Surveillance of Chagas disease among at-risk blood donors in Italy: preliminary results from Umberto I Polyclinic in Rome

Simona Gabrielli[1,4], Gabriella Girelli[2,5], Francesco Vaia[3], Mariella Santonicola[2], Azis Fakeri[2], Gabriella Cancrini[1,4]

[1]Parasitological Analyses Unit, [2]Immunohaematology and Transfusion Unit, [3]Sanitary District, Umberto I Teaching Polyclinic; [4]Department of Public Health and Infectious Diseases, [5]Department of Molecular Medicine, "Sapienza" University, Rome, Italy

Background. Chagas disease is a parasitic disease due to *Trypanosoma cruzi*, endemic in Central and Southern America, where the protozoon infects about 8-10 million people. In rural areas the infection is acquired mostly through reduviidae insect vectors, whereas in urban ones it is acquired mainly through the transfusion of blood products, vertical transmission and organ transplantation. The important migratory flows of the last decades have focused attention on possible *T. cruzi* transmission by transfusion also in non-endemic countries, and platelets have been recognised as the main origin of infection for recipients from serologically-positive Latino-American donors.

Materials and methods. In order to avoid the occurrence of transfusion-related cases, in 2010 systematic screening for anti-*T. cruzi* antibodies was started at the Umberto I Polyclinic in Rome, controlling blood donors born and/or coming from Latin-American countries in which the disease is endemic. The aim of this paper is to report the preliminary results achieved since the introduction of this screening.

Results. Anti-*T. cruzi* antibodies have been detected to date in 3.9% out of the 128 people examined. A seropositive subject also proved positive by polymerase chain reaction analysis and showed very light parasitaemia.

Discussion. The preliminary results are quite alarming. Indeed, serological findings exceed those reported in other non-endemic countries, and Italian travellers proved to be an insidious possible source of direct transmission. The need for systematic screening of at-risk blood donors also in non-endemic countries is emphasised.

Keywords: *Trypanosoma cruzi*, Chagas disease, screening, transfusion.

Introduction

Chagas disease, caused by the protozoon *Trypanosoma cruzi*, may be included among the neglected tropical diseases together with soil-transmitted helminthic infections, lymphatic filarioses, onchocercosis, dracunculosis, schistosomoses, leishmaniosis, human African trypanosomoses, Buruli ulcer, leprosy, and trachoma[1]. This anthropozoonosis is endemic in Latin America where it affects 8-10 million people[2]. Unlike the vectorial transmission (by means of *haematophagous reduviidae* insects) and oral transmission (through contaminated food) that prevail in endemic rural areas, vertical transmission of the parasite and infection through blood or organ transplantation can occur in any country[3]. As a consequence, the important migratory flows of the last decades have dramatically changed the traditional epidemiological pattern of Chagas disease. The lack of effective control measures and preparedness in most European countries facilitated the emergence of congenital and transfusion-related cases. In Spain (the country most involved, with 39,985-65,258 Latin Americans estimated to be infected by *T. cruzi*) at least six cases of transfusion-transmitted Chagas disease have been reported[4]. Although there is no evidence of transmission by red blood cells or frozen products, whole blood-derived platelets, including those from leucoreduced and irradiated products, have been recognised at the origin of infection in recipients from serologically-positive donors[5]. In order to prevent this risk, some non-endemic countries (among others France, Spain and the United Kingdom) have recently established legal requirements to ensure the safety of the blood supply and organ transplantation by monitoring them for Chagas disease[6]. Although Italy is the second country in Europe for number of Latin American residents, there are no systematic screening plans at a national level to assess the importance of this health problem and to avoid nosocomial transmission. Only two Centres (for Tropical Diseases in Negrar, Verona, and the Infectious and Tropical Diseases Unit, University of

Florence) started, in 1998, a specific programme to test all people at epidemiological risk (travellers, migrants, blood or organ recipients, neonates, adopted children), and evidenced that wider assessment of Chagas disease epidemiology is urgently required, as are measures to prevent its transmission and to improve its diagnosis and treatment[7]. In 2008 the two Centres, in collaboration with two of the authors of this paper (SG and GC), implemented a surveillance plan targeted to Latin American pregnant women and detected the infection in 6/151 of them and also in one vertically infected infant[8,9]. Finally, in order to avoid the occurrence of transfusion-related cases, in 2010 systematic screening for anti-*T. cruzi* antibodies was started at the Umberto I Polyclinic in Rome to control blood donors originating (or with mother originating) from endemic areas, and European donors who had lived in or travelled to Latin American countries in which the disease is endemic. The aim of this paper is to report the alarming preliminary results obtained since the introduction of this screening.

Materials and methods

Study population

During the years 2010-2012, blood donors who, during the questioning, reported histories suggesting a risk of *T. cruzi* infection were enrolled by the Immunohaematology and Transfusion Unit (Umberto I Polyclinic) and were included in the Chagas disease control schedule. Screened donors were subjects: (i) born in a Latin American endemic country (n=88); (ii) born from a mother who lived in an endemic country (n=14); or (iii) coming from Latin American endemic countries where they had spent more than 1 week for travel or employment (n=26). The screening plan was approved by the ethical committee (protocol number: 159/10-93/10) and followed the principles of the Helsinki Declaration and its subsequent modifications, as well as those of Italian legislation (Ministerial Decree, 18.03.98) and the Italian National Law n. 675.1996 concerning the protection of personal data. All the volunteers gave their written informed consent for the collection, analysis and storage of their blood samples.

