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Molecular Characterization of Agents of Cutaneous Leishmaniasis in Dutsin-Ma Local Government Area of Katsina State, Northwestern, Nigeria

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Abstract

Leishmaniasis is one of man's neglected tropical diseases (NTD), caused by a protozoan parasite of the Leishmania genus. The dixenous life cycle of Leishmania species consists of the vertebrate and vector stages resulting in the differentiation of metabolic processes, morphological forms, and remodelling of genes in the parasite. To identify the species of Leishmania responsible for cutaneous leishmaniasis (CL) in Dutsin-ma LGA of Katsina state, Nigeria, a purposive sampling technique was employed. Samples were collected from individuals with suspected CL lesions and subjected to molecular characterization to confirm the Leishmania species. Questionnaires were also used to gather socio-demographic and behavioral data. Molecular characterization involved amplifying the ITS rDNA genes and applying the restriction fragment length polymorphism (RFLP) technique. The results revealed that 85.7% of the individuals screened had lesions associated with Leishmania infection, with a higher prevalence in males (71.4%) compared to females (14.3%). Among the infected individuals, 71.46% were farmers, and one was a student. The lesions were predominantly found on the legs (57.1%) and hand/arm (28.6%), with no lesions detected on other body parts. The ITS rDNA genes were successfully amplified from six patients, confirming Leishmania major as the predominant species causing CL through RFLP analysis. In conclusion, CL caused by L. major is endemic in Dutsin-ma LGA, Katsina state, in the Northwestern zone of Nigeria.

Keywords: Leishmaniasis, NTD, patients, species, molecular characterization

INTRODUCTION

Leishmaniasis is caused by a protozoan parasite belonging to the Trypanosomatidae family and genus *Leishmania*. The disease is considered one of the neglected tropical diseases (NTD) of major public health concern in many underdeveloped countries, including Nigeria. Leishmaniasis is ranked among the top ten diseases in the tropical region and is considered one of the most recent and uncontrollable diseases (Reithinger, 2007). The hosts for the parasite can be humans, rodents, dogs, or hamsters, while the vectors responsible for transmission are female phlebotomines in the old world and lutzomyia sandflies in the new world. There are three clinical subtypes of leishmaniasis: cutaneous, mucocutaneous, and visceral. In the old world, species such as *L.* (Leishmania) major and *L. (L) tropica* are the primary causes of cutaneous leishmaniasis, whereas in the new world, species like *L. (L.)* mexicana, L. (L.) amazonensis, L. (Viannia) braziliensis, L. (V.) guyanensis, and *L. (V.)* peruviana are major contributors to the disease (Desjeux, 2001; Desjeux, 2004).

Typical cutaneous leishmaniasis (CL) is characterized by localized refractory skin ulcers or nodules at infection sites that heal spontaneously, leaving permanent scars. The complications and deformities resulting from the untreated ugly scars of cutaneous leishmaniasis pose a significant social and public health

problem in various regions worldwide, especially in Africa.

Leishmaniasis recidiva cutis (LRC) is characterized by the development of satellite nodules in or around the scar of a clinically healed lesion over time, leading to diffuse cutaneous leishmaniasis (DCL). DCL presents as non-ulcerating chronic nodules across the body, resembling skin lesions seen in lepromatous leprosy. Post-kala-azar Dermal Leishmaniasis (PKDL) manifests on the skin of individuals who have recovered from visceral leishmaniasis (Desjeux, 2001; Desjeux, 2004; Opara and Ameh, 2005).

Leishmaniasis is prevalent throughout the tropical, subtropical, and temperate regions of the Americas, Europe, Asia, and Africa, with an estimated 12 million people affected and over 350 million people at risk (Mandell, 2010). Leishmaniasis is considered one of the ten most important diseases in tropical regions, and the Tropical Diseases Research Department of the World Health Organisation counts it as one of the three first line diseases (African Trypanosomiasis, Chagas disease, and Leishmaniasis) and one of the new and uncontrollable diseases (Reithinger, 2007). Although various clinical manifestation are exhibited by the same species of the parasite, it appears that these manifestations may be influenced by certain factors which include *Leismania species*, genetic mutation in the parasites and immune response of the host, species of the sandfly e.t.c (Khalil *et al*., 2008; Lauren *et al*., 2009; Sakthianandeswaren *et al*., 2009; Ben-Ahmed *et al*., 2010). According to the WHO (2020) classification, there are 20 diseases considered as NTDs: African Trypanosomiasis, Buruli ulcer, Chagas disease, Cysticercosis, Chikungunya and Dengue fever, Deep mycoses, Dracunculiasis, Echinococcosis, Fascioliasis, Leishmaniasis, Leprosy, Lymphatic filariasis, Onchocerciasis, Rabies, Schistosomiasis, Soil-transmitted helminthiasis, Scabies and other ectoparasites, Snakebite envenoming, Trachoma, Mycetoma, and Yaws. Leishmaniasis has become a serious health problem worldwide due to coinfection with HIV/AIDS, drug resistance, virulence, and new transmission routes.

