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Final Report

1- Project Information

ID no SGS 14/14		
Country		**TUN**
Organization		**Pasteur Institute of Tunisia, Tunis**
Title of the project		**Development and evaluation of a Loop Mediated Isothermal Amplification method for the diagnosis of Old world *Leishmania* in Tunisia**
Principal Investigator		**Dr. Souha Ben Abderrazak**
Proposed duration (months)		**12**
Reporting Period		**Start date: 15/4/2015**	**Ending date:15/03/2016**
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2- Main objectives of the study

The general objectives of this study are related to the development and improvement of simple, rapid and reliable DNA tests for the diagnosis of cutaneous leishmaniasis in order to improve the management of patients and to provide a more accurate reporting, alert or counselling to the leishmanioses control program.

3- Specific objectives

More specifically, the aims are to:

1. Develop a set of polymorphic genomic markers for diverse *Leishmania* species encountered in Tunisia and in the Afro-Mediterranean region
2. Develop additional strategies to deliver affordable, simple DNA based tools and protocols for the rapid, sensitive and specific *Leishmania* parasites detection concomitant to their identification/ differentiation. We will focus here on the development of isothermal amplification of *Leishmania* DNA markers (LAMP)
3. Validate and transfer the tools and protocols developed to partner laboratories.

4- Progress:

The work was developed according to the work plan established with no significant difficulties, except the fact that the number of samples is less than expected.

4-1: Leishmaniasis patients' parasites and samples:

Cutaneous leishmaniasis (CL) patients were recruited from primary healthcare (Kairouan and Tataouine) during January 2016.

4-2: Selection of potential targets for molecular diagnosis and DNA identification of parasites.

It is aimed to identify species-specific sequences, which could be exploited to develop Loop Mediated Isothermal Amplification based assays for the selective and/or specific identification of, and discrimination among the two (2) *Leishmania* species encountered in Tunisia. Two approaches were developed to identify unique sequences to *L. major* in the first case and to *L. tropica* in the second.

4-2-1: Comparative genome analysis for the selection of *L. major* & *L. tropica* markers:

It is aimed here to identify species-specific sequences, which could be exploited to develop LAMP based assays for the selective and/or specific identification and discrimination among the 2 *Leishmania* species encountered in Tunisia. Regions of the *L. killicki* (*L. tropica*) and *L. major* genes sequences were scanned using BLAST program and subsequently compared using appropriate bioinformatic tools Chromas and MEGA. This allowed us to identify regions of dissimilarities between the two species. We have already initiated the construction of a local *Leishmania* DNA data base using the latest updates of the *Leishmania* genome projects resources and the sequences of Tunisian samples obtained after sequencing; allow proceeding to the *in silico* comparative genomics analysis of *Leishmania* DNAs and annotating the sequences identified. We opted for conserved sequences between species, presenting enough sequence heterogeneities to be considered for the design of species-specific LAMP primers: such primers would allow the selective amplification of the DNA targets. In previous studies, we have identified cysteine protease b sequences, which are specific to *L. infantum*. It is likely to identify unique sequences to *L. major* of this type.

As the information related to *L. tropica* genome is missing, we used an experimental approach that was coupled to the comparative genome analysis to identify specific targets to the species

L. killicki. According to a procedure that has been previously validated for the identification of species - specific targets among which some fragments would fit into the second category of sequences expected. The sequences were analyzed *in silico* by BLAST searching for homologies with other DNAs deposited in the local and public data bases. In case of homologies identified with the other *Leishmania* species, these sequences were compared to *L. major* and/or *L. tropica* using MEGA software.

4-2-2: Design of LAMP primers

The objective here is to develop LAMP assays specific for each of the *Leishmania* species so far encountered in Tunisia. It is ultimately aimed at identifying the causal agents using one reaction.

The sequences identified in the previous section of the work were used to design a combinations of 6 primers in 8 distinct regions of the target sequences for the amplification of a selection of reference DNAs belonging to the different Old World *Leishmania* species. First of all we proceeded to an alignment using the CLUSTAL W program to each *Leishmania* species and for the selected gene (cysteine protase b) (the alignment use published sequences and sequences which were sequenced in our laboratory). Then a consensus sequence was selected for each alignment with which we design the LAMP primers. This consensus sequence is uploaded to the Primer Explorer software, after running the software; a list of primer sets is generated. From those generated primer sets we chose which is most specific and suitable for our study.

