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PCR-RFLP Fails to Reveal Variability Within *Schistosoma haematobium* Detected in Loum (Cameroon) by Isoelectrofocusing Technique

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Abstract: *Schistosoma haematobium*, which causes urinary schistosomiasis in humans is responsible for the largest number of infections in the word. Genetic variability among parasite populations is an important factor in their potential for producing harmful effects on the human populations they infect. In many areas, *S. haematobium* is sympatric with related schistosome parasites (most of other mammals) (i.e., *S. bovis, S. mattheei, S. curassoni, S. intercalatum, S. guineensis* and *S. margrebowiei*). PCR-RFLP analysis of ITS-2 rDNA loci is an usefull tools to detect hybrids amongs *Schistosoma haematobium* group. Many studies have been carry out in the town of Loum (Cameroon) in order to characterize *Schistosoma haematobium* species from this locality. However, no study based on PCR-RFLP analysis succeeds to detect any genetic variability as reported before using electrofocusing (IEF) technique. PCR-RFLP analysis realised on 10 isolates of *Schistosoma haematobium* from Loum reveals a DNA fragment of 501 bp after amplification of ITS2 ribosomal gene. For all the samples, the enzymatic digestion of the mentioned DNA fragment gene with *Taq* I reveals two DNA bands of 158 bp and 199 bp which is characteristic of *Schistosoma haematobium.* In summary, molecular characterization of *S. haematobium* in Loum using PCR-RFLP approach reveals once more the absence of hybrids and no genetic variability. Further studies on a larger geographic scale involving many schools in Loum should be encouraged to screen more parasite isolates with different primers and molecular toolS. Information from such studies would provide better insight into the local lineages of *S. haematobium*. This knowledge might play a major role in establishing control strategies for urogenital schistosomiasis in Loum.

Keywords: *Schistosoma haematobium*, Genetic Variability, PCR-RFLP, Loum, Cameroon

1. Introduction

Schistosomiasis is a neglected tropical disease, caused by parasitic flatworms of the genus Schistosoma (subclass Digenea). Worldwide, approximately 779 million people are at risk of infection in endemic areas and more than 250 million people are estimated to be currently infected with *Schistosomiasis haematobium*. [1]. More people are infected with *S. haematobium* than with all the other schistosome species combined. Of the 110 million cases of *S. haematobium* infection in sub-Saharan Africa, 70 million are associated with hematuria, 18 million with bladder wall pathology, and 10 million with hydronephrosis leading to severe kidney disease [2, 3] and even bladder cancer [4].

Although morphologically *S. haematobium* is considered to be a uniform species, differences between parasite strains or populations have been observed in a number of biological characteristics such as infectivity [5], virulence [6], response to treatment [7] or fecundity [6]. The observed phenotypic variations have been attributed to genetic heterogeneity of the parasite [8].

The diversity of *S. haematobium* has been the subject of many molecular studies [9, 10] with one of the earlier study using enzyme analyses by isoelectric focusing in polyacrylamide gels [11] showing some variation and suggested mixing of parasite strains due to human population movementS. In Cameroon, many studies have been published on the strain of *S. haematobium* [12] which has replaced *Schistosoma guineensis* in the town of Loum through an exclusive competition [13] and introgressive hybridization [14]. Previous study indicates that *S. haematobium* found presently in Loum is a recombinant [12], more recently Luogbou and collaborators [15] used very few sample to indicate that there was no hybrid found in Loum. The current study aims to use more samples and investigate the presence of hybrids using PCR-RFLP approach.

2. Materials and Methods

2.1. Ethical Considerations

The study was approved by the National Ethics Committee of Cameroon (reference no. 072/CNE/DNM08) and was a public health intervention conducted by the Ministry of Health and the Ministry of Education. Stool and urine samples were collected from children in schools with the approval of the administrative authorities, school inspectors, directors and teachers. The objectives of the study were explained to the schoolchildren and to their parents or guardians from whom written informed consent was obtained. Children willing to participate were registered. Each child was assigned a unique identification number and results were entered in a database and kept confidential. All children who participated in the study were treated with PZQ at a dose of 40 mg/kg. Other children were treated during the mass drug administration campaign implemented by the National Programme for the Control of Schistosomiasis and Intestinal Helminthiasis (NPCSIH).

