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Short Communication

Comparison of methodologies for detecting *Trypanosoma cruzi* parasites by microscopic observation of microhematocrit capillary tubes

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Abstract

Introduction: The microscopic examination of microhematocrit tubes (mHCT) has been proposed as the gold standard for acute and congenital Chagas disease diagnosis. We compared different mHCT methodologies detecting *T. cruzi* parasites in the blood. **Methods:** The rotating method, water mount, and immersion oil methods were compared for their suitability, sensitivity, and specificity. **Results:** The rotating method was easier, faster, and more sensitive than the others with 100% specificity. **Conclusions:** The rotating method is feasible for laboratory technicians with standard training in microscopic techniques and is recommended for the diagnosis of acute Chagas disease in primary health care facilities.

Keywords: Microhematocrit test. *Trypanosoma cruzi*. Capillary tube test. *T. cruzi* diagnostic methods.

The American trypanosomiasis or Chagas disease caused by the protozoan parasite *Trypanosoma cruzi.* is endemic in Latin America, where its congenital transmission is a health problem. The World Health Organization (WHO) estimates 5,750,000 individuals to be infected with *T. cruzi*, of which 1,125,000 are women of fertile ages. Mexico has one of the highest percentages of the infected population (15.3%)[1], and maternal-fetal transmission occurs in an average of about 4.7% of infected mothers[2]. The microscopic examination of the buffy coat from centrifuged heparinized microhematocrit tubes (mHCT), which is a relatively simple, easy and cheap method to detect live parasites in blood, is very important because of its higher sensitivity than other methods like microscopic examination of fresh blood samples, fixed blood smears or thick smear[3]. In the mHCT, the parasites are concentrated by centrifugation, providing an easier detection of the moving trypomastigote[4]. This technique, invented by Devignat & Resse (1955)[5], has been improved[6] and later applied for the diagnosis of Chagas disease[7,8], particularly for the detection of congenital infections[4,8,9,10,11] and is recommended as the gold standard for early diagnosis[3]. The aim of this study is to compare the suitability, sensitivity, specificity and the optimal number of tubes used to detect and quantify *T. cruzi* parasites in the blood in three different mHCT methodologies: Method 1 (Rotating method), Method 2 (Immersion oil method), Method 3 (Wet mount method).

T. cruzi parasites were previously isolated from a human case in Yucatan, Mexico (H9 strain) and are routinely maintained in mice (ICR strain). The mouse blood was collected 24 days after inoculation (acute phase of infection), at the time of the highest parasitemia and the parasites were counted using a Neubauer

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chamber. This sample was further mixed with human peripheral blood to obtain suspensions of 10,000, 1000, 500, 300, 50, and 10 parasites per 50 µL.

Thirty heparinized capillary tubes were loaded with 50 µL of human blood previously inoculated with each of the six parasite suspensions (10000, 1000, 500, 300, 50 and 10 parasites per 50 µL), and another 30 tubes were filled with human blood without parasites (blind control). After sealing one end of the tubes with plasticine, the tubes were centrifuged at 12,000 rpm (322 g)/10 minutes at room temperature in a microcapillary centrifuge (LW-M24, LW-Scientific). Subsequently, the tubes were mounted on slides using three different procedures.

Method 1 (Rotating). The mHCT is held laterally over one edge of a slide using masking tape previously placed at both ends with a small space between the tape and the carrier, to be able to rotate the tube to get different angles of observation[9,12] in the microscope using the 40× objective (**Figure 1**, Row A).

Method 2 (Immersion oil). As in the technique described before, the mHCT is fixed laterally over one edge of a slide using masking tape, but without the possibility of rotation and a drop of immersion oil is deposited to cover the interphase zone. Observation was performed using the 100× immersion objective[6,7] (**Figure 1**, Row B).

Method 3 (Wet mount). Two plasticine stripes were placed on the short sides of a slide and two mHCT were placed parallelly on the slide with their ends resting on the plasticine stripes. Then, a cover slip was placed over the interphase of the two tubes, one to two drops of water were deposited between the slide and the cover slip, and the observation was made using the 40× objective (**Figure 1**, Row C).