4-3: LAMP Assays

We started by testing our specific primers for *L. major* and *L. tropica* complex with Real-Time PCR machine. The reactions are conducted in a total volume of 25 µl containing 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 1.4 mM dNTPs, 0.1% Tween 20, 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH4) 2SO4, 8 mM MgSO4, 0.1% Triton X-100, 8 units of Bst DNA polymerase wide Fragment, 1U fluorescence reagent Syto-9, and 2.5µl of DNA.

The reaction conditions was set on three decreasing amounts (one limiting) of one representative DNA for each species, by looking at different combinations of primers, varying their relative concentration in the reaction, incubation temperature (60-65°C) or incubation time (30-45min). The retained conditions were evaluated against a panel of *Leishmania* DNAs

to assess criteria of specificity and consistency. The primers showing specific/ selective amplification for each of the species targeted, *L. major* and *L. killicki* were selected for further evaluation on a large panel of well defined parasite isolates and species.

It is aimed to have at least one test for each of the species. The use of six different primers ensures the specificity of the assays while the intrinsic properties of the polymerase (continuous amplification at the same temperature) will ensure high yield of the amplified target in a relatively short time.

In total 53 DNA extracted from parasitic cultures of different species were tested (*L.infantum*, *L.donovani*, *L.major*, *L.tropica*, *L.turanica*, *L.gerbilii*, *L.tarentolea*, *T.cruzi*).

⇨ This allows us to appreciate the high level of sensitivity and specificity of the LAMP technique

4-4 LAMP assays results:

- The *L.major* LAMP has a good reproducibility, good specificity (no cross reactions with different species) (Fig.1), and finally a sensitivity of about 50 fg.
- The addition of the Loop primers, have reduced by almost half the reaction time.

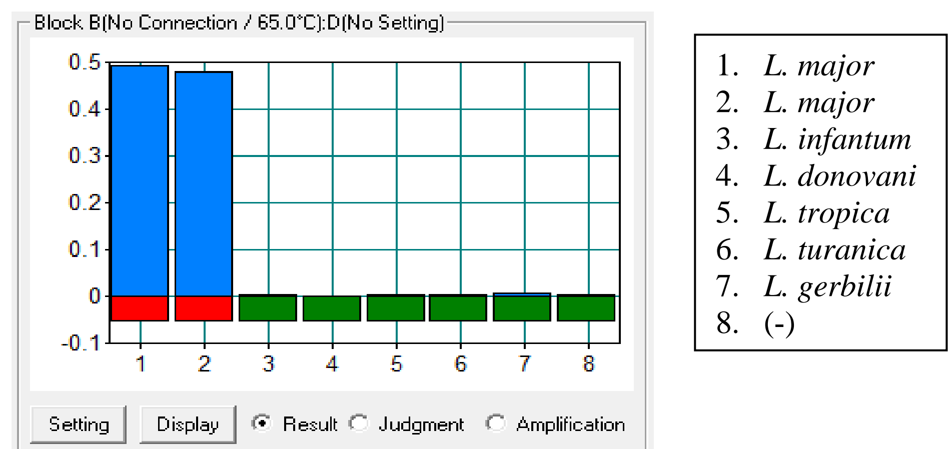

Fig.1: *L. major* LAMP specificity

- The *L. tropica* LAMP has a good reproducibility, good specificity (no cross reactions with different species), and finally a sensitivity of about 500 fg (Fig.2).