Laboratory testing

The World Health Organisation (WHO) established that a donor positive to one serological test is excluded from the possibility of bloodletting, and that two positive results are necessary to make a clinical diagnosis of Chagas disease[10]. Transfusion is, therefore, safe if a serological control is included among routine analyses. Given the lack of a widely accepted standard for serological diagnosis of chronic *T. cruzi*-infected patients, we applied three tests to compare their diagnostic efficiency. In detail, in all cases donors were screened using two kinds of serological tests: an immunochromatographic assay (ICT) (Chagas Quick Test, Cypress Diagnostics, Langdorp, Belgium) associated with an enzyme-linked immunosorbent assay (ELISA) based on recombinant antigens (BioELISA Chagas, Biokit S.A., Barcelona, Spain or NovaLisa Chagas ELISA test, Nova Tec Immunodiagnostica, GmbH, Dietzenbach, Germany). This algorithm was chosen based on the results we obtained during a pilot study on a sample of Bolivian people (n=226) in whom ICT proved more sensitive than the aforementioned first available ELISA (accordance=92.4%, k=0.83) at evidencing infections that were subsequently confirmed by positive polymerase chain reaction (PCR) analysis carried out on blood samples of subjects showing discordant results[11]. The NovaLisa Chagas ELISA test was used to compare the sensitivity of the two ELISA.

Serologically positive blood samples are thoroughly investigated by means of microscopy and molecular diagnostics. Giemsa stained peripheral blood smears were prepared after triple centrifugation of 10 mL of blood and observed at 40X magnification. Genomic DNA was isolated from 300 µL of whole blood (NucleoSpin® tissue, Macherey-Nagel, Düren, Germany) and submitted to a N-PCR for the repetitive area of nuclear DNA using primers TCZ1/TCZ2 and TCZ3/TCZ4, which yield products of 188 and 149 base pairs, respectively[12]. The first PCR amplification was performed in 25 µL volumes under the following final conditions: 1x Buffer including 1.5 mM $MgCl_2$, 0.2 mM of each dNTP, 1 µM each of forward and reverse primers, and 1 unit of polymerase (BIOTAQ™ DNA Polymerase, Aurogene, Rome, Italy). The thermal profile we used was: 94 °C for 30 sec, 60 °C for 30 sec, and 72 °C for 1 min for 30 cycles, following by a final extension for 7 min at 72 °C. One microliter of the reaction was used for the second amplification, in which primers TCZ3 and TCZ4 amplified a 149-nucleotide internal sequence of the same repetitive sequence. The N-PCR conditions were the same as for the first amplification. Cycles were differentiated: 94 °C for 40 sec, 55 °C for 40 sec, 72 °C for 1 min and 30 sec (30 cycles) and a final extension for 7 min at 72 °C. Ten microliters of the first and second reactions were electrophoresed in gel containing 2.0% agarose (Agarose, Molecular Grade, Aurogene) stained with ethidium bromide and visualised in ultra-violet light.

Amplicons were purified (SureClean Bioline, Aurogene) and then sequenced (Eurofins MWG Operon, Ebersberg, Germany). Sequences, corrected by visual analysis of the electropherograms and aligned using ClustalW (http://www.genome.jp/tools/clustalw), were compared with those available in the GenBank (http://www.ncbi.nlm.nih.gov/genbank/) dataset by BLAST analysis.

Results

A total of 128 subjects (74 males and 54 females) were included in the Chagas disease control plan. The mean age of the population was 37.5 years (range, 19-66 years). A slight predominance of males (58.26%) was observed. Countries of origin, age and laboratory results are shown in Table I. Most of the people enrolled (102 out of 128) were Latin Americans coming from endemic areas or born from Latin American mothers living in endemic areas (such as donors from Cuba and the Dominican Republic), whereas the remaining 26 subjects (20.31%) were Italian donors who travelled to endemic areas.

Five (3.9%) of the 128 donors proved positive to at least one serological test, one (0.8%) was positive to two tests and one (0.8%) was positive to PCR and one serological test. The seropositive individuals were three Latin American immigrants from Brazil, Bolivia and Colombia and two Italian from Rome. The first Italian donor is a 38-year old man, a backpacker globetrotter who, during 2008-2009, travelled through Mexico and in 2012 offered a first blood donation. The other one is a 46-year old engineer researcher, who worked in Mexico and, more recently, in impervious internal areas of Brazil, where he stayed until 2011 (6 months before his first blood donation that evidenced his positivity to ICT). The blood of this donor proved PCR-positive and showed a very mild parasitaemia in Giemsa-stained thick blood smears. BLAST analysis of the amplicon showed 100% identity to *T. cruzi* accession number AY520069, strain Y, discrete typing unit (DTU) TcII[13].

