Testing the prevalence of Leishmania Donovani DNA in the Blood of Sudanese blood donors

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ABSTRACT:

— ***Objective:*** Leishmaniasis is an endemic disease in almost ninety countries. The diagnosis of leishmaniasis should be ideally confirmed by demonstration of parasites, although some forms of Leishmaniasis are extremely difficult to diagnose by parasitology. Other alternative methods include culture, immune-based methods such as enzyme-linked immunosorbent assay -ELISA and direct agglutination test. Molecular techniques, particularly PCR, are now taking places in the diagnostics of Leishmaniasis, and prove high sensitivity and specificity.

— ***Materials and Methods:*** Fifty blood donors who were deemed fit to donate blood in were chosen. Blood samples were taken, and nucleic acids of the specimens were extracted. The PCR mix was supplemented in a 200 μl tube. The PCR was performed in an Eppendorf (EP) Gradient S thermo-cycler. 5 μl of the products were analyzed on a 2% Agarose gel stained with ethidium bromide; the extracts were tested with the SSU PCR assay (18s rRNA). It was amplified with primers: M18S-L-F and M18S-L-R.

— ***Results:*** None of the 50 donors' DNA showed a positive test result with SSU PCR assay.

— ***Conclusions:*** It's still not clear why there were no positive results: is it due to the failure of detection of parasite DNA by SSU-PCR, or simply because there were no parasites in the donors' blood.

— ***Keywords:*** Leishmaniasis, PCR, Blood donors.

— ***Abbreviations:*** CL: Cutaneous Leishmaniasis; DAT: direct agglutination test; DCL: Diffuse cutaneous leishmaniasis; ELISA: enzyme-linked immunosorbent assay; HIV: Human Immunodeficiency virus; IFAT: Indirect fluorescent antibody test; kDNA: kinetoblast DNA; a network of ring-structured mitochondrial DNA; rK-39: rapid Kinesin protein of 39 amino acids; an antigen ok Leishmania major; rKE16: rapid Kinesin protein cloned in E. coli bacterium; MCL: Muco-cutaneous leishmaniasis; PCR: polymerase chain reaction; RNA: Ribonucleic acid; SSU PCR-OC: Small subunit ribosomal RNA-(18S rRNA) PCR-Oligochromatography; TTL: transfusion transmitted Leishmaniasis; VL: Visceral Leishmaniasis.

INTRODUCTION

Leishmaniasis is a public health problem in at least 88 countries, of which 67 are in the old world and 21 in the new world[1]. About 1-1.5 million new cases are reported annually worldwide[1]. The trypanosomatid parasite of the genus *Leishmania* is the etiological agent of a variety of disease manifestations, collectively known as Leishmaniasis. The parasitic protozoa of the genus Leishmania is mainly transmitted to humans by sand flies. Over 20 species and subspecies infect humans. Humans are infected via the bite of

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sand flies (subfamily *phlebotominae*) - tiny sand-colored blood-feeding flies that breed in forest areas, caves, or the burrows of small rodents. Sand flies become infected by ingesting blood from infected reservoir hosts or from infected people[2-4]. Other than the insect route, transmission through placenta[5], semen[6], injection needles[7], and laboratory acquired infections have also been reported[8], though rarely.

Leishmaniasis acquired through blood bank products, particularly whole blood and RBCs, was mentioned in several researches and case reports[9-14]. HIV global phenomenon incited the endorsement of strict protocols in concern with blood transfusion. Several pathogens, however, still use the transfusion route as an infection route[15-18], and this is because most of the blood banks around the world only validate the screening protocol only for Hepatitis and HIV viruses. From the early 1900s, VL has been among the most important health problems in Sudan, particularly in the main endemic area in the eastern and central regions[19,20]. Several major epidemics have occurred, the most recent--in Western Upper Nile province in southern Sudan, detected in 1988-claiming over 100,000 lives[21].