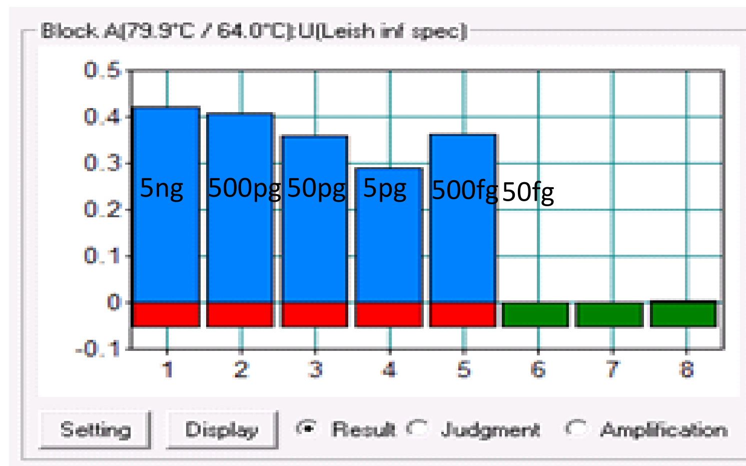

Fig.2: *L. tropica* LAMP sensitivity

5- Molecular assays and LAMP validation for the diagnosis of the Dermal Leishmaniasis disease in humans

The ultimate objective is to have simple, rapid and reliable DNA tests for the diagnosis of cutaneous leishmaniasis. After various regulations of reaction conditions, the LAMP tools were evaluated for human patients' diagnosis as regards two criteria: detection and identification. For these purposes, the assays were applied on the patients samples collected through this study (N=47). DNA was extracted by using the Qiamp DNA Blood Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's recommendations and performed directly on dermal scraping diluted in 200 µL phosphate-buffered saline. The PCR performed is species-specific for the *Leishmania* complex. The PCR primer targets a region of the cpb gene. The PCR reactions were conducted using a 25 µL final volume containing 5 µL of purified DNA sample or control, 1.5 mM MgCl2, 0.2 mM of each deoxynucleotide, 1.25U of Taq DNA polymerase and 50 pmole of each primer. Reactions were carried out in a TECHNE model TC512 using the following cycling conditions: an initial denaturation step at 94°C for 5 min, 35 cycles at 94°C for 1 min, 62°C for 1 min and 72°C for 1 min and a final elongation step at 72°C for 10 min. Amplicons were electrophoresed in 1% agarose gels stained with ethidium bromide and visualized under UV light. Standard DNA fragments (100 bp ladder, Fermentas) were used to size PCR products.

We tested our specific primers for *L. major* and *L. tropica* complex on the extracted DNA. The reactions are conducted in a total volume of 25 µl containing 40 pmol each of FIP and BIP primers, 5 pmol each of F3 and B3 primers, 1.4 mM dNTPs, 0.1% Tween 20, 20 mM Tris-HCl, pH 8.8, 10 mM KCl, 10 mM (NH4) 2SO4, 8 mM MgSO4, 0.1% Triton X-100, 8 units of Bst DNA polymerase wide Fragment, and 2.5µl of DNA, the reaction lasts 40 min at a temperature of 65 ° C. The results were confronted to the classical techniques for parasite detection and for identification. Smear examination and PCR amplification constitute the standard techniques for detection.

⇨ In total 48 samples were tested:

- 15 samples from the area of Tataouine in southern Tunisia
- 11 samples from the area of Kairouan in the area of Tunisian center
- 22 samples from the different areas in southern Tunisia

The following table details the results of the samples obtained with molecular techniques (LAMP and PCR)

