

QBC® for the diagnosis of human and canine american visceral leishmaniasis: preliminary data

QBC® para o diagnóstico de leishmaniose visceral americana humana e canina: dados preliminares

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Resumo O "Quantitative Buffy Coat" (QBC®) é um método direto e rápido de fluorescência, utilizado na identificação de parasitas hemáticos. Porque *Leishmania chagasi* circula no sangue, resolvemos testá-lo em leishmaniose visceral americana (LVA). Foram analisados medula óssea (MO) e sangue periférico (SP) de 49 pessoas e SP de 31 cães. O QBC® foi positivo na MO de 11/11 pacientes com LVA e em 1/6 com outras doenças. Foram identificados amastigotas no SP de 18/22 pacientes com LVA e em nenhum de 10 sem LVA. O teste foi positivo em 30 dos 31 cães sororreagentes e em 28/28 cães que tiveram *Leishmania* identificada em outros tecidos. O QBC® é um método promissor para o diagnóstico de LVA humana, e possivelmente para exame do SP de pacientes com LVA/AIDS, para controle de cura ou para identificação de portadores assintomáticos. Devido à facilidade da coleta, execução e rapidez, deveria ser avaliado para programas de controle de reservatórios.

Palavras-chaves: Calazar. Leishmaniose visceral. *Leishmania chagasi*. Cão. QBC®.

Abstract "Quantitative Buffy Coat" (QBC®) is a direct and fast fluorescent method used for the identification of blood parasites. Since *Leishmania chagasi* circulates in blood, we decided to test it in American visceral leishmaniasis (AVL). Bone marrow (BM) and peripheral blood (PB) of 49 persons and PB of 31 dogs were analyzed. QBC® was positive in BM of 11/11 patients with AVL and in 1/6 patients with other diseases. Amastigotes were identified in PB of 18/22 patients with AVL and in none without AVL. The test was positive in 30 out of the 31 seropositive dogs and in 28/28 dogs with *Leishmania* identified in other tissues. QBC® is a promising method for diagnosis of human AVL, and possibly for the exam of PB of patients with AVL/AIDS, for the control of the cure and for the identification of asymptomatic carriers. Because it is fast and easy to collect and execute, QBC® should be evaluated for programs of reservoir control.

Key-words: Kala-azar. Visceral leishmaniasis. *Leishmania chagasi*. Dog. QBC®.

The diagnosis of human and canine American visceral leishmaniasis (AVL) may be confirmed by serologic tests or by the demonstration of the protozoa *Leishmania chagasi* with parasitological or molecular methods. Both approaches present drawbacks that may be improved. While false-positive results may appear with serology due to cross-reactivity or due to the development of specific antibodies to past asymptomatic infections, parasitological methods are invasive because they usually require bone marrow (BM) or spleen puncture, and polymerase chain reaction (PCR) from peripheral blood still lacks feasibility and reliability for routine diagnosis¹⁷.

Stimulated by the recent description of high proportion of *Leishmania* DNA in the peripheral blood (PB) of patients with AVL²⁵ and by the need of a diagnostic method for canine AVL which directly identifies the parasites¹⁰, we have proposed the use of the Quantitative Buffy Coat (QBC®). The method, which is largely used for diagnosis of malaria^{15,32}, was intended for rapid demonstration of amastigotes in the BM and PB of persons with AVL and in dogs infected with *L. chagasi*. In this paper we report the preliminary but larger data of our previous observations of QBC® for human and canine AVL in Teresina, Brazil²⁰.

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SUBJECTS AND METHODS

BM and PB of persons and dogs were examined using QBC®. Persons' samples were taken from hospitalized patients with AVL, from hospitalized patients with other diseases and from healthy volunteers. The diagnosis of AVL was established when typical symptoms and reactive immunofluorescent antibody test (IFAT) (Biomanguinhos-RJ) or amastigotes in the BM aspirate were present. Seropositive dogs screened with IFAT (dilutions 1/40 or more) were examined by a veterinarian (ILM). Because they should be killed according to the recommendations of the Ministry of Health, they could be autopsied. PB was examined using QBC® and samples of BM, spleen, liver, lymph nodes and kidneys were stained with Giemsa and cultivated in the Novy-MacNeal-Nicolle (NNN) media.