Discussion

The preliminary results of this surveillance, even if based on the screening of only 128 donors, are quite worrisome. In fact, serology evidenced IgG against *T. cruzi* in 3/102 (2.94%) Latin American immigrants (including individuals born from mothers living in endemic areas), a value exceeding that expected from data reported in the literature[3,14] and, furthermore, with the exception of the Bolivian donors, revealed infections in people from countries in which the prevalence of Chagas disease is -currently- low. The findings in Italian donors are even more alarming: 2/26 (7.69%) subjects at risk of infection were really infected, and one of them even had parasitaemia. The findings should not, however, be surprising: travel or employment in endemic areas expose (of course for shorter times) foreigners to the same risk of infection as that of the local population, as demonstrated by further cases of Chagas disease reported in people usually resident in Italy, France and Japan[7,15,16], often associated with oral infection. Acute Chagas disease is frequently asymptomatic also when orally acquired (mainly through consumption of crude sugar-cane juice); moreover, the classical signs of the disease, such as Romaña's sign, chagoma and enlarged lymph nodes, can be missed in cases of oral transmission. The diagnosis of recent infections can, therefore, easily be missed, as too can cases of chronic disease, when the long past travel may be overlooked. The engineer researcher in our series with very mild parasitaemia was asymptomatic, was not aware of any insect bites and his history was negative for Romaña's

Table I - Nationality and age of blood donors enrolled in the screening programme, and results of serological and molecular findings.

Donors' origin	Examined N°	Age <40 years	Age >41 years	N. positive to BioELISA	N. positive to NovaLisa	N. positive to ICT	N. positive to PCR
Argentina	14	6	8	0	0	0	0
Bolivia	5	4	0	0	1	1	0
Brazil	7	7	0	1	0	0	0
Chile	3	2	1	0	0	0	0
Colombia	9	5	4	0	0	1	0
Cuba	3	2	1	0	0	0	0
Ecuador	15	9	6	0	0	0	0
El Salvador	1	1	0	0	0	0	0
Mexico	5	5	0	0	0	0	0
Nicaragua	1	0	1	0	0	0	0
Paraguay	1	1	0	0	0	0	0
Peru	29	17	12	0	0	0	0
Dominican Rep.	1	0	1	0	0	0	0
Venezuela	8	6	2	0	0	0	0
Italy	26	12	14	A: 1 B: 0	A: 0 B: 0	A: 0 B: 1	A: 0 B: 1
Total	128	78	50	2	1	3	1

Legend A: travelled through Mexico; B: worked in Mexico and Brazil.

sign; he could, therefore, have become infected by this route.

In conclusion, international travellers, although attributed only an anecdotal role in imported Chagas disease, may represent a more insidious source of infection than migrants. In addition, like Latin American people, they are developing a disease that requires further immediate medical checks (to whom the engineer has been submitted with negative preliminary results) and appropriate therapy, which is more effective if instituted promptly.

From a technical point of view, we applied three serological tests to detect *T. cruzi*-infected patients and, in addition, to compare their diagnostic efficiency. Besides immunoenzymatic tests, we used a rapid ICT assay, which proved the most sensitive test (it detected 3 positive donors). The two ELISA gave matching results in 98.43% of sera. The Novalisa showed excellent concordance with the ICT (k=0.99), whereas no donor was positive to the other ELISA and to ICT. However, the low number of positive subjects prevents interpretation of not-matching positive results; we can only hazard a guess that ICT is a more useful marker of relatively recent infections, when PCR may still confirm it, which is, in our opinion, a valid reason for its application.

Moreover, in the case of any positive serology, microscopic analysis of blood and molecular screening followed by sequencing were added to evaluate the possible risk of transmission and, even more, to assess the *T.cruzi* strain responsible for the infection. The use of molecular diagnostics to identify the possible source of infection is controversial: although some studies underlined the performance of PCR analysis in detecting infections[12], several screening schedules, following WHO recommendations[17], did not include it[18]. In our opinion, although prior standardisation of PCR techniques is mandatory to ensure reliable results, this powerful diagnostic technique should be included in future screening programmes, at least as regards identifying genotypes involved.

Molecular characterisation revealed the presence of the DTU TcII in the PCR-positive donor, the major *T. cruzi* lineage associated with human infections of varying severity[19-22], although TcI has been isolated from the myocardial tissue of a patient with chronic Chagas disease with end-stage heart failure[23].

In conclusion, up to now only the scientific community directly involved has started to pay attention to blood safety concerning trypanosomosis; however, these preliminary results, notwithstanding their methodological limitations, present a scenario that should be considered worrisome. They suggest that Chagas disease is an emerging problem also in Italy and highlight the need for systematic screening plans to control at-risk blood donors, mainly temporary residents in endemic areas, who constitute a possible insidious source of person-to-person *T. cruzi* transmission. That being so, a thorough pre-donation interview for the proper identification of at-risk donors must be considered of fundamental importance.

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Correspondence: Simona Gabrielli
Department of Public Health and Infectious Diseases
Sapienza University
Piazzale Aldo Moro 5
00185 Rome, Italy
e-mail: simona.gabrielli@uniroma1.it