The parasite suspensions were prepared in different days, to get 30 mHCT with parasites and 30 without parasites for each suspension (with a total of 360 mHCT per method). The microscopic observations were performed using a regular microscope (Motic DMBA-210) and carried out within 6 hours after the preparation of the parasite suspensions to avoid a decrease in sensitivity due to parasite lysis[3]. The observations were performed by a reader technician with experience in microscopic examinations. Blind control samples were subjected to observation as well. Moving trypomastigotes were searched for at the buffy coat layer level. The observation in each tube was performed by the technician within 10 minutes and the results were annotated either as the presence (+) or the absence (-) of parasites. The technician was able to count parasites only in the mHCT suspension with 1000 parasites per 50 µL.

The results obtained were compiled to establish the sensitivity and the specificity of the techniques.

This study used blood samples from *T. cruzi*-infected mice routinely maintained in the animal facility laboratory of the Regional Research Centre "Dr. Hideyo Noguchi" of the Autonomous University of Yucatan, and leftover blood samples from the haematology laboratory obtained after performing routine tests on healthy persons.

The advantages of method 1 are as follows: the sample tube is placed and retired easily from the tape of the slide and

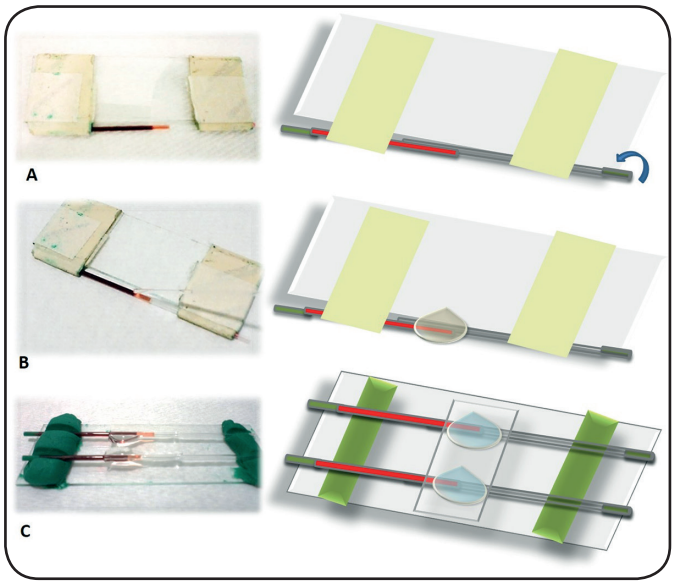

FIGURE 1: Row A. Method 1 (rotating the mHCT, dry mount): the masking tape is holding the tube making it easier to handle and rotate (observation under 40× standard objective). **Row B.** Method 2 (mHCT using immersion oil): the masking tape is holding the tube, without possibility of rotation and oil is deposited on the interface area (observation under 100× immersion objective). **Row C.** Method 3 (mHCT in water mount): the plasticine is holding 2 tubes, without possibility of rotation and water is deposited on the interface area under the cover slide (observation under 40× standard objective). Real photos of each method are shown on the left side and schematic pictures of each method are shown on the right side.

when rotated it can be seen from all angles. A trained technician can determine the presence or absence of parasites within ten minutes. The taped slide is prepared only once and can be reused with a small risk of the tube breaking during placement on the taped slide. The disadvantage was that only one tube can be placed on the taped slide and the tubes must be studied one at a time.

The advantage in the method 2 was that the detected parasites were clearly visible; however, similar to method 1, only one tube could be placed at a time and the immersion oil had to be placed carefully and was frequently spilled over the observation area, delaying the analysis of the next sample. The observation area is significantly smaller than in the other methods, reducing the probability of detecting parasites in 10 minutes.

The advantages of method 3: Two tubes can be placed simultaneously on the slide. The disadvantages are that placing tubes on the plasticine beds of the slide is not easy, and the beds must be prepared at every usage since they lose their form with usage. Besides, there is a high risk of breaking the tube when placing in the plasticine beds; also, the water must be deposited carefully for a clear sample observation.

It is important to note that, the immersion oil method, was useful only for the observation of parasites at the highest concentration (10,000 parasites), but not for lower parasitemia.

Preparing tapes to enable rotating observation of mHCT (method 1) required 2:00 minutes and after the first mounting onto the slide, easy observation of further mHCT was made

TABLE 1: Numbers of tubes detected positive and negative and sensitivity and specificity determination using the rotating and wet mount methods.