BM and PB were placed on a 75-mm (55ml) microhematocrit capillary tube previously coated with DNA stain acridine orange and potassium oxalate. Centrifugation at a preset speed (14,000 x g) (Parafuge, Becton Dickinson) allowed the separation of blood cells, with the help of a polystyrene float for expansion of the white cell layer. To adapt a benchtop microscope into a fluorescent microscope, the oil immersion objective (Paralens UV Microscope Adapter, Becton Dickinson) was screwed to a portable light source in the range of 480nm by a fiberoptic cable. Microphotographs were

taken using a Zeiss® microscope with epi-immunofluorescence HBO-50 and with a microphotographic system MC 80 DX and 1,000x amplification. A fluorescent microscopic was also occasionally used, although a little more cumbersome to get the focus.

The first part of the study consisted in practice and training. A skilled technician on malaria diagnosis with QBC® (FCOL) examined human and dog samples while teaching the Teresina team. At the same time, the pattern of fluorescence of the *L. chagasi* nuclei and kinetoplast on QBC® of promastigotes on NNN culture tubes was observed and characterized. When these flagellated forms found in sand flies and in culture change into the non-flagellated amastigotes found on host tissues, there is no morphological modification of the nuclei or the kinetoplast. Therefore, both forms are undistinguishable at QBC. Due to the initially high false-positive rate, a set of guidelines for amastigote identification was used in order to increase stringency; to be taken as positive, a sample must show all of the following 5 characteristics: parasite nuclei looking less than 1/5 the size of nuclei of host cells (1), not presenting cytoplasmic fluorescence (2) and showing the presence of the kinetoplast, which must border the parasite nuclei (3), being brighter than it (4) and seeming not to be more than 1/3 of its size (5).

RESULTS

The typical aspects of PB extracellular amastigotes and BM intracellular forms are seen in figure 1. Parasites were identified in the less dense cell layer. While PB preparations were clear and neat, BM tubes yield longer cell layers and were full of nucleated cells. Only one intracellular parasite was clearly seen on PB, but collections of parasites suggestive of being extracellular, were identified.

Before the establishment of the guidelines for parasite identification, initial PB examination of patients with AVL detected amastigotes in 16/19 (84%). Only 16/26 (62%) of the BM tested were positive. Four patients thought not to have AVL presented false-positive results on PB.

Table 1 presents the results of studies on 49 persons after the parameters for taking the samples as positive were used. All patients with AVL had positive QBC® on BM examination. One patient with splenomegaly had positive QBC® on BM but histopathology lately defined the diagnosis as paracoccidioidomycosis.

All 28 dogs in which parasites were found presented positive QBC® on PB. One dog with suggestive symptoms but without parasites identified on autopsy had a negative result. The overall sensitivity of the test was 97% (Table 2). Because no trial was designed for it, specificity of QBC® on PB for canine AVL was not evaluated in this study.

DISCUSSION

A simple fluorescent body on QBC® may not represent the nuclei of extracellular amastigotes, as the initially high false positive rate has shown. In order to increase the specificity, typical morphological characteristics were required, which, in fact, improved the test. When the pattern of fluorescence, the size of the nuclei and the presence of the kinetoplast were considered, the proportion of false-positive tests was significantly reduced. Only one patient tested false-positive. This patient presented fever, anemia and splenomegaly with the disseminated form of the South American deep mycosis caused by *Paracoccidioides brasiliensis*. The fungi was mistaken for *Leishmania* on BM QBC®, but this is not surprising since the shape of

a fluorescent yeast forming the characteristic bud might mimic the shape of an amastigote. However, although caution should be taken on the interpretation of a BM positive QBC®, the high sensitivity on BM and the high pre-test probability of AVL in patients with typical symptoms would balance the moderate specificity and allow the indication of BM QBC® for routine use.

