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TECHNICAL REPORT

METHYLENE BLUE VITAL STAINING FOR *Trypanosoma cruzi* TRYPOMASTIGOTES AND EPIMASTIGOTES

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SUMMARY

The morphological identification of *Trypanosoma cruzi* is currently considered to have a high specificity, but its sensitivity, which depends on the volume of the sample examined, is rather low. Trypanosome developmental stages suspended in blood, reduviid feces, and culture media are routinely searched for by means of fresh film examination (about 2 µL). High speed centrifugation of blood samples separates the buffy coat, where most trypomastigotes concentrate. As the parasites are transparent and colorless, their detection is mostly dependent on their motility. The fluorescent vital stain acridine orange has been used to enhance image contrast, as exemplified by the QBC (Quantitative Buffy Coat) technique. Staining blood, buffy coat, reduviid feces, and culture media samples with methylene blue (also a vital dye) is a means of producing sharp, well contrasted images of motile or non-motile *T. cruzi* developmental stages, only standard laboratory microscopes being required. Slides previously coated with a thin layer of methylene blue are used to stain fresh blood films. Photomicrographs exemplify the results of methylene blue staining applied to living and fixed parasites.

KEYWORDS: Chagas disease; Morphological identification of *Trypanosoma cruzi*; Fresh blood films; Buffy coat; Methylene blue vital staining.

The morphological identification of the etiological agent of Chagas disease*, Trypanosoma cruzi*, in fresh preparations of peripheral blood (about 2 µL) collected with anticoagulant has been for a long time standard technique. Such films are examined under a microscope fitted with ×10 and ×40 dry objectives. As the objects of our investigation are transparent, colorless motile protozoa, their detection is to a great extent dependent on their motility, which causes the red blood corpuscles to move[6], most frequently the first indication of their presence. A time lag between collection and examination of blood samples is one of the factors that can, by affecting the motility of trypomastigotes, be the cause of false-negative diagnoses. Image-enhancing means have been recommended for routine use, viz: reduction of the numerical aperture of the condenser diaphragm; dark field; phase-contrast microscopy[6], and staining. The use of dark field and phase-contrast techniques requires microscopes with expensive components. The reduction of the numerical aperture of the condenser diaphragm is not effective enough when the parasites are not motile. Romanowsky-stained films usually produce sharp and easily recognized images of blood parasites. This applies especially to thin films. However, they require much time and expertise; their use is restricted to selected cases.

On account of such characteristics as cost effectiveness and high specificity, wet blood films are still used, without major modifications, in routine clinical and epidemiological investigation. Other factors remaining constant, the sensitivity of techniques involving the direct examination of blood films depends on the volume of the sample. As the volume of blood which can be examined between slide and cover slip is very restricted, positive results are not expected when parasitemias are low. Indirect parasitological techniques, such as xenodiagnosis and hemoculture[2] can significantly increase the chances of correctly diagnosing the infection in parasite carriers, but are cumbersome and repeated microscopic examinations along considerable lengths of time are necessary to confirm negativity.

High speed centrifugation mechanically separates blood cellular constituents in layers. The lowest layer is that of packed red blood corpuscles; immediately above it, a narrow region, buff in hue (the buffy coat), concentrates the leucocytes and platelets[3,5]. After centrifugation, *T. cruzi* trypomastigotes should be searched for in a region which includes the buffy coat and the uppermost layer of red blood corpuscles.

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The Centrifugal Quantitative Buffy Coat (QBC)[1,5], a highly elaborate technique evolved from that of WOO[8], was devised to permit the direct observation of fluorochrome-stained parasites concentrated in the buffy coat. Blood samples are collected in capillary tubes previously coated with anticoagulant and the fluorochrome acridine orange[7]. The buffy coat within the tube is directly examined. For this purpose, the tube is put on a carrier designed to reduce, by using immersion oil, the image distortion produced by the curvature of its surface. The examination is made through an epi-fluorescence microscope (λ = 490 nm) fitted with a ×60 long working distance immersion objective. This technique was intended at first for the diagnosis of *Plasmodium* spp. only. In Brazil, AMATO NETO *et al.* evaluated in 1996[1] the practical use of QBC for the parasitological diagnosis of *T. cruzi* with good results as to sensitivity and practicality.

