagous female of a dipteran insect known as the "phlebotomus" [2]. Infection is manifested by lesions affecting the dermis or Cutaneous Leishmaniasis (CL) or the internal organs or Visceral Leishmaniasis (VL).

The disease is classified by the WHO as an important neglected parasitic disease, second only to malaria in terms of parasitic disease mortality [2]. According to WHO estimates, there are 1.3 million new cases and between 20,000 and 30,000 deaths per year.

In 2021, according to the WHO, leishmaniasis is endemic in over 98 countries, including those in tropical and subtropical zones. More than a billion people are at risk of contracting the infection and around 0.7 to 1 million cases of LC are reported each year [3].

In contexts of peri-domestic and anthroponotic transmission, children account for a significant number of cases. In 2016 for the Americas, among the 48915 reported LC cases, 15.5% were children ≤ 10 years of age [4]. In the Eastern Mediterranean region, 100,000 new cases of LC are reported each year and previous reports from Iran note that paediatric patients account for 7%-10% of cases [5,6].

Indeed, children constitute a particular population of LC cases [7]. It causes chronic, often ulcerative, skin lesions that leave permanent scars on the face or other visible areas [8].

LC is responsible for the ninth highest burden of disease among infectious diseases, but remains a much-neglected tropical disease, largely ignored in discussions of tropical disease priorities [9].

In Africa, according to available data, Cameroon, Chad and Nigeria have been identified as endemic foci of cutaneous and visceral leishmaniasis. Congo Brazzaville lies close to Cameroon, a leishmaniasis-endemic country. Its landscape and vegetation are similar to those of Cameroon, but published data on the disease are lacking.

Conventional methods for diagnosing leishmaniasis, such as parasitological or serological tests, still have their limitations [10,11]. Identification of the causal agent by these tests is difficult even in well-equipped facilities [12]. Polymerase Chain Reaction (PCR) has now revolutionized contemporary etiological diagnosis of infectious diseases, making it viable to use the method for the diagnosis of leishmaniasis [13,14]. However, certainty diagnostic tools for leishmaniasis are very limited in developing countries such as Congo Brazzaville.

Despite the fact that the vector has been reported in Congo, the country has still not reported any cases of endogenous leishmaniasis [15]. In addition, every year during the rainy season between January and May, there is an epidemic of skin sores among children aged 8 to 15 in the Tchiamba-Nzassi health district of Pointe-Noire. No epidemiological data have been reported on this phenomenon, let alone an etiological diagnosis. Given that Tchiamba-Nzassi is home to several lakes, rivers and oil pollution activities, the potential vectors of leishmaniasis, sandflies, find their habitat in these lakes. It is in this context that we conducted this study, with the overall aim of contributing to the diagnosis of cutaneous leishmaniasis in a population of elementary school pupils in the Tchiamba-Nzassi Health district presenting with suspicious chronic lesions in the Pointe-Noire department.

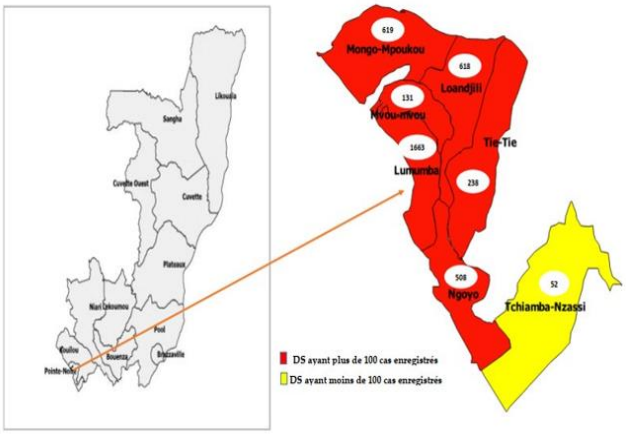
## MATERIALS AND METHODS

We carried out a descriptive cross-sectional study from June 1st to November 1st 2022, *i.e.,* a duration of 6 months.