According to Igbe *et al*. (2006), Northern Nigeria is endemic for Cutaneous leishmaniasis, particularly in Sokoto, Gusau, Katsina, Maiduguri, and Azare. Katsina was reported to provide suitable habitat for *Phlebotomus duboscqi* and *P. orientalis* sand flies, which are hosts of *Leishmania major* and *L.* donovani (Isa *et al*., 2017). The study aims to molecularly characterize agents of cutaneous leishmaniasis in Katsina state, Northwestern zone, Nigeria.

MATERIALS AND METHODS

Study Sites

Katsina is located at coordinates 12° 59' 26.95" N and 7° 36' 6.37" E, with an annual high/low temperature of 36.07°C/23.06°C and a humidity level of 30.11%. Dutsin-ma is a Local government area (LGA) within Katsina state.

Ethical Approval and Informed Consent

The Ministry of Health in Katsina States issued Ethical approvals and informed written consent was obtained from all participating volunteers before sample collections.

Study Population

The study population includes individuals of all ages, both male and female, with suspected cutaneous leishmaniasis lesions. Individuals without suspected symptoms of cutaneous leishmaniasis were not included in the research.

Determination of *Leishmania* species in the study area

Collection of Demographic Data

Demographic information on all participating subjects was obtained through a structured questionnaire. Age, sex, occupation, duration of the lesion, and location of the injury were recorded.

Sample Collection for Molecular Identification

A total of seven samples were collected from lesions of individuals suspected to have

cutaneous leishmaniasis in the study areas. The lesions were cleansed with cotton wool soaked in 70% alcohol before a scalpel was used to scrape the skin until blood oozed out from the lesion. Subsequently, a sterile needle was intradermally inserted into the border of the lesion and rotated several times to aspirate the blood/tissue fluid into a bottle placed in an ice jar. The collected samples were then transported to the laboratory for analysis.

Molecular Characterization of *Leishmania* Species

Extraction of DNA

Leishmania DNA was extracted using a DNA extraction kit from Qiagen South Africa. We followed the procedure outlined in the manufacturer's instructions, and the purified *Leishmania* DNA was then stored at -20°C.

Amplification of Leishmania Species ITS1-rDNA Region

Amplification of ITS 1 region using polymerase Chain Reaction (PCR) technique from the DNA extracted from the blood samples aspirated from infected patients was carried out. Primers specific to ITS 1 Region of the genus *Leishmania* LITS R (5'-CTGGATCATTTTCCGATG-3') and L5.8S (5'-TGATACCACTTATCGCACTT-3') were used for amplification. The thermocycler was set according to the following conditions for the amplification of ITS1- rDNA. Denaturation for 1 minute at 94 ºC, annealing for 1 minute at 55 ºC, extension for 1 minute and 15 seconds at 72 ºC, and in the end, extension for 7 minutes at 72 ºC (Sogand, 2017).

Gel Electrophoresis of PCR Product

Agarose gel was prepared by adding 1.5% agarose in Tris Acetate EDTA (TAE) buffer with 0.5µl of ethidium bromide. Subsequently, 15µl of DNA ladder (100bp) and 15µl of each sample were loaded into individual gel wells. The samples were run through the gel for 23 minutes at 120 Volts and 500 Amperes. After the analysis, the gel was examined using Gel doc to visualize the band sizes.

Identification of L. major by Restriction Fragment Length Polymorphism (RFLP)

Leishmania major was confirmed through the performance of RFLP analysis using the HaeIII restriction enzyme at a concentration of 10 U (µmol/min) in each reaction solution of a 20 µL volume. The digested fragments underwent electrophoresis in 2% agarose at 100 V in 1x TAE buffer, were stained with ethidium bromide, and subsequently observed and captured on camera using a UV transilluminator (Al-Nahhas and Kaldas, 2013).

