Definition of efficient population selection strategy for an appropriate evaluation of a *Leishmania* vaccine candidate immunogenicity

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Abstract

Identification of phenotypes in relation to *Leishmania (L.)* infection (uninfected, infected but asymptomatic, healed, symptomatic) is a crucial step in the studies evaluating the effectiveness of human vaccine candidate. Here, we present a strategy to select and to precisely define human groups among individuals living in endemic areas, representative of visceral, cutaneous and mucosal leishmaniasis (VL, CL and ML) and of the different status of the disease, needed: i) to identify immunological factors of resistance, ii) to evaluate the immunogenicity of a *Leishmania* vaccine candidate and iii) to determine thresholds for PCR technique, an approach allowing both detection and estimation of the parasitic load.

The recruitment of symptomatic, asymptomatic and healed individuals from endemic areas for VL (India: *L. donovani*, Tunisia, Spain, France: *L. infantum*), CL (Tunisia: *L. major*, Peru: *L. braziliensis*) and ML (Peru: *L. braziliensis*), and of naïve individuals from endemic or non endemic areas (negative controls), was performed following approval and recommendations of local and external ethical committees and was based on defined inclusion and exclusion criteria, ensuring standardization of sampling. Well-characterized endemic foci, blood banks and medical structures have been identified. Healed individuals were characterized by presence of scars for CL/ML and previous history of parasitologically-confirmed disease for VL, no active disease and positive proliferation to soluble *Leishmania* Antigen (SLA). No previous disease and positive SLA proliferation were used to define asymptomatics. Patients were characterized by specific clinical symptoms and detection of parasites in Giemsa-stained dermal, mucosal or bone marrow smears and/or in parasite culture and/or by PCR. Naïve individuals were selected among individuals from no or low endemic areas or from blood banks, with no leihmaniasis history and no SLA proliferation. Exclusion criteria were presence of other immunosuppressive diseases, long-term treatment, and pregnancy. Epidemiological, clinical, parasitological and immunological data were collected according to a standard case report form allowing the constitution of a common database for result analysis. Such a strategy based on standardized protocols for selection of defined populations can help for the definition of key features associated with resistance against different *Leishmania* species which can facilitate the development of a human vaccine candidate.

Background

Human leishmaniasis is a disease caused by *Leishmania*, an intracellular protozoan parasite associated with considerable morbidity and mortality throughout the world. Depending on the parasite species and on the host immunological response, leishmaniasis ranges from an asymptomatic infection to a self-limiting cutaneous lesion(s) or a fatal visceral form. Healing is generally associated with the development of a life-long immunity to reinfection, indicating that a vaccine is feasible.The development of a human-compatible vaccine against *Leishmania* infection require data from humans and an appropriate clinical and diagnosis management for the identification of the different phenotypes of the disease. In endemic areas, infection is followed or not by the development of clinical symptoms. Reliable distinction of uninfected, asymptomatic, and healed individuals is necessary not only for a better evaluation of the immunogenicity of the *Leishmania* vaccine candidate but also to identify immunogenetic factors that determine whether an exposed individual will develop clinical symptoms (susceptible phenotype) or will be infected but remain asymptomatic or will remain uninfected (resistant phenotype). Moreover, it could help to assess protection in future vaccinated population. Symptomatic individuals are required for the establishment of approaches allowing both detection of the *Leishmania* causative species and estimation of the parasitic load, which could be of great help in diagnosis, treatment and vaccine monitoring. Appropriate procedure is therefore required to precisely define the different phenotypes related to *Leishmania* infection. Here, we present a strategy to select and to define human groups among individuals living in endemic areas, representative of visceral, cutaneous and mucosal leishmaniasis (VL, CL and ML) and of the different status of the disease, required for the development of a human vaccine candidate targeting most *Leishmania* species. This strategy is used in the context of a multi-disciplinary (molecular and cellular biology, molecular epidemiology, immunology, diagnosis…) consortium that includes leader groups in Europe (*L.infantum)* and concerned endemic countries (India (*L. donovani*), Peru (*L. braziliensis*, *L. guyanensis*) and Tunisia (*L. major, L. infantum*), where both VL and CL visceral are highly prevalent. We also report the results of the selection and recruitment process of the different human groups in endemic areas for *L. major* or *L. infantum*, in Tunisia.

Selection and recruitment process in endemic regions in Tunisia

1- Ethical Approval

Ethical approval have been obtained from the ethical committee of the Institut Pasteur de Tunis (IPT) and from an external ethical reviewer for the protocols involving children, before the beginning of the recruitment.

2- Characterization of endemic foci

Two endemic regions have been identified, one for *L. infantum* infection (Dkhila, Kairouan) and the other for *L. major* infection (Guetitir, Kairouan). IPT team has informed all persons involved in the recruitment and blood sampling process, present in these areas and in blood banks (Tunis and Kairouan) of scientific objectives of this project and of the different protocols related to sample collection and process.