The diagnosis of leishmaniasis should be ideally confirmed by demonstration of parasites, although some forms of leishmaniasis are extremely difficult to diagnose by parasitology. Parasitological diagnostic methods include microscopic examination, culture and inoculation of aspirates into animals. The method of choice depends on local resources. Other approaches include sero-diagnosis, which is based on detection of circulating antibody. It is highly sensitive for diagnosing VL and useful for diagnosing MCL disease, but less useful for diagnosing other forms of CL. Usually it is a support to a parasitological diagnosis but sometimes it is appropriate for use under field conditions. The techniques include aldehyde (formal gel), direct agglutination test (DAT), enzyme-linked immunosorbent assay (ELISA), Indirect fluorescent antibody test (IFAT) and recombinant antigens. Other laboratory investigations include the polymerase chain reaction (PCR): it is a sensitive and specific technique for detecting and identifying specific leishmania DNA sequences but it requires advanced laboratory facilities and technical expertise so it is not suitable for field conditions. Leishmania species identification is important and techniques to identify leishmania species include parasite biology, isoenzyme analysis, monoclonal antibodies, PCR, DNA or RNA hybridization to specific probes and analysis of kDNA[22,23]. Screening of donated blood by microscopic examination is not a sensitive tool and aspirates from the spleen or the bone marrow will be unethical. Immunodiagnostic testing, including ELISA using recombinant antigens such as rK-39 developed from *Leishmania chagasi* of the new world or a recently developed recombinant antigen rKE16 from *Leishmania donovani* from India[24,25], and PCR technology can be used for mass screening of donor blood samples. But these methodologies may have financial and technical difficulties. It may be suggested that, all donors be screened for specific anti-leishmania antibodies. A rapid test using rKE16 antigen is now commercially available at a very economic price[24].

To the knowledge of the authors, there were no studies about incidence or prevalence of leishmaniasis in blood donors in the endemic areas of Africa. There are, however, multiple studies from South America and Asia documenting the prevalence of leishmaniasis in screened blood donors. In Brazil, the prevalence of leishmania in blood donors is approximately 30.0% in highly endemic areas and less than 10% in low endemic areas[25]. The prevalence of Leishmaniasis in El Salvador, next to Brazil, was positive by ELISA in 5.4% of the asymptomatic blood donors[26]. In Iran, the prevalence of blood donors with positive serology of leishmaniasis in a cohort study was less than 4% [27], whereas in a Bangladeshi study of more than 1100 blood donors, the prevalence of asymptomatic Leishmaniasis test through PCR was positive in three donors only, which represents 0.3% of the study population[28]. Therefore, an estimation of prevalence of positive leishmaniasis in blood donors could be anywhere from 0.3% to 30%. This current research aimed to determine the prevalence of viseral leishmaniasis among asymptomatic healthy blood donors in the blood bank of Al- Gadarif Teaching Hospital, also to assess the use of molecular techniques in diagnosis of visceral leishmaniasis, also to evaluate the Small subunit ribosomal RNA-(18S rRNA) PCR Oligo-Chromatography, abbreviated as SSU PCR-OC, for diagnosis of Leishmaniasis among blood donors, and to determine whether blood donors should be screened for leishmaniasis in an endemic areas.

MATERIALS AND METHODS

This was a prospective cohort study conducted in Gedarif State-Eastern Sudan, a recognized endemic area for VL [29-31]. The samples were selected from fifty donors who were deemed fit to donate blood in the blood bank. The personal, epidemiological and clinical data were collected using a standard form. The sampling collection and questionnaire filling were carried out after taking the permission from the blood bank and hospital administration, and verbal consent will be taken from the donors for both blood sampling and questionnaire. Ethical clearance for the study was obtained from the Department of Biochemistry Faculty of Medicine University of Khartoum. From each blood donor, 200 µl anti-coagulated blood was mixed with 200 µl of L3 buffer (the trade name AngeroNA™, from Mallinckrodt Baker (Phillipsburg, NJ, USA), this buffer allows specimen storage without loss of DNA and RNA quality. Specimens were shipped at 4°C from the collection site to the central laboratory at Soba University Hospital laboratory, Khartoum University and stored at 4°C for a maximum of two weeks.