RESULTS

Demographic and Physical Characteristics of the sampled population

The samples collected in dutsin-ma, katsina were from individuals aged 14-40 years, as shown in Table 1. Six (85.7%) of the individuals were males, while only one (14.3%) was female. Five (71.4%) of the seven individuals were farmers, and two (28.6%) were students. All individuals sampled had lesions, with 57.1% occurring on the legs and 42.8% on the hands/arms. The lesions were observed across all age groups, with the duration of injury/lesion ranging from 10 months to 10 years. All lesions presented by the sampled individuals were ulcerated.

Band sizes of amplified ITS 1 Gene from Suspected Cutaneous Leishmaniasis individuals

Leishmania DNA ITS 1 gene was successfully amplified from six (85.7 %) of the seven samples obtained from individuals exhibiting symptoms of leishmaniasis. The result of the amplification of DNA ITS 1 gene of *Leishmania* sp. revealed band sizes that ranged between 300-350 bp (Plate 1). Restriction Fragment Length Polymorphism (RFLP) profile of the PCR (Plate 2) amplicons showed fragments of band sizes 220 bp and 80 bp corresponding to *Leishmania major*.

Table 1: Demographics and Physical characteristics of Study individuals with suspected *Leishmania* infection in Katsina State

Sex	Age	Occupation	Duration of injury	Status of injury/lesion	Site of Lesion on the body
Male	25	Farmer	8 years	Ulcerated	Hand
Male	40	Farmer	10 years	Ulcerated	Hand
Female	35	Farmer	10 Months	Ulcerated	Leg
Male	16	Student	1 years	Ulcerated	Leg
Male	39	Farmer	1.6 years	Ulcerated	Leg
Male	63	Farmer	5 years	Ulcerated	Leg
Male	14	Student	3 years	Ulcerated	Hand