3- Sampling and data collection

Cured CL group (CCL)

CODE	Endemic Area	Age	Gender	Clinical Data		CPA (SLA)
				glucantim treatement duration (Days)	Number of s cares	
CCL 6	Guetitir-Kairouan	31	F	90	3	3,3
CCL 7	Guetitir-Kairouan	30	F	7	2	3,7
CCL 9	Guetitir-Kairouan	56	F	60	3	28,3
CCL 10	Guetitir-Kairouan	26	F	15	2	9,3
CCL 11	Guetitir-Kairouan	38	M	ND	2	3,5
CCL12	Guetitir-Kairouan	18	F	10	2	4,4
CCL13	Guetitir-Kairouan	18	F	35	2	4,3
CCL15	Guetitir-Kairouan	28	F	21	2	24,8
CCL16	Guetitir-Kairouan	20	F	32	2	5,8
CCL 19	Guetitir-Kairouan	56	F	10	2	7,0
CCL21	Guetitir-Kairouan	53	F	10	2	12,8
CCL22	Guetitir-Kairouan	54	M	10	2	4,4
CCL23	Guetitir-Kairouan	46	F	60	3	3,3
CCL24	Guetitir-Kairouan	44	F	30	2	3,1
CCL28	Guetitir-Kairouan	42	F	60	3	3,6
CCL31	Guetitir-Kairouan	34	M	60	3	62,0
CCL 32	Guetitir-Kairouan	ND	F	30	2	17,8
CCL 33	Guetitir-Kairouan	50	F	150	4	1,9
CCL 38	Guetitir-Kairouan	17	M	ND	2	69,7
CCL 39	Guetitir-Kairouan	53	M	30	2	31,6
CLC40	Guetitir-Kairouan	54	F	ND	2	12,4

Asymptomatic VL group (AVL)

CODE	Endemic Area	Age	Gender	CPA (SLA)
AVL 1	Guetitir-Kairouan (Blood Bank)	23	F	2,3
AVL 3	Guetitir-Kairouan (Blood Bank)	ND	F	3,1
AVL 4	Dkhila-Kairouan	57	F	7,6
AVL5	Dkhila-Kairouan	50	M	6,0
AVL 6	Dkhila-Kairouan	37	M	18,7
AVL 7	Dkhila-Kairouan	51	F	8,9
AVL 8	Dkhila-Kairouan	34	F	12,7
AVL9	Dkhila-Kairouan	35	F	5,1
AVL 10	Dkhila-Kairouan	35	M	8,5
AVL 11	Dkhila-Kairouan	35	M	7,2
AVL12	Dkhila-Kairouan	21	M	14,7
AVL 13	Dkhila-Kairouan	44	F	4,0
AVL 14	Dkhila-Kairouan	28	M	5,3
AVL 15	Dkhila-Kairouan	43	F	10,4
AVL 16	Dkhila-Kairouan	47	F	23,7
AVL 17	Dkhila-Kairouan	23	F	30,9
AVL 18	Dkhila-Kairouan	48	M	6,3
AVL 19	Dkhila-Kairouan	25	M	6,8
AVL20	Dkhila-Kairouan	23	M	5,7
AVL21	Dkhila-Kairouan	ND	M	14,8
AVL 22	Dkhila-Kairouan	20	M	25,8
AVL 23	Dkhila-Kairouan	[illegible]	F	7,8

Asymptomatic CL group (ACL)

CODE	Endemic Area	Age	Gender	CPA (SLA)
ACL 1	Guetitir-Kairouan	32	Female	6,2
ACL2	Guetitir-Kairouan	19	Female	3,3
ACL3	Guetitir-Kairouan	53	Male	4,1
ACL4	Guetitir-Kairouan	33	Male	43,6
ACL6	Guetitir-Kairouan	ND	Male	3,8
ACL7	Guetitir-Kairouan	ND	Male	2,1
ACL8	Guetitir-Kairouan	ND	Male	4,7
ACL12	Guetitir-Kairouan	48	Male	6,0
ACL14	Guetitir-Kairouan	ND	Male	7,1
ACL15	Guetitir-Kairouan	33	Female	2,3
ACL16	Guetitir-Kairouan	33	Male	3,0
ACL17	Guetitir-Kairouan	44	Male	3,4
ACL19	Guetitir-Kairouan	41	Female	8,3
ACL20	Guetitir-Kairouan	ND	Female	20,9
ACL25	Guetitir-Kairouan	ND	Female	8,4
ACL27	Guetitir-Kairouan	43	Female	13,4

Naive group

CODE	Current state	Age	Gender	CPA (SLA)
N 11	Blood bank Kairouan	32	M	1,9
N12	Guetitir-Kairouan	40	F	0,8
N14	Dkhila-Kairouan	38	M	0,3
N15	Dkhila-Kairouan	33	F	0,2
N16	Dkhila-Kairouan	33	M	1,1
N17	Dkhila-Kairouan	33	F	0,4
N18	Dkhila-Kairouan	38	F	1,6
N19	Dkhila-Kairouan	45	F	1,4

Strategy of the human group recruitment

1- Establishment of an ethical report

The ethical report includes:
- a description of human groups based on inclusion and exclusion criteria
- identification of endemic and non-endemic areas
- informed consent process in mother tongue to inform the donor on: i) freedom of participation ii) purpose and aim of the study and iii) sampling procedures, potential risks, safety and confidentiality of all clinical and biological exams.