QBC® had a satisfactory performance on human blood examination. Indeed, although these data are not new, in recent years *Leishmania* has been repeatedly demonstrated, either directly, culturing or via PCR®, in PB from asymptomatic persons infected with anthroponotic and zoonotic viscerotropic *Leishmania*^{13 27} and from immunocompromised^{11 22} and immunocompetent^{1 28 30 31} Old

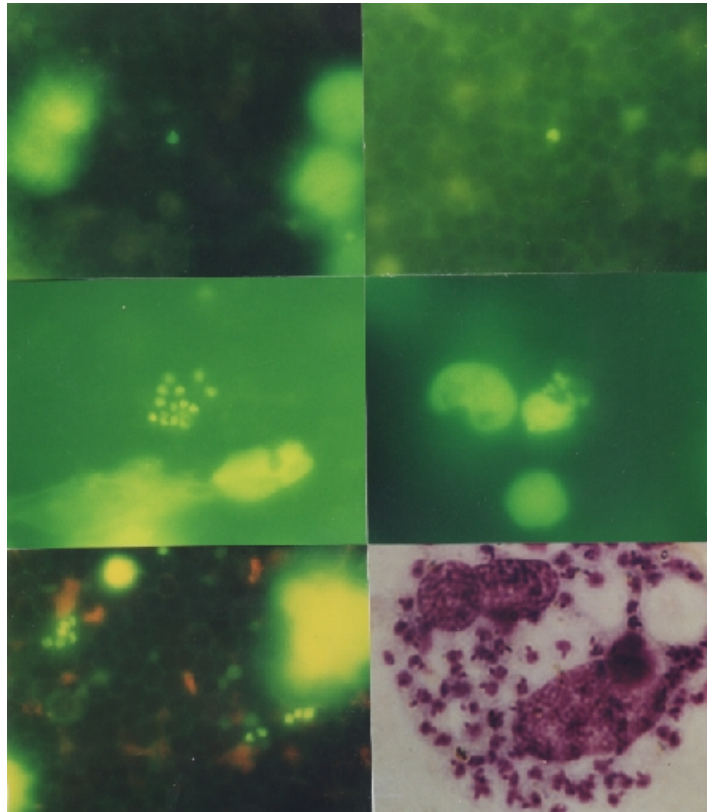


Figure 1 - Microphotographs of QBC® and Giemsa stain from bone marrow and peripheral blood of humans and dogs taken in a microscope of epifluorescence. **Top left:** Human bone marrow. A single amastigote is visible among many nucleated cells; the kinetoplast is situated at the position of 12:00 o'clock in relation to the parasite nuclei. **Top right:** Human peripheral blood with one isolated amastigote; the kinetoplast is found at the position of 5:00 o'clock. **Middle left:** Human peripheral blood with a group of extracellular amastigotes; the kinetoplast is visible in some of them. **Middle right:** Cluster of intracellular amastigotes from human peripheral blood. **Bottom left:** Dog peripheral blood with amastigotes; the kinetoplast is discernible in some parasite cells. **Bottom right:** Giemsa stain of dog bone marrow with several amastigotes, to highlight the similarity and differences of both staining methods. Magnification 1,000x.

Table 1 - QBC® results from bone marrow and peripheral blood in persons with and without American visceral leishmaniasis.

QBC® result	Clinical diagnosis at discharge				Total
	visceral leishmaniasis		other diagnosis/healthy		
	bone marrow (%)	peripheral blood (%)	bone marrow (%)	peripheral blood (%)	
Positive	11 (100.0)	18 (81.8)	1 (16.7)	-	30 (61.2)
Negative	-	4 (18.2)	5 (83.3)	10 (100.0)	19 (38.8)
Total	11 (100.0)	22 (100.0)	6 (100.0)	10 (100.0)	49 (100.0)

Table 2 - QBC® results in seropositive dogs accordingly to the identification of Leishmania chagasi at autopsy and to the clinical status.