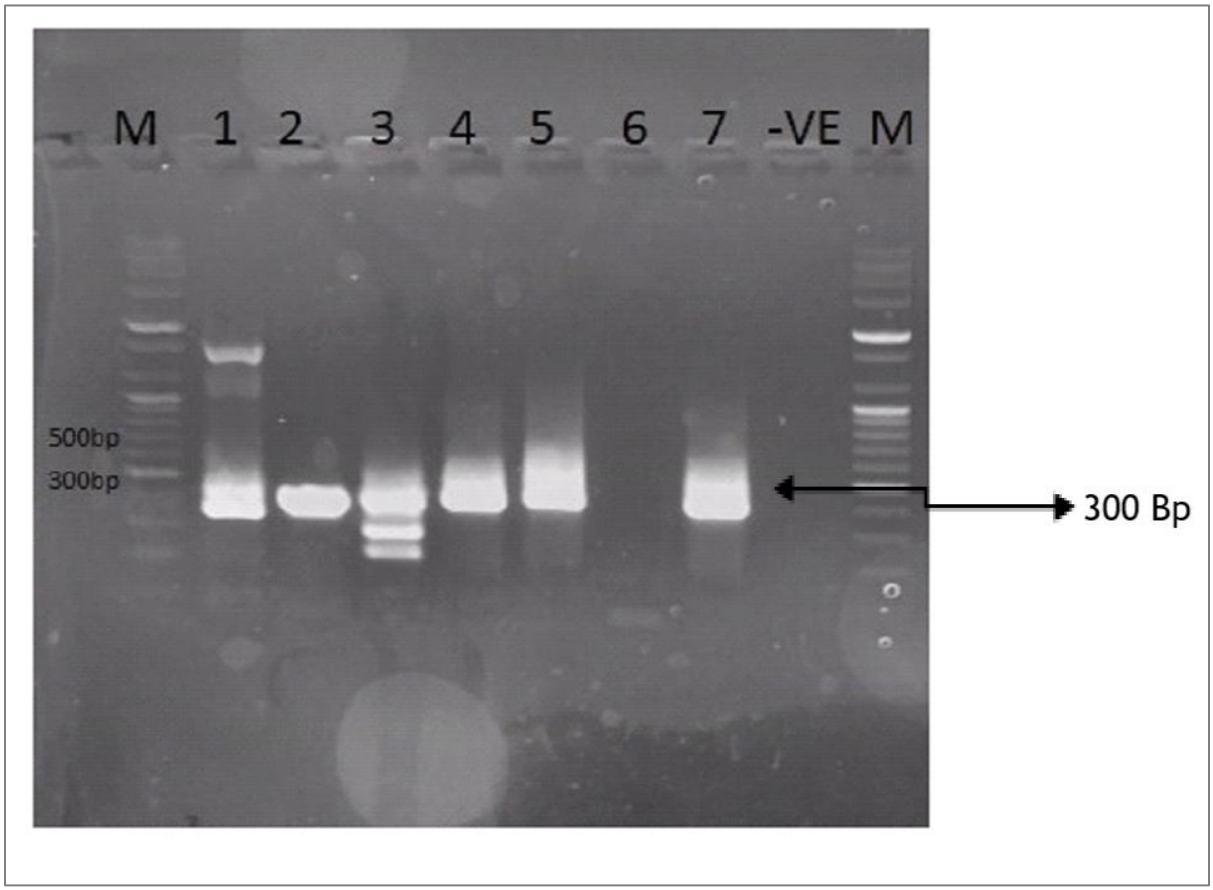

Plate I: Result of amplification of *Leishmania* DNA ITS 1 gene from blood samples of suspected cutaneous leishmaniasis infected persons in Dutsin-ma, Katsina state.

Key
M- Marker
lane 1-7 –Sampled Individuals
-ve – Negative control
Bp- Base pair

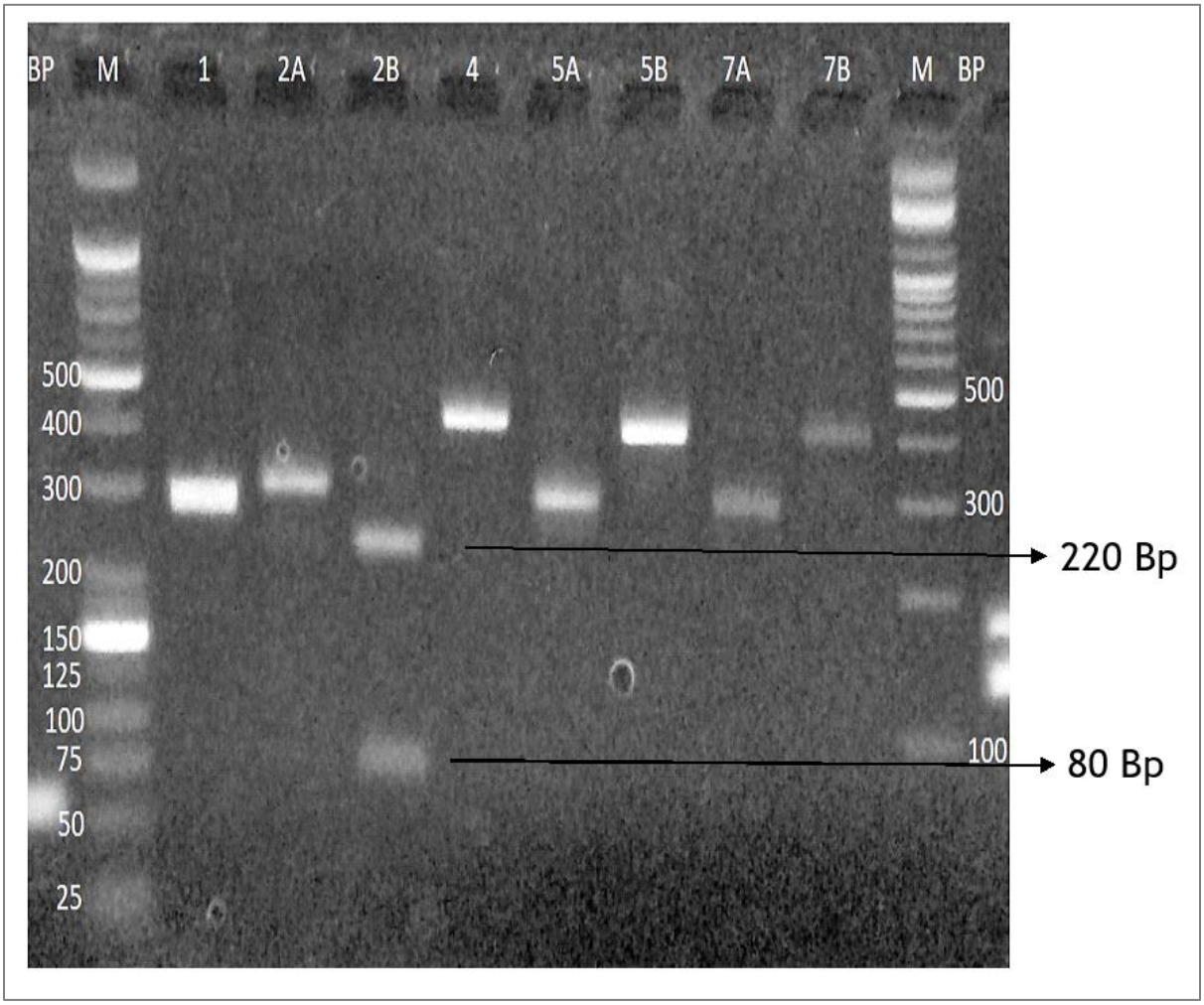

Plate II: Result of restriction fragment of *Leishmania* DNA ITS 1 amplified genes from samples of infected persons in Dutsin-ma, Katsina state.
Key
M- Marker
Lane 1, 2, 4, 5, and 7 – Sampled Individuals
Bp- Base pair

