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Utility of the microculture method for *Leishmania* detection in non-invasive samples obtained from a blood bank

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ABSTRACT

In recent years, the role of donor blood has taken an important place in epidemiology of Leishmaniasis. According to the WHO, the numbers of patients considered as symptomatic are only 5–20% of individuals with asymptomatic leishmaniasis. In this study for detection of *Leishmania* infection in donor blood samples, 343 samples from the Capa Red Crescent Blood Center were obtained and primarily analyzed by microscopic and serological methods. Subsequently, the traditional culture (NNN), Immunochromatographic test (ICT) and Polymerase Chain Reaction (PCR) methods were applied to 21 samples which of them were found positive with at least one method. Buffy coat (BC) samples from 343 blood donors were analyzed: 15 (4.3%) were positive by a microculture method (MCM); and 4 (1.1%) by smear. The sera of these 343 samples included 9 (2.6%) determined positive by ELISA and 7 (2%) positive by IFAT. Thus, 21 of (6.1%) the 343 subjects studied by smear, MCM, IFAT and ELISA techniques were identified as positive for leishmaniasis at least one of the techniques and the sensitivity assessed. According to our data, the sensitivity of the methods are identified as MCM (71%), smear (19%), IFAT (33%), ELISA (42%), NNN (4%), PCR (14%) and ICT (4%). Thus, with this study for the first time, the sensitivity of a MCM was examined in blood donors by comparing MCM with the methods used in the diagnosis of leishmaniasis. As a result, MCM was found the most sensitive method for detection of *Leishmania* parasites in samples obtained from a blood bank. In addition, the presence of *Leishmania* parasites was detected in donor bloods in Istanbul, a non-endemic region of Turkey, and these results is a vital importance for the health of blood recipients.

1. Introduction

Leishmaniasis is a zoonotic disease caused by the obligate intracellular protozoa of the genus *Leishmania* (Riera et al., 2008; Rioux et al., 1990; World Health Organization, 2010). The World Health Organization (WHO) identified six major tropical diseases, where leishmaniasis was listed as the second disease after malaria (Allahverdiyev et al., 2010; Wirth et al., 1986). Leishmaniasis is found in 98 countries, including 72 developing countries, threatening 350 million people (World Health Organization, 2010). It is estimated that there are 12 million *Leishmania* cases in the world, every year 500 thousand people added to this number, and, 75 thousand people died from this disease every year (Barbosa-De-Deus et al., 2002; Mary et al., 1992). According to WHO, these data are just for symptomatic individuals, which constitute only 5–20% of the current leishmaniasis. The remaining 80–95% of affected people is asymptomatic leishmaniasis (Cardo, 2006a; Romero et al., 2009). For many years, despite the fight against leishmaniasis, the main reasons of the increasing numbers of the disease in recent years are the resistance development by the disease vectors against insecticide, and the drugs used against pathogens. In addition, there has been a lack of effective vaccine against leishmaniasis until now (Allahverdiyev et al., 2010). With global warming, the disease is expected to increase gradually in countries around the world, including European countries. Furthermore increase in AIDS and other immune deficiencies, travels, wars, and problems in laboratory diagnosis plays an important role in the spread of the disease (Dujardin et al., 2008). Leishmaniasis is also a serious public health problem in Turkey (Bodur et al., 2003). The most common species seen in Turkey is *Leishmania infantum* for visceral leishmaniasis and *Leishmania tropica* for cutaneous leishmaniasis (World Health Organization, 2010). *Leishmania* parasites are being transmitted in

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different ways such as *Phlebotomus* bites (Cardo, 2006a; Roberts et al., 2000), blood transfusion (Cardo, 2006a; Chung et al., 1948; Kostman et al., 1963; Kubar et al., 1997; Mathur and Samantaray, 2004; Mauny et al., 1993; Singh et al., 1996), laboratory accidents (Cruz et al., 2002; Sundar and Rai, 2002), sexual transmission (Cardo, 2006a; Symmers, 1960), congenital transmission (Caldas et al., 2003; Meinecke et al., 1999), *Leishmania*-infected hamster bites (Manson-Bahr, 1987), organ (Kotton and Lattes, 2009) and stem cell transplantation (Zuk, 2011). In addition, there is a high risk for transmission by donor blood (Cardo, 2006a; Colomba et al., 2005; le Fichoux et al., 1999; Otero et al., 2000; Riera et al., 2004; Sharma et al., 2000; Silva et al., 2011). Additionally, *Leishmania* parasites were shown to retain the vitality and infectivity in the long-term storage of blood in blood banks (Cardo, 2006a; Grogl et al., 1993; Meinecke et al., 1999). Although it is difficult to determine if the disease is transmitted by blood transfusion of blood donors in endemic areas, there is evidence that the *Leishmania* parasite has spread. At the same time, the transmission of the disease with the blood of the donor is a vital importance for the health of blood recipients (Cardo, 2006a; Cardo et al., 2006; Grogl et al., 1993). Although there are studies on *Leishmania* parasites in donor blood in the various endemic regions of the world (Cardo, 2006a; Colomba et al., 2005; le Fichoux et al., 1999; Otero et al., 2000; Riera et al., 2004; Sharma et al., 2000; Silva et al., 2011), these studies are very limited due to the invasive methods used. As is known, various methods are used in the diagnosis of leishmaniasis. These current methods are generally intended for the diagnosis of patients with symptomatic leishmaniasis. However, the methods used in the diagnosis of symptomatic leishmaniasis are quite inadequate for the diagnosis of asymptomatic leishmaniasis, including testing for the parasite in the blood donors (Michel et al., 2011). Leishmaniasis is an endemic disease for the most of the regions of Turkey, but it is a sporadic disease for Istanbul and neighborhood and also it is known the reported cases are both for cutenuous and visceral leishmaniasis (Cakan et al., 2010; Gurel et al., 2012).