		LAMP cpb			PCR cpb		
	Isolates	*L.major*	*L.infantum*	*L.tropica*	*L.major*	*L.infantum*	*L.tropica*
TATAOUINE	1	*	*	-	+	-	-
	2	*	*	-	-	-	-
	3	*	*	-	-	-	-
	4	*	*	-	-	-	-
	5	*	*	+	-	-	-
	8	*	*	+	-	-	-
	9	*	*	-	-	-	-
	10	*	*	-	-	-	-
	11	*	*	-	-	-	-
	12	*	*	-	-	-	-
	13	*	*	-/+	-	-	-
	14	*	*	-	-	-	-
	15	*	*	+	-	-	-
	16	*	*	-	-	-	-
	17	*	*	-	-	-	-
KAIROUAN	18	*	*	-	-	-	-
	19	*	*	-	-	-	-
	20	*	*	-	-	-	-
	21	*	*	-	+	-	-
	22	*	*	+/-	-	-	-
	23	*	*	-	-	-	-
	24	*	*	-	-	-	-
	25	*	*	-	-	-	-
	26	*	*	-	-	-	-
	27	*	*	-	-	-	-
	28	*	*	-	-	-	-
CENTRAL AND SOUTHERN TUNISIA	LCT25	+	-	-	+	-	-
	LCT02	+	-	-	+	-	-
	Tat24	+	-	-	+	-	-
	T28	+	-	-	+	-	-
	LC 08/09	+	-	-	+	-	-
	LCT 03	+	-	-	+	-	-
	LCT 05	+	-	-	+	-	-
	LCT 04	+	+/-	-	+	-	-
	T178	-	+	-	+	-	-
	Tat4	+/-	-	-	+	-	-
	LCT14	+	-	-	+	-	-
	T180	+	-	-	-	-	+
	Tat 5	-	+	-	-	-	+
	LCT18	+	-	-	-	-	+
	LCT19	-	+	-	-	-	+
	LCT 06	-	-	-	-	-	+
	Tat01	+	-	-	-	-	+
	T176	+	-	-	-	-	+
	LC12/08	+/-	-	-	-	+	-
	LV	-	-	+	-	+	-
	LC14/08	-	-	-	-	+	-
	T101	+	-	-	-	+	-

*not yet performed.

Conclusion

The sampling campaign realized during this project in central and southern Tunisia, were finalized and allowed us to obtain a total of 48 samples. During the reported period, we have developed new collaborations with dermatologists and medical technicians affiliated with regional basic health care centers in the center and south part of the country. This has the advantage of ensuring a larger interest of clinicians in the approach and tools developed.

Samples were tested by both PCR and LAMP. The specificity and sensitivity of the LAMP assays were compared to conventional cpb PCR and routine microscopy that were commonly used for cutaneous leishmaniasis diagnosis. The results obtained by the developed LAMP shows superior performance compared to other conventional techniques for diagnosis of cutaneous leishmaniasis. The relative stability of the reagents indicates that LAMP could be a good alternative to the conventional PCR.. Indeed, LAMP amplification of the multicopy gene cpb, is more reliable than conventional PCR and more convenient and less expensive than the q-PCR or nested PCR. The objective of the present work remains to position the LAMP assay in the basket of diagnosis options currently used. LAMP can be used to develop rapid on site molecular diagnostic tests which represent an important aspect for the early diagnosis and prompt treatment of *Leishmania*. Being able to detect *Leishmania* parasite with a technique that combines the reliability of molecular techniques together with the low cost opens horizons for production of high precision LAMP-based devices.

Due to a reduced number of patients who attended the basic health care center wich is a common problem in this type of project. Sample collection has not achieved our goals, a second campaign to reach the number of patient that will allow a better analysis of the results, is already planned. The final payment will be used to complete the work and write the paper. The risk was anticipated and expected as mentioned in our TDR application (Risk Management, Section 10).

As prospects, it is necessary to increase the number of patient samples to validate the different specific LAMP. Thereafter, we believe we can validate the detection and visualization of results by the colorimetric assay of hydroxy naphthol blue (HNB), which will facilitate the use of LAMP technology by the various stakeholders, mainly for obtaining quick and reliable results.

What we can retain in practice, and as recommendation, this type of project should be extended over a minimum of 2 years; the size of the sampling is closely related to the

dissemination of disease and the numbers of patients who consult the health centers. So to have a sufficient number of samples which allows adequate analysis, it requires that the recruitment campaign takes place over a minimum period of 2 seasons, and to optimize transfer and the LAMP technique on the field more 6 months are necessary.

Once well established and validated, the LAMP assays will be transferred to the Parasitology laboratory, which will be equipped to be able to achieve *Leishmania* identification directly on the patients' samples. This will also necessitate the training of the personnel of this laboratory to conduct the assays. Two steps are envisaged: first, the staff members of the Parasitology laboratory will be trained in Tunis to the application of the assays, and will introduce them in the laboratory. Second, a member of the team of Tunis will visit the laboratory to assist in the installation of the tests and in solving potential problems. Furthermore, the laboratory in Tunis will provide all scientific support needed to sustain the application of these tests.