2.2. Study Area

This study was conducted in LOUM Health District, Moungo Division, Littoral region of Cameroon), a semi-urban setting situated at 70 Km west of Douala the economic capital city of Cameroon (Figure 1). The coordinates of LOUM ranged from 180 m to 235 m, latitude 4°43′ N and longitude 9°43′ E. The city is surrounded by high mountains, hills and plateauS. It is crossed by the river Mbette which takes its source on Mount Koupé (2070 m) located to the north of the city. The soils are of volcanic origin and black texture over a large part of the communal territory. The climate is equatorial with a wet season of about 9 months (March to November) and a short dry season (December to February). Precipitation is abundant (a mean of 2699 mm per year), while the mean monthly temperature fluctuates from 19°C to 25°C. The soil type is very diverse but all from volcanic origin, that have developed to produce fertile soilS. There has been serious deforestation due to the activities of large plantations and small farmS. This is a farming area, with food crops and fruits farms adjacent to major industrial banana. This city is known as a high endemic zone for schistosomiasis and several authors have already taken urine or stool samples from schoolchildren in this city over the past forty years to study schistosome infections [16, 17]. According to Tchuem-Tchuenté et *al*. [16] the main sites of schistosomiasis transmission have been identified in areas adjacent to the Mbette river and its tributarieS.

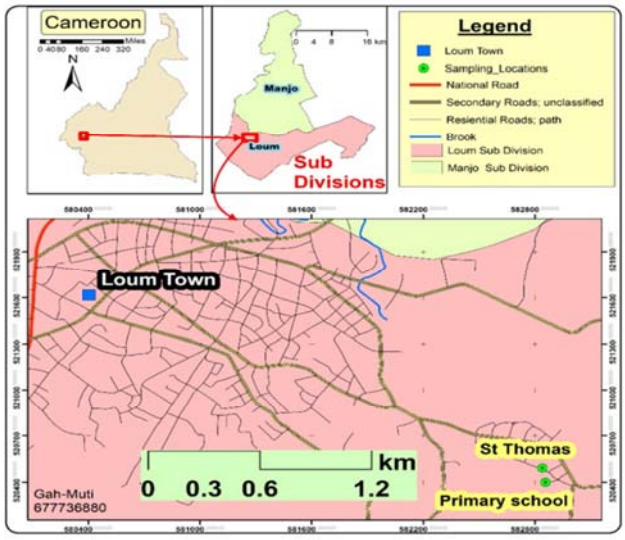

Figure 1. *Map of LOUM showing the school where the samples analysed in this study were collected.*

2.3. Samples Collection

2.3.1. Collection of Adult Worms

From 2008 to 2010, a cross-sectional survey was launched across the country to determine the prevalence of Schistosoma infection among school-aged children. Among the samples tested positive as described in Luogbou et *al,* [15], urine samples from 5 randomly selected infected children were chosen for further analysis in two schools: Primary School Bonkeng (n =3), and "St Thomas" Primary School (n = 2) (Table 1). Miracidia were hatched from eggs obtained from individual urine samples by sedimentation according to DBL (Danish Bilharziasis Laboratory) standard methods [18] and used to infect laboratory bred snailS. Adult worms were obtained through the passage on mice in laboratory as described in Luogbou et *al.* [15]. For each sample, two adult worms male were analysed separately.

2.3.2. DNA Extraction

Genomic DNA extraction from all collected samples was performed as described by Luogbou and collaborators [15].

For each sample, one adult worm male was gently dried for 1 hour in a tank containing a desiccant. Next, 20 µl of 250 mM NaOH were added to each tube. After a 15 min incubation period at 25°C, the tubes were heated at 99°C for 2 min. Then, 10 µl of 250 mM HCl, 5 µl of Tris-HCl (500 mM) and 5 µl of TritonX-100 (2%) were added. A second heat shock at 99°C for 2 min was performed before products were stored at-20°C.

2.3.3. DNA Amplification

The ITS2 subunit (including most of the 5.8 S gene and 40 bases of the 5' of the 28 S gene) was amplified using two ''universal primers'' (Ransom Hill Bioscience Inc. Ramona, CA.) ITS-3 (5'-GCA TCG ATG AAG AACGCA GC-3') and ITS-4 (5´-TCC TCC GCT TAT TGA TAT GC-3') [19]. PCR was undertaken in 25 µl volumes consisting of 250 µM of each dNTP, 2.5 µL of 10x PCR buffer 4 (New England Biolabs), 0.5 µM of ITS-3 and 0.5 µM of ITS-4 primers, 0.625 U of *Taq* DNA polymerase (New England Biolabs), 18.25 µL of DdH_2O and 10 ng/µl of template DNA. Amplifications were done with the following cycling parameters: 94°C for 4 min, 30 cycles of 94°C for 1 min, 50°C for 30 sec, 72°C for 45 sec, and a final extension of 7 min at 72°C and then held at 4°C. One percent agarose gel electrophoresis with ethidium bromide staining was used to visualize and identify ITS-2-PCR productS.