SSU PCR test was done at the Department of Biochemistry, Faculty of Medicine, Khartoum University, and Soba University Hospital. Nucleic acids of the specimens were extracted according to the method described by Boom et al [32]. Elution was done in 50 µl of pure water and stored at -20°C until analysis. The extracts were tested with the SSU PCR assay (18s rDNA)[24]. It was amplified with primers (M18S-L-F and M18S-L-R); (5'CGTAGTTGAACTGTGGGCTGTGC3') and (5' ACTCCCGTGTTTCTTGTTTCTTTGAA 3').

The primers concentrations were 0.8 μM and they were ordered from Sigma-Aldrich (Bornem, Belgium). The total reaction volume was 25 μl of 1x PCR buffer (Qiagen, Venlo, The Netherlands), including 1 mM of $MgCl_2$ buffer (Qiagen, Venlo, The Netherlands), 200 μM of each dNTPs from Eurogentec (Seraing, Belgium) and 0.5 unit HotStarTaq Plus DNA polymerase (Qiagen, Venlo, The Netherlands) was used to give 0.02 u/ul concentration in the final PCR mix, 5 ul of extracted DNA added to the mix. The PCR mix was supplemented in a 200 μl tube. An initial denaturation step at 94°C for 15 min to activate the HotStar Taq polymerase was followed by 40 cycles of 94°C for 30 s, 60°C for 1 min, 72°C for 1 min, and a single final extension at 72°C for 5 min. The PCR was performed in an Eppendorf EP Gradient S (Eppendorf, Wesseling-Berzdorf, Germany) thermocycler. 5 µl of the products were analyzed on a 2% agarose gel stained with ethidium bromide, and scored positive when an amplicon of the expected size was observed (100 bp).

RESULTS

None of the 50 donors' DNA showed a positive test result with SSU PCR assay (Figure 1).

DISCUSSION

Molecular diagnostics are attractive alternatives to conventional parasite detection as they combine sensitivity with specificity. Recently, the Leishmania SSU- PCR (18s) was developed as standardized formats for molecular detection of Leishmania parasites [33]. In this study the SSU-PCR was used to screen the blood of 50 donors from Gedarif for *leishmania* DNA. No sub clinical cases could be detected in this study although high prevalence of asymptomatic *L. donovani* carriers in VL endemic areas has been reported from the different VL endeminc areas in the world and in Sudan subclinical infection has been reported in Gedarif state [30,31]. There were no positive results. However, knowing that PCR detection of leishmaniasis has higher sensitivity than ELISA (nine times) and Western blot (three times) [34], and the false negative is a remote possibility, in contrast to the false positive result which is the more common to occur when the primer is not appropriately prepared [35], the SSU-PCR have higher sensitivity when compared with other PCR methods[36-38]. Therefore, this negative results is very likely to be valid.

The automatic sample calculator software was used to estimate an adequate sample size. Using the 95% confidence level and knowing that the general population of Gedarif state in Sudan was 1,400,000 (according to the year 2000 census), the calculated confidence interval was 13.86 putting a percentage of 50% (for the possibility of being positive of leishmaniasis). The sample size needed accordingly was 50 participants. Therefore, it is fair to say that the research sample size is adequately representative. Saad et al[39] reported 10% of asymptomatic *L. donovani* carriers but those healthy control were from a highly endemic village outside the town of Gedarif. Another point is, unlike this current study, they used internal controls which allowed them to check for consistent amplification across different runs, and PCR sample inhibition. Failure to amplify this control in a negative *Leishmania* sample points to the presence of inhibitors or an erroneously prepared PCR mix, in which case no conclusions must be drawn from the PCR. There are only 42 documented cases of TTL in published medical journals [40]. Ten of these cases were sporadic case reports, and thirty-two were in patients undergoing hemodialysis in Brazil[14]. In the ten cases, only one was for an Indian adult female of thirty years of age, while all the remaining nine cases are children within different ages (Table 1).