There is an urgent need for new methods with higher sensitivity for the detection of asymptomatic leishmaniasis. In recent years, it is known that MCM has a high sensitivity in the diagnosis of symptomatic leishmaniasis and it is still applied in various parts of the world. But there is no study in asymptomatic leishmaniasis particularly in donors blood for the sensitivity of MCM.

Accordingly, this study for the first time is to determine the *Leishmania* parasites in samples obtained from blood bank donors, and the sensitivity of MCM was compared to other methods (smear, IFAT, ELISA, NNN, ICT and PCR)used in the diagnosis of leishmaniasis.

2. Materials and methods

2.1. Donors

The study was performed in Istanbul, Turkey, between November 2008 and April 2011. Blood samples of 343 healthy blood donors from the CAPA Turkish Red Crescent Blood Bank (Istanbul, Turkey) were examined in the study. Of these donors, 39 (11%) were females and 304 (89%) were males. The age ranges were between 18 and 65. Informed consent was obtained from all participants, and the Ethics Committee of Istanbul University approved the study.

2.2. Blood collection

Peripheral bloods (PBs) were obtained by sterile venipuncture, and two different samples were separated. First the 4 mL of PB was collected without EDTA, and the serum was separated by centrifugation and stored at −40 °C for use in IFAT, ELISA and ICT methods; second the 4 mL of PBs was collected in ethylenediaminetetraacetate (EDTA)-coated polypropylene tubes and washed for several times by centrifugation to obtain the buffy coat (BC). The obtained BC was used for preparation of smears, NNN and MCM. PCR methods were applied to the samples obtained from MCM.

2.3. Microscopic methods

2.3.1. Giemsa staining of smears

Smears were prepared as described in our previous study (Allahverdiyev et al., 2005, 2012). The slides were observed under light microscopy at ×1000 magnification using immersion oil.

2.4. Culture methods

2.4.1. Traditional culture (NNN medium) preparation

This method were applied to the 21 samples which were detected positivi with at least one method of smear, IFAT, ELISA, or MCM and for this a second blood sample from these donors were obtained. The NNN medium was prepared as previously described with some modifications (Allahverdiyev et al., 2004, 2005; Riera et al., 2008). The defibrinated rabbit blood for NNN medium, kindly provided from Istanbul University, Institute of Experimental Medical Research-DETAE. The collected 40–50 μL of BC, which includes PBs was mixed with 100–200 μL of Schneider's *Drosophila* medium (S0146, Sigma Chemical Co., St. Louis, MO) supplemented with 30% fetal bovine serum (FBS) (S0115, Biochrom AG, Berlin, Germany) and whole mixed volume inoculated to NNN medium for cultivation. Prepared cultures were maintained at 27 °C and examined for 1–10 days.

2.4.2. Microculture method

PBs of donors were collected into sterile EDTA-coated polypropylene tubes and mixed. The mixed samples were washed 2–3 times with PBS (in the ratio of 1:3) by centrifugation for 10 min at 1500 × *g*. After the last wash, the supernatant was removed and the sediment resuspended with RPMI-1640 medium and centrifuged for 30 min at 2000 × *g*. After removing the supernatant, 40–50 μL of the BC which includes peripheral blood mononuclear cells were filled to 2 or 3 sterile hematocrit capillary tubes and centrifuged for 10 min at 800 × *g* (Heraeus Pico 17). The BC samples in the capillary tubes were collected and mixed with 100–200 μl Schneider *Drosophila* medium supplemented with 30% FCS and gentamicin (G1397, Sigma Chemical Co., St. Louis, MO). The mixed solution was dispensed into 2–4 sterile hematocrit capillary tubes and the ends of the capillary tubes were sealed with melted sterile paraffin. Intracellular amastigotes can become extracellular promastigotes in a microaerophilic environment, and these motile promastigotes can be detected under the inverted microscope by MCM (Allahverdiyev et al., 2012). Prepared microculture tubes were incubated at 27 °C under atmospheric conditions and then examined under the inverted microscope at ×200 magnification for not only 1–7 days (Allahverdiyev et al., 2005) but also were examined for 1–12 days. After the 12th day, the microcapillary tubes were stored at −40 °C for use in PCR.

2.5. Serological methods

2.5.1. Immunofluorescent antibody test (IFAT)

The antigens for IFAT were prepared from *L. infantum* (MON1/EP126) promastigotes culture and the strain was kindly provided by the Department of Parasitology, Faculty of Medicine, Ege University, Izmir, Turkey. The IFAT method was performed as described at our previous study (Ates et al., 2012). The presence of IgG antibodies was investigated in different dilutions (1/32, 1/64, and 1/128) against *Leishmania* parasites. Titers of 1/20 or over