2.3.4. Restriction Fragment Length Polymorphism (RFLP) Analysis of the ITS-2 Regions

The positive ITS-2 PCR products were digested with *Taq*1 (New England Biolabs). Each RFLP reaction included 1 µL of *Taq*1, 2 µL of 10x Thermopol buffer, 0.2 µl of BSA (10 mg/mL), 7 µL of PCR product (approximately 300 ng/µL of DNA) and 9.8 µL of DdH_2O, for a total volume of 20 µL per reaction. The *Taq*1 reactions were incubated at 65°C overnight [19]. The reactions were inactivated at 80°C for 20 min. 10 µL of PCR-RFLP products were separated on 2% agarose gels, with molecular size markers of a 100-bp DNA Ladder (New England Biolabs). Fragments were sized by calculating their rate of flow in the gel and interpolation on the standard curve of rate of flow versus logarithm of molecular weight of the ladder.

3. Results

PCR-RFLP analysis

An ITS-2 DNA fragment of about 501 bp was amplified from all the isolates of *S. haematobium* group and no difference in product size was observed as indicated in Figure 2.

The *TaqI* enzymatic digestion of the ITS-2 DNA fragment revealed only one profile as shown in Figure 3: Profile A (typical of *S. haematobium*) which constituted of 2 bands (158 bp and 199 bp (Table 1).

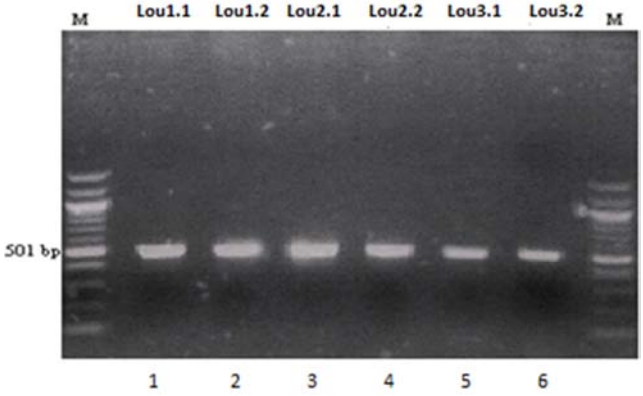

Figure 2. *ITS2-PCR Profiles obtained from the samples analysed.*

Legend: Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS-2) region of Schistosoma sp strains amplified by PCR. M = 100 bp ladderS. Lou1.1, Lou1.2, Lou2.1, Lou2.2, Lou3.1 and Lou3.2 are all isolates from Primary School of Bonkeng in Loum. All samples from "St Thomas" Primary School gives the same results (not showing here).