QBC® result	Parasitological diagnosis at autopsy				Total
	Leishmania present		Leishmania absent		
	Signs of disease	no signs of disease	signs of disease	no signs of disease	
Positive	26 (100.0)	2 (100.0)	-	2 (100.0)	30 (96.8)
Negative	-	-	1 (100.0)	-	1 (3.2)
Total	26 (100.0)	2 (100.0)	1 (100.0)	2 (100.0)	31 (100.0)

World and New World patients with visceral leishmaniasis. Therefore, QBC® testing in the PB of patients with AVL was straightforward. Although the sensitivity was not as high as serology, parasite identification has the advantage of being a direct demonstration of *L. chagasi*, which may be critical when the force of infection is high. In these situations, a significant proportion of seropositive persons is represented by those with asymptomatic past infections, which reduces the specificity of serology.

The simplicity of finger-prick and rapid diagnosis makes this approach specially helpful during epidemics in the most remote areas. Due the easy finding of *Leishmania* in the PB of VL patients co-infected with HIV²², the method could be the best way to identify these patients. Also, evaluation of the response to therapy could eventually be attempted with QBC®, as proposed for PCR^{19,26}. Besides these applications, the identification of antibody-negative asymptomatic carriers of *L. chagasi*⁹ could be more easily performed by direct examination of PB with QBC®. The previous suggestion of a more important role of humans as reservoirs of VL than it is commonly accepted⁸ is highlighted by the easy finding of morphologically preserved parasites in the blood of most patients with QBC® and by another report of frequent cutaneous parasitism in patients with AVL³³.

QBC® has its most promising role for canine AVL. Currently, control of human AVL by killing seropositive dogs on a national scale is used in Brazil. Among other suppositions, this program relies on the assumption that serology is efficient to detect infective dogs. However, many infected dogs are seronegative¹² and many seropositive dogs are not infective when they are followed-up (Courtenay O, Quinnel RJ, Garcez LM, Dye C: unpublished data), characterizing serology as a poor

screening test. Direct detection of circulating parasites or its antigens would be a better indicator of the present infection and also of the infectivity to sand flies, which is the intent of such a screening program. Our results demonstrated a QBC® sensitivity of 97%, and that the test was able to detect the two infected but asymptomatic dogs. These promising results make QBC® a candidate for larger studies on the impact of dog killing on the transmission of AVL to humans. However, experiments on uninfected dogs from endemic and non-endemic areas should be performed to evaluate the specificity of QBC®. Moreover, because only seropositive dogs were studied and antibody levels may be related to the presence of active infection¹², evaluation of sensitivity on infected but seronegative dogs should also be done.

Besides malaria, QBC® has been tested in blood subjects with relapsing fever⁷, leptospirosis¹⁸, animal babesiosis⁵, sleeping sickness², Chagas disease²⁴ and several human and animal filariasis^{4, 6, 14, 21} and even in vaginal fluid of patients with trichomoniasis¹⁶. Most results are promising. Actually, it was a surprise that QBC® has not yet been used for the diagnosis of canine AVL because, similarly to humans, recent studies have shown high proportions of circulating parasites in infected dogs^{23, 29} using PCR and in view of staining of *Leishmania* promastigotes from culture media with acridine orange has already been published³. Blood for QBC® can easily be obtained from ear-pricks from dogs, and if the blood in the capillary tube is soon centrifuged, it can be examined within weeks. Examination takes less than five minutes and the images are usually very good. If in the future dog killing is proved to benefit people by preventing infection with *L. chagasi*, QBC® is a candidate method to take part of a set of serial screening tests – possibly as the sensitive test prior to a specific antigen capture assay.