A kit with all the equipment necessary to carry out the examination, including specifically designed disposable capillary tubes, a 12,000 rpm centrifuge, epifluorescence microscope and halogen ultraviolet radiation source was supplied ready for use. Obviously, this equipment was rather costly and not adequate for institutions lacking ample means. As the main drawback to the use of QBC was its cost, alternatives were suggested for the use of fluorochrome-stained preparations with standard equipment[5]. QBC kits have not been any more available in Brazil.

As a matter of fact, the problem of sensitivity of the techniques for the direct demonstration of *T. cruzi* trypomastigotes in blood samples can be reduced to: first, concentrating the parasites, and second, enhancing their images so as to identify them without effort. The examination of fresh buffy coat smears placed between a slide and a cover slip is current practice in many laboratories. Dark field or phase contrast microscopy and fluorescent vital staining with acridine orange are widely accepted image-enhancing techniques, but even their simplified versions require the acquisition of expensive equipment.

A quite simple process for staining trypanosomes in fresh or fixed blood preparations, reduviid feces or culture media is proposed. It can be used with an ordinary laboratory microscope equipped with standard objectives and eyepieces. Methylene blue is an alternative to fluorochromes. It is a vital stain[4] which has been used for a very long time and is readily available. A thin coat of methylene blue is produced by evaporating a drop of an aqueous solution of this stain (1 to 10,000 or 1 to 100,000) over an area around the center of the slide. Trypanosomes suspended in films of fresh blood, buffy coat, reduviid feces or culture media, rapidly acquire a blue color and become clearly visible under the microscope, their motility being preserved. As an alternative to Romanowsky staining, films previously fixed with methanol can also be rapidly stained with methylene blue. The buffy coat can be obtained by centrifugation in ordinary microhematocrit capillary tubes (length = 75.00 mm; internal diameter, 1.25 mm) at 12,000 rpm in a hematocrit centrifuge. The total volume of these capillary tubes is about half that used in QBC. To obtain comparable results, two tubes should be centrifuged.

T. cruzi evolutionary stages from reduviid feces or hemoculture media can be also promptly stained by using the same process.

The important change introduced in the preparation of fresh blood

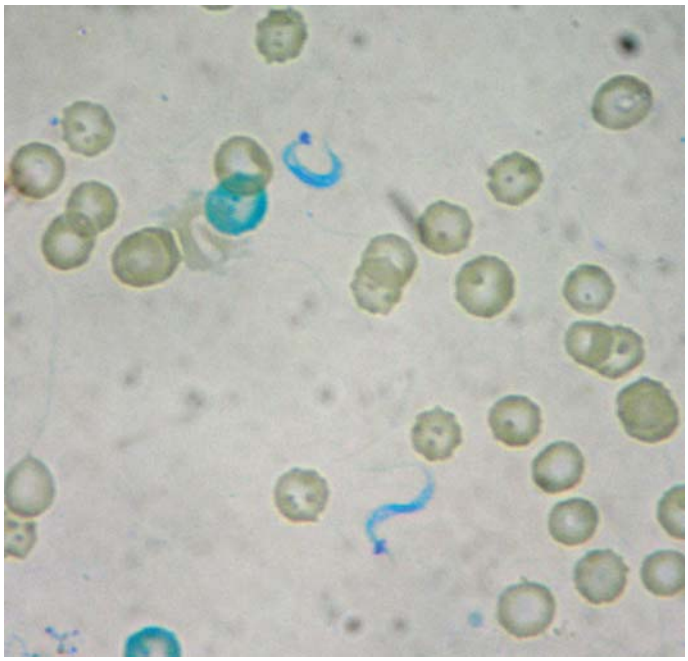

Fig. 1 - *Trypanosoma cruzi* fixed with methanol (buffy coat film) and stained with methylene blue. ×100 objective.