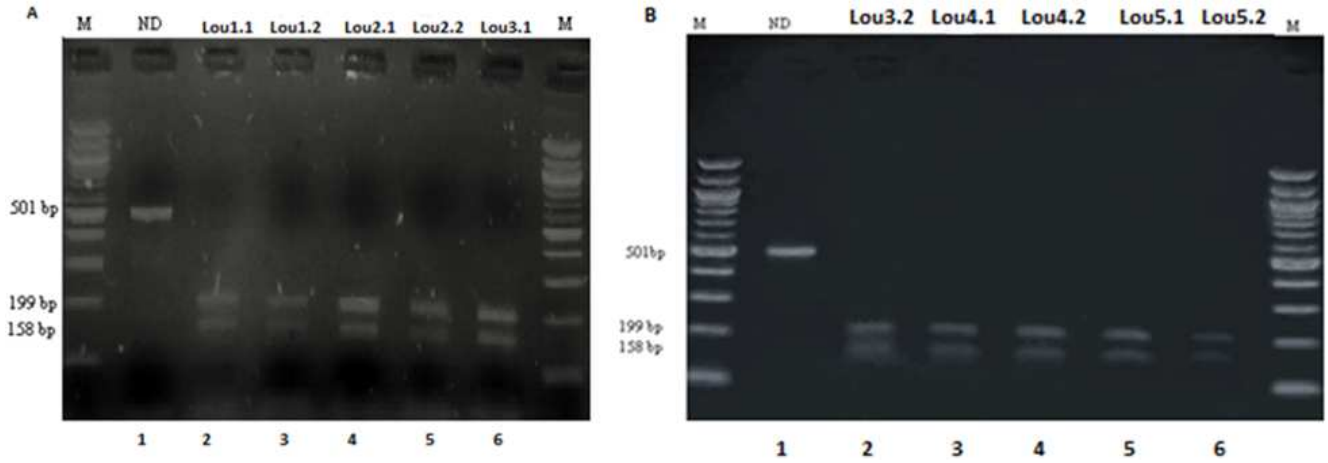

Figure 3. *ITS-2 RFLP profile (A) obtained from the samples analysed.*

Legend: Gel electrophoresis of the amplified rDNA internal transcribed spacer (ITS-2) region of Schistosoma sp strains digested with the TaqI. ND on lane A1 and B1 = Non digested, M = 100 bp ladderS. Lou1.1, Lou1.2, Lou2.1, Lou2.2, Lou3.1, Lou3.2 and Lou4.1, Lou4.2, Lou5.1, Lou5.2 are sets of isolates from Primary School of Bonkeng and "St Thomas" Primary School in Loum respectively.

Table 1. *DNA Bands size of ribosomal gene ITS2 after digestion using Taq I restriction enzyme.*

School	Geographic Coordinnate	Isolate Code	Bands size after digestion with Taq I		Ribotypes	Classification
			158 bp	199 bp		
		Lou1.1	1	1	A	*S. haematobium*
		Lou1.2	1	1	A	*S. haematobium*
Primary School	Alt 242 m, N04.70772°	Lou2.1	1	1	A	*S. haematobium*
Bonkeng	E009.74710°	Lou2.2	1	1	A	*S. haematobium*
		Lou3.1	1	1	A	*S. haematobium*
		Lou3.2	1	1	A	*S. haematobium*
		Lou4.1	1	1	A	*S. haematobium*
"St Thomas" Primary	Alt 232 m, N04.70852°	Lou4.2	1	1	A	*S. haematobium*
School	E009.74696°	Lou5.1	1	1	A	*S. haematobium*
		Lou5.2	1	1	A	*S. haematobium*

1 = Presence of DNA Fragment.

4. Discussion

In this study, we used RFLP analysis of ITS-2 rDNA loci to identify adult schistosome worms isolated from Loum in Cameroon and to compare the results with previously obtained IEF (Isoelectrofocusing) results in the same locality. All the samples analysed indicate that only *S. haematobium*is present in Loum with no hybrids found. This result agrees with a previous study [15] that reported the absence of schistosoma hybrid in Loum using the same RFLP approach. However, this study shows contradicting results with our previous study that showed genetic variability of *S. haematobium* using IEF. A total of 10 adult worms from 5 samples were analysed in this study compaired to the most recent study [15] where only 2 samples were analysed indicating that the size of the sample is not the justification.

Unlike our findings, other studies based on G6PD pattern showed in Loum, the genetic variability of *S. haematobium* [20] and the presence of *S. haematobium –S. guineensis* hybrids [21].

Our result is not also in line with the study of Webster and collaborators [12] which demonstrates the presence of pure *S. haematobium* and the recombinant in Loum using a single-strand conformation polymorphism (SSCP) approach for investigating genetic interactions of *Schistosoma haematobium* and *Schistosoma guineensiS.* The absence of genetic variability in this study may be due to the limitation of PCR-RFLP approach which is not the suitable technique for intraspecifique variation.

Our study, in spite of its limitations, expresses the need to assess the genetic variability of schistosoma population. In fact, the level of genetic variability is a significant measure for assessing the effects of selective pressure generated by drug therapy control measureS. Hence, high genetic variability may provide a sizable genetic basis for selection to increase the rate of development of praziquantel resistance [22].

5. Conclusion

In summary, our study on molecular characterization of *S. haematobium* in Loum using PCR-RFLP approach reveals that they were neitheir hybrids nor genetic variability. Further studies on a larger geographic scale involving many schools from study area are necessary in order to isolate and screen more parasite using different primers and molecular toolS. Results from such studies might provide better insight into the local lineages of *S. haematobium* and might play a major role in establishing control strategies for urogenital schistosomiasis in Loum.

Competing Interests

The authors declare that they have no competing interestS.

Author Contributions

LND and PMN conceived the study. LND and PMN wrote the first draft of the manuscript. LND and PMN performed the molecular analyseS. LND, PMN and NNF revised the manuscript. All authors read and approved the final manuscript prior to submission.

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