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REFERENCES

1. Adhya A, Chatterjee M, Hassan MQ, Mukherjee S, Sen S. Detection of *Leishmania* in the blood of early kala-azar patients with the aids of the polymerase chain reaction. Transactions of the Royal Society of Tropical Medicine and Hygiene 89: 622-624, 1995.
2. Bailey JW, Smith DH. The use of the acridine orange QBC technique in the diagnosis of African trypanosomiasis. Transactions of the Royal Society of Tropical Medicine and Hygiene 86:630, 1992.
3. Barreca GS, Berlinghieri MC, Foti F, Matera G, Foca A. Microscopic observation of progressive immobilization of *Leishmania* promastigotes in acridine orange stain. Journal of Clinical Microbiology 35: 1867-1869, 1997.
4. Bawden M, Slaten D, Malone J. QBC: rapid filaria diagnoses from blood -*Mansonella ozzardi* and *Wuchereria Bancrofti*. Transactions of the Royal Society of Tropical Medicine and Hygiene 86: 66, 1994.
5. Bose R, Jorgensen WK, Dalgliesh RJ, Friedhoff KT, De Vos AJ. Current state and future trends in the diagnosis of babesiosis. Veterinary Parasitology 57 :61-74, 1995.
6. Brown SA, Barsanti JA. Quantitative buffy coat analysis for hematologic measurements of canine, feline, and equine blood samples and for detection of microfilaremia in dogs. American Journal of Veterinary Research 49: 321-324, 1988.
7. Chatel G, Gulletta M, Matteelli A, Marangoni A, Signorini L, Oladeji O, Caligaris S. Diagnosis of tick-borne relapsing fever by the quantitative buffy coat fluorescent method. The American Journal of Tropical Medicine and Hygiene 60: 738-739, 1999.
8. Costa CHN, Gomes RBB, Gonçalves MJO, Garcez LM, Ramos PKS, Santos RS, Shaw JJ, David JR, Maguire JH. Competence of human host as reservoir for *Leishmania chagasi*. Journal of Infectious Diseases 182: 997-1000, 2000.
9. Costa CHN, Stewart JM, Gomes RB, Garcez LM, Ramos PKS, Bozza M, Santoskar A, Dissenayake S, Santos RS, Silva MRB, Shaw JJ, David JR, Maguire JH. Asymptomatic human carriers of *Leishmania chagasi*. The American Journal of Tropical Medicine and Hygiene, 2001.