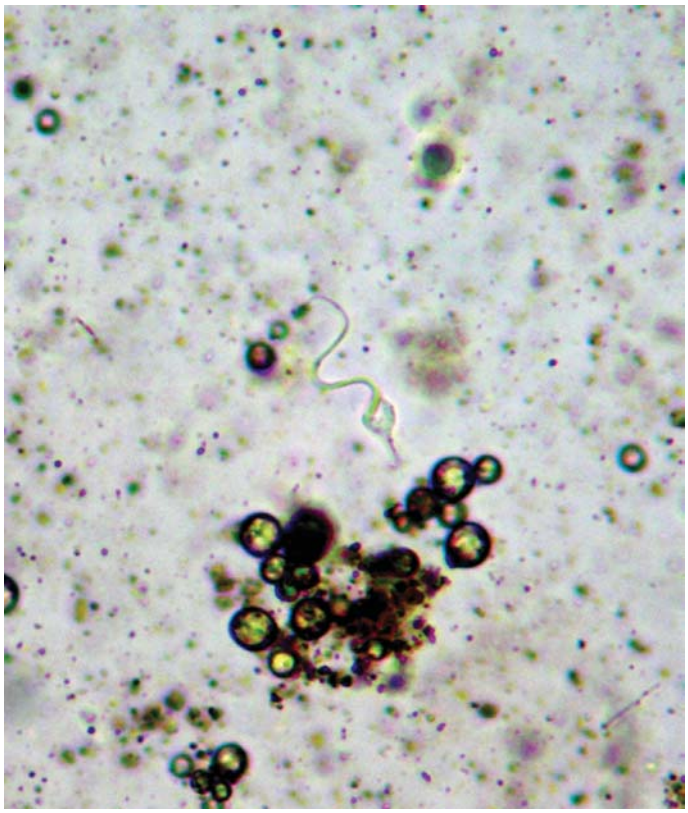

Fig. 2 - *Trypanosoma cruzi* living, motile (reduviid feces) stained with methylene blue. ×40 objective.

films for the diagnosis of Chagas disease was the use of methylene blue instead of a fluorochrome as a vital stain to cause living or dead trypanosomes to be easily detected in wet films. Methylene blue-stained films do not require expensive equipment; an ordinary laboratory microscope is adequate to permit the prompt identification of the parasites. The eye strain caused by the observation of poorly contrasted images is avoided. Laboratory personnel in charge of the examination of fresh blood films will not require any extra training to prepare and examine methylene blue-stained films. It is expected that such simple procedure will produce a reduction of the number of cases of false-negative results.

As an example of the effect of this simple procedure, two photographs are included (Fig. 1 and 2), which show, respectively, methylene blue-stained *T. cruzi* developmental stages in (previously fixed) buffy coat and (living, motile) in reduviid feces.

RESUMO

Coloração vital com azul de metileno aplicada a tripomastigotas e epimastigotas de *Trypanosoma cruzi*

A identificação morfológica de *Trypanosoma cruzi* tem alta especificidade, segundo é geralmente aceito; entretanto, sua sensibilidade, dependente do volume da amostra examinada, é baixa. Formas evolutivas de *T. cruzi* suspensas em sangue, fezes de reduviídeos e meios de cultura são rotineiramente pesquisadas em esfregaços a fresco (cerca de 2 μL). Centrifugação de amostras de sangue a altas velocidades produz a separação do creme leucocitário, onde se concentram as formas tripomastigotas. Em preparações a fresco, a motilidade das formas tripomastigotas e epimastigotas de *T. cruzi*, protozoário transparente e incolor, facilita sua detecção. Laranja de acridina, corante vital fluorescente, tem sido usada para acentuar o contraste das imagens de parasitas. Disto é exemplo a técnica QBC (Quantitative Buffy Coat). A coloração por meio de azul de metileno (também um corante vital), de amostras de sangue, de fezes de reduviídeos ou de meios de cultura permite obter imagens nítidas e contrastadas de formas evolutivas de *T. cruzi* com ou sem motilidade. Microscópios de uso geral em laboratórios permitem o exame dos parasitas corados. Uma camada bem delgada de azul de metileno colocada sobre a parte central da lâmina limpa (por meio da evaporação de solução diluída do corante) é usada para corar as preparações a fresco. O aspecto dos parasitas corados em materiais frescos ou previamente fixados pode ser observado em fotomicrografias.

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