The study took place in Pointe Noire, Republic of Congo, in the Tchiamba-Nzassi health district (DS/T), where we took the samples and in the molecular biology laboratory of the Marie Madeleine GOMBES foundation for sample conservation, parasitological analysis and molecular analysis.

The Tchiamba-Nzassi health district comprises 21 villages. It is bordered to the north by the districts of Hinda and Mvouti, to the south by the Atlantic Ocean, to the east by Cabinda and to the west by the Commune of Pointe-Noire. It covers an area of 1,032 km$^2$ (Figure 2).

**Figure 1.** Map of the Tchiamba- Nzassi health district.




Study participants were DS/T elementary school pupils with suspected chronic skin lesions referred by health staff. DS/T elementary school pupils with suspected chronic skin lesions referred by health staff and for whom parental consent had been obtained were included. Patients for whom extraction was unsuccessful. Students with skin lesions but not referred by health staff were excluded. We carried out non-exhaustive probability sampling among the samples that met the selection criteria. Data collection was based on an information sheet. The following data were collected. Age, sex, site of lesion. For each patient, a slide swab was taken with a sterile swab for parasitological examination and a second swab was discharged into NEST transport medium for molecular qPCR analysis targeting the mspC gene for GSP63 of *Leishmania tropica*.

Parasitological analysis of the first sample involved staining the slide with May Grunwald Giemsa. Direct Examination (DE) followed by staining with May Grunwald Giemsa.

DNA extraction was performed using the Canvax Biotech, S.L, (Spain) extraction kit according to the manufacturer's protocol. Next, amplification was undertaken using the Leishmania Pro qPCR Reagents TECHNE amplification kit, (USA) by real-time quantitative PCR (qPCR). The 20 µl reaction volume consisted of 10 µl master mix, 1 µl Leishmania primer/probe mix, 1 µl internal extraction control primer/probe mix, 3 µl RNAse/DNAse free water, 5µl DNA sample. The amplification protocol for each cycle was 95°C for 2 minutes, 95°C for 10 seconds and 60°C for 60 seconds. The number of cycles was 50. Results were interpreted according to the manufacturer's recommendations. DNA amplification controls for each sample were performed by qPCR.

A positive slide result was obtained when amastigote forms were observed under microscopy. An amplification curve was considered positive when the FAM CT was less than 35 and the VIC internal control indicated the presence of *Leishmania tropica* DNA.

The variables studied were age, sex, site of skin lesion, result of parasitological analysis of slides and result of *Leishmania tropica* DNA amplification. Data were entered and analyzed using Excel 2013 software. Results were expressed as mean ± standard deviation for quantitative variables and as headcount and percentage for qualitative variables.

The study was conducted in compliance with the rules of ethics and anonymity. Consent was obtained from the schools and the students' parents.

## RESULTS

23 students were selected for the study.

### Socio-demographic characteristics

Table 1 shows the age distribution of the study population. The mean age of our subjects was 11.7 ± 2 years, with extremes of 8 and 15 years. Table I shows that over 87% of the study population was at least 11 years old. 65.2% of patients were male. The sex ratio (M/F) was 1.87. None of the slides examined by light microscopy were positive.

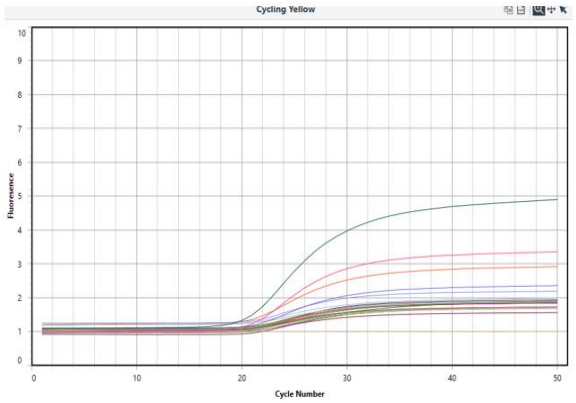
**Table 1.** Breakdown of students by age group and gender.