10. Costa CHN, Vieira JBF. Changes in the control program of visceral leishmaniasis in Brazil. *Revista da Sociedade Brasileira de Medicina Tropical* 34: 223-228, 2001.
11. Costa JM, Durand R, Deniau M, Rivollet D, Izri M, Houin R, Vidaud M, Bretagne S. PCR enzyme-linked immunosorbent assay for diagnosis of visceral leishmaniasis in human immunodeficiency virus-infected patients. *Journal of Clinical Microbiology* 34: 1831-1833, 1996.
12. Dye C, Vidor E, Dereure J. Serological diagnosis of leishmaniasis: on detecting infection as well as disease. *Epidemiology and Infection* 103:647-656, 1993.
13. Fichoux Y, Quaranta JF, Aufeuvre JP, Lelievre A, Marty P, Suffia I, Rousseau D, Kubar J. Occurrence of *Leishmania infantum* parasitemia in asymptomatic blood donors living in an area of endemicity in Southern France. *Journal of Clinical Microbiology* 37: 1953-1957, 1999.
14. Freedman DO, Berry RS. Rapid diagnosis of Bancroftian filariasis by acridine orange staining of centrifuged parasites. *The American Journal of Tropical Medicine and Hygiene*, 47: 787-793, 1992.
15. Garin B, Salun JJ, Peyron F, Vigier JP, Busangu I, Perrone J. Rapid *in vivo* detection of chloroquine resistance by the quantitative buffy coat malaria diagnosis system. *The American Journal of Tropical Medicine and Hygiene* 47: 446-449, 1992.
16. Hammouda NA, Hegazy IH, Tawfik TA. A rapid diagnostic test for *Trichomonas vaginalis* infection. *Journal of the Egyptian Society of Parasitology* 27: 341-347, 1997.
17. Herwaldt BL. Leishmaniasis. *Lancet* 2: 354(9185):1191-1199, 1999.
18. Kramer KJ, Pang LW, Minette HP, Perrone JB. Evaluation of the quantitative buffy coat analysis (QBC) system for the detection of *Leptospira* in human blood. *The Southeast Asian Journal of Tropical Medicine and Public Health* 25: 788-789, 1994.
19. Lachaud L, Dereure J, Chabbert E, Reynes J, Mauboussin JM, Oziol E, Dedet JP, Bastien P. Optimized PCR using patient blood samples for diagnosis and follow-up of visceral leishmaniasis, with special reference to AIDS patients. *Journal of Clinical Microbiology* 38: 236-240, 2000.
20. Liarte DB, Mendonça IL, Luz FCO, Farias TJC, Ferreira AFB, Millington MA, Tosta CE, Costa CHN. QBC for the diagnosis of visceral leishmaniasis. *In: Abstract Book of WorldLeish2, Hersonissos, Greece, p.119, 2001.*
21. Long GW, Rickman LS, Cross JH. Rapid diagnosis of *Brugia malayi* and *Wuchereria bancrofti* filariasis by an acridine orange/microhematocrit tube technique. *Journal of Parasitology* 76: 278-281, 1990.
22. López-Vélez, R, Laguna F, Alvar J, Pérez-Molina JA, Molina R, Martínez P, Villarrubia J. Parasitic culture of buffy coat for diagnosis of visceral leishmaniasis in human immunodeficiency virus-infected patients. *Journal of Clinical Microbiology* 33: 937-939, 1995.
23. Mathis A, Deplazes P. PCR and *in vitro* cultivation for detection of *Leishmania spp.* in diagnostic samples from humans and dogs. *Journal of Clinical Microbiology* 33: 1145-1149, 1995.
24. Nisida IV, Amato Neto V, Braz LM, Duarte MI, Umezawa ES. A survey of congenital Chagas disease, carried out at three health institutions in Sao Paulo City, Brazil. *Revista do Instituto de Medicina Tropical de São Paulo* 41: 305-311, 1999.
25. Nuzum E, White III F, Thakur C, Dietze R, Wages J, Grogl M, Berman J. Diagnosis of symptomatic visceral leishmaniasis by use of the polymerase chain reaction on patient blood. *Journal of Infectious Diseases* 171: 751-754, 1995.
26. Osman OF, Oskam L, Zijlstra EE, El-Hassan AM, El-Naiem DA, Kager PA. Use of the polymerase chain reaction to assess the success of visceral leishmaniasis treatment. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 92: 397-400, 1998.
27. Otero AC, Silva VO, Luz KG, Palatnik M, Pirmez C, Fernandes O, Palatnik de Sousa CB. Occurrence of *Leishmania donovani* DNA in donated blood from seroreactive Brazilian blood donors. *The American Journal of Tropical Medicine and Hygiene* 62: 128-131, 2000.
28. Qiao Z, Miles MA, Wilson SM. Detection of parasites of the *Leishmania donovani*-complex by a polymerase chain reaction-solution hybridization enzyme-linked immunoassay (PCR-SHELA). *Parasitology* 110: 269-275, 1995.
29. Reale S, Maxia L, Vitale F, Glorioso NS, Caracappa S, Vesco G. Detection of *Leishmania infantum* in dogs by PCR with lymph node aspirates and blood. *Journal of Clinical Microbiology* 2931-2935, 1999.
30. Rohrs LC. Leishmaniasis in the Sudan Republic. XVIII. Parasitemia in kala-azar. *The American Journal of Tropical Medicine and Hygiene* 13: 265-271, 1964.
31. Schaefer KU, Schoone GJ, Gachihi GS, Muller AS, Kager PA, Meredith SEO. Visceral leishmaniasis: use of the polymerase chain reaction in an epidemiological study in Baringo District, Kenya. *Transactions of the Royal Society of Tropical Medicine and Hygiene* 89: 492-495, 1995.
32. Spielman A, Perrone JB, Teklehaimanot A, Balcha F, Wardlaw SC, Levine RA. Malaria diagnosis by direct observation of centrifuged samples of blood. *The American Journal of Tropical Medicine and Hygiene* 39: 337-342, 1988.
33. Vasconcelos IAB, Sousa AQ, Vasconcelos AW, Diógenes MJN, Momem H, Grimaldi Jr. G, Menezes DB, Sleigh AC. Parasitisme cutané par *Leishmania (Leishmania) chagasi* au cours de la leishmaniose viscérale sud-américaine. *Bulletin de la Société de Pathologie Exotique* 86: 101-105, 1993.