	Parasites/ tube	Total tubes	Tubes with parasites	Tubes detected positive	False negative tubes	Tubes without parasites	Tubes detected negative	False positive tubes	Sensitivity	Specificity
Rotating tube technique	**10**	60	30	3	27	30	30	0		
	50	60	30	3	27	30	30	0		
	300	60	30	3	27	30	30	0		
	500	60	30	9	21	30	30	0	78/(78+102)=0.43	180/(180+0)=1.00
	1000	60	30	30	0	30	30	0		
	10000	60	30	30	0	30	30	0		
	Total	360	180	78	102	180	180	0		
Wet mounted tube technique	**10**	60	30	0	30	30	30	0		
	50	60	30	0	30	30	30	0		
	300	60	30	0	30	29	29	1		
	500	60	30	2	28	30	30	0	62(62+118)=0.34	179/(179+1)=0.99
	1000	60	30	30	0	30	30	0		
	10000	60	30	30	0	30	30	0		
	Total	360	180	62	118	179	179	1		

possible. By contrast, observation of mHCT in water mount needed 2:45 minutes to prepare the plasticine stripes on the slide and this had to be repeated at each observation.

Table 1 shows the tubes detected positive or negative for each parasite suspension in the rotating and the wet mount techniques; sensitivity and specificity of the rotating technique calculated from this data are 43% (95% CI: 0.36-0.51) and 100% (95% CI: 1-1), respectively, and those of the wet mount technique are 34% (0.28-0.41) and 99% (0.98-1.00) respectively. Based on the results obtained, we observed a better probability of detection of parasites than in other studies which use a lower number of tubes (4, 6 and 10 tubes)[9,10] in the rotating and the wet mount techniques, are shown in **Table 2.** Our results indicate that by using the rotating technique, 10 capillary tubes were necessary to detect 10 parasites/tube, whereas the use of 4 or 6 tubes allowed the detection of 500 parasites/tube. By contrast, only 1000 parasites/tube could be detected with the wet mount method regardless of the number of tubes used.

The count of parasites observed per tube with 1000 parasites, showed an average of 1% to 2% detection of the actual number of parasites. Hence, it can be concluded that the rotating method can significantly detect more parasites than the wet mount technique (Mean [SD]: 18.37 [1.343] vs. 9.33 [0.9818], respectively; t' student test: $P<0.01$).

Our results indicate that among the mHCT methodologies for detecting *T. cruzi* in blood, the rotating method is easier to manipulate, less time consuming, and has higher sensitivity than the other methods.

The specificity of mHCT rotating method is 100% since observation of a motile trypomastigote in blood indicates a current infection. The sensitivity of this method is equivalent to examining parasites after tube dissection[8]. Different studies have shown a lack of sensitivity of the mHCT parasitological detection in congenital infection[13]; however, the sensitivity of the rotating method increases significantly by using more tubes, and repeating blood examination at different times after birth, since neonatal parasitic loads can increase up to 1 to 3 months after delivery, allowing the correct diagnosis of most cases of congenital infection[9,12].

Under this basis, we recommend the rotating method for microscopic detection of live *T. cruzi* in blood. Its simplicity of manipulation by trained laboratory technicians, in addition to the certainty of the diagnosis, makes the rotating mHCT method an important tool for the diagnosis of acute Chagas disease in primary health care facilities in endemic areas.

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TABLE 2: Parasite detection probabilities using 4, 6 or 10 tubes with the rotating and wet mount methods.

	Parasites/mHCT	% of detected positive mHCT (observed from 30 positive tubes) *	Calculated number of positive mHCT		
			using 4 tubes	using 6 tubes	using 10 tubes
Rotating tube method	10	10.0 (+)	0.4 (-)	0.6 (-)	1.0 (+)
	50	10.0 (+)	0.4 (-)	0.6 (-)	1.0 (+)
	300	10.0 (+)	0.4 (-)	0.6 (-)	1.0 (+)
	500	30.0 (+)	1.2 (+)	1.8 (+)	3.0 (+)
	1000	100.0 (+)	4.0 (+)	6.0 (+)	10.0 (+)
	10,000	100.0 (+)	4.0 (+)	6.0 (+)	10.0 (+)
Wet mount tube method	10	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)
	50	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)
	300	0.0 (-)	0.0 (-)	0.0 (-)	0.0 (-)
	500	6.6 (+)	0.3 (-)	0.4 (-)	0.7 (-)
	1000	100.0 (+)	4.0 (+)	6.0 (+)	10.0 (+)
	10,000	100.0 (+)	4.0 (+)	6.0 (+)	10.0 (+)

*% results derived from table 1; **(+):** at least one positive tube; **(-):** absence of positive detected tube.

Conflict of Interest

The authors declare no conflict of interests.

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