| | Number (n) | Frequency (%) |
|---|---|---|
| **Age range (years)** | | |
| (5-10 years) | 3 | 13 |
| (11-15 years) | 20 | 87 |
| **Gender** | | |
| Male | 15 | 65.2 |
| Female | 8 | 34.8 |
| Total | 23 | 100 |

### Relative frequency of cutaneous leishmaniasis

Leishmaniasis DNA was detected in 3 of the 23 patients studied, a frequency of 13.04%. The CT values for leishmaniasis-positive cases were 23.9, 22.0 and 28.0. Figure 2 shows the amplification curves for the various samples. The P1, P2 and P3 curves are those of the students with skin lesions, while the CN and CP curves are those of the negative and positive controls respectively.

**Figure 2.** DNA amplification control curves for samples.



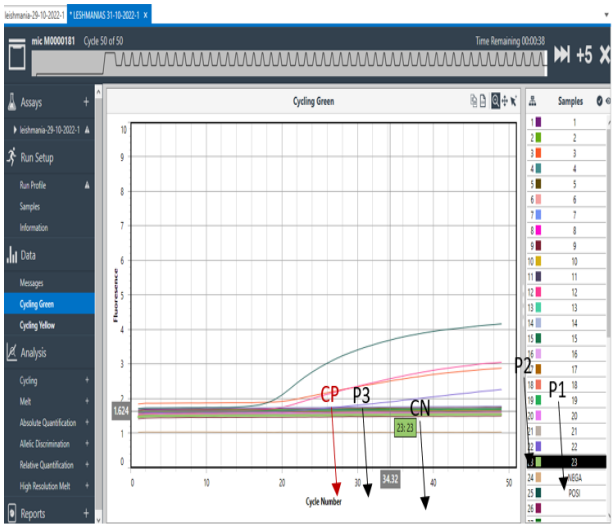

### Location of suspected chronic lesions

The chronic leishmaniasis skin lesions of the 3 positive patients were located on the legs in 66.7% of cases and on the toe in 33.3% of cases. Carriage was predominant on the legs, with a frequency of over 66.66%.

## DISCUSSION

The results obtained in this study may be of great importance as providing evidence of the existence of cutaneous leishmaniasis in our country. However, aspects such as the lack of use of genus primers to detect the presence of *Leishmania* and of a single species primer (*Leishmania tropica* in our case) represent a limitation of this work. As the primers are specific to *Leishmania tropica*, only a sample containing this DNA can be amplified.

**Figure 3.** Amplification curves showing CT values of cutaneous leishmaniasis-positive patients.




Biological diagnosis of cutaneous leishmaniasis is based on microscopic parasitological examination and molecular analysis. Microscopic parasitological examination is a simple, rapid and inexpensive technique Gunlzaz Culha, et al. [16]. But it has a low positive predictive value compared with molecular amplification techniques, mainly qPCR, which are recognized as more sensitive Luz, et al. because these techniques directly search for the parasite's genetic material [17]. Although this technique is faster than real-

time PCR, it is by far less efficient and less specific. The approach used in the present study has also been applied by authors such as Herman, et al., and Sharifeh, et al. [18,19].

The study involved school-age subjects aged between 8 and 15 years, with an average age of 11.7 ± 2 years. This target population of children was also the one worked on by Nassiri A, et al, 2020, but they worked on children under 5, whereas Achour N, et al., 2009 worked on children under 10 [21]. On the other hand, Fendri, et al. carried out their study on subjects whose modal class was between 20 and 30 years of age [22]. Issouf, et al., conducted their study on subjects aged 15 to 49 [23]. This proves that leishmaniasis is a pathology that affects all ages, but also both sexes. This was also found in the present study, in which a male predominance was observed, with a ratio of around 2 men to 1 woman. This male predominance was also found in the studies by Kebe M, et al., where the sex ratio was 2 (2H/1F) and by Omar, et al., where the sex ratio was 1.66 (30H/18F) [24,25]. The predominance of male children can be explained by their greater freedom of action. They are more likely than girls to frequent certain rural areas where they engage in farming activities.

