

Letter to Editor

A highly sensitive *Leishmania infantum chagasi* isolation method from bone marrow and peripheral blood of adults and children

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Key words: Visceral leishmaniasis; culture isolation; diagnostic; *Leishmania infantum chagasi*; peripheral blood.

J Infect Dev Ctries 2016; 10(11):1275-1277. doi:10.3855/jidc.8022

(Received 18 December 2015 – Accepted 5 April 2016)

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Dear Editor,

Visceral leishmaniasis (VL) is the most severe form of a neglected tropical diseases group transmitted by phlebotomine sandflies harboring promastigotes forms of donovani complex *Leishmania* species with distinct eco-epidemiological patterns. The anthroponotic *L. donovani* in East Africa and India; and the zoonotic *L. infantum* in Europe and North Africa and in America after its introduction during European colonization, being named *L. infantum chagasi* [1]. Promastigotes regurgitated by sandfly vector into the mammalian host dermis are phagocytosed by monocyte cells lineage, replicating as amastigotes [2]. Infected macrophages migrate through lymphatic and vascular systems infiltrating lymph nodes (LN) bone marrow (BM), spleen (S) and liver, causing a strict fatal systemic disease, if untreated.

According to World Health Organization last review on global VL incidence, 90% of 0.2 to 0.4 million cases per year, worldwide, occur in six countries: India, Bangladesh, Sudan, South Sudan, Ethiopia and Brazil (90% of American human cases); with a mortality rate of 10-20% [3]. In endemic and new affected areas, early and accurate VL diagnosis are shown to decrease mortality rates [4].

VL classical diagnosis correspond to microscopic examination of amastigotes in BM, LN and S cells smears, Giemsa or Leishman stained, obtained through aspiration; a painful, invasive and risky procedure, principally in children. Accuracy of this method depends on the tissue examined, the quality of reagents used and the technician identification ability [5].

Serologic and nucleic acid based VL diagnostic methods present several drawbacks like cross reaction with others American endemic trypanosomiasis including tegumentar leishmaniasis species [6]. Presence of macrophages in peripheral blood (PB) makes culture isolation suitable, as showed for children and adult patients infected with *L. infantum* from Mediterranean basin [7-9]. Most prominent *L. infantum* culture isolation techniques from PB presenting high sensitivity depends on sample washing with culture medium and concentration, or isolation of peripheral blood mononuclear cells (PBMC) [7,9], which are hard to be performed in laboratory routine, principally due to contamination risk during sample manipulation. Low efficiency of *L. infantum* isolation methods are probably related to immune complexes mediated parasite lysis [10,9]. Therefore, by using a BM and PB cells extensive saline washing approach, we obtained a 100% sensitive *L. i. chagasi* isolation method directly from adult and children patients samples.

The Study

From January through August of 2015, 29 adults (10 female and 19 male, 22-77 years) and five children (one female and 4 male, 2-9 years) consecutive patients with clinical signs of VL, resident in the west region of the Brazilian São Paulo State, were attended at the Hemocenter of Marília Medical School and submitted to routine VL tests. BM aspiration and PB with and without heparin or EDTA were collected for parasitological, serological and hematological examination, respectively. Serological test was

performed through the Kalazar Detect (Inbios International, Seattle, USA), a rapid strip immunochromatography on the base of rK39 antigen. Optical microscopic detection of amastigotes was carried out by two specialized groups of three technicians in hematology and parasitology, respectively, on two different BM Leishman stained smears. For each smear, after technicians consensus, parasitological test was expressed as absence, presence, rare, and suggestive forms of amastigotes.

Subsequent to routine procedures, the discarding BM and PB samples of all 34 patients included in the study were used for *L. i. chagasi* isolation by culture. The protocol was approved by Marilia Medical School Human Experimental Ethical Committee (CAAE 50128015.5.0000.5413). From each patient, 500 microliter of BM aspirate and one to two milliliters of PB, were centrifuged for 10 minutes at 4°C, 2000xg. Liquid phase was discarded, and blood cells were washed with chilled phosphate saline (PBS) (Thermo Fisher Scientific, Waltham, USA) until clearance of the supernatant. Afterwards, one volume of M199 medium (Thermo Fisher Scientific, Waltham, USA) enriched with 10% of bovine fetal serum (Thermo Fisher Scientific, Waltham, USA) and 2% of filter sterilized male urine, were added to the washed cells following incubation at 28°C for 20 days or until promastigotes emergence.

Six adult and four children patients were positive for VL diagnosis (table 1). BM and PB cultures and parasitological tests presented agreement of 100%. From these samples one was negative by serologic assay, confirming its manufacturer informed sensitivity of 90%. High sensitivity and specificity of

parasitological assay was probably reached due to the number of specialized technicians examiners, not always available in VL diagnostic services. BM and PB parasite isolation was generally obtained at the same day, except for one patient with BM being positive at day 10 and PB at day 20, probably due to increased amount of parasites in the former. *L. infantum* specificity of all isolates was confirmed by Sanger's sequencing of the V7V8 variable segment of trypanosomatides 18S rRNA encoding gene, as previously described [11] [GenBank: KT762389-KT762398]. *L. i. chagasi* negative patients presented hematologic alterations related to VL clinic and laboratorial characteristics, whose discussion is out of this report proposal.

Conclusions

Considering the simplicity, low cost and high accuracy of BM and PB *L. i. chagasi* isolation method, associated to the possibility to perform biological studies, asymptomatic infection investigation [12], and drug resistance test, we suggest its routine application. Adaptation of this method to the microculture scale using microplates [7] can also be tested in order to facilitate the visualization of parasites during isolation period. Also, to investigate asymptomatic *L. i. chagasi* infection, PB can be used for culture isolation, since BM aspiration is only recommended for symptomatic patients. Potential anthroponotic transmission of *L. i. chagasi*, supported by parasite PB circulation in adults and children, and by evidence of its principal vector *Lutzomyia longipalpis* urbanization and feeding in human blood [13], should be considered in VL control programs in South America.

Table 1. Demographic data and diagnostic methods outcome of *Leishmania infantum chagasi* infected patients

Patients			Parasitological exam		Serology	Culture isolation			
Number	Gender	Age	Smear hematology	Smear parasitology	ELISA	BM [‡]	Day [§]	PB [¶]	Day
A*03	M	34	Presence	Presence	+	+	10	+	10
A10	M	47	Rare	Rare	+	+	12	+	NA
A21	M	60	Presence	Presence	+	+	10	+	10
A24	M	68	Rare	Rare	+	+	10	+	20
A28	M	29	Suggestive	Inconclusive	+	+	7	+	7
A34	M	75	Suggestive	Suggestive	-	+	10	+	NA
C†08	M	5	Presence	Presence	+	+	6	+	6
C11	M	2	Presence	NA#	+	+	5	+	5
C23	M	6	Presence	Presence	+	+	9	+	9
C29	F	2	Presence	Presence	+	+	6	+	6

*Adult, †Children, ‡Bone marrow, §Day at promastigotes emergence, ¶Peripheral blood, #Sample not available.

Acknowledgements

This work was financed by Fundação de Amparo a Pesquisa do Estado de São Paulo (FAPESP), research grant 2012/20221-9, and post-doctoral fellowship to ADC 2013/26096-4.

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Conflict of interests: No conflict of interests is declared.