To be included in the study, all donors have to sign the consent. For minor donors, consent from legal representatives and from the donor itself, is necessary.

2- Identification of endemic foci

-Identification of well-characterized endemic foci, blood banks and medical structures (hospitals, medical departments and public health centers)
- Establishment of contacts with persons who will be involved in the recruitment and blood sampling of human groups, by the organisation of specific meetings.
- Validation of the process needed to provide the samples from the collection spot to the processing lab. In some cases, this implied pilot activities to optimize the sample collection and transportation because some study sites are far from institution.

3- Definition of inclusion and exclusion criteria

Inclusion criteria

Cured Cutaneous , Mucosal or Visceral Leishmaniasis individuals (CCL, MCL, CVL):
- selected among donors living in an endemic focus for CL, ML or VL
- presence of *Leishmania*-specific scars (assessed by clinical examination) for CCL/MCL
- previous history (5 to 10 years after healing) of parasitologically-confirmed VL for CVL
- no active skin or mucosal lesion for CCL/MCL. no mucosal involvement for CCL
- positive proliferation to SLA

Asymptomatic Cutaneous or Visceral Leishmaniaisis (ACL, AVL)

-selected among donors living in an endemic focus for CL, ML or VL where there is no overlapping *Leishmania* species, or in blood banks.
- absence of scars (assessed by clinical examination), no history of leishmaniasis
- positive proliferation to SLA

Active Cutneous, Mucosal or Visceral Leishmaniasis (CL, ML, VL)
- Identified in the different health structures in charge of patient management
- Diagnosis: clinical examination and detection of parasites in Giemsa-stained dermal or bone marrow smears and/or parasite culture and/or PCR (for diagnosis and parasite typing) and, alternatively by serology

Naive individuals
- selected in low or non endemic foci or in blood banks
- Without history of leishmaniasis
- No proliferation to SLA

Exclusion criteria

- immunosuppressive diseases, long-term treatment, pregnancy

4- Establishment of a common Case Report Form (CRF) document.

A CRF document is established for individual data collection in order to built a common database for result analysis. This document includes sections dealing with individual identification, socio-demographic information (an example below), clinical and epidemiological data (an ex below) and immunological results.

Individual baseline
2-Socio-demographic information

Gender □ Male □ Female Date of birth
|_|_|/|_|_|/|_|_|_|_|
Pregnancy □ Yes □ No
Have you ever been in this area?
□ Yes □ No
If "no" how long have you been in the area ? |_|_| Days |_|_| Months |_|_| Years
Have you visited other areas during the last 10 years ? □ Yes □ No
If "yes" :
area :_____ __ ___ date |_|_|/|_|_|/|_|_|_|_|
DISTRICT PROVINCE STATE
area :_____ __ ___ date |_|_|/|_|_|/|_|_|_|_|
DISTRICT PROVINCE STATE
area :_____ __ ___ date |_|_|/|_|_|/|_|_|_|_|
DISTRICT PROVINCE STATE
Comment _____
Occupation □ not applicable □ no job □ labor □ agriculture □ liberal job □ public sector (middle) □ public sector (high) □ mining □ other *(specify)* _____
Education □ illiterate □ preschool □ primary □ secondary □ higher

Individual baseline
5-Cutaneous Leishmaniasis (CL)

Previous CL history □ Yes □ No *if yes :* HEALED CL OR ACTIVE ML

If yes
Presence of scars □ Yes □ No
If "yes", location of scars

Mucosal symptoms associated □ Yes □ No

Medical record □ Yes □ No
If "yes" date |_|_|/|_|_|/|_|_|_|_| Hospital _____

Parasitological confirmation □ Yes □ No □ Not known
If "yes" date |_|_|/|_|_|/|_|_|_|_|
□ Direct smears □ Culture □ PCR

Treatment □ Yes □ No
type:_____
duration:_____

Conclusion

The strategy we presented here for the selection and recruitment of different phenotypes related with *Leishmania* infection, is used in the context of the RAPSODI project whose goal is to develop a human-compatible vaccine based on PSA antigens. The precise definition of these human groups using fully ethical compliant procedures and according to inclusion and exclusion criteria is associated with several benefits such as improvement of surveillance and early detection of the disease, reduction and control of potential risks (blood testing, skin biopsy of CL lesions..) and establishment of some procedures allowing a better control of biological material transfer between the different partners. Furthermore, this could help to better distinguish between the two "resistant" phenotypes: asymptomatic and cured, which is not always easy to establish, especially in the case of dermotropic *Leishmania* species. A precise definition of these groups is of great help for the identification of immunologic factors that control susceptibility vs resistance to *Leishmania* infection, which are still not fully clarified.