However, our results differ from those of Zouari, et al., 2020 who, in their study of the epidemiology of cutaneous leishmaniasis in the El Oued region, showed that the disease struck both sexes indiscriminately. Qasmi S, et al., 2008 highlighted the predominance of females in their study of cutaneous leishmaniasis in children in a dermatology unit in Morocco.

The 3 chronic skin lesions were caused by *Leishmania tropica*. This enabled us to determine a relative frequency of cutaneous leishmaniasis caused by *Leishmania tropica* of 13.04%. These results are similar to those of Omari H, et al., in Morocco, who found a frequency of 12.5% for *Leishmania tropica* in the Meknes prefecture in central Morocco [26-28]. But Baghad B, et al, in the same country, found a frequency of 72.5% of *Leishmania tropica* cases in their study of the clinical and epidemiological features of cutaneous leishmaniasis in children [29]. These data bear witness to the variability of the disease's distribution from one country to another and within the same country.

But there is also variability in the species involved in leishmaniasis. Thus, depending on the region and continent, the etiological agents may be different. We therefore understand the difference between our results and those of Kebe M, et al., where the L. major species was identified.

However, the use of only a genus primer explains the fact that we highlighted only *Leishmania tropica*, whereas Kebe M, et al., 2019 did not use a species-specific kit but rather a genus-specific one.

Parasitological examination of appositions after staining with MGG was carried out, but no positive results were revealed. This confirms the low sensitivity of conventional diagnostic methods, as described by Hernan Aviles, et al., Aoun K, et al., in Tunisia, Omar, et al., Sharifeh Khosravi, et al. [30] Thanks to their high sensitivity, molecular amplification techniques are increasingly preferred as a means of diagnosing LC. The positive results we obtained were obtained using the real-time PCR technique.

Indeed, several authors in the literature have used real-time PCR as an essential diagnostic tool. This is the case of Kahima Bourdache, et al., 2007, Yasmione Almaboudi, et al., 2016 [31]. This made up for leishmaniasis cases that were negative on light microscopy. However, the species identified in our study *Leishmania tropica* has been observed in the literature by several authors, such as Omari H, et al., in Morocco and Baghad B, et al., 2020 in Morocco.

Indeed, several authors in the literature have used real-time PCR as an essential diagnostic tool. This is the case of Kahima Bourdache, et al., Yasmione Almaboudi, et al. [32,33]. This made up for leishmaniasis cases that were negative on light microscopy. However, the species identified in our study *Leishmania tropica* has been observed in the literature by several authors, such as Omari H, et al., in Morocco, Baghad B, et al, 2020 in Morocco.

Our subjects' lesions were located on the lower limbs, with 66.7% on the leg and 33.3% on the toe. This observation is similar to that found by Edhaouia, et al., in the El-Oued region of Algeria in their study of the epidemiological and clinical aspects of leishmaniasis. Unlike other studies such as those by Sharirif I, et al., in Iran, Abrha Brast, et al., in northern Ethiopia, Dakhia Abed Djali Ferhat Ahmed, et al., in the Birska region, Ahlam Jaafari, 2021 in her study on the role of the pharmacist in the prevention of cutaneous leishmaniasis [34,35]. Each of the above authors found lesions all over the body, including the upper limbs, neck and face. This tropism of lesions can be explained by the fact that these parts of the body are most often uncovered and therefore most exposed to sandfly bites.

## CONCLUSION

Although the frequency of leishmaniasis found in this study cannot be generalized, it nevertheless indicates that the pathology is indeed rampant in our country. *Leishmania tropica* species isolated may not be the only one in the Tchamba Nziassi health district in Pointe Noire in particular and in Pointe Noire in general. It was diagnosed using qPCR. The widespread use of PCR in our health facilities will enable the diagnosis of certain parasitoses for which microscopic observation is recognized to be of little effectiveness.

## CONFLICT OF INTEREST

None.

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