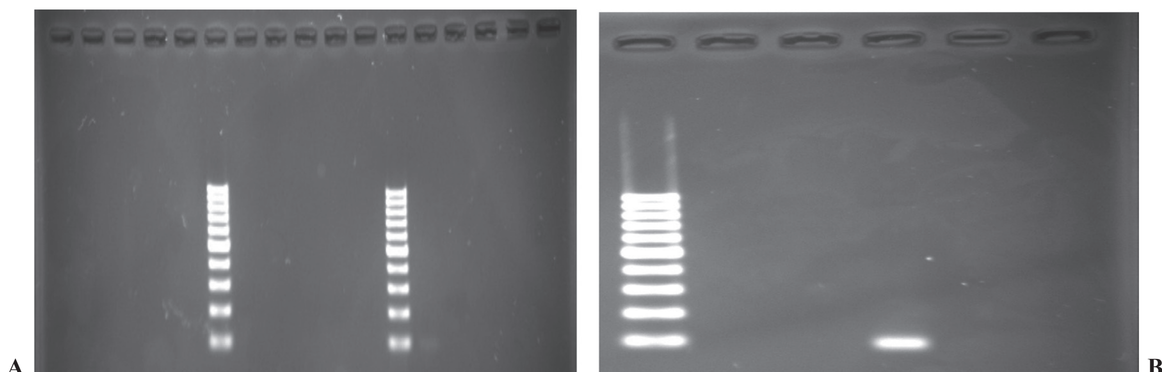

Figure 1. SSU PCR assay *(A)*: on blood bank donors' samples. *(B)*: on leishmania positive control and negative control.

Table 1. A summary of the characteristics of the 10 individual case reports of transfusion transmitted Leishmaniasis (TTL) in the literature.

Numer of Cases	Country	Year of report	Case description	Comments	References
2	China	1948	Mom donated to each of her two daughter 20 ml of blood, in the form of IM injection	This was a method for measles prevention	41
2	France	1955	Blood was received from an adult donor recently arriving from Spain. Two infants received the infected blood	One infant died within three months, while the other treated from VL	9
2	Sweden	1963	Blood donated to two newborns by an asymptomatic donor. He was travelling outside Sweden the last few years before the donation	Symptoms appeared in the newborns after six months from donation	10
1	Belgium	1991	Baby of eleven month received blood from four different donors. All donors and the mother were both healthy and seronegative for leishmania	The history lacks the information of the travelling history of blood donors; transfusion transmitted leishmaniasis couldn't be ruled out	11
1	India	1993	The patient is a child aged six years, a known case of ALL, and received multiple blood transfusions	Both 1993 cases from India received blood from the same bold bank	13
1	India	1993	A female of 30 years, transferred blood in two occasions due to severe anemia.	Both 1993 cases from India received blood from the same bold bank	13
1	UK	1995	A child was hospitalized for cardiac surgery, and blood was transfused fifteen times to him, in addition to one plasma unit. He and his mother have no travelling history outside UK	He was diagnosed with TTL by exclusion of other routes of transmission	12

The first two cases were two Chinese sisters received infected blood from their own mother[41], consequently, two French infants[9], two Swedish newborns[10], an eleven month Belgian baby[11], a six years old Indian boy[13], a 30 years old Indian lady[13], and two years old British baby[12] were added to the TTL list of reports.

The American Association of Blood Banks, Arlington, suggested that TTL can be prevented by applying a blanket policy of not drawing blood from individuals who are at potential risk of being infected with the parasite [42]. The authors recommend introducing internal control in this SSU-PCR assay. They also recommend performing appropriately designed studies to detect the prevalence and cost-benefit ratio are needed to determine whether screening of donor blood for anti-leishmania antibodies needs to become a routine procedure in Sudanese and African blood banks.

CONCLUSIONS

This research presented a prospective cohort to measure the prevalence of *Leishmania Donovani* parasite in blood donor in a well-known Leishmania endemic region: Gedarif, eastern Sudan. A DNA-based technique – Small subunit ribosomal RNA-(18S rDNA) PCR Oligo-Chromatography, was used. There were no positive results in this experiment. The high sensitivity of the SSU-PCR in detection of leishmania DNA and the adequate sample size go in favor of the validity of the result.

Conflict of Interest:
The Authors declare that they have no conflict of interests.

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