DISCUSSION

Protozoan parasites from the Trypanosomatidae family cause leishmaniasis and are among the most common Neglected Tropical Diseases (NTDs) declared by the World Health Organization (WHO). These diseases are maintained in different parts of the world as a result of human/animal migration, population growth, availability of insect vector species, and suitable environmental conditions (Agha-Maybodi *et al.*, 2018).

The presence of Leishmania species among individuals in Dutsinma, Katsina State may be associated with several factors such as variations in the level of urbanization, deforestation, and the socioeconomic status of individuals, among other factors. Additionally, Dutsinma in Katsina State has been identified as an endemic area for cutaneous leishmaniasis (Isa *et al.*, 2017).

In Katsina state, our research findings suggested that males were infected more than females. Our results expanded on previous clinical-epidemiological evidence of sex-biased cutaneous leishmaniasis incidence, with males typically having a greater case frequency than females (Weigle *et al.* 1993; Calvopiña and Hashiguchi 2004; Guerra-Silveira and Abad-Franch 2013). According to Travis *et al.* (2002), the discrepancy may be attributed to dissimilar risks of exposure because of the diverse activities of males and females, or whether

gender-related changes in the host immune response play a role in resistance and susceptibility to infection.

Human behavior has been suggested to influence the location of lesions on the body. In a study of cutaneous leishmaniasis in the Peruvian Andes, Davies *et al*. (1997) observed more lesions on the head of patients living in cooler areas and equally frequent lesions on the extremities of individuals in hotter regions. This observation is consistent with the findings of our research, where all screened individuals had lesions on their hands or legs. These extremities are likely the most exposed body parts, as the use of headgear/veils is a common cultural practice in the area. This practice may have prevented exposure of the head/face to the insect vector, resulting in the absence of lesions on those parts of the body.

Leishmania major was successfully identified molecularly using the ITS 1 and PCR-RFLP approach to confirm positive cases among the seven samples studied in Katsina State, which is similar to the work of Remadi *et al*. (2020). However, sequencing and BLAST results of the ITS1 amplicon (LITSR/L5.8S) produced sequences that did not align with *Leishmania* species. This limitation of the techniques used has been noted in previous works. Research has shown that RFLP digestion of the ITS 1 PCR amplicon is the optimal approach for ITS 1–based characterization of *Leishmania* (Mouttaki *et al*., 2014; Remadi, 2020). The restriction fragments obtained from the digestion of the 300–350 bp *Leishmania major* ITS 1 amplicon by the Hae III enzyme, as described by Mouttaki *et al*. (2014), provided a more accurate method of characterizing the parasite compared to morphological, culture, and molecular amplification of 13A/13B or Lmj4/Uni21.

In this study, the RFLP restriction enzyme HaeIII digestion resulted in band sizes of 220 bp and 80 bp, which are indicative of L. *major*. These band sizes, however, differed slightly from those reported by Slami *et al*. (2020), Hijjawa *et al*. (2016), and Remadi *et al*. (2020), who observed restricted fragment bands of sizes 220 bp and 127 bp, 145 bp and 126 bp, and 200 bp and 132 bp, respectively, for RFLP enzyme digestion using the same enzyme. The variation in band sizes of restriction fragments obtained during RFLP enzyme digestion has been suggested to be influenced by the sizes of the PCR products. *Leishmania major* was confirmed as the predominant species in Dutsin-ma LGA, in line with the findings identifying *Phlebotomine duboscqi* species of sandflies, which serve as hosts for *Leishmania* major, as the predominant sandfly in the LGA (Isa *et al*., 2017).

CONCLUSION

Our study reveals that the duration of cutaneous leishmaniasis infection in individuals ranged from 1 to 10 years. Lesions were predominantly located on the legs rather than the hands. All examined samples showed ulcerated lesions, with a higher prevalence of cutaneous leishmaniasis in males than females. *Leishmania major* was identified as the primary species affecting the human population in the Dutsin-ma LGA of Katsina state, located in the north-western region of Nigeria.

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